Materials and Methods

3.0 MATERIALS AND METHODS

The methodology involved in the present investigation entitled, **"Antidiabetic and Antioxidant effect of *Aristolochia bracteolata* on Streptozotocin induced Albino rats".** The steps involved in the experiments are as follows.

COLLECTION OF PLANT SAMPLE

The plant *Aristolochia bracteolata* was collected for the present investigation from **SKM Siddha and Ayurvedic Medicines India Private Limited**. Stem and leaves of *Aristolochia bracteolata* were washed and air dried in the shade at room temperature for seven days. The dried samples were separately powdered and used for further investigation.

PREPARATION OF PLANT EXTRACT

The powdered *Aristolochia bracteolata* was extracted with methanol (40^{o} C) by the method of continuous hot extraction using a Soxhlet apparatus. The methanol extract solution was distilled, and dried in vacuum (Mazunder *et al.,* 2005). The extract was dissolved in dimethyl sulphoxide and used for investigation of Antidiabetic activity and estimation of antioxidant status of the plant.

PLATE 1

PLANT SELECTED FOR THE STUDY *Aristolochia bracteolata*

PLATE 2

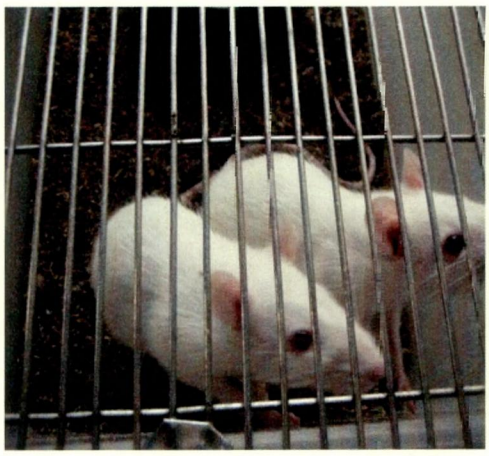
EXTRACTION OF THE PLANT SAMPLE IN SOXHLET APPARATUS

DETERMINATION OF ANTIDIABETIC ACTIVITY

SELECTION OF ANIMALS

Male albino Wistar rats (weighing 180-200 g) were procured from Agricultural University, Trissur and Kerala. All animals were allowed free access to water and pellet diet and maintained at room temperature in plastic cages. All procedures described were reviewed and approved by the university Animal Ethical Committee (Reg no: 623/02/b/CPCSEA).

PLATE 3 EXPERIMENTAL ANIMALS



EXPERIMENTAL INDUCTION OF DIABETES

Diabetes was induced in the experimental animals by an intraperitoneal injection of streptozotocin (STZ, 60 mg/kg body weight) in a freshly prepared citrate buffer (0.1M, pH 4.5) in a volume of 1 ml/kg after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemic mortality. Diabetes was confirmed by measuring the fasting blood glucose concentration. After 96 hours of streptozotocin administration, rats with moderate diabetes having hyperglycemia (i.e. with blood glucose of 200-300 mg/dl) were taken for the experiment (Veeramani. *et al.*, 2008).

EXPERIMENTAL DESIGN

The animals were randomly divided into five groups of six animals each. The extract was suspended in dimethylsulphoxide (vehicle solution) and was orally given to animals.

Group	Treatment
Control	Stock diet
G I	Dimethyl sulphoxide
G II	*Aristolochia bracteolata* extract (500mg/kg)
GIII	Streptozotocin control
GIV	Streptozotocin + *Aristolochia bracteolata* extract (250mg/kg)
G V	Streptozotocin + *Aristolochia bracteolata* extract (500mg/kg)

After 30 days of treatment, the animals were deprived of food overnight and were anaesthised using chloroform. Blood was collected in tubes with ethylenediaminetetraacetic acid (EDTA) for the estimation of blood glucose. Liver was immediately dissected out, washed in ice cold Phosphate buffered saline, patted dry, weighed and analyzed for various antioxidant assays and Histopathological examination.

ESTIMATION OF GLUCOSE

The collected blood samples were analyzed for glucose levels by the glucose oxidase peroxidase (GOD/POD) method as described earlier and serum glucose levels were expressed in mg/dl (Deshmukh *et al.*, 2008). The blood glucose was estimated by kit method as described in Appendix I.

DETERMINATION OF ANTIOXIDANT ACTIVITY

The human body has a complex system of natural enzymic and nonenzymic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Thirugnanasampandan *et al.*, 2008).

ENZYMIC ANTIOXIDANTS

The major ROS scavenging activities include complex enzymic antioxidants like Catalase (CAT), Superoxide dismutase (SOD), Peroxidase (POD), Glutathione S- Transferase (GST) (Jayasri *et al* ., 2008)

ESTIMATION OF CATALASE (CAT)

Catalase is a heme protein which catalyses the reduction of hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chandramohan *et al.*, 2009). The activity of catalase was determined by the method of Luck, (1974) which is detailed in Appendix II.

ESTIMATION OF PEROXIDASE (POD)

Nonheme peroxidases are ubiquitous and are divided into two major subfamilies: peroxiredoxins (PRXs) and glutathione peroxidases (GPXs) peroxidases catalyzing the reduction of hydrogen peroxide, alkylhydroperoxides, and peroxynitrite to water (Dayer *et al* 2008). Peroxidase activity was estimated by the method of Reddy *et al.* (1995) as described in Appendix III

ESTIMATION OF SUPEROXIDE DISMUTASE

Superoxide dismutase scavenges the superoxide radical by converting it to H_2O_2 and molecular oxygen (Rajasekaran *et al.*, 2005). SOD activity was estimated by the method described by Mishra and Fridovich (1972) and is explained in Appendix IV.

ESTIMATION OF GLUTATHIONE –S- TRANSFERASE

Glutathione S-transferases (GSTs) are a family of intracellular isoenzymes with broad substrate specificities that catalyze the conjugation of the tripeptide, glutathione (GSH) to many compounds bearing a sufficiently electrophilic center (Selvam *et al* , 2008).The activity of Glutathione- S- Transferase was determined by the method proposed by Habig *et al.*, (1974) and the procedure is described in Appendix V.

NON-ENZYMIC ANTIOXIDANTS

The nonenzymic antioxidant defense system includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH). There is a balance between both the activities and intracellular levels of these antioxidants that is essential for the survival of organisms and their health (Raygani *et al.*, 2007).

ESTIMATION OF ASCORBIC ACID

Ascorbate is one of the most extensively studied antioxidant and has been detected in the majority of plant cell types, organelles and apoplast. Ascorbic acid is readily oxidised to monodehydroascorbic acid as part of its antioxidant function (Nair *et al.*, 2009). Appendix VI describes the detailed method followed for the estimation of ascorbic acid as proposed by Roe and Kuether, (1953).

ESTIMATION OF α- TOCOPHEROL

Vitamin E is one of the most important free radical scavenging chain-breaking antioxidant within biomembrane. Vitamin E present in the lipid residue reduces ferric ion to ferrous ions (Pavana *et al.*, 2007). Emmeric – Engel method given by Rosenberg, (1992) was followed for the estimation of α – tocopherol which is explained in the Appendix VII.

ESTIMATION OF REDUCED GLUTATHIONE

A tripeptide glutathione (r- glutamylcysteinyl glycine) is abundant compound in plant tissues in all cell compartments and executes multiple functions. I act as potent detoxifier and serve as precursor of phytochelatins (Blokhina, *et al.*, 2003). The content of reduced glutathione of the sample was determined by the method of Moron *et al.* (1979) as given in Appendix VIII.

ESTIMATION OF PROTEINS

Proteins, a fundamental component of all living cells act as enzymes. They interact with each other and other substrates and finally feedback into the gene to regulate the synthesis of other proteins (Poitout *et al.*, 2006).The total protein of samples was estimated by the method of Lowry *et al.*, (1951) which is in Appendix IX.

DETERMINATION OF INVITRO LIPID PEROXIDATION

Oxidation of lipids produces lipid peroxides that can reduce membrane fluidity, inactivate membrane-bound proteins and decompose into cytotoxic aldehydes (Li *et al.*, 2007).Quantitative measurement of lipid peroxidation was performed by the method of Okhawa *et al.* (1979) as given in Appendix X.

DETERMINATION OF NITRIC OXIDE GENERATION INVITRO

Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity (Nagulendran *et al.*, 2007). Appendix XI explains the procedure given by Green *et al.*, (1982) which was used to estimate the extent of nitric oxide inhibition by plant samples and crude extracts.

DETERMINATION OF SUPEROXIDE GENERATION INVITRO

Superoxide anion is one of the most representative free radicals. In cellular oxidation reactions, superoxide radicals have their initial effects magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents, hydroxyl radicals (Shyur *et al.*, 2005). Inhibition of Superoxide generation in plant samples was determined by the method of Mc cord and Fridovich (1995) as explained in Appendix XII.

DPPH ASSAY

2, 2- Diphenyl-1- Picrylhydrazyl (DPPH) is a stable free radical and often used to evaluate the antioxidant activity of several natural compounds. Antioxidants on interaction with DPPH, either transfer electron or hydrogen atom to DPPH, thereby neutralizing its free radical character (Singh *et al., 2009*). Appendix XIII describes the detailed method followed for DPPH assay as proposed by Blois (1958).

ABTS ASSAY

ABTS [2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay was based on the activation of hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. The assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants (Re *et al.*, 1999). ABTS assay was determined by the method as described in Appendix XIV.

HISTOPATHOLOGICAL EXAMINATION

A histopathological study of the liver samples of all the experimental rats was carried out to observe the modulatory effect of *Aristolochia bracteolata* on hepatocytes by using the method of Culling, (1979) as given in Appendix XVI.