3. MATERIALS AND METHOD

3.1 Collection and preparation of the plant material:

The *Murraya koenigii* Spreng., leaves were collected from the Department of Horticulture, Division of Aromatic and Medicinal plants. University of Agricultural Sciences, GKVK, Bangalore. Authentication of the plant was done by the Department of Botany, University of Agricultural Sciences, Bangalore.

The leaves were shade dried and powdered. The powder was stored in airtight containers till the use. Photos depicted through Plate No. 1 to 3

3.2 Preparation of Extracts:

3.2.1 Preparation of solvent extracts:

Four solvents namely aqueous, chloroform, methanol and petroleum ether were used to prepare the extracts from *Murraya koenigii* dried leaf powder.

Procedure: To obtain the aqueous extract, one kilogram of *Murraya koenigii* powder was extracted with water at room temperature till the exhaustion. The extract was filtered through buchner funnel using vacuum pump connected to side arm flask.

The filtrate thus collected was concentrated under reduced pressure at the temperature 40-50°C.

Chloroform, Methanol and Petroleum ether extracts were also obtained using the same procedure by using fresh *Murraya koenigii*
powder for each extract. The extracts thus obtained were stored in the freezer till the use.

### 3.2.2 Freeze dried aqueous extract

One hundred grams of *Murraya koenigii* powder was dissolved in 1 liter of water and kept on the automatic shaker for 24 hours for extraction of water-soluble compounds. The extract was filtered through buchner funnel using vaccume pump connected to side arm flask. The filtrate thus collected was centrifuged at 3,000 rpm for 10 min to remove the particulate substances. The clear supernatant was freeze dried to obtain the fine powder. The freeze-dried powder was stored in the freezer till the use.

### 3.3 Experimental Animals:

Adult Wistar albino rats aged 5-6 months and weighing 250±15 grams were used as an animal model in this study. The rats were obtained from the Department of Livestock Production and Management, Veterinary College, Bangalore. The animals were given standard commercial feed and clean drinking water. They were maintained under 14:10 h light:dark conditions throughout the experimental period.
3.3.1 Animal Ethics Committee permission

Institutional animal ethics committee permission was taken wide No.25 LPM/IAEC/2001-2003. Dated 19/11/2003

3.3.2 Induction of diabetes mellitus:

The overnight fasted rats were made diabetic with Alloxan hydrate (S.d. Fine-Chem Ltd, Boisar) by intraperitoneal administration at the dose 150mg/kg b.w. Diabetes was confirmed in the Alloxan-treated rats by measuring the fasting blood glucose concentration 72-h post injection. The rats with fasting plasma glucose level above 250 mg /dl were considered diabetic and were used for the experiment. The animals were given free access to pelleted rat feed and clean drinking water throughout the experiment.

3.4 Experimental Design

3.4.1 Experiment-I

Aqueous extract, chloroform extract, methanol extract and ether extract were evaluated for their antidiabetic activity.

Animal model: Alloxan-Diabetic Wistar albino rats

Duration of study: 2 months (8 weeks)

Extracts: Aqueous, chloroform, methanol and ether leaf extracts of Murraya koenigii

Vehicle: All the extracts were dissolved in 10% DMSO to make the solution of required concentration.
Route of administration: oral administration once daily

Dose: Each extract tested at 2 doses 100 and 1000mg/kg. b.w.

(Which are randomly selected to cover a wide range)

Grouping: Rats were grouped with 10 rats with equal number of

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Details of administration</th>
<th>Oral dose (mg /kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>DC</td>
<td>Diabetic Control rats</td>
<td>10% DMSO</td>
<td>1ml</td>
</tr>
<tr>
<td>Group-2</td>
<td>A1</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>100</td>
</tr>
<tr>
<td>Group-3</td>
<td>A2</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>1000</td>
</tr>
<tr>
<td>Group-4</td>
<td>C1</td>
<td>Alloxan-diabetic rats</td>
<td>Chloroform extract</td>
<td>100</td>
</tr>
<tr>
<td>Group-5</td>
<td>C2</td>
<td>Alloxan-diabetic rats</td>
<td>Chloroform extract</td>
<td>1000</td>
</tr>
<tr>
<td>Group-6</td>
<td>M1</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>100</td>
</tr>
<tr>
<td>Group-7</td>
<td>M2</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>1000</td>
</tr>
<tr>
<td>Group-8</td>
<td>E1</td>
<td>Alloxan-diabetic rats</td>
<td>Ether extract</td>
<td>100</td>
</tr>
<tr>
<td>Group-9</td>
<td>E2</td>
<td>Alloxan-diabetic rats</td>
<td>Ether extract</td>
<td>1000</td>
</tr>
</tbody>
</table>

males and females in each group as follows:

3.4.1.1 Blood sampling

Blood samples were collected on the Day 0, 7, 14, 21, 28, 35, 42, 49 and 56 of the experiment. The animals were fasted minimum of 16 hours before the blood collection. The blood samples were collected from retro-orbital venous plexus through medial canthus of eye from light ether anaesthetized rats. One mililitre of blood samples were collected in the fresh vials
containing heparin (5IU/ml) or sodium fluoride (10mg/ml) and immediately after the collection blood samples were centrifuged at 3000 rpm for 10 min to obtain the clear plasma for the estimation of plasma insulin and glucose levels respectively.

3.4.1.2 Parameters studied for:

3.4.1.2a Antidiabetic activity

Plasma glucose and plasma insulin

3.4.2 Experiment -II

Based on the results obtained from experiment-I, the aqueous and methanol leaf extracts of *Murraya koenigii* were considered for experiment-II. Objectives of this study were to evaluate the antidiabetic, antioxidant and hypolipidemic activity of the extracts at different dose levels and to evaluate hypoglycemic efficacy in comparison with the known oral hypoglycemic drug one drug from Sulfonylurea group (Glibenclamide) and another from Biguanide (Metformin).

Animal model: Alloxan-Diabetic Wistar albino rats

Duration of study: 3 months (12 weeks) Days

Route of administration: Oral administration once daily

Plant extracts: Aqueous and Methanol leaf extracts of *Murraya koenigii*

Vehicle: All the extracts were dissolved in 10% DMSO to make the solution of required concentration.
Dose: each extract was tested at 3 doses: 200, 400 and 800 mg/kg b.w.

Grouping: Rats were grouped with 10 rats with equal number of males and females in each group as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Details of administration</th>
<th>Oral dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>DC</td>
<td>Diabetic Control rats</td>
<td>10% DMSO</td>
<td>1 ml</td>
</tr>
<tr>
<td>Group-2</td>
<td>DGL</td>
<td>Alloxan-diabetic rats</td>
<td>Glibenclamide</td>
<td>0.25</td>
</tr>
<tr>
<td>Group-3</td>
<td>DME</td>
<td>Alloxan-diabetic</td>
<td>Metformin</td>
<td>10</td>
</tr>
<tr>
<td>Group-4</td>
<td>DA1</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>200</td>
</tr>
<tr>
<td>Group-5</td>
<td>DA2</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>400</td>
</tr>
<tr>
<td>Group-6</td>
<td>DA3</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>800</td>
</tr>
<tr>
<td>Group-7</td>
<td>DM1</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>200</td>
</tr>
<tr>
<td>Group-8</td>
<td>DM2</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>400</td>
</tr>
<tr>
<td>Group-9</td>
<td>DM3</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>800</td>
</tr>
</tbody>
</table>

### 3.4.2.1 Blood sampling

Blood samples were collected on the day 0, 15, 30, 45, 60, 75 and 90 of the experiment. The animals were fasted minimum of 16 hours before the blood collection. The blood samples were collected from retro-orbital venous plexus through medial canthus of eye from light ether anaesthetized rats. One millilitre of blood samples were collected in the fresh vials containing heparin (5 IU/ml) or sodium fluoride (10 mg/ml) and immediately after the collection blood samples were centrifuged at 3000 rpm for 10 min to obtain the clear plasma for the estimation of plasma insulin and glucose levels respectively. Another set of one milliliter blood samples were collected in fresh vials and allowed to clot in slanting position. The
serum separated was utilized for the estimation of lipid profiles like serum Total-cholesterol, HDL-C, LDL-C, VLDL-C, Serum phospholipids, Serum total lipids and Serum triglycerides and biochemical parameters like Serum creatinine, Blood Urea Nitrogen (BUN), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and bilirubin

**3.4.2.2 Parameters studied for:**

**3.4.2.2a Antidiabetic activity**

Plasma glucose and plasma insulin

**3.4.2.2b Hypolipidemic activity**

Serum Total-cholesterol, HDL-C, LDL-C, VLDL-C, Serum phospholipids, Serum total lipids and Serum triglycerides.

**3.4.2.2c Antioxidant activity**

On the last day of experiment the animals were sacrificed using ether anaesthesia and tissues like pancreas, liver, kidney and heart were collected for estimation of antioxidant parameters like superoxidedismutase, catalse and lipid peroxidation.

**3.4.2.2d Biochemical parameters**
Serum creatinine, Blood Urea Nitrogen (BUN), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and bilirubin

3.4.2.2e Histology

On the last day of experiment, the pancreas, liver, kidney and heart samples were collected from the ether-anesthetized animals. The organs were fixed in formal buffer saline for histological examination.

3.4.3 Experiment-III

Freeze dried aqueous extract of *Murraya koenigii* leaves was considered in this study with the objective to evaluate its antidiabetic, antioxidant and hypolipidemic activity upon intraperitoneal administration to Alloxan-diabetic rats.

Animal model: Alloxan-Diabetic Wistar albino rats

Duration of study: 1 month (4 weeks)

Route of administration: Intraperitoneal administration once daily.

Plant extract: Freeze dried aqueous extract of *Murraya koenigii* leaves dissolved in pyrogen free normal saline to make the solution of required concentration.

Dose: 2 doses: 25 and 50mg/kg b.w.
Grouping: Rats were grouped with 10 rats with equal number of males and females in each group as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Details of administration</th>
<th>I/P dose (mg /kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>FC</td>
<td>Diabetic control rats</td>
<td>Saline</td>
<td>1ml</td>
</tr>
<tr>
<td>Group-2</td>
<td>F1</td>
<td>Alloxan-diabetic rats</td>
<td>Freeze dried extract</td>
<td>25</td>
</tr>
<tr>
<td>Group-3</td>
<td>F2</td>
<td>Alloxan-diabetic rats</td>
<td>Freeze dried extract</td>
<td>50</td>
</tr>
</tbody>
</table>

### 3.4.3.1 Blood sampling

Blood samples were collected on the day 0, 7, 14, 21 and 28 of the experiment. The animals were fasted minimum of 16 hours before the blood collection. The blood samples were collected from retro-orbital venous plexus through medial canthus of eye from light ether anaesthetized rats. One millilitre of blood samples were collected in the fresh vials containing heparin (5IU/ml) or sodium fluoride (10mg/ml) and immediately after the collection blood sample was centrifuged at 3000 rpm for 10 min to obtain the plasma for the estimation of plasma insulin and glucose levels respectively. Another set of one milliliter blood samples were collected to in fresh vials and allowed to clot in slanting position to separate the serum. The serum separated was utilized for the estimation of lipid profiles like serum Total-cholesterol, HDL-C, LDL-C, VLDL-C, Serum phospholipids, Serum total lipids and Serum triglycerides.
**3.4.3.2 Parameters studied for:**

**3.4.3.2a Antidiabetic activity**

Plasma glucose and plasma insulin.

**3.4.3.2b Hypolipidemic activity**

Serum total-cholesterol, High Density Lipoprotein Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VLDL-C), serum phospholipids, Serum total lipids and Serum triglycerides.

**3.4.3.2c Antioxidant activity**

On the last day of experiment the animals were sacrificed using ether anaesthesia and tissues like pancreas, liver, kidney and heart were collected for estimation of antioxidant parameters like superoxide dismutase, catalase and lipid peroxidation.

**3.4.3.2d Histological studies**

On the last day of experiment, the pancreas, liver, kidney and heart samples were collected from the ether-anesthetized animals. The organs were fixed in formal buffer saline for histological examination.
3.4.4 Experiment –IV

Oral Glucose tolerance test was carried out in Alloxan induced diabetic rats using aqueous, methanol and freeze-dried aqueous leaf extract of *Murraya koenigii*.

Animal model: Alloxan-Diabetic Wistar albino rats

Route of administration: aqueous and methanol extract were administered by oral gavage, whereas freeze dried aqueous extract was administered by Intraperitoneal route.

Plant extract: aqueous and methanol extract were dissolved in 10% DMSO whereas freeze dried aqueous extract was dissolved in pyrogen free normal saline to make the solution of required concentration.

Dose: aqueous and methanol extract were used at the rate of 800mg/kg b.w and freeze-dried aqueous extract used at the rate of 50mg/kg b.w.

Glucose: 3g/kg b.w. by oral gavage

Grouping: Rats were grouped with 10 rats with equal number of

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Details of administration</th>
<th>Dose and route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>GC</td>
<td>Diabetic control rats</td>
<td>Saline</td>
<td>1 ml oral</td>
</tr>
<tr>
<td>Group-2</td>
<td>G1</td>
<td>Alloxan-diabetic rats</td>
<td>Aqueous extract</td>
<td>800mg/kg oral</td>
</tr>
<tr>
<td>Group-3</td>
<td>G2</td>
<td>Alloxan-diabetic rats</td>
<td>Methanol extract</td>
<td>800mg/kg oral</td>
</tr>
<tr>
<td>Group-4</td>
<td>G3</td>
<td>Alloxan-diabetic rats</td>
<td>Freeze dried aqueous extract</td>
<td>50mg/kg I/P</td>
</tr>
</tbody>
</table>
males and females in each group as follows:

Experimental procedure: Alloxan-diabetic rats were fasted for 16 hours prior to oral glucose tolerance test (OGTT). One milliliter of saline was administered orally to control group GC. Aqueous and methanol extracts were administered at the rate of 800mg/kg b.w orally to groups G1 and G2 respectively. Freeze dried aqueous extract was administered intraperitoneally to G3 group at the rate of 50mg/kg b.w. Thirty minutes later glucose was administered to all the groups at the rate of 3g/kg b.w.

3.4.4.1 Blood sampling

Blood samples were collected from retro-orbital venous plexus through medial canthus of eye from light ether anaesthetized rats at -30 min (just before administration of saline and extracts to respective group), 0 min (just before the oral administration of glucose), 30, 60 and 120 min after the oral glucose administration. Blood samples (0.5 ml each) were collected in the fresh vials containing sodium fluoride (10 mg/ml) and centrifuged at 3000 rpm for 10 min to obtain the plasma for the estimation of plasma glucose.
3.4.5 Experiment- V

This study was carried out to investigate the protein profile in pancreatic beta cells in the animals treated with *Murraya koenigii* extracts.

3.4.5.1 Collection of Pancreas

Pancreas was collected from diabetic rats of experiment-II, which were treated with aqueous extract (800mg/kg b.w), methanol extract (800 mg/kg b.w) and diabetic control rats.

3.4.5.2 Isolation of pancreatic islets

Islets were isolated from rat pancreas according to a method Metrakos *et al.* (1993). In brief, Pancreas was collected in RPMI 1640 medium and minced well. The Minced pancreas was digested with collagenase (0.7mg/ml) at 37° c for 30 min, then centrifuged at 3000 rpm for 10 min and supernatant is discarded. Islet purification was achieved using a two-step, discontinuous density gradient of BSA. Islets were collected from the interface between the 1·000 and 1·081 g/ml layer. To ensure purity, islets were handpicked under an inverted microscope.
3.4.5.3 Preparation of cell lysate

Pancreatic islet cell lysate was prepared by homogenization in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM sodium ethylenediaminetetraacetate, 1 mM phenylmethysulfonyl fluoride, 5 µg/ml of aprotinin, 5 µg/ml of leupeptin). Cell debris was removed by centrifugation. Protein concentration in cell lysate was determined according to lowry et al. (1957).

3.4.5.4 SDS- PAGE

The pancreatic islet cell lysate was boiled for 5 min in 1 x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% SDS, 0.01% bromophenol blue) containing 5% b-mercaptoethanol. lysate was loaded on SDS-PAGE at 10 µg per lane (Biorad, USA).

3.4.6 Experiment-VI

Aqueous and methanol extracts used in the Experiment-II of the investigation were subjected to 28 days repeated dose toxicity study in the non-diabetic rats.

Animal model: Wistar albino rats

Duration of study: 28 Days

Route of administration: oral administration once daily through oral gavage
Plant extracts: Aqueous and Methanol extract

Dose: Low dose (200mg/kg b.w), Intermediate dose (400mg/kg b.w), High dose (800mg/kg b.w)

Rats were grouped with 10 rats with equal number of males and females in each group as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Details of administration</th>
<th>Oral dose [mg /kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>TC</td>
<td>Control</td>
<td>Saline</td>
<td>1ml</td>
</tr>
<tr>
<td>Group-2</td>
<td>TA1</td>
<td>Low dose</td>
<td>Aqueous extract</td>
<td>200</td>
</tr>
<tr>
<td>Group-3</td>
<td>TA2</td>
<td>Medium dose</td>
<td>Aqueous extract</td>
<td>400</td>
</tr>
<tr>
<td>Group-4</td>
<td>TA3</td>
<td>High dose</td>
<td>Aqueous extract</td>
<td>800</td>
</tr>
<tr>
<td>Group-5</td>
<td>TM1</td>
<td>Low dose</td>
<td>Methanol extract</td>
<td>200</td>
</tr>
<tr>
<td>Group-6</td>
<td>TM2</td>
<td>Medium dose</td>
<td>Methanol extract</td>
<td>400</td>
</tr>
<tr>
<td>Group-7</td>
<td>TM3</td>
<td>High dose</td>
<td>Methanol extract</td>
<td>800</td>
</tr>
</tbody>
</table>

3.4.6.1 Blood sampling

Blood samples were collected on the day 0, 7, 14 and 28 of the experiment. The blood samples were collected from retro-orbital venous plexus through medial canthus of eye from light ether anaesthetized rats. One millilitre of blood samples were collected in the fresh vials containing heparin (5IU/ml) for haematology. Another set of one milliliter blood samples were collected to in fresh vials and allowed to clot in slanting position. The serum separated was utilized for the estimation of biochemical parameters.

3.4.6.2 Safety study parameters:
3.4.6.2a Biochemical parameters:
Serum creatinine, Blood Urea Nitrogen (BUN), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Bilirubin,

3.4.6.2b Haematological parameters
RBC (Red Blood Cells count), WBC (White Blood Cell count), DLC (Differentail Leukocytes Count), Hb (Haemoglobin).

3.4.6.2c Histology
On the last day of experiment, pancreas, liver, kidney and heart samples were collected from the ether-anesthetized animals. The organs were fixed in formal buffer saline for histological examination.

3.5 Assay of Parameters:
3.5.1 Antidiabetic Parameters
3.5.1a Plasma Insulin
Insulin concentration in the plasma was determined by using Radioimmuno Assay kit obtained from Board of Radiation and Isotope Technology, BARC, Mumbai, India.
RIA involved competitive binding of radiolabelled antigen and unlabelled antigen to a high affinity antibody (Cam and Neil, 1996).

3.5.1b Plasma glucose
Plasma glucose concentrations were estimated using spectrophotometer (2101 Systronic, Type 081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore.

### 3.5.2 Hypolipidemic Parameters

#### 3.5.2a Serum Total Cholesterol

Serum Cholesterol concentrations were estimated using spectrophotometer (Systronic instruments, S081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore., as per the procedure of Allain et al. (1974).

Cholesterol esters are hydrolyzed by cholesterol esterase. The free cholesterol was oxidised to release hydrogen peroxide in the presence of cholesterol oxidase and peroxidase. Hydrogen peroxide reacts with aminoantipyrine to form red coloured quinoneimine complex. The intensity of the colour was proportional to cholesterol concentration and was measured by spectrophotometer at 510 nm (Allain et al., 1974).

#### 3.5.2b High Density Lipoproteins-Cholesterol (HDL-C)

High Density lipoprotein Cholesterol levels were estimated using spectrophotometer (Systronic instruments, S081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore., as per the procedure of Allain et al. (1974).
Serum treated with polyanionic-divalent cation precipitates chylomicrons, VLDL and LDL fractions. The supernatant on centrifugation contain only HDL. An aliquot of the supernatant was quantitatively transferred to another dry test tube and the cholesterol was estimated as described for serum total cholesterol (Allain et al., 1974)

3.5.2c Very Low Density Lipoprotein Cholesterol (VLDL-C)

The serum VLDL-C content was calculated by employing the Friedewald formula, i.e., VLDL-C = Serum triglyceride / 5 (Friedewald et al., 1972).

3.5.2d Low Density Lipoprotein cholesterol (LDL-C)

LDL-C level was calculated as the difference between Total Cholesterol and the sum of VLDL-C and HDL-C concentration (Friedewald et al., 1972).

3.5.2e Serum Triglycerides

Serum triglycerides was estimated using spectrophotometer (Systronic insruments, S081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore., as per the procedure of Footsati and Prencipe (1982). Triglycerides were hydrolyzed by lipase and the liberated glycerol was phosphorylated in the presence of ATP to glycerol-3-phoshphate.
This was then oxidized in presence of glycerol phosphate oxidase and peroxidase to produce red coloured quinoneimine complex. The intensity of the colour was proportional to triglyceride concentration and was measured by spectrophotometer at 510 nm (Footsati and Prencipe, 1982).

3.5.2f Phospholipids

Serum phospholipids was estimated using spectrophotometer (Systronic instruments, S081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore., according to the method of Ackermann and Toro (1963). The lipid extract obtained by trichloracetic acid precipitation of plasma is digested with sulphuric acid-perchloric acid mixture to convert organic phosphorus into inorganic phosphate. The inorganic phosphate reacts with ammonium molybdate to give ammonium phosphomolybdate, which is reduced to molybdate blue by a reducing agent, aminonaptholsulphonic acid. The intensity of colour is proportional to amount of inorganic phosphorus present which is measured by spectrophotometer at 660 nm. The phospholipid concentration is calculated as 25 times the content of lipid phosphorus (Ackermann and Toro, 1963).

3.5.2g Total lipids

Total lipids concentration in serum was calculated as the sum of serum phospholipids, total cholesterol and triglycerides
3.5.3 Antioxidant Parameters

Tissue samples of pancreas, liver, kidney and heart were collected on ice and 10% homogenate was prepared in normal saline. Homogenate was centrifuged at 3000 rpm for 10 min in refrigerated centrifuge (Remi, India) and the supernatant collected was maintained at 4°C till the assay of superoxide dismutase, catalase and lipid peroxidation.

Protein concentration in the supernatant was estimated according to Lowry et al, 1951

3.5.3a Superoxide Dismutase

Superoxide dismutase activity was measured by the method of Mahadesh and Balasubramanian (1998). The enzyme activity was expressed in U/mg of protein. One unit of superoxide dismutase means enzyme concentration required to inhibit the MTT formazen by 50%.

3.5.3b Catalase

Catalase activity was measured by the method of Moehly and Chance, 1954. The enzyme activity was expressed as CAT Unit/mg protein. One unit of CAT is equal to amount of enzyme concentration required to decompose one mmol of hydrogen peroxide per seconds.

3.5.3d Lipid peroxidation

Lipid peroxidation in tissues was measured by Ohkawa et al (1979). It was based on the estimation of the content of the thiobarbituric acid reactive substances (TBARS) such as
malondialdehyde (MDA) which is an autolytic intermediate product of fatty acid peroxidation. The result was expressed as nmol of MDA /g of tissue /min.

3.5.4 Biochemical parameters

Serum creatinine, Blood Urea Nitrogen (BUN), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and bilirubin were analysed using spectrophotometer (Systronic instruments, S081) and ready to use kits supplied by M/S Swemed Diagnostics, Bangalore.

3.5.5 Histopathological examination:

Organs were fixed in buffered neutral formalin solution and processed according to the standard method followed by paraffin embedding and tissue sectioning of 3 um thickness. Tissue sections were mounted on to glass slides and stained with Haematoxylin and Eaosin (Menzies, 1962).

3.6 Phytochemistry:

*Murraya koenigii* leaf extracts aqueous, methanol, chloroform and ether extracts were subjected to physical characteristics like colour, nature and %yield on wt/wt basis. Solubility was tested at 100mg/ml by turbidity method. The qualitative
phytochemicals tests were performed as per the standard procedures mentioned in Indian pharmacopoeia (1994) and British pharmacopoeia (1991).

Tests for steroids: Salkowski Test and Liebermann Burchardt test

Tests for Triterpenes: Salkowski test, Liebermann Burchardt test and Tschugajen test

Tests of Alkaloids: Mayer’s test, Wagner’s test, Hager’s Test and Dragendorff’s test

Tests for Tannins: Ferric chloride test and Gelatin test

Tests for Flavonoids: Ferric chloride test and Lead acetate test

Test for Lactones: Legal’s test, Feigel’s test and Baljel’s test

Tests for Diterpenes: Copper acetate test

Tests for Glycosides: Sodium hydroxide reagent, Kellar Killani’s test and Picrate paper test

Tests for Saponins: Foam test and Haemolysis test

3.7 Statistical analysis

The data were statistically analyzed by One-way ANOVA with Dunnett’s post test, using GraphPad Prism version 4.00 (2006). The results were presented as mean ± S.E.M.