CHAPTER – III
MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Eight-week old adult male albino rats of wistar strain, weighing approximately 150 to 160 g, were acclimatized for 7 days at room temperature (28 ± 3°C) and relative humidity (55%) in a 12-hour light/dark cycle in a room under hygienic condition. The animals reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Male rats only were used throughout the investigation to avoid complications due to the estrous cycle. The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal feed, Pranav Agro Industries Ltd., Bangalore, India). The study protocol was approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 1084/2014/CPCSEA). The animals were handled and cared in accordance with the “Committee for the purpose of control and supervision on experimental animals” (CPCSEA, 2014).
3.2 CHEMICALS

Streptozotocin (STZ) was purchased from Sigma-Chemical Co. Bangalore. All other chemicals and reagents used for this study were of analytical grade.

3.3 PLANT MATERIAL

*Pimenta dioica* leaves were collected from Kumuli, Kerala State, India.

3.4 PREPARATION OF EXTRACT

The *Pimenta dioica* leaves were dried at room temperature and then were powdered using dry grinder and passed through sieve. Hundred grams of *Pimenta dioica* were packed in a soxhlet apparatus and extracted with methanol. The methanolic extracts were concentrated on a rotary evaporator.

3.5 EXPERIMENTAL INDUCTION OF DIABETES

The animals were rendered diabetes by a single intraperitoneal injection of streptozotocin (55 mg/ BW) in freshly prepared citrate buffer (0.1M, pH 4.5) after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemic mortality. STZ injected animals exhibited massive glycosuria (determined by Benedict’s qualitative test) and hyperglycemia (by glucose oxidase method). Diabetes in STZ rats was
confirmed by measuring the blood glucose concentration, 96 h after injection with STZ. The animals with blood glucose of more than 220mg/dL were considered diabetic and used for the further experiments.

3.6 EXPERIMENTAL DESIGN

Preliminary study for dose fixation

Preliminary study was carried out to determine effective dose of *Pimenta dioica* on the plasma glucose level in diabetic rats. *Pimenta dioica* at the doses of 75, 150 and 300 mg/kg body weight was given to diabetic rats fed by intubation. Blood was collected by retinalorbital puncture at 0 h and 2 h after the administration of *Pimenta dioica*. The plasma glucose was estimated by the method of Trinder using the reagent Kit. *Pimenta dioica* at the doses of 75 and 150 mg/kg body weight lead to the maximum reduction in plasma glucose level. To confirm the effective dose, a 15-day study was carried out. *Pimenta dioica* at the doses of 75, 150 and 300 mg/kg body weight for 15 days were administrated to diabetic rats and after 15 days the animals were tested for plasma glucose level. Administration of *Pimenta dioica* at all the three doses, gave a significant reduction of plasma glucose in diabetic rats. Since the *Pimenta dioica* at the doses of 75 and 150 mg/kg body weight gave the maximum reduction of plasma glucose
level, it was fixed as the optimum dosage for further studies. The following groups were categorized for the preliminary study.

Group I : Control
Group II : Diabetic control
Group III : Diabetic + *Pimenta dioica* (75 mg/kg BW/day)
Group IV : Diabetic + *Pimenta dioica* (150 mg/kg BW/day)
Group V : Diabetic + *Pimenta dioica* (300 mg/kg BW/day)

3.7 LONG TERM STUDY (45 DAYS)

The animals were randomly divided into five groups of eight animals each. *Pimenta dioica* and glibenclamide were administrated post orally by intubation once in a day in the morning hours for 45 days. The experimental animal groups were fixed as follows.

Group I : Control
Group II : Diabetic control
Group III : Diabetic + *Pimenta dioica* (75 mg/kg BW/day)
Group IV : Diabetic + *Pimenta dioica* (150 mg/kg BW/day)
Group V : Diabetic + glibenclamide (0.6 μg/kg BW/day)

After 45 days, the animals were anaesthetized using ketamine (24mg / kg/ body weight, intramuscular injection), and sacrificed between 8:00 am and 9:00 am by cervical dislocation. Blood was collected in tubes with a mixture of potassium oxalate and sodium
fluoride (1:3) for the estimation of plasma glucose and ethylene diamine tetra acetic acid for the estimation of various biochemical parameters. Tissues (pancreas and liver) were surgically removed and washed with cold physiological saline. Erythrocytes from the blood were also separated for the estimation of various biochemical parameters.