CHAPTER III
Materials and methods

3.1. Study area and ethnographic background

Karbi Anglong is situated in the central part of Assam, bounded by Golaghat district in the East, Meghalaya & Morigaon district in the West, Nagaon & Golaghat district in the North and NC Hills and Nagaland in the South. The district with dense tropical forest covered hills and flat plains is situated between 25° 33' N to 26° 35' N Latitude and 92°10'E to 93° 50'E Longitude.

The district consists of three subdivisions namely, Diphu Sadar, Bokajan Civil and Hamren Civil. Our study areas consisted of four villages which lie in the Boithalangsu PS of Hamren subdivision (Figure 1.2). Bothol is about 26 km towards east, Rongjami is 35 km towards east, Jenkha is 35 km towards north-east and Belapara is about 6 km east of Hamren.

Due to variation in topography, the district experiences different climates in different parts. The winter commences from October and continues till February. During summer, the atmosphere becomes sultry. The temperature ranges from 6 degree to 12 degree in winter and 23 degree to 32 degree Celsius in summer. The average rainfall is about 2,416 mm.

The population in Karbi Anglong is predominantly tribal. Major ethnic groups are Karbis, Bodos, Kukis, Dimasas, Garos, Rengma Nagas, Tiwas, Man (Tai-speaking) and large number of non tribals. The present study areas are Bodo tribe dominated villages with approximately more than 5000 Bodo families in Rongjami, about 500 families in Borthol, more than 40 families in Jenkha and about 15 families in Belapara (according to the village chiefs, 2003).

The economy of this district is essentially based on agriculture. Paddy is the main crop, except for the valleys, where the people follow Jhum cultivation. Traditional medicines still remain the main recourse for a large majority of people for treating health problems as people can not afford conventional treatments.
3.1.1. Methods of survey. The ethno-botanical explorations were conducted from October 2003 to October 2008. The preliminary survey of the villages (Fig. 3.2.) was made from October 2003 to October 2004 with the help of two local volunteers (Fig. 3.3.). Total 64 informants (48 males and 16 females) (Fig. 3.4. and 3.5.) were involved in the data collection. Conversations were built on trust with the common goal to improve the health situation in the country and to preserve and increase the knowledge on medicinal plants. Further, total 7 most knowledgeable informants were short listed and more information was collected from them.

The field works were done in the months of June, July, October, November, January and February. The methods used for ethnobotanical data collection were semi structured interviews, field observation, preference ranking and direct-matrix ranking (Martin GJ, 1995; Cotton CM, 1996; Jain SK, 2004, Pesek T, 2005). These interviews were conducted in local language (Bodo and Assamese). Six different field trips were conducted. Table 3.1. Indicates relevant data collected on: age, sex, and occupation of informants as well as animal and human health indications treated, vernacular plant names, growth form, plant part used, methods of preparation, dosages, and route of administration. These interviews were done in the field in order to avoid the probable confusions with regard to the identity of the medicinal plants. The morphological characteristics, habitats and habits of medicinal plants were observed, photographed and recorded during and after the interviews.

Knowledgeable informants were taken to the field and along with collection of plants for voucher specimens; uses of the plants as mentioned by them were recorded. Occasionally, the plants were collected from the surrounding areas and showed to them one by one and information was recorded accordingly (Jain SK, 2004). Voucher specimens were collected and deposited at Hojai College Botany herbarium. Few specimens were deposited in Botanical Survey of India, Shillong for future reference (Jain SK, 2004).
Fig. 3.1. Map of Karbi Anglong district of Assam (India). Arrow head: study area.
Fig. 3.2. Study area.

Fig. 3.3. Local volunteers.

Fig. 3.4. Most revered traditional healer of Borthol, Karbi Aglong.

Fig. 3.5. An informant from Borthol, Karbi Anglong.
Table 3.1. Data acquisition questionnaire for utilization and conservation of medicinal plants in Karbi Anglong district, Assam.

**QUESTIONNAIRE**

**PART 1: Details of informant.**

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<thead>
<tr>
<th>Name</th>
<th>Sex</th>
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<td>M/F</td>
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<th>Approximate dosage</th>
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<th>Response of Patient Good</th>
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<th>Poor</th>
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Any adverse effects:

**PART 2: Informant consent agreement.**

Hereby agree to participate in this study with my full consent and conscious and declare that to the best of my Knowledge the information that I have provided is true, accurate and complete.

Signature/Thumb print: Date: Dec/2007

**PART 3: Researchers’ Declaration.**

1. The following research will be undertaken with respect to the indigenous knowledge and intellectual proprietary of the Bodo Community.

2. We will at no given time initiate or conduct practices that are deemed to obtain information from the respondents by intimidation, coercion or false pretence.

3. The respondents will be informed of the intended project elaborately prior to questionnaire administration and in confidential to eliminate any degree of conspiracy.

4. We will be no under any obligation to edit or tamper the information provided by the respondents.

5. The information collected will be used for the described research purpose and not any undisclosed intention.

**Signatory Researchers:**

1. C. Basumata
2. M. Saikia
3. J.C. Kalita
3.1.2. Collection of plant materials. The herbarium specimens were collected and pressed with proper labels and field notes (Jai and Rao, 1977; Jain SK, 2004). *Trichosanthes bracteata* (Cucurbitaceae), *Leucas pleukenetii* (Lamiaceae) and *Cissampelos pareira* Linn. (Meispermaceae) plants were collected in the month of June from a remote village in Karbi Anglong district of Assam and planted in the Botanical garden of Hojai College for future use. A voucher specimen of *Cissampelos pareira* was deposited in the herbarium of Botanical Survey of India, Shillong (No.1399/BSI, EC) for further reference.

3.2. Plant materials

The leaves of *Trichosanthes bracteata* (Cucurbitaceae), *Leucas pleukenetii* (Lamiaceae) and *Cissampelos pareira* Linn. (Meispermaceae) were collected in the months of May, June and July in sunny days, washed properly and air dried. The dried leaves were then powdered in a grinder and kept in plastic containers, sealed with proper labeling and kept in a refrigerator at 2-8°C for future use (Sabu MC and Kuttan R, 2003; Babu V *et al.*, 2003).

3.2.1. Preparation of the extract and administration. The leaf powder (10g/100ml) was extracted with water in the experiment I and with water-methanol (1:1) in rest of the experiments with constant shaking for 24 hours (with short break in between) and filtered. The filtrate was evaporated to dryness in a rotary evaporator below 50°C, weighed and reconstituted with purified water and used (the yield was about 10%).

The animals were first trained to feed from syringe for one to two weeks continuously. When they became accustomed to it, different doses (30mg/kg and 100mg/kg and 300 mg/kg body weight) of plant extract was continuously fed to the animals between 9 a.m. to 10 a.m. each day during the study period.
3.3. Chemicals
Anesthetic drug was purchased from Neon Laboratories Ltd.; aloxan and RPMI-1640 media were procured from Sigma Chemicals (St. Louis, Mo, USA). The chemical for glucose (Glucose Oxidase- Peroxidase Method), triglyceride (Enzymatic Method), Cholesterol (Enzymatic Method), Serum Glutamate Oxalate Transaminase (SGOT, UV Kinetic IFCC Method), Serum Glutamate Pyruvate Transaminase (SGPT, UV Kinetic IFCC Method) and Alkaline Phosphatase (AP, PNPP Method) were of analytical grade and were purchased from Bayer Diagnostics India Ltd. The chemicals for histological procedures were purchased from Merck Chemicals. The reagent for percent glycosylated haemoglobin determination was purchased from Transasia Bio-medicals Ltd. and ERBA Diagnostics Mannheim/Germany and the Immuno-radiometric kit for Insulin estimation was procured from M/S Immunotech, France.

3.4. Anaesthesia
Rats were anaesthetized with ketamine hydrochloride anaesthesia (Ready-to-use). Each animal was given intra-peritoneal injection of 0.4 to 0.6 ml of ketamin hydrochloride (100 mg/kg bw) using 26 gauge needle.

3.5. Animals
Male Wistar albino rats (*Rattus norvegicus alvinus*) weighing 150-200g (about 6-8 weeks old) were used with the approval of the Institutional Animal Ethical Committee, and were maintained at standard laboratory condition as per the committee’s guidelines. Animals were fed standard pellet diet (American Agro.vet. India Ltd.) and water ad-libitum.

3.5.1. Experimental animal groups. The rats exhibiting blood glucose levels in the ranges of 200-250mg/dl and triglyceride levels more than 200mg/dl were randomly selected for the studies and were subdivided into following groups:

- **Group II**: Hyperglycaemic control (FR).
- **Group III**: Hyperglycaemic Pioglitazone (Pioz, 3 mg/kg bw)-treated (FR+Pioz).
- **Group IV**: Hyperglycaemic CLE-treated. This group was further subdivided into different groups according to different dose levels and type of herbal extract used. (Group I: normal control (NC). All the groups consisted of 6 animals).
3.5.2. Collection of blood samples from the animals. The area over the tail, 3 cm from the base of the tail, was cleaned with antiseptic scrub. The blood samples were collected by using a syringe equipped with 25 gauge needle inserted at a 45 degree angle towards the vein applying a gentle negative pressure (IACUC Guidelines, Annex VIII). The blood samples were collected in 1.5 ml sterile microcentrifuge tubes and allowed to clot at room temperature. The sera were separated from the clot within 30 minutes of blood collection and the biochemical parameters were assayed immediately. For insulin determination, 0.1 ml of serum from each animal was aliquoted in the microcentrifuge tubes and kept frozen at -20° C. For Percent Glycosylated Haemoglobin, 0.1 ml of whole blood from each animal was collected in the microcentrifuge tubes containing EDTA.

3.6. Biochemical parameters

Prior to conducting the experiments, body weight of the animals was taken and Glucose estimations were done in order to rule out congenital glucose intolerance. All the biochemical parameters were performed each week during the study periods.


(i) Test Principle. Glucose was oxidized by glucose oxidase (GOD) into Gluconic Acid and Hydrogen Peroxide. Chromogen 4-aminoantipyrine/phenolic compound was oxidized to a red colored compound by hydrogen Peroxide in presence of Peroxidase (POD). The intensity of the red colored compound was proportional to the glucose concentration and was measured at 505 nm (490-530 nm). (The final colour was stable for 2 hours).

\[
\begin{align*}
\text{GOD} & \\
\text{Glucose} + \text{O}_2 & \rightarrow \text{Gluconic Acid} + \text{H}_2\text{O}_2 \\
\text{POD} & \\
\text{H}_2\text{O}_2 + \text{Phenolic} + 4\text{-Amino Compd.} & \rightarrow \text{Red} + 2\text{H}_2\text{O} \\
\text{Antipyrine} &
\end{align*}
\]
(ii) Procedure.

**Reagent preparation.** The reagent 1 containing Phosphate buffer, 4-Aminoantipyrine, p-Hydroxy Benzoic acid, Glucose Oxidase and peroxidase was dissolved with 20ml of distilled water with continuous stirring in a beaker and transferred into a dark bottle and labeled. The mixture was allowed to stand for 5 minutes before use.

**Dispensing.** The reconstituted reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 10μl of standard (Glucose 100 mg/dl) or 10μl of samples (sera of rats) were dispensed successively to properly marked test tubes with the reagent and mixed gently.

**Incubation.** The reagent and standard/sample mixtures were incubated at 37° C for 15 minutes.

**Reading.** The instrument was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 30° C, zero setting was done with reagent blank. Then the final red coloured compound developed after the incubation was measured at 505 nm (490-530).

**Calculation:** Concentration of glucose in serum (c):

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 \text{ (mg/100 ml)}
\]


**(i) Test Principle.** Triglycerides in the sample were hydrolyzed by lipase to glycerol and fatty acids. The glycerol was then phosphorylated by adenosine-5-triphosphate (ATP) to Glycerol-3-phosphate (G₃P) and Adenosine-5-diphosphate in a reaction catalyzed by Glycerol kinase (GK). Glycerol-3-phosphate was then converted to dehydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerolphosphate oxidase (GPO). The hydrogen peroxide then reacted with 4-aminoantipyrine (4-AAPO and ADPS) in a reaction catalyzed by peroxidase to yield a purple coloured quinoneimine dye. The
intensity of the colour produced was directly proportional to the concentration of Triglycerides in the sample when measured at 546nm.

\[
\begin{align*}
\text{Lipase} & \\
\text{Triglycerides} + \text{H}_2\text{O} & \rightarrow \text{Glycerol} + \text{Fatty Acid} \\
\text{GPO} & \\
\text{Glycerol} + \text{ATP} & \rightarrow \text{Glycerol-3-Phosphate} + \text{ADP} \\
\text{Glycerol-3-Phosphate} + \text{O}_2 & \xrightarrow{\text{Peroxidase}} \text{Dehydroxyacetone Phosphate} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{ADPS} & \rightarrow \text{Red quinone} + 4\text{H}_2\text{O} \\
\text{GPO} &= \text{Glycerol-3-Phosphate Oxidase} \\
\text{ADPS} &= \text{N-Ethyl-N-Sulfopropyl-n-anisidine}
\end{align*}
\]

(ii) Procedure.

Reagent preparation. The reagents were allowed to attain room temperature, and the contents of reagent 1 containing Lipoprotein lipase, Glycerol kinase, Glycerol-3-Phosphate Oxidase, Peroxidase, 4-Aminoantipyrine and ATP was dissolved with reagent 2 containing Pipes buffer, pH 7.50, ADPS and Magnesium salt and were mixed by gentle swirling. The mixture was allowed to stand for 5 minutes before using.

Dispensing. The reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 10μl of standard (Triglyceride 200mg/dl) or 10μl of samples (sera of rats) were dispensed successively to properly marked test tubes with the reagent and mixed gently.

Incubation. The reagent and standard/sample mixtures were incubated at 37° C for 5 minutes.

Reading. The instrument (digital colorimeter) was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 30° C, zero setting was done with reagent blank. Then the final purple colored compound developed after the incubation was measured at 546 nm (520-570).
Calculation: Concentration of Triglyceride in serum (c):

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 \text{ mg/dl}
\]


(i) Test Principle. Cholesterol esters were hydrolysed by cholesterol esterase to free cholesterol and fatty acids. Free cholesterols were oxidized to cholest-4-en-3-one and liberated hydrogen peroxide then coupled with 4-aminoantipyrine and phenol in the presence of peroxidase to form a coloured compound. The intensity of the colour developed was proportional to the cholesterol concentration and was measured photometrically at 500nm wavelength (490 to 550 nm) or with Green filter.

\[
\begin{align*}
\text{Cholesterol Esterase} \\
\text{Cholesterol esters} \rightarrow & \text{Cholesterol + Fatty acids.} \\
\text{Cholesterol Oxidase} \\
\text{Cholesterol} \rightarrow & \text{Cholest-4-en-3 one + H}_2\text{O}_2. \\
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \rightarrow & \text{Red Quinone + H}_2\text{O}.
\end{align*}
\]

(ii) Procedure.
Reagent preparation. The reagents were allowed to attain room temperature, and the contents of reagent 1 containing cholesterol Esterase, Cholesterol Oxidase, Peroxidase and 4-Aminoantipyrine was dissolved with reagent 2 containing Pipes buffer, pH 6.90, Phenol and Sodium Cholate and were mixed by gentle swirling. The mixture was allowed to stand for 5 minutes before use.
Dispensing. The reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 10μl of standard (Cholesterol 200 mg/dl) or 10μl of samples (sera of rats) were dispensed successively to properly marked test tubes with the reagent and mixed gently.

Incubation. The reagent and standard/sample mixtures were incubated at 37° C for 5 minutes.

Reading. The instrument was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 30° C, zero setting was done with reagent blank. Then the final red-colored compound developed after the incubation was measured at 500 nm (492-550).

Calculation: Concentration of Cholesterol in serum (c):

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 \text{ mg/dl}
\]


(i) Principle of the assay. The immuno-radiometric assay of insulin was a ‘sandwich’ type assay. Mouse monoclonal antibodies directed against two different epitopes of insulin and hence not competing were used. Serum, controls and calibrators were incubated in tubes coated with the first monoclonal antibody in the presence of the second monoclonal antibody which was labeled with iodine 125. After incubation, the content of the tubes was rinsed so as to remove unbound ¹²⁵I-labeled antibody. The bound radioactivity was then determined in a gamma counter. The insulin concentrations in the samples were obtained by interpolation from the standard curve. The concentration of insulin in the samples was directly proportional to the radioactivity.
(ii) **Procedure.**

**Reagent preparation.** All the reagents were brought to room temperature and prepared in the following manners:

**Control samples.** The vials containing insulin lyophilized in human serum was reconstituted with the volume of distilled water indicated in the label and allowed to stand for 10 minutes. The content of the vials were mixed gently before dispensing.

**Wash solution.** The content of the vial (50ml) was diluted with 950ml of distilled water and homogenized before using.

**Calibrators (Ready-to-use):** The vials consisted of zero to 300μIU/ml of insulin in buffer with bovine serum albumin and preservatives.

**Anti-insulin monoclonal antibody coated tubes (2x50, ready-to use).**

**Dispensing.** 50 μl of calibrator, control and samples were added successively to antibody coated tubes. This was followed by addition of 100μl of tracer to each tubes and gentle mixing. 500μl of tracer was added to two additional tubes to obtain total counts per minute (cpm).

**Incubation.** The tubes with the above mixture were incubated two hours at 25° C with constant shaking (300 rpm).

**Counting.** The contents of the tubes except the two tubes containing only tracer were aspirated carefully which was followed by washing twice with 2 ml of wash solution and aspirated once again. Finally, the radioactivity (cpm) was determined for 1 minute in a gamma counter.

3.6.5. **Determination of percent glycohaemoglobin (%HbA1c).**

(ii) **Test Principle.** A haemolysed preparation of the whole blood was mixed continuously for 5 minutes with a weak binding cation exchange resin. During this time, HbA₀ were bound to the resin. After the mixing period, a filter was used to separate the supernatant containing the glycohaemoglobin from the resin.
The Glycohaemoglobin percent is determined by measuring the absorbances at 415nm of the glycohaemoglobin fraction and the total haemoglobin fraction. The ratio of the two absorbances gives the percentage Glycohaemoglobin.

(iii) Procedure

Reagents. Glycohaemoglobin Ion Exchange Resin: Ready-to-use and pre-filled in plastic tubes (Stable till the expiry date at 15-30°C. Turbidity or discoloration of the supernatant liquid above the resin indicate deterioration); Glycohaemoglobin Lysing Reagent: Ready-to-use; Glycohaemoglobin Calibrator: After attaining room temperature, the contents of each vial was dissolved in 1ml of deionised water, aliquoted in microcentrifuge tubes and frozen at -18°C.

The reagents were allowed to attain room temperature and following steps were followed:

Step I: Haemosylate Preparation: 500μl of lysing reagent was mixed with 100μl of calibrator and 100μl of test sample in appropriately marked tubes, and were allowed to stand for 5 minutes at room temperature till lysis was complete.

Step II: Separation of Glycohaemoglobin. 1ml of the haemosylate from Step I was added into the appropriately marked Ion Exchange Resin tubes. The filter separators were positioned approximately 2cm above the liquid level in the tubes. The tubes were placed on the shaker and allowed to mix continuously for 5 minutes. After removing the tubes from the shaker, the filter separators were pushed down until the resin was firmly packed. Then, the supernatant of each tube was poured into appropriately marked tubes, and the absorbance of each tube for Glycohaemoglobin was read and recorded at 415nm (405nm-420m) against deionised water blank.

Step III: Total Haemoglobin Fraction. 20μl of calibrator haemosylate and sample haemosylate were dispensed into appropriately marked test tubes containing 5.0ml of
deionised water. After mixing gently, the absorbance of calibrator and samples were read against a deionised water blank at 415 nm (405nm-420nm) for total haemoglobin readings.

Calculation.

\[
R_c = \frac{\text{Absorbance of calibrator (Glyco)}}{\text{Absorbance of calibrator (Total)}}
\]

\[
R_u = \frac{\text{Absorbance of Unknown (Glyco)}}{\text{Absorbance of Unknown (Total)}}
\]

\[
\% \text{ Glycohaemoglobin of Unknown} = \frac{R_u}{R_c} \times \text{Value of Calibrator.}
\]

3.6.6. Estimation of Glutamate Oxalate Transaminase activity in serum according to the recommendations of International Federation of Clinical Chemistry (IFCC).

(i) Principle.

\[
\begin{align*}
\text{GOT} & \\
\text{L-Aspartate + } \alpha \text{ketoglutarate} & \rightarrow \text{ Oxaloacetate + L-Glutamate} \\
\text{MDH} & \\
\text{Oxaloacetate + NADH + H}^+ & \rightarrow \text{ L-Malate + NAD}^+ \\
\end{align*}
\]

MDH=Malate dehydrogenase

There was decrease in absorption at 340nm when NADH was converted to NAD. The rate of decrease measured was proportional to GOT activity in the sample.

(ii) Procedure.

Reagent preparation. The reagents were allowed to attain room temperature and the contents of one bottle of reagent 1 containing enzyme Malate dehydrogenase, NADH and \( \alpha \) Ketoglutarate was dissolved with one bottle of reagent 2 containing Tris buffer, pH
7.80 and L-Aspartate and mixed by gentle swirling. The mixture was allowed to stand for 5 minutes before using.

**Step I (Dispensing).** The reconstituted reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 100 μl of samples (sera of rats) were dispensed successively to properly marked test tubes with the reagent, mixed gently and reading was taken immediately.

**Step II (Reading).** The instrument was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 37° C, zero setting was done with distilled water. The rate of decrease in absorption was measured at 340 nm.

3.6.7. Estimation of Glutamate Pyruvate Transaminase activity in serum according to the recommendations of International Federation of Clinical chemistry (UV Kinetic IFCC method, Bayer Diagnostic India Ltd).

**(i) Test Principle.**

\[
\begin{align*}
\text{GPT} & \quad \text{L-Alanine + } \alpha\text{-ketoglutarate} \quad \text{L-Glutamate + Pyruvate} \\
\text{LDH} & \quad \text{Pyruvate + NADH + } H^+ \quad \text{L-Lactate + } NAD^+ 
\end{align*}
\]

The rate of decrease in absorbance at 340nm as NADH was converted to NAD was measured and was proportional to GPT activity in the sample.

**(ii) Procedure.**

**Reagent preparation.** The reagents were allowed to attain room temperature and the contents of one bottle of reagent 1 containing enzyme Lactate dehydrogenase, NADH and α Ketoglutarate was dissolved with one bottle of reagent 2 containing Tris buffer, pH 7.50 and L-Alanine and mixed by gentle swirling. The mixture was allowed to stand for 5 minutes before using.

**Dispensing.** The reconstituted reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 100 μl of samples (sera of rats) were
dispensed successively to properly marked test tubes with the reagent, mixed gently and reading was taken immediately.

**Reading.** The instrument was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 37° C, zero setting was done with distilled water. The rate of decrease in absorption was measured at 340 nm.

3.6.8. Estimation of Alkaline Phosphatase in serum according to the recommendations of the German Society for Clinical Chemistry.

(i)**Test Principle.** p-Nitro phenyl Phosphate (PNPP) was hydrolysed by alkaline phosphatase into p- Nitro phenol and Phosphate. At the alkaline pH of the buffered medium, p-Nitrophenol turned yellow. The colour developed by hydrolysis was proportional to the alkaline phosphatase activity and was measured at 405 nm.

\[
P\text{-Nitrophenyl phosphate} + \text{H}_2\text{O} \xrightleftharpoons{\text{ALKP}} \text{p-Nitrophenol} + \text{Phosphate.}
\]

(ii)**Test Procedure.**

**Reagent preparation.** The reagents were allowed to attain room temperature and the contents of one bottle of reagent 1 containing substrate p-Nitrophenyl phosphate was dissolved with 3ml of reagent 2 containing buffer, Diethanolamine and Magnesium chloride and mixed by gentle swirling till complete dissolution. The mixture was allowed to stand for 5 minutes before using.

**Dispensing.** The reconstituted reagent was once again mixed gently and 1.0 ml of this reagent was dispensed into clean and dry test tubes. 30µl of samples (sera of rats) were dispensed successively to properly marked test tubes with the reagent, mixed gently and reading was taken immediately.

**Reading.** The instrument was switched on 30 minutes prior to taking the reading. When the flow-cell temperature attained 37° C, zero setting was done with distilled water. The rate of decrease in absorption was measured at 405 nm.
3.7. Induction of diabetes in the animals

They were maintained in a temperature-controlled room at 23 °C, with a fixed 12-hour light: 12-hour darkness cycle, and initially fed standard rat laboratory pellet diet (American Agro.vet. India Ltd.) to standardize the nutritional status. After one week, the rats were randomly divided into two groups: the experimental group received Fructodex (FR, 50% w/v), and high carbohydrate-source diet, while the control rats (NC) received the normal pellet diet. The animals had free access to food and water and were maintained on their respective diets for 24 weeks. The weight of each animal was recorded twice per week, weight gain of at least 10 animals in each group were assessed twice per week during the experimental period. The food intake of the animals was observed throughout the experimental period. On the day of the experiment, food was removed at 09:00 hour, and experiments were performed between 09:00 and 12:00 hour.

3.8. Investigation on the effect of aqueous leaf extract of and Cissampelos pareira (CLE), Trichosanthes bracteata (TLE) and Leucus pleukentitii (LLE) on the Serum glucose and triglyceride levels of 50% fructose- induced hyperglycaemic rats.

To evaluate effect of CLE, TLE and LLE on the glucose and triglyceride levels of hyperglycaemic animals, they were divided into following groups and the extract was administered to the different treated groups continuously for 3 weeks and blood samples were collected each week for estimation of glucose and triglyceride levels. Oral Glucose Tolerance Test (OGTT) was also conducted in the animals after treating them with CLE leaf extract for four weeks.

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>NC</td>
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<tr>
<td>II</td>
<td>FR</td>
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<tr>
<td>III</td>
<td>FR+Pioz-treated</td>
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<tr>
<td>IV1</td>
<td>FR+CLE (30 mg/kg bw)-treated</td>
</tr>
<tr>
<td>IV2</td>
<td>FR+CLE (100 mg/kg bw)-treated</td>
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<tr>
<td>IV3</td>
<td>FR+TLE (30 mg/kg bw)-treated</td>
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<tr>
<td>IV4</td>
<td>FR+TLE (100mg/kg bw)-treated</td>
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<tr>
<td>IV5</td>
<td>FR+LLE (30mg/kg bw)-treated</td>
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<tr>
<td>IV6</td>
<td>FR+LLE (100mg /kg bw)-treated</td>
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3.8.1. Oral Glucose Tolerance Test. Glucose-D (3g weight/volume) was administered to each animal and blood glucose levels were estimated at 0, 30, 60 and 120 minutes. The group IV (1 and 2) of rats was subjected to this test before and after 4 weeks of daily treatment with CLE (Bajaj S and Srinivasan BP, 1999; Galipeau D, et al., 2001).

3.9. Toxicity Evaluation of CLE

To rule out any adverse effect of the extract of *C. pareira*, acute and sub-acute toxicity studies were conducted as per standard protocol with minor modifications (Babu V, et al., 2003).

The animals were divided randomly into 6 groups (3 animals in each). Group I received normal saline orally and Group II, III, IV, V, VI and VII received *C. pareira* extract at the dose rate of 0.5g, 1g, 2g, 4g and 8g and 10g orally. The animals were observed for 72 hours for any mortality and behavioural changes. The parameters observed were: grooming, hyperactivity, and sedation, loss of sighting reflex, convulsion and aggressiveness.

For sub-acute toxicity evaluation, animals were divided into 5 groups (6 animals in each). Four doses (10, 30, 100 and 300mg/kg body weight, p.o.) were administered to the treated groups continuously for 14 days. Control group received only normal saline. On the 15th day, blood was collected from these animals for estimation of serum Glutamate Oxalate Transaminase, Glutamate Pyruvate Transaminase and Alkaline Phosphatase. Two animals from each group were then sacrificed and the following organs were collected for routine histo-pathological examinations: Liver, Heart, Kidney and Pancreas.

3.9.1. Histo-pathological Examinations

(i) Tissue Preparation and sectioning. Immediately after the collection blood samples for the above biochemical tests, the tissues (Heart, Kidney, Liver and Pancreas) were excised, washed with normal saline, blotted dry and immediately fixed in Bouin’s solution for 24 hours before being transferred to 30% alcohol. The tissues were then dehydrated via a series of alcohol (50%, 70%, 90% and 100 %) and cleared with xylene (100% alcohol/xylene, xylene), before being infiltrated with hot paraffin wax (xylene &
paraffin-2:1, xylene & paraffin-1:1, xylene and paraffin-1:2 and finally with only paraffin) for 2-3 hours. Subsequently, the tissues were embedded transversely in paraffin wax and cut into 5μm sections using a rotary microtome.

(ii) Staining. The sections were stained with haematoxylin and eosin. Prior to staining, the sections were de-waxed by immersion in xylene. The xylene was then removed by immersion in 100% alcohol and then in distilled water. The tissues were placed in hematoxylin for 5 minutes before being rinsed in tap water and dipped in 1% Hcl. Haematoxylin is a nuclear stain which colours the Phosphate groups of DNA blue, and Hcl removes the stain from the other parts of the tissues. The tissues were then rinsed thoroughly under running tap water for 5 minutes before placing them in 1% eosin and 70% alcohol for 3 minutes. Eosin stains the cytoplasm pink. The sections were subsequently dehydrated again by immersion in 100% alcohol and finally in xylene before being mounted in DPX.

3.10. Evaluation of the effect of CLE on the serum glucose, insulin, triglyceride and cholesterol levels of fructose-induced hyperglycaemic rats.

To evaluate the effect of CLE on these biochemical parameters, the hyperglycaemic animals were divided into following groups and CLE was administered to the treated groups continuously for 6 weeks and blood samples were collected each week for estimation of glucose, insulin, triglyceride and cholesterol levels.

Group I : NC.
Group II : FR
Group III : FR+Pioz-treated.
Group IV 1 : FR+CLE (100mg/kg bw)-treated.
Group IV 2 : FR+CLE (300 mg/kg bw)-treated.
3.11. Evaluation of the immune status of the hyperglycaemic control and CLE treated rats.

The in vivo effect of water-methanol extract of the plant leaf on macrophage phagocytes was studied as described by earlier workers (Subramaniam A, et. al., 1996; Aderem A, 1999) with little modifications. Further, the influence of the extract on antibody response of the different groups of rats was also evaluated.

3.11.1. Studies of phagocytosis of macrophages. Different doses of extract (10, 30, 100 and 300 mg/kg bw.) were administered to different groups of rats (daily p.o.) for 6 days. On the 7th day, peritoneal exudate cells were collected in RPMI-1640 medium. Macrophages were separated by adhering on glass surface at 37°C in heparin free medium. The adhered cells were collected in cold RPMI-1640 medium and used for the study.

Phagocytosis of macrophages was assayed using opsonized Sheep RBCs (SRBCs). The SRBCs were opsonized with anti-SRBC immunoglobulin G. The SRBCs (10^7 cells) were incubated with 10^6 Macrophages in 1 ml RPMI-1640 medium for 20 minutes at 27°C in a Leiten tube. After incubation undigested erythrocytes were lysed with distilled water and stained with Leishman’s stain. Macrophages engulfing more than 2 SRBCs were considered positive. Phagocytosis was determined by counting the number of macrophages positive out of 100 macrophages counted.

3.11.2. Evaluation of Haemagglutination Antibody Titre. The different groups of animals were immunized by injecting (i.p. with 23 gauze needle) 0.2ml of SRBCs (5×10^9 cells/ml) on 4th day of drug administration. On 7th day after immunization and continuous treatment with the extract, blood samples were collected from tail vein for evaluating Haemagglutination Antibody titre.

Two fold dilutions of sera were performed in 0.15 Phosphate Buffered Saline (pH 7.2) and 50μl of each dilution was aliquoted into 96-well microtitre plates. A 25μl of fresh 1% SRBC suspension in the above buffer was dispersed into each well and mixed well. The plates were incubated at 28°C for 2 hours and examined visually for
agglutination. The value of the highest serum dilution causing haemagglutination was taken as the Antibody Titre.

3.12. Evaluation of the effect of CLE on glycaemic control in 50% fructose (FR)-induced and Fructose plus aloxan (FR-ALX, 40mg/kg, i.p.) induced-hyperglycaemic rats.

To evaluate the effect of CLE on the glycaemic control, the hyperglycaemic animals were divided into following groups and they were treated with CLE continuously for 12 weeks and blood samples were collected each week to estimate glucose, percent glycosylated haemoglobin and insulin levels.

Diabetes is associated with characteristic and progressive changes in the structure of the pancreatic islets. Such changes include depletion of insulin-containing secretory granules in β-cells, loss of definition of islet boundary and displacement of exocrine cells into the islets (Diani A.R. et al., 2003). Therefore, in order to observe these changes Gomori’s Aldehyde Fuchsin staining method was used to evaluate changes in islets of Fructose- induced hyperglycaemic rats and Fructose- Aloxan- induced hyperglycaemic rats.

Group I : NC
Group II : FR
Group III : FR+Pioz-treated.
Group IV 1 : FR+CLE (100mg/kg bw)-treated.
   IV 2 : FR+CLE (300mg/kg bw)-treated.
   IV 3 : FR-ALX +CLE (100mg/kg bw)-treated.
   IV 4 : FR-ALX+CLE (300mg/kg bw)-treated.


(i) Tissue preparation: Splenic region of pancreas was taken and fixed in Bouin’s solution for 24 hours. The tissues were then washed several times with distilled water till the yellow colour of the fixative was completely removed. After dehydrating the tissues in different grades of alcohol (30%, 50%, 70%, 90% and 100%) and clearing with xylene, they were embedded in paraffin blocks and cut in 5 μm thickness and stained with Eosin-Haematoxyline for preliminary observation and finally with Gomori’s aldehyde-fuchsin
stain. The stained section of highest quality on each slide was chosen for analysis on the basis of lack of artifacts and staining. Each islet was examined by an observer who was unaware of the animal's treatment allocation. Each islet was given a score between 1 (least) and 4 (greatest) for β-cell granulation observed with aldehyde-fuchsin staining.

(ii) Gomori's aldehyde-fuchsin staining method.

**Preparation of the stain.** 1ml of Concentrated HCl and 1ml of acetaldehyde was added to 100ml of 0.5% basic fuchsin in 70% alcohol. The stain was kept at room temperature until the shade of the mixture darkened to a deep violet (Buehner TS, et al., 1979).

**Procedure.** The pancreatic tissue sections were oxidized in Lugol's iodine for 30 minutes and after immersing the sections in 5% thiosulphate for 1 minute to remove the iodine, the sections were washed well in water. The sections were then given three changes of 70% alcohol before being immersed in aldehyde fuchsin stain. After 30 minutes, the sections were rinsed in several changes of 70% alcohol and counter stained in orange G. The sections were subsequently dehydrated again by immersion in 70% and 100% alcohol and finally in xylene before being mounted in DPX.

**Scanning of the pancreatic tissues.** Aldehyde fuchsin stained sections from randomly selected pancreases were scanned using micrometer component quantitator (Hoftiezer V and Carpenter AM, 1973). Islet volume, expressed as % of the total splenic region of pancreas was determined at low power (10× objective) with traverse interval of 1mm. The total linear scan for each determination was 500mm or more. The volumes of various components of the islets, expressed as percent of total islet, were measured at higher magnification (40× and oil immersion) with traverse intervals of 20μm.

**Identification of islet components** (Kvistberg, et al., 1966; Diani AR, et al., 2003). β-cells containing insulin stain purple by Gomori's aldehyde fuchsin stain. In aldehyde fuchsin stained material, β-cells containing varying number of granules, were classified on a scale from 1+ to 4+ granulation. Cells without granules, but with pink cytoplasm were identified as non-granular cells. The vessels identified included the lumen and the endothelial lining of the capillaries within and surrounding the islets.
Because the number of micrometer limited the number of components that could be quantitated per scan to five, the designations were: (1) 3-4+ β-cells (β-cells containing more than half of the full component of granules), (2) 1-2+ β-cells (β-cells containing less than half of the full component of granules), (3) Non-granular cells, and (4) Vessels.

3.14. Statistical analysis. All data were presented as mean ±SEM. For data with multiple time points, variables were analyzed by the general linear model ANOVA in the experiment II and by one way ANOVA (Tukey-Kramer multiple comparison test) by using SPSS version 10.0 in rest of the experiments. An unpaired ‘t’ test was also used to compare effect of fructose feeding on the different biochemical parameters and area under the curve (AUC) values in OGTT. Mean values were considered significant at p<0.05, 0.01 and 0.001.