CHAPTER - 3
MATERIALS & METHODS
The materials and methods used in the present study entitled “In vivo antidiabetic and antioxidative studies of selected herbal plants extract in albino rats” are as follows-

3.1 Chemicals

Alloxan monohydrate, Glimiprex MF drug, Heparin di-sodium salt, Double distilled water, Absolute ethanol (100%), Di ethyl ether, Methyl alcohol, 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Quercetin, Gallic acid, tert-butyl-4-hydroxy toluene (BHT), Folin Ciocalteu reagent (FCR), Ascorbic acid, Reduced glutathione, 5, 5’-dithiobis (2-nitrobenzoic acid) DTNB, Ethylene diamine tetraacetate (EDTA), β-carotene, Aluminium chloride, Potassium acetate, Potassium hydroxide, Sodium carbonate, Catechol, Oxalis acid, Sodium bi carbonate, 2, 6-dichloro phenol indophenols (DCPIP dye), Sulfo salicylic acid, Potassium di-hydrogen phosphate, Di-potassium hydrogen phosphate, Potassium chloride, Tris-HCL, Tris-EDTA, Di-thio thretol (DTT), Hydrogen per oxide (H2O2), Riboflavin, Nitro blue tetrazolium (NBT), Guaicol, CDNB, Tri-chloro acetic acid (TCA), Thio barbituric acid (TBA), Tri-methoxy propane (TMP), Hydrochloro acetic acid, Anthrone reagent, Standard glucose, Sodium di-hydrogen phosphate, Di-sodium hydrogen phosphate, Sodium chloride, Soluble starch, Formaldehyde, Sodium hydroxide, Sodium tungstate, Potassium iodate, Potassium Iodide, Xylene, Eosin solution, Haematoxyline solution. All chemicals were of analytical reagent grade and products of Sigma Aldrich, Merk, Fischer Scientific, Himedia, Loba Chemie.

3.2 Diagnostic Kits

Glucose kit (GOD/POD method), Glycosylated haemoglobin kit (Ion Exchange Resin method), Total protein kit (Biuret method), Total cholesterol kit (CHOD/PAP method), Triglyceride kit (GPO/PAP method), HDL cholesterol kit (PEG/CHOD PAP method), Alkaline phosphatase kit (Modified Kind & King’s method), Serum/plasma glutamate pyruvate transminase (SGPT) kit (Reitman & Frankel’s method), Serum/plasma glutamate oxalo acetate transminase (SGOT) kit (Reitman & Frankel’s method), Lipase kit (Turbidimetric method), Urea kit (Modified Bertholot method), Uric acid kit (Uricase/ PAP method), Creatinine kit (Alkaline picrate method). All kits are under licenced and manufactured by Coral/Clinical system Pvt. Ltd., Verna, Goa and marketed by Crest Biosystem.
3.3 Glass wares and Equipments

All the glass wares were washed properly with detergent, rinsed with distilled water and autoclaved prior to use.

The equipments were used in present experimental study are listed below-

- Hot air oven (Tempo)
- Incubater (MAC)
- Autoclave (MAC)
- Weighing balance (Citizen)
- Rotary evaporater (MAC)
- Soxhlet unit (MAC)
- pH meter (Century)
- Cooling centrifuge (REMI, VCAK-430)
- Laminar air flow, UV Lamp (MAC)
- Colorimeter (AIMIL Photochem)
- Spectrophotometer (Thermo scientific UV/VIS)
- Micropipette with tips, 100 and 1000µl, (Tarsons)
- Water bath (NSW 128)
- Deep freezer (TERUMO PENPOL)
- Microtome machine (Thermo scientific Microm HE 340E)
- Compound light microscope (Magnus)

3.4 Plant materials

The test plants were collected from Allahabad district which was identified using flora of Northern India. Brief introduction of test plants are given below-

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>English name</th>
<th>Part(s) used</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oxalis corniculata</em> L.</td>
<td>Wood sorrel</td>
<td>Whole plant</td>
<td>Oxalidaceae</td>
</tr>
<tr>
<td><em>Phyllanthus fraternus</em> L.</td>
<td>Bhoomi amlaki</td>
<td>Whole plant</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td><em>Trichosanthes cucumerina</em> L.</td>
<td>Snake gourd</td>
<td>Fruits, leaves, stems, roots</td>
<td>Cucurbitaceae</td>
</tr>
</tbody>
</table>
Figure 3.1: *Oxalis corniculata* (Creeping wood sorrel)

Figure 3.2: *Phyllanthus fraternus*  
(Bhoomi amlaki)

Figure 3.3: *Trichosanthes cucumerina*  
(Snake gourd)
3.5 Preparation of ethanolic extracts

Ethanolic extracts of plants and their parts were prepared by the method, followed by Devendra et al. (2009); Koffuor and Amoateng (2011). Accordingly, O. corniculata, P. fraternus plants and different parts (like fruits, leaves, stem and roots) of T. cucumerina were collected freshly, washed, dried under shade and powdered into fine particles. The 50g powder of each was macerated in 600 ml of 95% ethanol separately at room temperature for 48 hours with occasional shaking at 8 hours. These were then filtered by Whatmann filter paper (No.1) and the filtrates were evaporated on rotary evaporator to concentrate in crude extract form at or below 40°C. There, 6.56 gm light green residue (13.12% w/w) of O. corniculata, 6.50 gm dark green residue (13% w/w) of P. fraternus, 10.26 gm reddish residue (20.52% w/w) of T. cucumerina’s fruit, 3.99 gm dark green residue (7.98% w/w) of T. cucumerina’s leaves, 3.50 gm light green residue (7.0% w/w) of T. cucumerina’s stem and 4.0 gm brownish residue (8% w/w) of T. cucumerina’s roots, were obtained respectively. These were kept in air tight bottle in a refrigerator until used. The extracts were used as such for screening of the following phytochemicals and for their antioxidant potentials.

A. Total antioxidant activity (Yen and Duh, 1994)

B. Phytochemical constituents

- Total phenols (Bray and Thorpe, 1954)
- Total flavonoids (Chang et al., 2002)
- Total carotenoids (Mahadevan and Sridhar, 1986)
- Non protein thiols (Ellman, 1995)
- Ascorbic acid (Harris, 1935)
- Total sugar (Sadasivum and Manickam, 1992)

3.6 Acclimatization of Animals

Charles foster strain albino rats of either sex weighing 200 ± 20 g were used for animal experiments that were performed in animal house of Mahavir Cancer Sansthan and Research Center, Phulwari sharif, Patna, Bihar, India. Rats were acclimatized under standard rat house conditions for 21 days before the trial was initiated. These rats were housed in steel wired poly propylene cages and maintained under controlled temperature at 25±2°C with 12h light/dark cycle. The animals were maintained on standard pellet diet and water ad libitum. The physical environmental condition and rest of the guidelines for experimentation on rats were strictly eyed by Ethics Committee. The study was approved as IAEC/2011/12/03 by the Institutional Ethics
Committee for Animal Experimentation, MCSRI, Patna, Bihar, which was registered by CPCSEA as Regd. No.: 1129/PO/bc/07/CPCSEA.

Figure 3.4: Charles foster strain albino rats

Figure 3.5: Acclimatization of rats
3.7 **Determination of Lethal dose (LD$_{50}$) and toxicity of plants extract**

Ethanolic crude extracts of test plants were dissolved in distilled water to form suspension of extracts. Its different doses ranges from 200-2000 mg/kg BW/day were assigned to male albino rats for one week and evaluated percentage of mortality and LD$_{50}$ values. Further two doses as 200 and 400 mg/kg BW/day were supplied to male albino rats for 7 days to check out any sign of behavioural and physiological toxicity. The hepatotoxicity and renotoxicity of these doses were also checked out by assessing of hepatorenal biochemical markers and histopathological observations.

3.8 **Induction of Diabetes Mellitus Type-2 in experimental rats**

Overnight fasted rats were injected with Alloxan monohydrate (140mg/kg body weight, dissolved as 70mg/ml in distilled water) by intra peritoneal route. The control rats received the same volume of 0.1 M sodium citrate buffer. Rats were treated with 30% glucose solution orally at different time intervals after 6 hour of Alloxan induction to overcome the drug-induced hypoglycemia. After 7 days, rats showed plasma glucose level $> 250$ mg / dl hyperglycemic condition that was checked by Glucometer in tail vein blood sample (Dallak and Bin-Jaliah, 2010).

![Figure 3.6: Intraperitonial injection of alloxan to induce Diabetes mellitus](image)
3.9 Grouping of experimental rats

In experimental design the rats were divided randomly into 4 major groups such as:

- Group I- normal controlled healthy rats
- Group II- diabetic controlled rats
- Group III- diabetic rats treated with plants extract
- Group IV- diabetic rats treated with commercial drug Glimiprex as standard at a dose of 100 mg/kg BW.

Group III was divided further into eight sub groups to treat the rats with four different plants extract at the dose of 200 & 400 mg/kg BW. Each group contained 4 rats. Normal control rats and diabetic control rats had no treatments.

3.10 Dose design for treatment of diabetic rats

According to the lethal dose, the alloxan induced diabetic CF strain albino rats were treated with ethanolic plant extracts as mentioned below in the table –

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Dose(s)</th>
<th>Frequency</th>
<th>Route of administration</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. corniculata</em> (whole plant)</td>
<td>200 mg/kg BW, 400 mg/kg BW</td>
<td>Daily</td>
<td>oral</td>
<td>28 days</td>
</tr>
<tr>
<td><em>P. fraternus</em> (whole plant)</td>
<td>200 mg/kg BW, 400 mg/kg BW</td>
<td>Daily</td>
<td>oral</td>
<td>28 days</td>
</tr>
<tr>
<td><em>T. cucumerina</em> (fruits)</td>
<td>200 mg/kg BW, 400 mg/kg BW</td>
<td>Daily</td>
<td>oral</td>
<td>28 days</td>
</tr>
<tr>
<td><em>T. cucumerina</em> (leaves)</td>
<td>200 mg/kg BW, 400 mg/kg BW</td>
<td>Daily</td>
<td>oral</td>
<td>28 days</td>
</tr>
</tbody>
</table>

3.11 Collection of blood samples and vital organs

Blood samples were drawn from all group rats at the end of the experiment by cardiac puncture using fine needle syringe and collected in centrifuge tubes having heparin di-sodium salt solution as anticoagulant. All animals were sacrificed by cervical dislocation under light ether anesthesia. The vital organs such as liver, kidney, pancreas and spleen of all group rats were collected in potassium phosphate buffer (50mM, pH-7.6) and further rinsed with 150mM KCL before use. Both samples were kept in ice box at controlled temperature.
Figure 3.7: Oral dosing of plants extract to rats via orogastric intubation

Figure 3.8: Rat having ether anaesthesia
Figure 3.9: Cervical dislocation of rat

Figure 3.10: Collection of blood from rat’s heart
Figure 3.11: Pancreas removal for histopathological study

Figure 3.12: Liver removal for biochemical & histopathological study
Figure 3.13: Kidney removal for histopathological study

Figure 3.14: Work in progress at biochemical study
3.12 Preparation of plasma and liver tissue homogenate samples

Plasma samples were got by centrifugation of all blood samples at 1500X g for 10 minutes at 4°C temperature and stored at -40°C in deep freezer until ready for analysis. After weighing of all collected organs, only liver was homogenized in 1:2 ratio (w/v) in Tris-HCL buffer (pH-7.6) at controlled temperature. All crude tissue homogenate samples were centrifuged at 14000X g for 5 minutes at 4°C temperature. Supernatant were collected and stored at -40°C in deep freezer (Sindhu et al., 2010).

3.13 Biochemical studies in plasma and liver tissues:

(I) Plasma glucose (Trinder, 1969)

(II) Glycosylated haemoglobin (Nathan, 1984)

(III) Total protein (Doumas, 1975)

(IV) Lipid profile

➢ Total cholesterol (Richmond, 1973)
➢ Total triglycerides (Fossati and Prencipe, 1982)
➢ HDL- cholesterol (Flegg, 1973)

(V) Liver function markers

➢ Glutamate Oxaloacetate Transaminase (GOT) (Reitman and Frankel, 1957)
➢ Glutamate Pyruvate Transaminase (GPT) (Reitman and Frankel, 1957)
➢ Alkaline phosphatase (ALP) (Kind and King, 1954)

(VI) Kidney function markers

➢ Urea (Fawcett and Scott, 1960)
➢ Uric acid (Fossati et al., 1980)
➢ Creatinine (Bonsnes and Taussky, 1945)

(VII) Pancreas function markers

➢ α-Amylase (Sheriff, 2004)
➢ Lipase (Teitz and Shuey, 1993)

(VIII) Enzymatic antioxidants markers
Catalase (CAT) (Beers and Sizer, 1952)
Superoxide dismutase (SOD) (Winterbourn et al., 1975)
Glutathione peroxidase (GSH-POX) (Miyake et al., 1991)
Glutathione- S transferase (GST) (Habig and Jakoby, 1981)
Guaicol peroxidase (GPX) (Hemeda and Klein, 1990)

(IX) Non enzymatic antioxidants markers

- Reduced glutathione (GSH) (Hu, 1994)
- Lipid peroxidation (LPO) (Draper and Hadley, 1990)
- Vitamin-A (Rutkowski and Grzegorczyk, 2007)
- Vitamin-C (Rutkowski and Grzegorczyk, 2007)

Protocols -

3.14 Total antioxidant activity assay

Total antioxidant activity was assayed by % scavenging of the DPPH free radicals as the method mentioned by Yen and Duh (1994).

Reagents:
- Methyl alcohol (95%)
- Ascorbic acid as standard
- 1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

Assay: DPPH solution (0.004% w/v) was prepared in 95% methanol. The crude extracts were mixed with 95% methanol to prepare solution of known concentration as 20μg/ml, 40μg/ml, 60μg/ml, 80μg/ml and 100μg/ml respectively in five test tubes. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes and after 10 minutes, the absorbance was taken at 517nm wavelength. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10mg/100ml or 100μg/ml) of extracts. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. The % scavenging of the DPPH free radical was calculated using the following equation \( [(A_{\text{control}} - A_{\text{Test}})/A_{\text{control}}] \times 100 \). Where, A is absorbance.
3.15 Total Phenols estimation

Quantitative estimation of total phenol was done by the method Bray and Thorpe (1954).

Reagents:

- 50% Methanol
- 1N Folin ciocalteu reagent (FCR)
- Na$_2$CO$_3$ (20%)
- Gallic acid as standard

Assay: Total phenols in crude extracts were determined by Folin ciocalteu reagent method. 5 mg of each crude extract were dissolved in 3 ml of (1:1) solution of methanol and distilled water. 0.5 ml of FCR solution (1:10 diluted) and 3 minute after 2 ml of 20% Na$_2$CO$_3$ solution were added. Slightly heated the mixture in boiling water bath for exactly one minute, then cooled and measured the absorbance at 650 nm wavelength against the reagent blank. The calibration curve was prepared by Gallic acid equivalent at conc. 1mg/ml (1:10 diluted).

3.16 Total flavonoids estimation

Aluminium chloride colorimetric method was used for total flavonoids determination given by Chang et al. (2002).

Reagents:

- Methanol
- Aluminium chloride
- Potassium acetate
- Quercetin

Assay: Each plant crude extracts (0.5mL of 1:10g/mL-1) in methanol were separately mixed with 1.5mL of methanol, 0.1mL of 10% aluminium chloride, 0.1mL of 1M potassium acetate and 2.8mL of distilled water. It remained at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415nm wavelength with a single beam Systronics UV/Visible spectrophotometer (India). The calibration curve was prepared by standard quercetin solutions at concentrations 12.5 to 100g/ml in methanol.
3.17 Total Carotenoids estimation

Total carotenoids were estimated by the method of Mahadevan and Sridhar (1986).

Reagents:

- Ethyl alcohol
- Potassium hydroxide
- Di ethyl ether

Assay: 50 mg of each crude extracts were dissolved in 3 ml absolute ethanol. Then 0.3 ml of 60% aqueous KOH were added and kept overnight at room temperature. After that they were washed with 5% ice-cold saline water to remove alkali and collective saline washings were extracted with ether (3:15 v/v). The ether extract from both were mixed together followed by washing with cold water till alkali free. The alkali free ether extracts were evaporating to crude residue form. These residues were dissolved in minimum volume of ethanol and absorbance was measured at λmax 450 nm by using ethanol as blank. The calibration curve was prepared by pure β carotene solution (1mg/ml).

3.18 Non Protein Thiol assay

Non protein thiol was assayed by the method of Ellman (1995).

Reagents:

- Sulfosalicylic acid ice cold (5% w/v)
- Phosphate buffer (0.1M)
- 5, 5’-dithiobis (2-nitrobenzoic acid) DTNB
- Ethylene diamine tetraacetate (EDTA)
- Reduced glutathione

Assay: Non-protein thiols (NPT) were extracted by dissolving of 5 mg crude extract sample in 1 ml ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at 10,000 rpm at 4°C for 30 min, 300μl of the supernatant was mixed with 1.2 ml of 0.1M PBS (pH 7.6). After a stable absorbance reading of 412 nm was obtained, 25μM DTNB solution (6mM DTNB dissolved in 5mM EDTA, 0.1M PBS, pH 7.6) was added, and the increase in absorbance at 412 nm was monitored. The calibration curve was prepared by using reduced glutathione as standard (3 to 12μg/ml) and results were expressed in μmol/ 100 mg of extract.
3.19 Ascorbic acid estimation

Ascorbic acid was estimated using the method described by Harris (1935).

Reagents:

- 2, 6-dichloro phenol indophenols dye
- Oxalic acid
- Ascorbic acid

Assay: Titration method with 2, 6-dichloro phenol indophenols dye was used for ascorbic acid assay in ethanolic extract. 50 mg of each crude extract were dissolved in 15 ml of 4% oxalic acid solution and titrated against dye (V₂). Stock solution of pure ascorbic acid in 4% oxalic acid (as 1 mg/ml) diluted 10 times. 5 ml of this solution dissolved in 10 ml of 4% oxalic acid and titrated against dye (V₁). The ascorbic acid content (mg/100mg) was calculated by using formula EV × V₁ × 100 / V₂ × W. Where EV is ascorbic acid equivalent of dye and W is weight of sample (in mg).

3.20 Estimation of total sugar

Total sugar was estimated by using the method given by Sadasivum and Manickam (1992).

Reagents:

- HCL (2.5 N)
- Anthrone reagent
- Sodium carbonate
- Glucose standard solution

Assay: 10 mg of crude extract was hydrolyzed with 0.5 ml of 2.5N HCL and cooled to room temperature. It was neutralized by Sodium carbonate until the effervescence ceased. Volume made up to 10 ml with distilled water and centrifuged. Took 1 ml aliquots from supernatant and add 4 ml Anthrone reagent. Heated 5-8 minute in boiling water bath and cooled rapidly to dark green colour. Absorbance was measured at λmax 630 nm against blank. The calibration curve was prepared of standard glucose solution (1mg/ml). Total sugar (in 100 mg) was calculated using formula as [sugar value from standard graph (mg) / sample aliquot (ml)] × [total volume of extract (ml) / wt. of sample (mg)] × 100.
3.21 Determination of LD$_{50}$

The LD$_{50}$ value of ethanolic crude extracts of test plants were determined by using the arithmetic method of Karber as modified by Aliu and Nwude (1982). Charles foster rats were divided into six groups of four rats each given graded doses (200, 400, 800, 1200, 1600, and 2000 mg/kg body weight) of the extract by oral route while the control group received 2 ml of normal saline by the same route. The animals were then observed firstly for 24 h and secondarily for 7 days to check toxicity signs and death. It was calculated by using formula as LD$_{50} = \text{Least dose that killed all animals} - \sum (\text{Dose difference} \times \text{Mean dead}) / (\text{No. of animals per group}).$

3.22 Estimation of plasma glucose

Plasma glucose estimation was based on GOD/POD method described by Trinder (1969).

Reagents:
- L1: glucose reagent
- S: Glucose standard

Assay: Pipetted 1.0 ml of glucose reagent in blank (B), standard (S), test (T) labelled test tubes. 0.01 ml of distilled water in blank, 0.01 ml of glucose standard in standard, 0.01 ml of plasma sample in test was added. Mixed well and incubated at 37ºC for 10 minutes. The optical density was read on colorimeter at 505 nm wavelength or green filter. The total plasma glucose (mg/dl) was calculated by using formula (O.D. of Test / O.D. of Standard) $\times$ 100.

3.23 Blood glycosylated haemoglobin, GHb

Glycosylated haemoglobin in blood was measured by Ion exchange resin method described by Nathan (1984).

Reagents:
- Ion-Exchange Resin
- Lysing reagent
- Control (10% GHb)
- Resin separators

Assay: Dispensed 0.5 ml lysing reagent into test tubes labelled as control (C) & test (T). 0.1 ml of reconstituted control and well mixed fresh whole blood samples collected in EDTA vials were added into appropriately labelled tubes and mixed until complete haemolysis happened. Allow to
stand for 5 minutes. Again Ion-Exchange Resin tubes were labelled as C & T. 0.1 ml of haemolysate added into appropriately labelled Ion-Exchange Resin tubes. After inserting resin separator they were mixed on vortex mixer for 5 minute. After settling of resin the supernatants (GHb fraction) were measured for absorbance at 415nm wavelength on spectrophotometer against distilled water. Again 0.02 ml of haemolysate was added to 5.0 ml of distilled water into two separate test tubes labelled as control and test. Mixed well and read the absorbance against distilled water (THb fraction). GHb in % = [ratio of test (RT) / ratio of control (RC)] × 10 (value of control).

3.24 Estimation of total proteins

Total proteins in plasma and liver tissues homogenate samples were assayed by biuret method described by Doumas (1975).

Reagents:

- L1: Biuret Reagent
- S: Protein Standard (8 g/dl)

Assay: Pipetted 1.0 ml of Biuret reagent in blank (B), standard (S), test (T) labelled test tubes. 0.02 ml of distilled water in blank, 0.02 ml of glucose standard in standard, 0.02 ml of plasma/tissue homogenate sample in was added. Mixed well and incubated at 37°C for 10 minutes. The optical density was read on colorimeter at 550 nm wavelength or yellow green filter. The total proteins in plasma and tissue homogenate (gm/dl) samples were calculated by using formula (O.D. of Test / O.D. of Standard) × 8.

3.25 Total cholesterol assay

Total cholesterol was assayed in plasma sample based on CHOD/PAP method given by Richmond (1973).

Reagents:

- L1: Enzyme Reagent 1
- L2: Enzyme Reagent 2
- S: Cholesterol Standard (200 mg/dl)

Assay: Working reagent was prepared by mixing of both L1 and L2 in ratio of 4:1. Three test tubes were labelled as blank (B), standard (S), test (T) and filled with 1ml of working reagent.
0.01ml of DW in blank, 0.01ml of cholesterol standard in standard and 0.01ml of plasma sample in test were added. Mixed well and incubate for 5 minute at 37°C. Optical density was recorded on colorimeter at 546nm wavelength. Total Cholesterol (mg/dl) = (O.D. of test / O.D. of standard) × 200.

3.26 Total triglyceride assay

Total triglyceride was assayed in plasma sample based on GPO/PAP method described by Fossati and Prencipe (1982).

Reagents:
- L1: Enzyme Reagent 1
- L2: Enzyme Reagent
- S: Triglycerides Standard (200 mg/dl)

Assay: Working reagent was prepared by mixing of both L1 and L2 in ratio of 4:1. Three test tubes were labelled as blank (B), standard (S), test (T) and filled with 1ml of working reagent. 0.01ml of DW in blank, 0.01ml of triglyceride standard in standard and 0.01ml of plasma sample in test were added. Mixed well and incubate for 5 minute at 37°C. Optical density was recorded on colorimeter at 546nm wavelength. Total Cholesterol (mg/dl) = (O.D. of test / O.D. of standard) × 200.

3.27 HDL cholesterol assay

HDL cholesterol was assayed in plasma sample based on GPO/PAP method described by Flegg (1973).

Reagents:
- L1: Enzyme Reagent 1
- L2: Enzyme Reagent 2
- L3: Precipitating Reagent
- S: HDL Standard (25 mg/dl)

Assay: Working reagent was prepared by mixing of both L1 and L2 in ratio of 4:1. Other cholesterol like LDL and VLDL were separated by addition of 0.1ml of precipitation reagent L3 to 0.1ml of plasma sample. After 5 minute incubation it was centrifuged at 2500 rpm. Three test tubes were labelled as blank (B), standard (S), test (T) and filled with 1ml of working reagent. 0.5 ml of each DW, HDL standard and supernatant was added B, S and T respectively. Mixed
well and incubated at room temperature for 15 minute. Optical density was read on colorimeter at 546nm wavelength. HDL Cholesterol (mg/dl) = (O.D. of test / O.D. of standard) × 25 × 2.

**3.28 Glutamate Oxaloacetate Transaminase (GOT) activity assay**

GOT activity in plasma and liver tissue homogenate samples were assayed by method of Reitman and Frankel (1957).

**Reagents:**

- L1: Substrate Reagent
- L2: DNPH Reagent
- L3: NaOH solution
- S: Standard Pyruvate solution

**Assay:** Working NaOH reagent was prepared by addition of 1.0 ml of NaOH Reagent (L3) and 9.0 ml of distilled water. Pipetted 0.50 ml substrate reagent (L1) in test tubes labelled as B (Blank), T (test) and incubated at 37°C for 3 minutes. Added 0.10 ml sample in T labelled test tube. Mixed and incubate for 60 minutes at room temperature. After that 0.50 ml of DNPH reagent (L2) was added. Mixed and allow to standing for 20 minutes at room temperature. Added 0.10 ml of distilled water in B labelled test tube and 5.0 ml of working NaOH reagent (L3) in both blank and tests. Mixed well and allow standing for 10 minutes at room temperature. The Optical Density (OD) was read against blank on colorimeter at green filter and matched with given standard reference curve.

**3.29 Glutamate Pyruvate Transaminase (GPT) activity assay**

GPT activity in plasma and liver tissue homogenate samples were assayed by method of Reitman and Frankel (1957).

**Reagents:**

- L1: Substrate reagent
- L2: DNPH Reagent
- L3: NaOH solution(4N)
- S: Standard Pyruvate solution

**Assay:** Working NaOH reagent was prepared by addition of 1.0 ml of NaOH Reagent (L3) and 9.0 ml of distilled water. Pipetted 0.50 ml substrate reagent (L1) in test tubes labelled as B
(Blank), T (test) and incubated at 37°C for 3 minutes. Added 0.10 ml sample in T labelled test tube. Mixed and incubate for 30 minutes at room temperature. After that 0.50 ml of DNPH reagent (L2) was added. Mixed and allow to standing for 20 minutes at room temperature. Added 0.10 ml of distilled water in B labelled test tube and 5.0 ml of working NaOH reagent (L3) in both blank and tests. Mixed well and allow standing for 10 minutes at room temperature. The Optical Density (OD) was read against blank on colorimeter at green filter and matched with given standard reference curve.

3.30 Alkaline Phosphatase (ALP) activity assay

ALP activity in plasma and liver tissue homogenate samples were assayed by method of Kind and King (1954).

Reagents:
- L1: Buffer reagent
- L2: Substrate reagent
- L3: Colour reagent
- S: Phenol Standard

Assay: Pipetted 1.0 ml distilled water into four clean dry test tubes labelled as Blank (B), Standard (S), Control (C) and Test (T). 0.05ml of distilled water added extra in blank (B). Then 1.0 ml of buffer reagent (L1) and 0.10 ml of substrate reagent (L2) added to each test tube. Mixed well and allowed to stand for 3 minutes at 37°C. 0.05 ml of phenol standard into S test tube and 0.05 ml of sample into T test tubes added. Mixed well and allowed to stand at 37°C for 15 minutes. Then 1.0 ml of colour reagent (L3) added to each test tube. Again 0.05 ml of sample is added to control (C) test tube. Mixed well and OD was read on colorimeter at 510 nm wavelength / Green filter against distilled water. Activity of ALP (in King-Armstrong Units/ml, OR, KA Units/ml) was calculated by using formula, \([\text{Abs (T)} – \text{Abs (C)} / \text{Abs (S)} – \text{Abs (B)}] \times 10.\)

3.31 Assay of plasma urea

Urea in plasma sample was assayed by the method described by Fawcett and Scott (1960).

Reagent:
- L1: Buffer Reagent
- L2: Enzyme Reagent
Assay: Pipetted 1 ml buffer reagent (L1) in test tube labelled blank (B), standard (S), test (T) and added 0.1 ml enzyme reagent in each test tube. Then 0.01 ml distilled water was added in test tube labelled B, 0.01 ml urea standard in test tube labelled S and 0.1 ml of plasma in test tube labelled T. Mixed well and incubated for 5 minutes at 37°C. Added chromogen reagent (L3) in each test tube, mixed well and incubated for 5 minutes. The Optical density was read in colorimeter at 600 nm, after adjusting for zero using blank. Urea (in mg/dl) = \[\frac{\text{Abs. (test)}}{\text{Abs. (standard)}}\] × 40.

3.32 Assay of plasma uric acid

Plasma uric acid was assayed by Fossati et al. (1980) method.

Reagents:
- L1: Buffer reagent
- L2: Enzyme reagent
- S: Standard Uric acid

Assay: Working reagent was prepared by pouring 1 ml of enzyme reagent (L2) into 4 ml of buffer reagent is (L1). Pipetted 1 ml working reagent into test tubes labelled as blank (B), standard (S), test (T). It was added 0.02 ml of distilled water in blank (B), 0.02 ml of uric acid standard in standard and 0.02 ml of plasma was added in test tube labelled test (T). Mixed well and incubated at 37°C for 5 minutes. The Optical density was read in colorimeter at 520 nm, after adjusting for zero using blank. Plasma uric acid (in mg/dl) = (O.D. Test / O.D. Std.) × 8.

3.33 Assay of plasma creatinine

Plasma creatinine was quantified by alkaline picrate method detailed by Bonsnes and Taussky (1945).

Reagents:
- L1: Picric Acid Reagent
- L2: Buffer Reagent
- S: Standard Creatinine
**Assay:** Pipetted 2 ml picric acid reagent (L1) and 0.2 ml of plasma into a clean dry test tube. Mixed well and centrifuged at 2500-3000 rpm for 10 min. to obtain a clear supernatant. Pipetted 1ml supernatant in test tube labelled (T), added 1 ml picric acid reagent (L1) in test tube labelled (B) and (T). Then, added 0.1 ml of distilled water in test tube labelled blank (B), and added 0.1 ml of creatinine standard in test tube (S). Added 0.1 ml of Buffer reagent (L2) to the test tube mixed well and kept the test tube at room temperature for 20 minutes. The Optical density was read in colorimeter at 520 nm, after adjusting for zero using blank. Plasma Creatinine (mg/dl) = (O.D. of test / O.D. of standard) × 2.

### 3.34 α-Amalyase activity assay

Assaying of α-Amalyase activity in plasma samples was done by method of Sheriff (2004) based on starch-iodine interaction.

**Reagents:**

- Anhydrous Na$_2$HPO$_4$, KH$_2$PO$_4$
- Soluble starch
- Formaldehyde solution
- KIO$_3$ and KIO$_2$
- HCL and saline

**Assay:** α-Amalyase activity was assayed by Starch-Iodine method. Buffered Starch Substrate was prepared by mixing of Anhydrous Na$_2$HPO$_4$, KH$_2$PO$_4$ and soluble starch in 150 ml of normal saline in amount of 2.825g, 1.560g and 0.050g respectively, adjusted pH 7.0 ± 0.2. Stock Iodine solution was prepared by dissolving of 0.007g of KIO , 0.070g of KIO and 14µl HCL in 2ml of distilled water. It was diluted 10 times to obtain working iodine solution. 2.5 ml of buffered starch substrate was taken in 25ml volumetric flask labeled as control (C) and test (T) and 0.05 ml of plasma added to test while control has same amount of DW. Incubated 7½ minute in water bath at 37ºC. 2.5ml of working iodine solution was added into test and control. Final volume was made up to 25ml by adding DW. Absorbance was read immediately at 660nm wavelength on spectrophotometer. Activity of α-Amalyase (Somogyi units/100ml) = (control – test / control) × 100.
3.35 Lipase activity assay

Assaying of lipase activity in plasma samples was done by method of Teitz and Shuey (1993).

Reagents:

- L1: Lipase reagents
- C: Calibrator

Assay: Reconstituted the calibrator with 1 ml of DW. 1.0 ml of lipase reagent was taken in two test tubes labeled as calibrator (C) and test (T). 0.04ml of calibrator and sample were added respective tubes. Mixed well and read the initial absorbance A₁ for both. Incubated for 5 minutes at 37°C and read another absorbance A₂ immediately. For calibrator Δ AC = A₂C – A₁C, for test Δ AT = A₂T – A₁T. Activity of Lipase (units/L) = (Δ AT / Δ AC) × 266.

3.36 Catalase (CAT) activity assay

CAT activity in plasma and liver tissue homogenate samples were determined by method of Beers and Sizer (1952).

Reagents:

- Potassium phosphate buffer (PBS, 50 mM, pH 7.4)
- Hydrogen peroxide (30% H₂O₂, 59 mM in PBS)

Assay: The assay was based on the degradation of H₂O₂ at 240 nm spectrophotometrically. One unit decomposes one micromole of H₂O₂ per minute at 25°C, pH 7.0 under the specified condition. The reaction medium contains 1.9ml of 50 mM PBS (pH 7.4), 1ml of 59 mM H₂O₂ and 0.1 ml of enzyme aliquot. The decrease in absorbance was monitored at 240 nm for 3-4 minutes. Catalase activity (Unit/minute/ml) was calculated as (Δ A₂₄₀/minute × Total volume of assay × dilution factor) / (molar extinction coefficient × volume of sample). One unit of CAT activity was defined as μmole of hydrogen peroxide consumed per minute per ml of sample.

3.37 Superoxide dismutase (SOD) activity assay

SOD activity in plasma and liver tissue homogenate samples were determined by method of Winterbourn et al. (1975).

Reagents:

- Potassium phosphate buffer (67 mM, pH 7.8)
- Ethylene diamine tetra acetic acid (EDTA, 0.1M)
- Riboflavin (0.12 mM)
- Nitro blue tetrazolium chloride (NBT, 1.5 mM)

**Assay:** For the assay of SOD, the reaction medium containing 0.2 ml EDTA (0.1M), 3.0 ml phosphate buffer (67 mM, pH 7.8), 0.1 ml NBT (1.5 mM) and enzyme aliquots of different concentration in ascending order. After adding of 50 μl riboflavin (0.12 mM) they were incubated for 10 minutes in light box having light intensity 400 μM m⁻² s⁻¹. Absorbance was read at 560nm wavelength on spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT reduction in one minute.

### 3.38 Glutathione peroxidase (GSH-POX) activity assay

GSH-POX activity in plasma and liver tissue homogenate samples were resolute by method of Miyake *et al.* (1991).

**Reagents:**
- Potassium phosphate buffer (50 mM, pH 7.4)
- Ethylene diamine tetra acetic acid (EDTA, 0.40M)
- Reduced glutathione (GSH, 200 mM)
- Di-Thio Thretol (DTT, 1 mM) in 10 mM Potassium phosphate buffer (w/v)
- Hydrogen peroxide (0.042% H₂O₂, w/w)

**Assay:** The assay was based on oxidation of H₂O₂ by Glutathione peroxidase (GSH-POX), which was going on reduced to oxidation of Reduced Glutathione (GSH). The reaction medium was containing 2.7 ml of phosphate buffer with EDTA, 0.1 ml of GSH and 0.1 ml of H₂O₂. 0.1 ml of enzyme aliquot and 0.1 ml of DTT were added into test and blank. The reaction was started on addition of enzyme extract. Change in absorbance per minute was recorded on spectrophotometer at 265nm wavelength. GSH-POX activity (Unit/minute/ml) was calculated as $(Δ A_{265/minute} \times \text{Total volume of assay} \times \text{dilution factor}) / (\text{molar extinction coefficient} \times \text{volume of sample})$. One unit of GSH-POX activity was defined as μmole of GSH oxidized in one minute per ml of sample.

### 3.39 Glutathione-S-Transferase (GST) activity assay

GST activity in plasma and liver tissue homogenate samples were determined by method of Habig and Jakoby (1981).
**Reagents:**

- Potassium phosphate buffer (100 mM, pH 7.4)
- Ethylene diamine tetra acetic acid (EDTA, 1 mM)
- Reduced glutathione (GSH, 1 mM)
- 1-chloro 2, 4-dinitrobenzene (CDNB, 1 mM)
- Distilled water

**Assay:** The enzyme is assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm. The reaction medium contained 1.5 ml of phosphate buffer, 50 µl of EDTA, 30 µl of CDNB, 300 µl of GSH and 1.12 ml of DW in a total volume of 2.9ml. The reaction was initiated by the addition of 0.1ml of enzyme aliquot. The absorbance was recorded per minute for 3-4 minute at 340nm against distilled water blank on spectrophotometer. GST activity was calculated as $(\Delta A_{340/\text{minute}} \times \text{Total volume of assay} \times \text{dilution factor}) / (\text{molar extinction coefficient} \times \text{volume of sample})$. One unit of GST activity was defined as µmole of CDNB-GSH conjugate formed in one minute per ml of sample.

### 3.40 Guaiacol Peroxidase (GPOX) activity assay

GPOX activity in plasma and liver tissue homogenate samples were assayed by method of Hemeda and Klein (1990).

**Reagents:**

- Potassium phosphate buffer (PBS, 50 mM, pH 7.4)
- Hydrogen peroxide ($\text{H}_2\text{O}_2$, 10 mM )
- Guaiacol (0.2 mM, or 1% v/v)
- Distilled water

**Assay:** The assay was based on rate of formation of Guaiacol dehydrogenation product by $\text{H}_2\text{O}_2$ with help of Guaiacol Peroxidase. The reaction medium was containing 2.4 ml of phosphate buffer, 0.3 ml of Guaiacol and 0.3 ml of $\text{H}_2\text{O}_2$. 0.1 ml of enzyme aliquot and 0.1 ml of DW were added into test and blank. The reaction was started on addition of enzyme extract. Change in absorbance per minute was recorded on spectrophotometer at 340nm wavelength. GPOX activity (Unit/minute/ml) was calculated as $(\Delta A_{340/\text{minute}} \times \text{Total volume of assay} \times \text{dilution factor}) / (\text{molar extinction coefficient} \times \text{volume of sample})$. One unit of GPOX activity was defined as µmole of guaiacol oxidized in one minute per ml of sample.
3.41 Reduced Glutathione, GSH

Reduced Glutathione content in plasma and liver tissue homogenate samples were estimated by the method of Hu (1994).

**Reagents:**
- Tris-EDTA buffer (0.25 mM Tris base with 20 mM EDTA, pH 8.2)
- 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB, 10 mM)
- Standard solution of Reduced Glutathione

**Assay:** Total thiol concentration or sulfhydryl groups (SH) in reduced glutathione (GSH) form, in test samples were measured by the methods described by Hu M. L. The assay of thiols is based on its interaction with 5, 5'-dithiobis-(2 nitrobenzoic acid) (DTNB), forming a highly colored anion with maximum peak at 412nm. An aliquot of 25µl fresh sample was mixed with 1mL Tris-EDTA buffer (0.25mmol/L Tris base, 20mmol/L EDTA, pH 8.2), and initial absorbance (A1) was read at 412nm. Next, a 25µl aliquot of DTNB stock solution (10mmol/L in absolute methanol) was added to the solution. After 15 minutes incubation at ambient temperature, the absorbance was read again (A2) together with a DTNB blank (B). The concentration of sulfhydryl groups was calculated by using reduced glutathione as sulfhydryl group standard and the result was expressed in µmol/L.

3.42 Lipid Per Oxidation, LPO

LPO level in both plasma and liver tissue homogenate samples were determined by the method of Draper and Hadley (1990).

**Reagents:**
- Tri chloro acetic acid (TCA, 10%)
- Thio-barbituric acid (TBA, 0.675%)
- Standard solution of 1, 1, 3, 3- Tetramethoxy propane (TMP)

**Assay:** Since MDA is derived in lipid peroxidation process, so assay was based on formation of MDA (TBA)2 adduct which was quantified by spectrophotometric method at 532nm. 0.5 ml of plasma/tissue homogenate sample was taken in test tubes and 2.5 ml of 10 % TCA was added, mixed well and incubated for 15 minutes at 90ºC and followed by centrifugation for 10 minutes at 3000 rpm. 2 ml of supernatant was taken to which 1ml of 0.675 % TBA was added, mixed well and incubated for 15 minutes at 90ºC and cooled to room temperature. A pale pink colour
developed, whose intensity was measured at 530 nm against blank. Concentration of MDA was calculated by calibration curve using TMP as a standard in terms of nano mole/ml.

3.43 Vitamin – A

Vitamin- A was quantified in plasma and liver tissue homogenate samples by the method of Rutkowski and Grzegorczyk (2007).

Reagents:
- Xylene
- KOH solution (1M in 90% ethanol)

Assay: This assay was based on UV absorption coefficient of 1% solution of vitamin A (as the retinol form) in xylene medium at 335 nm wavelength. Took 1 ml of analysed sample and added 1 ml of 1M KOH solution into a test tube and shaken vigorously for 1 minute. Heated in water bath for 20 minutes at 60ºC and cooled. 1ml of Xylene was added and shaken vigorously for 1 minute, then after centrifuged at 1500Xg for 10 minutes at controlled temperature. The supernatant (separated extract upper layer) was separated and measured the absorbance A₁ at 335 nm wavelength against xylene blank. Irradiate the supernatant to UV light for 30 minutes, again measure the absorbance A₂. The concentration of Vitamin - A (in µM/ml) was calculated by using the formula – Cₓ = (A₁ - A₂) × 22.23.

3.44 Vitamin – C

Vitamin- C was quantified in plasma and liver tissue homogenate samples by the method of Rutkowski and Grzegorczyk (2007).

Reagents:
- Sodium tungustate, molybdenium free
- Sodium hydrogen phosphate
- Sulphuric acid
- Ascorbic acid standard
- Oxalic acid (50 mM)
- Distilled water

Assay: Phosphotungustate Reagent (PR) was made of 3.11 gm of sodium tungustate molybdenium free, 1.25 gm of sodium hydrogen phosphate and 5.84 ml of D.W. mixed by heat and cooled. 2.16 ml of conc. HCL was added and pH adjusted to 1. Standard solution of vitamin
C was made of 56.81µM L-ascorbic acid by using 50 mM of oxalic acid as solvent. 1 ml of the analysed sample and 1 ml of the PR was mixed thoroughly and leaved at room temperature for 30 minutes. It was centrifuged at 7000×g for 10 minutes and collected whole supernatant which was measured for absorbance on spectrophotometer at 700 nm against the mixture PR: 50 mM solution of oxalic acid = 1:1 (v/v) as a reference sample. Concentration of vitamin- C (µM/ml) was calculated by using the formula as given: (Abs. of test / Abs. of standard) × concentration of standard solution.

3.45 Histopathological Study

Histopathological study of liver, kidney and pancreas tissues were done by method of Lillie (1965).

3.45.1 Collection of Tissues

After sacrificing, the rat’s liver, pancreas and kidney tissues were dissected out and washed thoroughly in normal saline (0.85 %) and fixed in 10% formalin.

3.45.2 Fixation and embedding

Small pieces of liver, pancreas and kidney tissues from the sacrificed rats were fixed by the following procedure for subsequent histological studies under compound light microscope.

3.45.3 Tissue Processing

Tissues were fixed for 24 hrs. (Left in the fixative for longer periods when required, and then washed overnight under running tap water). The tissues were dehydrated in 30% alcohol, 50% alcohol, 70% alcohol, 90% alcohol and 100% absolute alcohol for 45 minutes each. The tissues were cleaned in acetone and absolute alcohol with xylene (1:1) and poured in xylene for 20 minutes. Then after passing through a mixture of xylene and molten wax (1:1) for 30 minutes, they were embedded in molten paraffin wax. Blocks were made using L- moulds.

3.45.4 Sectioning

Blocks were fixed on the holder of the microtome. Sections were cut at 5-6 µm thickness and fixed on Mayer’s albumin rubbed glass slides.
3.45.5 Staining

The sections were deparaffinised in xylene for 10-15 minutes and hydrated through descending series of 100% absolute alcohol, 90% alcohol, 70% alcohol, 50% alcohol and then 30% alcohol for 10 minutes. After washing in distilled water, these hydrated sections were stained in haematoxyline for 2-3 minutes with one dip in acid water (if over stained). The sections were washed under running tap water for 3-5 minutes and then rinsed in distilled water for differentiation. Then the sections were dehydrated in ascending series 30% alcohol, 50% alcohol, 70 % alcohol and counter stained in eosin, (since eosin is prepared in 70% alcohol) dehydrated further in 90 % and 100% absolute alcohol for 10 minutes. Thereafter sections were cleaned in xylene and mounted in DPX with clean glass cover slip. Slides were dried and viewed under compound light microscope at 200X and 400X.
Figure 3.15: Sectioning of processed tissues

Figure 3.16: Microscopic study of tissue slides
3.46 Statistical Analysis

All results were expressed as mean ± SD (Standard Deviation) of four replicates. Data ascertained from experiments were subjected to statistical analysis of variance using one way and two way classification with \( r = 4 \) observations per cell ([Snedecor and Cochran, 1980](#)). The value of probability less than 5% (\( P < 0.05 \)) was considered statically significant. Thereafter standard error of mean and critical difference at 5% probability level were also computed. The skeleton of the ANOVA table and the formulae of Standard Error (SE) and Critical Difference (CD) are given below:

**Table 3.1: Skeleton of two-way ANOVA**

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>d. f.</th>
<th>S. S.</th>
<th>M. S. S.</th>
<th>F (Cal)</th>
<th>F (Tab) 5%</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant</td>
<td>(p-1)</td>
<td>S. S. (p)</td>
<td>M. S. S. (p)</td>
<td>M. S. S. (p)/M. E. S. S.</td>
<td>( F_{3 \times 69} )</td>
<td>Sig./N.sig.</td>
</tr>
<tr>
<td>Treatment</td>
<td>(t-1)</td>
<td>S. S. (t)</td>
<td>M. S. S. (t)</td>
<td>M. S. S. (t)/M. E. S. S.</td>
<td>( F_{4 \times 69} )</td>
<td>Sig./N.sig.</td>
</tr>
<tr>
<td>Replicate</td>
<td>(r-1)</td>
<td>S. S. (r)</td>
<td>M. S. S. (r)</td>
<td>M. S. S. (r)/M. E. S. S.</td>
<td>( F_{3 \times 69} )</td>
<td>Sig./N.sig.</td>
</tr>
<tr>
<td>Error</td>
<td>† 69</td>
<td>E. S. S.</td>
<td>M. E. S. S.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>(ptr)-1</td>
<td>Total S.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Formulae for SE and CD**

For plant

\[
SE = \sqrt{2} \times \frac{M. E. S. S}{r \times t}
\]

\[
CD = SE \times t \text{ (5\%) on 69 d. f.}
\]

For treatment

\[
SE = \sqrt{2} \times \frac{M. E. S. S}{r \times p}
\]

\[
CD = SE \times t \text{ (5\%) on 69 d. f.}
\]