

## 4.1. MATERIALS

### 4.1.1. Equipments

**Table 10.** List of equipments

ELISA Microplate Reader (ELx800 Absorbance Microplate Reader)	BioTek Instruments, Winooski, USA
HPLC System	Waters Corporation, USA
Spectrophotometer	Shimadzu Analytical (India) Pvt. Ltd. New Delhi, India
FT-IR Spectrophotometer	Jasco corporation, Japan
Non-Invasive Blood Pressure System (Tail-cuff method)	Kent Scientific Corporation, Connecticut, USA
Incubator	York Scientific Industries Pvt. Ltd., India
Electronic Balance	Mettler-Toledo India Pvt. Ltd., Mumbai, India
Hot Air Oven	Grover Enterprises, India
Laboratory Centrifuge (R-8C DX)	REMI Instruments Ltd., Mumbai, India
Cooling Centrifuge (C-24)	REMI Instruments Ltd., Mumbai, India
Homogenizer	REMI Instruments Ltd., Mumbai, India
pH Meter	Mettler-Toledo India Pvt. Ltd., Mumbai, India
Ultra-low Temperature Freezer	New Brunswick Scientific, CT, USA
Micropipette	Thermo Fisher Scientific Inc., USA
Rotary Evaporator (Rotavapor)	BUCHI Corporation, DE, USA
ACCU-Check Glucosemeter	Roche Diagnostics GmbH, Mannheim, Germany

**4.1.2. Drugs****Table 11.** List of drugs

Orlistat	Gift sample from Ranbaxy Laboratories Ltd. Gurgaon, India
Embelin	Sigma-Aldrich Co., Bangalore (Bengaluru), India
Streptozotocin	Sigma-Aldrich Co., Bangalore (Bengaluru), India
Metformin	Gift Sample from Panacea Biotech Ltd., Chandigarh, India

**4.1.3. Chemicals and reagents****Table 12.** List of chemicals and reagents

1-Chloro-2,4-dinitrobenzene	SDFCL, Mumbai, India
5, 5'-Dithiobis(2-nitrobenzoic acid)	SDFCL, Mumbai, India
Adenosine triphosphate	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Ammonium molybdate	Central Drug House (P) Ltd., New Delhi, India
Anthrone reagent	SDFCL, Mumbai, India
Ascorbic acid	SDFCL, Mumbai, India
Bovine serum albumin	Himedia Laboratories, Mumbai, India
Calcium chloride	Merck Ltd., Mumbai, India
Chloroform	Central Drug House (P) Ltd., New Delhi, India
Citric acid	SDFCL, Mumbai, India
Copper sulphate	SDFCL, Mumbai, India
Diethyl ether	Central Drug House (P) Ltd., New Delhi, India
Disodium hydrogen phosphate	SDFCL, Mumbai, India
Ethylenediaminetetraacetic acid	SDFCL, Mumbai, India
Folin-Ciocalteu reagent	Central Drug House (P) Ltd., New Delhi, India

Formaldehyde	Merck Ltd., Mumbai, India
Glacial acetic acid	Central Drug House (P) Ltd., New Delhi, India
Hydrochloric acid	Nice Chemicals Pvt. Ltd., Cochin, India
Hydrogen peroxide	Nice Chemicals Pvt. Ltd., Cochin, India
Magnesium chloride	Central Drug House (P) Ltd., New Delhi, India
Magnesium sulphate	Central Drug House (P) Ltd., New Delhi, India
Nicotinamide adenine dinucleotide phosphate	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Glutathione oxidized	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Glutathione reduced	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Potassium chloride	Central Drug House (P) Ltd., New Delhi, India
Potassium dihydrogen phosphate	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Potassium hydroxide	Central Drug House (P) Ltd., New Delhi, India
Sodium arsenite	Central Drug House (P) Ltd., New Delhi, India
Sodium azide	Central Drug House (P) Ltd., New Delhi, India
Sodium carbonate	Merck Ltd., Mumbai, India
Sodium chloride	Central Drug House (P) Ltd., New Delhi, India
Sodium citrate	SDFCL, Mumbai, India
Sodium hydroxide	Central Drug House (P) Ltd., New Delhi, India
Sodium potassium tartrate	Merck Ltd., Mumbai, India
Sucrose	SDFCL, Mumbai, India

Sulphuric acid	Merck Ltd., Mumbai, India
Thiobarbituric acid	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Trichloroacetic acid	Central Drug House (P) Ltd., New Delhi, India
Tris buffer	SDFCL, Mumbai, India

#### 4.1.4. Diagnostic kits

**Table 13.** List of diagnostic kits

Rat Insulin ELISA Kit	Crystal Chem Inc.IL, USA
Rat Adiponectin EIA Kit	Ray Biotech Inc.,GA, USA
Rat Leptin ELISA Kit	Ray Biotech Inc.,GA, USA
Apolipoprotein-B	Randox Laboratories Ltd., UK
Glucose	Span Diagnostics Ltd., Surat, India
Lactate Dehydrogenase	Crest Biosystems, Goa, India
Albumin	Span Diagnostics Ltd., Surat, India
Total Protein	Span Diagnostics Ltd., Surat, India
Creatinine	Span Diagnostics Ltd., Surat, India
Alkaline Phosphatase	Span Diagnostics Ltd., Surat, India
Lipase	BioAssay Systems, CA, USA
Total Cholesterol	Span Diagnostics Ltd., Surat, India
Triglycerides	Span Diagnostics Ltd., Surat, India
High Density Lipoprotein Cholesterol	Span Diagnostics Ltd., Surat, India

## 4.2. PLANT MATERIAL

Dried fruits of *Embelia ribes* Burm.f. (Family: Myrsinaceae) were purchased from North Eastern India Ayurveda Research Institute, Barsjoi, Bhetapura, Guwahati, Assam, India, and authenticated by Dr. H. B. Singh, Head, Raw Materials and Herbarium, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Ref. NISCAIR/RHMD/Consult/-2010-11/1500/98, dated 26 August, 2010).

### 4.2.1. Preparation of ethanolic extract of *E.ribes*

The dried and coarsely powdered berries of *E.ribes* (1 kg) were evenly packed in Soxhlet apparatus and were subjected to extraction with 90% ethanol for 72 hours. The solvent was removed under reduced pressure to give a dried extract, 8.4% yield w/w with respect to the crude material stored at 4°C until the experiment.

### 4.2.2. Standardization of ethanolic extract of *E.ribes* (WHO, 1998; Indian Pharmacopoeia, 1996)

The following parameters were carried for the standardization of ethanolic extract of *E.ribes*:

#### 4.2.2.1. Physico-chemical standardization

##### 4.2.2.1.1. Extractive value

The extractive values (Hot extractive values) were determined according to the method described in Indian Pharmacopoeia 1996, Appendix 3.37. The dried and coarsely powdered fruits of *E.ribes* (10 gm) were packed in a Soxhlet apparatus and were subjected to extraction with different solvents such as ethanol, petroleum ether, chloroform, acetone, methanol and water for 72 hours. The total volume of extract was readjusted with same solvent to 100 ml. The extract was divided into 4 parts of 25 ml each. Then each part of 25 ml of extract was transferred to an evaporating dish and was evaporated to dryness on water bath, then weighed and their constant extractive values with different solvents were calculated.

$$\% \text{ Extractive value} = \frac{\text{Weight of extractable matter}}{\text{Weight of drug}} \times 100$$

#### **4.2.2.1.2. Ash values**

The percentage of total ash, acid-insoluble ash and water soluble ash were determined as follows:

##### Total ash

Ethanol extract of *E.ribes* (1 gm) was placed in the silica crucible and was incinerated at a temperature not > 450°C until free from carbon. It is then cooled and weighed to get the total ash content.

##### Acid insoluble ash

Total ash was boiled with 25 ml of dilute HCl (6N) for five minutes. The insoluble matter collected on filter paper, washed with hot water and ignited at a temperature not >450°C to constant weight.

##### Water soluble ash

Total ash was dissolved in distilled water and the insoluble matter collected on filter paper and ignited at 450°C to constant weight. By subtracting the weight of insoluble part from that of the total ash, the weight of soluble part of ash was obtained.

Percentage ash value was calculated from formula below:

$$\% \text{ Ash content} = \frac{\text{Weight of Ash}}{\text{Weight of extract}} \times 100$$

#### **4.2.2.1.3. pH values**

##### pH of 1% aqueous solution

1 gm of ethanolic extract of *E.ribes* was dissolved in 100 ml of distilled water, filtered and pH of the filtrate was checked with pH meter.

##### pH of 10% aqueous solution

1 gm of ethanolic extract of *E.ribes* was dissolved in 10 ml of distilled water, filtered and pH of the filtrate was checked with pH meter.

#### **4.2.2.1.4. Foaming Index**

1 gm of ethanolic extract of *E.ribes* was transferred to conical flask containing 100 ml of boiling water and maintained at moderate boiling for 30 minutes. After cooling, poured into 10 ml stopper glass tube (16 ×16 mm) in successive portions of 1 ml, 2 ml, 3 ml upto 10 ml and adjusted the volume of liquid in each tube with water to 10 ml. Stopped the tubes and were shaken then in a lengthwise motion for 15 sec, two shakes per second. Then allowed

to stand for 15 minutes and the height of foam were measured. The results of foaming index were assessed by following criteria:

1. If the height of foam in tube is <1 cm, the foaming index is less than 100.
2. If the height of foam in any tube is 1cm, the volume of plant material decoction in the tube is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.
3. If the height of foam is >1 cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of decoction of detection in order to obtain a result.

The foaming index was calculated by the formula =  $\frac{1000}{a}$

Where a= the volume in ml of decoction used for preparing the dilution in the tube where foaming to a height of 1 cm is observed.

#### 4.2.2.1.5. Microbial Contamination

1 gm of ethanolic extract of *E.ribes* was taken and suspended in 50 ml of sterile distilled water. The suspension was shaken well and then filtered. The filtrate was used as stock solution. Serial dilution (1:1, 1:100, 1:10000) of this stock solution were prepared and 1 ml each of different diluted solution were separately inoculated (by spreading method) on nutrient agar medium in petri plates and incubated at 37<sup>0</sup> C for 24 hours. After 24 hours, the petri plates with most clearly visible colonies were taken and number of colonies determined by colony counter. Composition of nutrient agar medium was as follows:

**Table 14.** Composition of nutrient agar medium

1. Agar	15.0 %
2. Peptic Digest of Animal tissue	5.0 %
3. Sodium Chloride	5.0 %
4. Beef Extract	1.5 %
5. Yeast Extract	1.5 %
6. pH	7.4 ± 0.2 at 25 <sup>0</sup> C
7. Distilled Water	1000 ml

The medium was autoclaved at 15 lbs/ inch<sup>2</sup> pressure at 121<sup>0</sup>C.

#### **4.2.2.2. Preliminary phytochemical screening (Ali, 1998; Evans, 2002)**

Preliminary phytochemical screening of the ethanolic extract of *E.ribes* has been performed to detect the presence or absence of different phytoconstituents such as alkaloids, carbohydrates, glycosides, phenolic compounds, flavonoids, protein, saponin, sterols, resins, and lipids/fats.

#### **4.2.2.3. Thin Layer Chromatography (TLC) analysis**

Ethanolic extract of *E.ribes* was subjected to thin layer chromatography analysis to find out the number of compounds present in it. The details of the procedure followed were as follows:

##### **Preparation of the plates**

The adsorbent used for thin layer chromatography was silica gel G. About 25 gm of silica gel G was taken in a glass mortar and about 35 ml of distilled water was added to it. This mixture was stirred with glass rod to make homogenous and allowed to swell for about 12 minutes. Then, an additional 15 ml of distilled water was added to it with stirring. The suspension was then transferred to a 150 ml conical flask with a plastic stopper, and was shaken vigorously for about 2 minutes. This suspension was then spread immediately on thin layer chromatographic plates with the help of a thin layer chromatography (TLC) applicator.

##### **Drying and storage of the plates**

The freshly coated plates were then air dried until the transparency of the layer had disappeared. The plates were then stacked in a drying rack and were heated in the hot air oven for 30 minutes, at 121<sup>0</sup>C. The activated plates were kept in a desiccator, till further use.

##### **Application of the sample**

For applying test samples on TLC plate, glass capillaries were used. The spot were applied with the help of a transparent template, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity.

##### **Chromatographic chamber, condition of saturation and the development of TLC plates**

Chromatographic rectangular glass chamber (16.5 cm × 29.5 cm) was used in the experiment. To avoid insufficient chamber saturation and the undesirable edge effect, a



smooth sheet of filter paper approximately 15 cm × 40 cm size was placed in the chromatographic chamber in a 'U' shape and was allowed to be soaked in the developing solvent. After being moistened, the paper was then pressed against the walls of the chamber, so that it adheres to the walls. The chamber was allowed to saturate for 2 hours before use.

#### **Spraying equipments**

Compressed air sprayer with a fine nozzle was used to detect the different constituents present on TLC plates. Air compressor was attached to a glass sprayer. The sprayer was filled with about 50 ml of the detecting reagent before use. After each spray, the sprayer was washed separately with water, chromic acid, distilled water and then acetone.

#### **Visualization**

The TLC was done for ethanolic extract of *E.ribes* on a pre-coated silica gel 60 F<sub>254</sub> plates (E. Merck, 0.20 mm thickness, 10 cm × 10 cm) using different mobile phases. Chromatograms were viewed under long wavelength UV light (520 nm and 365 nm). The R<sub>f</sub> values for different spots were recorded by HPTLC Apparatus (Reprostar Chromatography Documentation Apparatus, CAMAG, Switzerland).

#### **4.2.2.4. High Performance Thin Layer Chromatography (HPTLC) analysis**

For development of HPTLC fingerprint, ethanolic extract of *E.ribes* (20 mg) was suspended in 4 ml of ethanol. The extraction was done by soxhlet apparatus. The extracted material along with solvent was filtered through Whatman Grade No. 1 filter paper.

#### **Preparation of test solution**

4 mg of ethanolic extract of *E.ribes* was dissolved in 4 ml of ethyl acetate and 15 µl of this solution was applied on TLC plate as reference marker for TLC profiling.

#### **Preparation of standard solution**

8 mg of reference compound embelin was dissolved in 4 ml of ethyl acetate (2 µg/µl). From this solution, the different concentrations *i.e.* 0.25, 0.50, 0.75, 1.0, and 1.25 µg/µl were prepared.

#### **Solvent system**

Ethyl Acetate: Methanol (9:1)

**Procedure**

Ethanollic extract of *E.ribes* and standard solution of embelin were applied on pre-coated silica gel aluminium TLC plate of uniform thickness of 0.2 mm. After 10 min drying at room temperature, the plate was developed in a twin through TLC chamber (20 cm × 10 cm, CAMAG) saturated with respective solvent system. Solvent was allowed to run upto 75% of the plate height i.e. 75 mm above the line of band application. Again the plate was dried at room temperature for 30 minute and then, scanned in TLC scanner at 520 and 365 nm respectively.

**Visualization**

The results were visualized using win CATS software. Chromatograms were viewed under wavelength UV light (520 and 365 nm). The  $R_f$  values for different spots were recorded by HPTLC Apparatus (Reprostar Chromatography Documentation Apparatus, CAMAG, Switzerland)

**4.2.2.5. High Performance Liquid Chromatography (HPLC) analysis**

The HPLC analysis of ethanolic extract of *E.ribes* was carried out by the methodology of Shelar *et al.* (2009).

**Preparation of test and standard solutions**

Ethanollic extract of *E.ribes* (100 mg) was transferred into a 100 ml volumetric flask fitted with a glass stopper, and volume was made by methanol and sonicated for 5 min. The mixture was then filtered and the desired concentration (1 mg/ml) is obtained. Reference standard embelin (5 mg) was transferred into a 50 ml volumetric flask and dissolved in methanol to obtain solution with 0.1 mg/ml concentration of embelin. Then 10  $\mu$ l of the resulting solutions were subjected to HPLC analysis.

**HPLC analytical condition**

HPLC system (Waters Corporation, USA) equipped with Millennium Chromatography software version 2.15 was used in the present study and the chromatographic separations were performed using a Hyperchrome Column (250mm×4.6 mm), with a flow rate of 1ml/min and a sample size of 10  $\mu$ l. The mobile phase used was methanol (A) and 0.1% trifluoroacetic acid (B) with a ratio of 88:12 (A:B (v/v)). The sample analysis was performed at wavelength 288 nm using photodiode array (PDA) detector at ambient temperature.

The chromatographic peaks of ethanolic extract of *E.ribes* were confirmed by comparing its retention time and UV spectra with corresponding reference standard embelin. The result was obtained by comparison of peak area (at 288 nm) of ethanolic extract of *E.ribes* with that of embelin.

#### **4.2.3. Extraction and isolation of embelin from *E. ribes***

Coarsely powdered berries of *E. ribes* (1 kg) were exhaustively extracted in a Soxhlet apparatus and were subjected to extraction with diethyl ether for 72 h. The extract was concentrated under reduced pressure to give a dry extract (average yield 10.58% w/w). The extract so obtained was subjected to column chromatography over silica gel (60-120 mesh) and eluted using benzene. The eluted fractions were collected at intervals of 250 ml each. Elution of the column with benzene yielded an orange colored powder which on crystallization with diethyl ether afforded orange crystals of embelin (average yield 0.45% w/w of crude drug).

#### **4.2.4. Characterization of embelin isolated from *E.ribes***

The following parameters were carried for characterization of embelin isolated from *E.ribes*:

##### **4.2.4.1. Melting point**

Melting was determined in open capillary tube in a Hicon melting point apparatus.

##### **4.2.4.2. UV spectroscopy**

A double beam UV Spectrophotometer (Jasco Corporation, Japan) was used for analysis. 10 µg/ml embelin solution was prepared in ethanol and scanned at 200-400 nm using ethanol as a blank. The wavelength ( $\lambda$  max) corresponding to maximum absorbance was determined.

##### **4.2.4.3. High Performance Liquid Chromatography (HPLC) analysis**

The HPLC analysis of ethanolic extract of *E.ribes* was carried out by the methodology of Shelar *et al.* (2009) on HPLC system (Waters Corporation, USA) equipped with Millennium Chromatography software version 2.15, the chromatographic separations were performed using a Hyperchrome Column (250mm×4.6 mm) at a flow rate of 1ml/min and a sample size of 10 µl. The mobile phase used was methanol (A) and 0.1% trifluoroacetic acid (B) at a ratio of 88:12 (A:B (v/v)). The flow rate was set 1 ml/min and sample analysis was performed at a wavelength of 288 nm using photodiode array (PDA) detector at

ambient temperature. The chromatographic peaks of the embelin isolated from *E.ribes* were confirmed by comparing its retention time with standard embelin.

#### ***4.2.4.4. Fourier-transform infra-red (FT-IR) spectroscopy***

Fourier-transform Infra-red (FT-IR) spectra of isolated embelin and that of standard embelin were obtained using a FT-IR spectrophotometer (Jasco Corporation, Japan). A total of 2% (w/w) of sample, with respect to the potassium bromide (KBr), was mixed. The mixture was grounded or triturated into fine powder before compressing into KBr disc. Each KBr disc was scanned (at 4 mm/s at a resolution of 2 cm) over a wave number region of 4000–400  $\text{cm}^{-1}$ . The characteristic peaks were recorded for different samples and compared.

### 4.3. PLAN OF WORK

#### 4.3.1. Animals

Male Wistar rats (150-200 g) were procured from the central animal house facility of Hamdard University (New Delhi, India). The animals were housed in polypropylene rat cages and maintained under controlled room temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity ( $55 \pm 5\%$ ) with 12 h light and 12 h dark cycle. All the rats had free access to commercially available rat normal pellet diet (Amrut rat feed, Nav Maharashtra Chakan Oil Mills Ltd., Delhi, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethics Committee of Jamia Hamdard, New Delhi, India (Approval No.316), and performed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Rats were allowed to acclimatize to the experimental environment for 1 week prior to the dietary manipulation.

#### 4.3.2. High fat diet

A standardized high fat diet (HFD) was procured from National Centre for Laboratory Animal science (NCLAS) National Institute of Nutrition (NIN), Hyderabad, India. A composition of HFD is shown in Table 15. Rats were fed a HFD *ad libitum* in a pellet form for the period of 4 weeks to induce obesity.

**Table 15.** Composition of high fat diet (gm/kg)

Ingredients	Quantity (g/kg)
Casein	342.0
L-Cystine	3.0
Starch	172.0
Sucrose	172.0
Cellulose	50.0
Ground nut oil	25.0
Tallow	190.0
AIN Salt mix	35.0
AIN Vitamin mix	10.0
Total (gm)	999.0

\* AIN- American Institute of Nutrition

### **4.3.3. Preparation of drug solution/suspension**

#### ***4.3.3.1. Preparation of ethanolic extract of *E.ribes* suspension***

Suspension of ethanolic extract of *E.ribes* was prepared by triturating the weighed amount of extract [100- and 200 mg/kg body weight (bw)] in 1% gum acacia in normal saline (0.90% w/v of NaCl).

#### ***4.3.3.2. Preparation of embelin suspension***

Suspension of embelin was prepared by triturating the weighed amount of embelin (50 mg/kg bw) in 1% gum acacia in normal saline (0.90% w/v of NaCl).

#### ***4.3.3.3. Preparation of orlistat suspension***

Suspension of orlistat was prepared by triturating the weighed amount of orlistat (10 mg/kg, bw) in 1% gum acacia in normal saline (0.90% w/v of NaCl).

#### ***4.3.3.4. Preparation of streptozotocin solution***

Solution of streptozotocin was prepared freshly by dissolving the weighed amount of streptozotocin (35 mg/kg bw) in ice-cold 0.1 M Citrate buffer (pH 4.4). Citrate buffer was prepared by mixing 0.1 M sodium citrate and 0.1 M citrate acid. pH 4.4 was adjusted using 1 N NaOH.

#### ***4.3.3.5. Preparation of metformin suspension***

Suspension of metformin was prepared by triturating the weighed amount of metformin (180 mg/kg bw) in 1% gum acacia in normal saline (0.90% w/v of NaCl).

### **4.3.4. Basis for dose selection of ethanolic extract of *E.ribes*, embelin, orlistat, and metformin**

#### ***4.3.4.1. Ethanolic extract of *E.ribes* (100- and 200 mg/kg bw, p.o.)***

Bhandari *et al.*, (2007, 2008) reported the significant reduction in the levels of blood glucose, hemodynamic parameters, lipid peroxidation in pancreatic tissue following oral administration of ethanol *E. ribes* extract at dose of 100- and 200 mg/kg bw in streptozotocin induced-diabetes in rats. Further, *Embelia ribes* significantly enhances the antioxidant defense against reactive oxygen species produced under hyperglycaemic condition at 100 mg/kg bw dose level in rats. Insulin resistance, diabetes mellitus, dyslipidemia, hypertension and oxidative-stress are linked to obesity and related co-morbidities. Therefore, the 100- and 200 mg/kg bw doses of ethanolic extract of *E.ribes* were selected in the present study.

**4.3.4.2. Embelin (50 mg/kg bw, p.o.)**

This dose was selected on the basis of previous reports on embelin where a 50 mg/kg bw dose of embelin produced significant results compared to a 25 mg/kg bw dose in rats, which are as follows:

Mahendran *et al.*, (2011) showed the antihyperglycemic, lipid lowering and antioxidant activities of embelin at the doses of 25- and 50 mg/kg bw in alloxan induced diabetes in rats. They reported that among the two doses of embelin (25- and 50 mg/kg bw), 50 mg/kg bw dose produced significant antihyperglycemic, lipid lowering and antioxidant action.

Thippeswamy *et al.*, (2011) showed the protective effects of embelin at 25- and 50 mg/kg bw dose on ischemia/reperfusion-induced brain injury in rats. They reported that embelin pretreatment at 50 mg/kg bw exhibited that better protection against ischemic damage than 25 mg/kg dose.

**4.3.4.3. Orlistat (10 mg/kg bw, p.o.)**

The dose for orlistat (10 mg/kg bw, p.o.) was selected on the basis of previously reported experimental study by Caner *et al.*, (2005). They reported that oral administration of orlistat (10 mg/kg bw) for 7 days in Wistar rats significantly reduced serum glucose, triglycerides and cholesterol levels as compared to control animals.

**4.3.4.4. Metformin (180 mg/kg, p.o.)**

Gad *et al.*, (2010a, 2010b) reported effect of metformin (180 mg/kg bw, p.o.) on carbohydrate metabolism and insulin sensitivity in diabetic and glucose intolerant rats on high fat diet. Metformin (180 mg/kg bw) produced significant increase in glucose-6-phosphate dehydrogenase activity by ~20%, decrease in glucose-6-phosphatase activity by ~25%, serum glucose by ~30% and decreases in liver and kidney glycogen contents. Therefore, the 180 mg/kg bw dose of metformin was selected in the present study

#### 4. 4. EXPERIMENTAL DESIGN

The whole study was designed into **three experimental plans (Plan I, II and III)** as follows:

**Plan I: Anti-obesity potential of ethanolic extract of *E.ribes* in HFD-induced obesity in Wistar rats.**

**Plan II: Anti-obesity potential of embelin isolated from *E.ribes* in HFD-induced obesity in Wistar rats.**

**Plan III: Anti-diabetic potential of ethanolic extract of *E.ribes* in HFD-fed and low dose streptozotocin (STZ)-induced type 2 diabetes in Wistar rats.**

##### 4.4.1. PLAN- I

#### ANTIOBESITY POTENTIAL OF ETHANOLIC EXTRACT OF *E.RIBES* IN HFD-INDUCED OBESITY IN WISTAR RATS

##### 4.4.1.1. Baseline characteristic of HFD-induced obesity

After acclimatization of one week, the rats were divided into two groups as follows:

<b>Group</b>	<b>Treatment</b>	<b>No. of animals</b>
Normal Pellet Diet (NPD)	Rats fed with NPD from day 1 to day 7 <i>ad libitum</i>	10
High Fat Diet (HFD)	Rats fed with HFD from day 1 to day 7 <i>ad libitum</i>	10

After 1 week of dietary manipulation i.e. on 8<sup>th</sup> day, effect of NPD and HFD on body weights, body mass index and biochemical estimations (blood glucose, serum triglycerides, serum total cholesterol, and serum insulin) were carried out in Wistar rats.

##### 4.4.1.2. Treatment schedule

Rats were fed with normal pellet diet (NPD) for 1 week in the experimental environment before the experiments were conducted. Once they had adapted to the environment, rats were divided into five groups of 10 rats each as follow:



Group (n=10)	Treatment
<b>I</b> Normal Control	Rats fed with NPD for the period of 28 days + 1% gum acacia (1 ml/kg bw, p.o) from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>II</b> Obesity Control	Rats fed with HFD for the period of 28 days + 1% gum acacia (1 ml/kg, p.o) from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>III</b> HFD + Ethanolic extract of <i>E.ribes</i>	Rats fed with HFD for the period of 28 days + Ethanolic extract of <i>E.ribes</i> (100 mg/kg bw, p.o.) in 1% gum acacia from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>IV</b> HFD + Orlistat	Rats fed with HFD for the period of 28 days + Orlistat (10 mg/kg bw, p.o.) in 1% gum acacia from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>V</b> Ethanolic extract of <i>E.ribes per se</i>	Rats fed with NPD for the period of 28 days + Ethanolic extract of <i>E.ribes</i> (100 mg/kg bw, p.o.) in 1% gum acacia from 8 <sup>th</sup> day to 28 <sup>th</sup> day

Drug solutions were prepared freshly every day and administered by oral gavage at approximately 24 hour interval on each day. Food and water intake were measured daily while body weight was measured weekly for the period of 28 days. At the end of the experimental period (on 29<sup>th</sup> day), the animals were anesthetized with ether, following overnight fasting. After blood pressure measurement, blood was withdrawn by retro-orbital method into a tube and the serum was obtained by centrifugation (3000 RPM for 15 minutes). After collection of blood, rats were sacrificed; organs (heart, liver) and visceral fat pads (perirenal, epididymal, mesenteric) were excised immediately, rinsed with phosphate buffer saline and weighed. Heart and liver tissues were fixed in 10% neutral buffer formalin for histopathological analysis. The serum and organ samples were stored at -70°C until analysis.

#### 4.4.1.3. Parameters assessed

##### 4.4.1.3.1. Anthropometric measurements

- Food intake
- Water intake
- Body weight gain

- Body mass index (BMI)

#### ***4.4.1.3.2. Blood pressure measurement***

- Heart rate
- Systolic blood pressure
- Diastolic blood pressure
- Mean arterial (blood) pressure

#### ***4.4.1.3.3. Visceral fat pads weight (mesenteric, epididymal and perirenal)***

#### ***4.4.1.3.4. Organs weight (heart and liver)***

#### ***4.4.1.3.5. Biochemical investigations (Blood)***

- Lipid profile
  - A) Total Cholesterol
  - B) Triglycerides
  - C) Low density lipoprotein-cholesterol (LDL-C)
  - D) Very low density lipoprotein-cholesterol (VLDL-C)
  - E) High density lipoprotein-cholesterol (HDL-C)
  - F) Atherogenic Index (AI)
  - G) Coronary Risk Index (CRI)
  - H) Apolipoproteins-B (Apo-B)
- Blood glucose
- Insulin
- Homeostasis model assessment of insulin resistance (HOMA-IR)
- Leptin
- Lactate dehydrogenase (LDH)

#### ***4.4.1.3.6. $Na^+/K^+$ ATPase contents in hepatic and cardiac tissues***

#### ***4.4.1.3.7. Oxidative stress parameters in hepatic and cardiac tissues***

- Protein estimation
- Thiobarbituric acid reactive substances (TBARS)
- Glutathione (GSH)
- Superoxide dismutase (SOD)
- Catalase (CAT)
- Glutathione-S-transferase (GST) estimation
- Glutathione peroxidase (GPx) estimation
- Glutathione reductase (GR) estimation

#### ***4.4.1.3.8. Histopathological analysis of hepatic and cardiac tissues***

**4.4.2. PLAN- II****ANTIOBESITY POTENTIAL OF EMBELIN ISOLATED FROM *E.RIBES* IN HFD-INDUCED OBESITY IN WISTAR RATS****4.4.2.1. Treatment schedule**

Rats were fed with normal pellet diet (NPD) for 1 week in the experimental environment before the experiments were conducted. Once they had adapted to the environment, rats were divided into four groups of 10 rats each as follow:

<b>Group (n=10)</b>	<b>Treatment</b>
<b>I</b> Normal Control	Rats fed with NPD for the period of 28 days + 1% gum acacia (1 ml/kg bw, p.o) from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>II</b> Obesity Control	Rats fed with HFD for the period of 28 days + 1% gum acacia (1 ml/kg bw, p.o) from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>III</b> HFD+ Embelin	Rats fed with HFD for the period of 28 days + Embelin (50 mg/kg bw, p.o.) in 1% gum acacia from 8 <sup>th</sup> day to 28 <sup>th</sup> day
<b>IV</b> HFD+ Orlistat	Rats fed with HFD for the period of 28 days + Orlistat (10 mg/kg, bw, p.o.) in 1% gum acacia from 8 <sup>th</sup> day to 28 <sup>th</sup> day

Drug solutions were prepared freshly every day and administered by oral gavage at approximately 24 hour interval on each day. Food and water intake were measured daily while body weight was measured weekly for the period of 28 days. At the end of the experimental period (on 29<sup>th</sup> day), the animals were anesthetized with ether, following overnight fasting. After blood pressure measurement, blood was withdrawn by retro-orbital method into a tube and the serum was obtained by centrifugation (3000 RPM for 15 minutes). After collection of blood, rats were sacrificed; organs (heart, liver, kidney) and visceral fat pads (perirenal, epididymal, mesenteric) were excised immediately, rinsed with phosphate buffer saline and weighed. Heart, liver and kidney tissues were fixed in 10% neutral buffer formalin for histopathological analysis. The serum and organ samples were stored at -70°C until analysis.

**4.4.2.2. Parameters assessed****4.4.2.2.1. Anthropometric measurements**

- Food intake
- Water intake
- Body weight gain
- Body mass index (BMI)

**4.4.2.2.2. Blood pressure measurement**

- Heart rate
- Systolic blood pressure
- Diastolic blood pressure
- Mean arterial (blood) pressure

**4.4. 2.2.3. Visceral fat pads weight (mesenteric, epididymal and perirenal)****4.4. 2.2.4. Organs weight (heart, liver and kidney)****4.4. 2.2.5. Biochemical investigations (Blood)**

- Lipid profile
  - A) Total Cholesterol
  - B) Triglycerides
  - C) Low density lipoprotein-cholesterol (LDL-C)
  - D) Very low density lipoprotein-cholesterol (VLDL-C)
  - E) High density lipoprotein-cholesterol (HDL-C)
  - F) Atherogenic Index (AI)
  - G) Coronary Risk Index (CRI)
  - H) Apolipoproteins-B (Apo-B)
- Blood glucose
- Insulin
- Homeostasis model assessment of insulin resistance (HOMA-IR)
- Leptin

**4.4. 2.2.6.  $Na^+/K^+$ ATPase contents in hepatic and cardiac tissues****4.4. 2.2.7. Oxidative stress parameters in hepatic and cardiac tissues**

- A) Protein estimation

- B) Thiobarbituric acid reactive substances (TBARS)
- C) Glutathione (GSH)
- D) Superoxide dismutase (SOD)
- E) Catalase (CAT)
- F) Glutathione-S-transferase (GST) estimation
- G) Glutathione peroxidase (GPx) estimation
- H) Glutathione reductase (GR) estimation

***4.4. 2.2.8. Histopathological analysis of hepatic, cardiac and kidney tissues***

**4.4.3. PLAN- III****ANTIDIABETIC POTENTIAL OF ETHANOLIC EXTRACT OF *E.RIBES* IN HFD-FED AND LOW DOSE STREPTOZOTOCIN (STZ)-INDUCED TYPE 2 DIABETES IN WISTAR RATS****4.4.3.1. Development and characterization of HFD-fed and low dose STZ-treated type-2 diabetic rat model (Srinivasan *et al.*, 2005)**

After acclimatization of one week, the rats were divided into two groups as follows:

<b>Group</b>	<b>Treatment</b>	<b>No. of animals</b>
Normal Pellet Diet (NPD)	Rats fed with NPD from day 1 to day 14 <i>ad libitum</i>	20
High Fat Diet (HFD)	Rats fed with HFD from day 1 to day 14 <i>ad libitum</i>	80

After 2 weeks of dietary manipulation i.e. on 15<sup>th</sup> day, effect of NPD and HFD on body weights and biochemical estimations of blood glucose, serum triglycerides, serum total cholesterol, and serum insulin of Wistar rats were carried out. On 15<sup>th</sup> day (after estimations of biochemical parameters), a subset of the rats (10 rats from NPD-fed group and 70 rats from HFD-fed group) were injected intraperitoneally with low dose of STZ (35 mg/kg bw, single dose, pH 4.4 in citrate buffer). After 1 week of STZ injection i.e. on 21<sup>st</sup> day, body weights of all the rats were measured and biochemical estimations of blood glucose, serum triglycerides, serum total cholesterol, and serum insulin were carried out in all the rats (i.e. NPD+STZ and HFD+STZ groups) and effects were compared with NPD and HFD-fed rats. The rats with the fasting blood glucose level of  $\geq 200$  mg/dl in HFD+STZ group were considered diabetic and selected for further study. These rats were continued on HFD until the end of the study.

#### 4.4.3.2. Treatment schedule

For evaluation of antidiabetic potential of ethanolic extract of *E.ribes*, rats were divided into five groups of ten each:

Group (n=10)	Treatment
<b>I</b> Normal control (NC)	Normal control rats fed with NPD + 1% gum acacia (1 ml/kg bw, p.o) in normal saline daily for 21 days
<b>II</b> Diabetic control (DC)	Diabetic control rats administered HFD + 1% gum acacia (1 ml/kg bw, p.o) in normal saline daily for 21 days
<b>III</b> Diabetic control + Ethanolic extract of <i>E.ribes</i> (100 mg/kg bw)	Diabetic rats administered HFD + Ethanolic extract of <i>E.ribes</i> (100 mg/kg bw, p.o) in 1% gum acacia daily for 21 days
<b>IV</b> Diabetic control + Ethanolic extract of <i>E.ribes</i> (200 mg/kg bw)	Diabetic rats administered HFD + Ethanolic extract of <i>E.ribes</i> (200 mg/kg bw, p.o) in 1% gum acacia daily 21 days
<b>V</b> Diabetic control + Metformin (180 mg/kg bw)	Diabetic rats were administered HFD + Metformin (180 mg/kg bw, p.o) in 1% gum acacia daily 21 days

Drug solutions were prepared freshly every day and administered by oral gavage at approximately 24 hour interval on each day. Food and water intake were measured daily while body weight and blood glucose levels were measured weekly for the period of 28 days. At the end of the experimental period (on 29<sup>th</sup> day), the animals were anesthetized with ether, following overnight fasting. After blood pressure measurement, blood was withdrawn by retro-orbital method into a tube and the serum was obtained by centrifugation (3000 RPM for 15 minutes). After collection of blood, rats were sacrificed; organs (heart, liver, kidney, pancreas) were excised immediately, rinsed with phosphate buffer saline and weighed. Heart, liver and kidney and pancreas tissues were fixed in 10% neutral buffer formalin for histopathological analysis. The serum and organ samples were stored at -70°C until analysis.

**4.4.3.3. Parameters assessed****4.4.3.3.1. Anthropometric measurements**

- Food intake
- Water intake
- Body weight

**4.4.3.3.2. Blood pressure measurement**

- Heart rate
- Systolic blood pressure
- Diastolic blood pressure
- Mean arterial (blood) pressure

**4.4.3.3.3. Organs weight (heart, liver and kidney)**

- Heart Index [(Heart weight/body weight) × 100]
- Liver Index [(Liver weight/ body weight) × 100]
- Kidney Index [(Kidney weight/ body weight) × 100]

**4.4.3.3.4. Biochemical parameters (blood)**

- **Blood glucose estimation using glucometer**
- **Oral Glucose Tolerance Test (OGTT)**
- *Area under curve (AUC<sub>0-120 min</sub>) of OGTT*
- **Estimation of serum insulin levels in response to oral glucose challenge**
- *Area under curve (AUC<sub>0-120 min</sub>) of serum insulin levels in response to oral glucose challenge*
- Lipid profile
  - A) Total Cholesterol (TC)
  - B) Triglycerides (TG)
  - C) Low density lipoprotein-cholesterol (LDL-C)
  - D) Very low density lipoprotein-cholesterol (VLDL-C)
  - E) High density lipoprotein-cholesterol (HDL-C)
  - F) Atherogenic Index (AI)
  - G) Coronary Risk Index (CRI)
- Insulin



- Homeostasis model assessment of insulin resistance (HOMA-IR)
- Leptin
- Adiponectin
- Lipase
- Lactate dehydrogenase (LDH) estimation
- Creatinine
- Alkaline phosphatase
- Total protein
- Albumin

#### ***4.4.3.3.5. Biochemical parameters (hepatic tissue)***

- ***Determination of hepatic Glucose-6-Phosphatase (Glc-6-Pase) activity***
- ***Determination of hepatic glycogen content***
- ***Oxidative stress parameters in hepatic, cardiac and kidney tissues***
  - A) Thiobarbituric acid reactive substances (TBARS)
  - B) Reduced glutathione (GSH)
  - C) Superoxide dismutase (SOD)
  - D) Catalase (CAT)

#### ***4.4.3.3.6. Histopathological analysis of pancreatic, hepatic, cardiac and kidney tissues***

## 4.5. EXPERIMENTAL METHODS

### 4.5.1. Anthropometric measurements

#### *4.5.1.1. Measurement of food and water intake*

Food and water intakes were measured daily for the entire treatment period at the same time. Food intake and water intake were measured on per cage basis and the average food and water consumed were calculated. Unit for food consumption was gm/day/rat and unit for water consumption was ml/day/rat.

#### *4.5.1.2. Measurement of body weight and body mass index*

The body weights (g) were monitored weekly for the entire study period. Body mass index (BMI) was calculated from formula:  $BMI = \text{body weight (gm)} / \text{length}^2 \text{ (cm}^2\text{)}$

### 4.5.2. Blood pressure measurements

Blood pressure (systolic, diastolic, mean arterial blood pressure) and heart rate were measured by non-invasive blood pressure (NIBP) recorder using rat tail-cuff method (Kent Scientific Corporation, USA). Unit for heart rate was beats/min and unit for blood pressure was mmHg.



**Figure 7.** Non-invasive blood pressure recorder

#### *4.5.2.1. Animal preparation*

Rats were trained in rat restrainer for approximately 1 week, 15 minutes each day before beginning the experiment, so that trained rats could comfortably and quietly remain in the

holder during blood pressure measurement. Further, rats were placed in the rat holder at least 10-15 minutes prior to obtaining pressure measurements.

#### ***4.5.2.2. Animal body temperature***

Rats must have adequate blood flow in the tail to acquire a blood pressure signal. Thermoregulation is the method, by which the animal reduces its core body temperature, dissipates heat through its tail and generates tail blood flow. The room temperature should be at or above 26°C.

#### ***4.5.2.3. Volume pressure recording***

The non-invasive blood pressure methodology consists of utilizing a tail-cuff placed on the tail to occlude the blood flow. Upon deflation, one of the several types of noninvasive blood pressure sensors, placed distal to the occlusion cuff, can be utilized to monitor the blood pressure.

Volume pressure recording (VPR) sensor utilizes a specially designed differential pressure transducer to noninvasively measure the blood volume in the tail. VPR utilizes a volumetric method to measure the blood flow and blood volume in the tail, there are no measurement artifacts related to ambient light; movement artifact is also greatly reduced. Special attention is afforded to length of the occlusion cuff with VPR in order to derive the most accurate blood pressure readings. VPR is the most reliable, consistent and accurate method to non-invasively measure the blood pressure in mice as small as 10 grams to rats greater than 950 grams.

### **4.5.3. Organ and visceral fat pad weights**

At the end of experimental period, all the rats were euthanized, organs [heart and liver (Plan-I); heart, liver and kidney (Plan-II and III)] and visceral fat pads [mesenteric, epididymal, perirenal (Plan I and II)] were excised immediately, rinsed with phosphate buffer saline and weighed. Heart index [(heart weight/ body weight) × 100]; Liver index [(liver weight/ body weight) × 100] and kidney index [(Kidney weight/ body weight) × 100] were calculated (Plan-III).

#### ***4.5.3.1. Mesenteric fat pads***

Mesenteric fat is a fatty tissue inside the abdominal cavity, includes visceral fat and retroperitoneal fat. Mesenteric fat pads were collected from horizontal disposition of the peritoneum in the lower part of the abdomen of rats.

#### ***4.5.3.2. Epididymal fat pads***

Epididymal fat pad is a long, narrow, convoluted tube, part of the spermatic duct system that lies on the posterior aspect of each testicle connecting it to the vas deferens. Epididymal fat pads were collected from the region of the testis of rats.

#### ***4.5.3.3. Perirenal fat pads***

Perirenal fat is also known as ‘perinephric fat’ or ‘adipose capsule of kidney’. A structure between the renal fascia and renal capsule, and may be regarded as a part of the latter. A different structure, the perirenal fat, is the adipose tissue superficial to the renal fascia. Perirenal fat pads were collected from the region of both kidneys of rats.

### **4.5.4. Biochemical parameters (blood)**

#### ***4.5.4.1. Blood glucose measurement***

Fasting blood glucose levels were measured weekly on lateral tail vein blood samples using an ACCU-Check glucose meter (Roche Diagnostics, GmbH-Mannheim, Germany).

#### ***4.5.4.2. Oral glucose tolerance test***

Oral glucose tolerance test (OGTT) was also performed on the last day of the study using a methodology of Wang *et al.* (2011). After a 12-hour fasting, the rats of all groups were orally gavaged with 2 g/kg bw of glucose dissolved in distilled water (40% w/v). Blood glucose levels were measured at 0, 15, 30, 60, and 120 minutes after glucose administration with the glucose meter. The results were expressed as area under the curve (AUC<sub>0-120 min</sub>).

#### ***4.5.4.3. Estimation of serum insulin levels in response to oral glucose challenge***

Serum insulin levels were measured at the above indicated time points after glucose administration using ultra sensitive rat insulin ELISA kit (Crystal Chem Inc., IL, USA). The results were expressed as area under the curve (AUC<sub>0-120 min</sub>).

#### ***4.5.4.4. Serum Lipid profile***

Total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), concentrations in serum were measured using enzymatic method of diagnostic kit from Span Diagnostics Ltd., Surat, India. All experimental assays were done according to the manufacturer's instructions. Low density lipoprotein cholesterol (LDL-C) levels were calculated using Friedewald formula (Friedewald *et al.*, 1972):

$$\text{LDL-C} = \left[ \text{TC} - \left( \text{HDL-C} + \left( \frac{\text{TG}}{5} \right) \right) \right]$$

Very low density lipoprotein cholesterol (VLDL-C) concentration was calculated using formula:

$$\text{VLDL-C} = [\text{TC} - (\text{LDL-C} + \text{HDL-C})]$$

The atherogenic index (AI) was calculated using the formula (Dobiasova and Frohlich, 2001):

$$\text{AI} = \log (\text{TG}/\text{HDL-C})$$

Coronary risk index (CRI) was calculated as (Alladi and Shanmugasundaram, 1989):

$$\text{CRI} = \text{TC}/\text{HDL-C}$$

Atherogenic and coronary artery risk indices are strong and reliable indicators of whether or not cholesterol is deposited into tissues or metabolized and excreted.

***(A). Total Cholesterol and High density lipoproteins cholesterol estimation***

***(Span Diagnostics Ltd., Surat, India)***

*Assay Principle*

Cholesterol reacts with hot solution of ferric perchlorate, ethyl acetate and sulphuric acid and gives a lavender coloured complex which is measured at 560 nm.

High density lipoproteins cholesterol (HDL-C) is obtained in the supernatant after centrifugation. The cholesterol in the HDL fraction is also estimated by this method.

*Reagent (supplied in the kit)*

Reagent 1: Cholesterol reagent

Reagent 2: working cholesterol standard, 200 mg%

Reagent 3: Precipitating reagent

*Procedure*

1) *For Total Cholesterol*

<b>Pipette into tubes marked</b>	<b>Blank</b>	<b>Standard</b>	<b>Test</b>
Reagent 1: Cholesterol reagent	2 ml	2 ml	2 ml
Reagent 2: working cholesterol standard (200 mg%)	-	0.01 ml	-
Serum	-	-	0.01 ml

Mixed well and kept the tubes immediately in the boiling water bath exactly for 90 seconds.

Cooled them immediately to room temperature under running tap water. The O.D. of standard and test was measured against Blank on a spectrophotometer at 560 nm.

## 2) For HDL-Cholesterol

*(i) HDL-Cholesterol separation*

Pipette into centrifuge tube	Quantity
Sample	0.2 ml
Precipitating reagent	0.2 ml

Mixed well and kept at room temperature for 10 minutes and then centrifuged at 2000 RPM for 15 minutes to obtain a clear supernatant. Proceeded to step 2.

*(ii) HDL-Cholesterol estimation*

Pipette in to test tubes	Blank	Standard	Tests
Reagent 1: Cholesterol reagent	2 ml	2 ml	2 ml
Reagent 2: working cholesterol standard (200 mg%)	-	0.01 ml	-
Supernatant from step-1	-	-	0.12 ml

Mixed well and kept the tubes immediately in the boiling water bath exactly for 90 seconds. Cooled them immediately to room temperature under running tap water. The O.D. of standard and test was measured against reagent blank on a spectrophotometer at 560 nm.

Calculation

Calculated using formula given below and results were expressed as mg/dl.

## 1. Total cholesterol

$$\text{Total cholesterol } \left( \frac{\text{mg}}{\text{dl}} \right) = \frac{\text{O. D. of test}}{\text{O. D. of standard}} \times 200$$

## 2. HDL cholesterol

$$\text{HDL cholesterol } \left( \frac{\text{mg}}{\text{dl}} \right) = \frac{\text{O. D. of test}}{\text{O. D. of standard}} \times 50$$

***(B). Triglyceride estimation (Span Diagnostics Ltd., Surat, India)******Assay Principle***

Lipase hydrolyses triglycerides sequentially to Di and monoglycerides and finally to glycerol. Glycerol kinase (GK) using ATP as PO<sub>4</sub> source converts Glycerol liberated to Glycerol-3-phosphate (G-3-Phosphate). G-3-Phosphate oxidase (GPO) oxidises G-3-Phosphate formed to Dihydroxy acetone phosphate and hydrogen peroxide is formed. Peroxidase (POD) uses the hydrogen peroxide formed to oxidise 4-aminoantipyrine and TOOS (N-ethyl-N-sulphohydroxy propyl-m Toluidine) to a purple coloured complex. The absorbance of the coloured complex is measured at 546 nm which is proportional to Triglyceride concentration.

***Reagents (supplied in the kit)***

<b>Reagent No.</b>	<b>Reagent</b>	<b>Pack Size</b>
1	Triglycerides (Enzymes, Chromogen)	15 ml
2	Triglycerides (Buffer)	1 ml
3	Triglycerides standard (200 mg/dl)	1 ml

***Preparation of working reagent***

Content of one vial labelled 1 TRIGLYCERIDES was dissolved with 1.1 ml of 2 TRIGLYCERIDES (Buffer).

***Reaction parameters***

Type of Reaction	End Point
Wavelength	546nm
Flowcell temperature	30°C/37°C
Incubation	15 min. at 37°C
Standard Concentration	200 mg/dl
Sample Volume	10 µl

Reagent Volume	1 ml
Zero setting with	Reagent Blank
Light path	1 cm

Procedure

Pipette into test tubes	Procedure for 1 ml		
	Blank	Standard	Test
Working Reagent (ml)	1.0 ml	1.0 ml	1.0 ml
Standard (ml)	-	0.01 ml	-
Sample (ml)	-	-	0.01 ml

Mixed and incubate at 37°C for 15 minutes and read absorbance of test and standard against reagent blank at 546 nm.

Calculation

Calculated using formula given below and results were expressed as mg/dl.

$$\text{Triglycerides } \left( \frac{\text{mg}}{\text{dl}} \right) = \frac{\text{Absorbance of test}}{\text{Absorbance of std.}} \times 200$$

***(C). Apolipoprotein B estimation (Randox Laboratories Ltd., UK)***Assay Principle

This method is based on the reaction of a sample containing Apo-B and a specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm. By constructing a standard curve from the absorbance of standards the concentration of Apo-B can be determined.

Reagents (supplied in the kit)

Reagent No.	Reagent	Pack Size
R1	Assay buffer	4 × 50 ml
R2	Antibody Reagent	4 × 9 ml
CAL.	Calibrator	4 × 1 ml



Preparation of Reagents

- i) Buffer: Contents were ready for use.
- ii) Antibody Reagent: Contents were ready for use.
- iii) Calibrator: Opened the vial carefully, avoid any loss of the material and reconstituted with 1 ml of the distilled water. Replaced the rubber stopper; closed the vial and left to stand for 30 minutes before use. Ensured that all traces of dry material were dissolved by swirling gently.
- iv) Preparation of Standard:

	<b>S1</b>	<b>S2</b>	<b>S3</b>	<b>S4</b>	<b>S5</b>
Dilution	Saline	1+7	1+3	1+1	Nil

Procedure

It was recommended that all reagents and samples were equilibrated to room temperature prior to use. Pipetted into appropriate tube as followed:

	<b>Calibrators</b>	<b>Sample</b>	<b>Control</b>
Assay buffer	1.0 ml	1.0 ml	1.0 ml
Diluted calibrator	60 $\mu$ l	-	-
Control	-	-	60 $\mu$ l
Diluted Sample	-	60 $\mu$ l	-
Antibody reagent	200 $\mu$ l	200 $\mu$ l	200 $\mu$ l

Mixed and incubated at 37°C. Read the absorbance at 340 nm.

Calculation

The Apo-B standard curve was constructed by plotting the mean absorbance value for each calibrator on the Y axis versus the corresponding calibrator concentration on the X axis. Apo-B concentrations in the samples were interpolated using the standard curve and mean absorbance values for each sample. Following equation was obtained:

$$y = 0.993x + 3 \text{ [Correlation coefficient } R^2 = 0.986\text{]}$$

Where, y = Concentration of Apo-B in sample

x = Absorbance of sample at 340 nm

**4.5.4.5. Insulin estimation (Crystal Chem Inc., IL, USA)**Assay Principle

## 1. First reaction

Rat insulin in the sample is bound to the guinea pig anti-insulin antibody coated on the microplate well.

## 2. Washing

Unbound material is removed by washing.

## 3. Second reaction

Horse radish peroxidase (POD)-conjugated anti-insulin antibody is then bound to the guinea pig anti-insulin antibody/rat insulin complex immobilized to the microplate well.

## 4. Washing

Excess POD-conjugate is removed by washing.

## 5. Enzyme reaction

The bound POD conjugate in the microplate well is detected by the addition of the 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution.

## 6. Measurement of absorbance

## 7. Evaluation of results

The insulin concentration is determined via interpolation using the standard curve generated by plotting absorbance versus the corresponding concentration of rat insulin standard.

Reagents supplied

<b>Mark</b>	<b>Description</b>	<b>Amount</b>
A	Antibody-coated Microplate (One pack contains 6x8 well modules)	2 packs
B	Rat Insulin Standard, Lyophilized	2.56 ng/vial (for 100 µl)
C	Anti-Insulin Enzyme Conjugate Stock Solution	1 bottle (8 ml)
D	Enzyme Conjugate Diluent	1 bottle (4 ml)

E	Enzyme Substrate (TMB) Solution	1 bottle (13 ml)
F	Enzyme Reaction Stop Solution (1 N Sulfuric Acid)	1 bottle (13 ml)
G	Sample Diluent	1 bottle (30 ml)
H	Wash Buffer Stock Solution (20X Concentrate)	1 bottle (50 ml)
	Frame for affixing the microplate well module	1 piece
	Plastic microplate cover	1 piece

#### Storage and Stability

1. Ultra Sensitive Rat Insulin ELISA kit was stored at 2-8°C and avoided light exposure (not freeze the kit or held it at temperatures above 25°C).
2. The kit should not be used after the expiration date.

#### Preparation of reagents

Prior to use, all reagents were brought to room temperature (18-25°C), and were stored at 2-8°C immediately after use. Before use, reagents were mixed thoroughly by gentle agitation or swirling.

##### *1. Antibody-coated microplate*

Removed the “Antibody-coated Microplate” (marked “A”) from the sealed foil pouch after the pouch has been equilibrated to room temperature.

##### *2. Rat insulin stock solution*

Reconstitute the “Rat Insulin Standard, Lyophilized” (marked “B”) by careful addition of 100 µl of distilled or deionized water to the vial. Invert the vial gently until the contents are completely dissolved. This stock solution contained 25.6 ng/ml of rat insulin. The reconstituted rat insulin stock solution was stable for one week at 2-8°C.

##### *3. Anti-insulin enzyme conjugate*

For six modules, prepared the needed volume of anti-insulin enzyme conjugate solution by mixing 3.6 ml of “Anti-Insulin Enzyme Conjugate Stock Solution” (marked “C”) with

1.8 ml of “Enzyme Conjugate Diluent” (marked “D”), and mixed completely to ensure a homogeneous and clear solution. Avoided foaming during mixing.

#### *4. Enzyme substrate solution*

The “Enzyme Substrate Solution” (marked ‘E’) was provided as a ready-to-use preparation. Once the bottle is opened, the enzyme substrate solution was stable for one week at 2-8°C.

#### *5. Enzyme reaction stop solution (1 N sulfuric acid)*

The “Enzyme Reaction Stop Solution” (marked “F”) was provided as a ready-to-use preparation.

#### *6. Sample diluent*

The “Sample Diluent” (marked “G”) was provided as a ready-to-use preparation. Once the bottle was opened, the sample diluent was stable for one week at 2-8°C.

#### *7. Wash buffer*

The “Wash Buffer Stock Solution” (marked “H”) was brought to 1 litre with distilled or deionized water in a volumetric flask. Mixed the solution well before use. The wash buffer was stable for one week at 2-8°C.

#### Preparation of working rat insulin standards

1. 50 µl of sample diluent (marked “G”) and 50 µl of rat insulin stock solution (25.6 ng/ml) was pipetted into a polypropylene microtube labeled 12.8 ng/ml, and mixed thoroughly.
2. 50 µl of sample diluent was dispensed into seven polypropylene microtubes labeled 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/ml, respectively.
3. 50 µl of the 12.8 ng/ml standard was dispense into the 6.4 ng/ml microtube, and mixed thoroughly.
4. 50 µl of the 6.4 ng/ml standard was dispensed into the 3.2 ng/ml microtube, and mixed thoroughly.
5. This dilution scheme was repeated using the remaining microtubes.
6. 50 µl of sample diluent was dispensed into one polypropylene microtube labeled 0 ng/ml.

#### Assay Procedure

##### *First reaction:*

1. Removed the antibody-coated microplate modules (marked “A”) from the sealed foil pouch after the pouch has been equilibrated to room temperature. Microplate was affixed to the supporting frame.

2. In each well, 95  $\mu$ l of sample diluent (marked "G") was dispensed.
3. 5  $\mu$ l samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 ng/ml working rat insulin standards) were pipetted into the wells.
4. The microplate was covered with the plastic microplate cover and incubated for 2 hours at 4°C.

*Second reaction:*

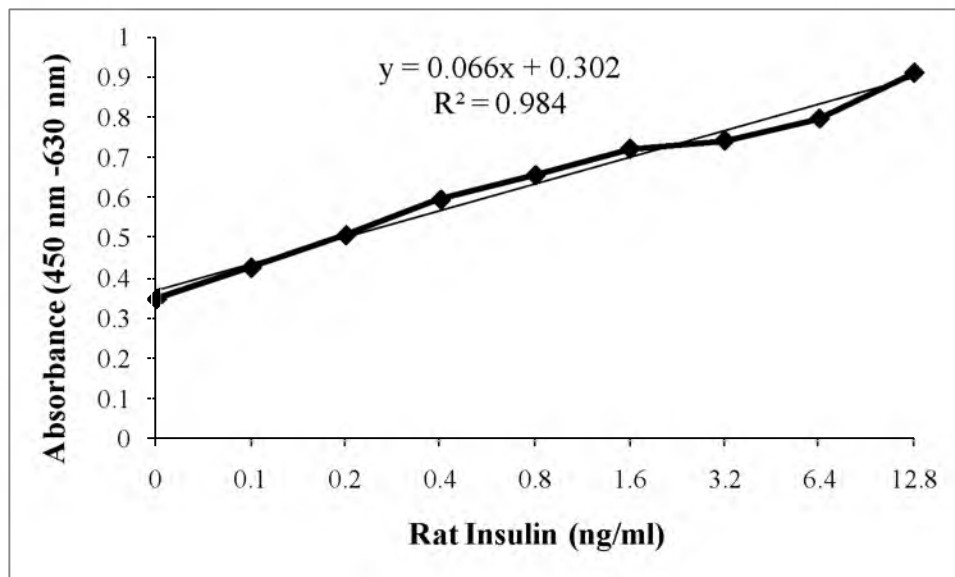
5. Well contents were aspirated and washed five times using 300  $\mu$ l of wash buffer per well. After each wash, any remaining solution was removed by inverting and tapping the plate firmly on a clean paper towel.
6. 100  $\mu$ l per well of anti-insulin enzyme conjugate was dispensed.
7. The microplate was covered with the plastic microplate cover and incubated for 30 minutes at room temperature.

*Third reaction:*

8. Well contents were aspirated and washed five times using 300  $\mu$ l of wash buffer per well. After each wash, any remaining solution was removed by inverting and tapping the plate firmly on a clean paper towel.
9. Immediately dispensed 100  $\mu$ l per well of enzyme substrate solution and allowed to react for 40 minutes at room temperature. During the enzyme reaction, exposing the microplate to light was avoided.
10. The enzyme reaction was stopped by adding 100  $\mu$ l per well of enzyme reaction stop solution (marked "F")
11. Absorbance was measured within 30 minutes using a plate reader. (Measured A450 values and subtract A630 values).

*Determining the insulin concentration*

1. The mean absorbance for each set of duplicate standards or samples was determined.
2. Using linear graph paper, the insulin standard curve was constructed by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat insulin concentration on the X axis.
3. Rat insulin concentrations in the samples were interpolated using the standard curve and mean absorbance values for each sample.



**Figure 8.** Standard calibration curve for insulin

#### 4.5.4.6. Homeostasis model assessment of insulin resistance (HOMA-IR)

HOMA-IR, an index of insulin resistance (IR), was calculated by following formula:

$$\text{HOMA} = \frac{\text{Insulin } (\mu\text{U/ml}) \times \text{glucose (mM)}}{22.5}$$

#### 4.5.4.7. Leptin estimation (Ray Biotech Inc., GA, USA)

##### Assay Principle

The RayBio® Rat Leptin ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of rat Leptin in serum, plasma and cell culture supernatants. This assay employs an antibody specific for rat Leptin coated on a 96-well plate. Standards and samples are pipetted into the wells and Leptin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-rat Leptin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Leptin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Reagents supplied

<b>Kit Components</b>	<b>Quality</b>
Anti-rat Leptin Coated Microtiter Strips (Item A)	96 wells
Wash Buffer concentrate (20X) (Item B)	25 ml
Standards (Item C); recombinant rat Leptin	2 vials
Assay Diluent D (Item K); 5x concentrated buffer. For Standard/Sample (serum/plasma samples/cell culture medium) diluents	15 ml
Assay Diluent B (Item E); 5x concentrated buffer. For detection antibody and HRP-Streptavidin diluents	15 ml
Detection Antibody Leptin (Item F); Biotinylated anti-rat Leptin (each vial is enough to assay half microplate)	2 vials
HRP-Streptavidin concentrate (Item G); 10,000x concentrated HRP-conjugated streptavidin	8 $\mu$ l
TMB One-Step Substrate Reagent (Item H); 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution	12 ml
Stop Solution (Item I); 2 M sulfuric acid	8 ml

Storage

The kit was stored at 2° to 8° C. Standard (recombinant protein) was stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Unused wells were returned to the pouch containing desiccant pack, reseal along entire edge.

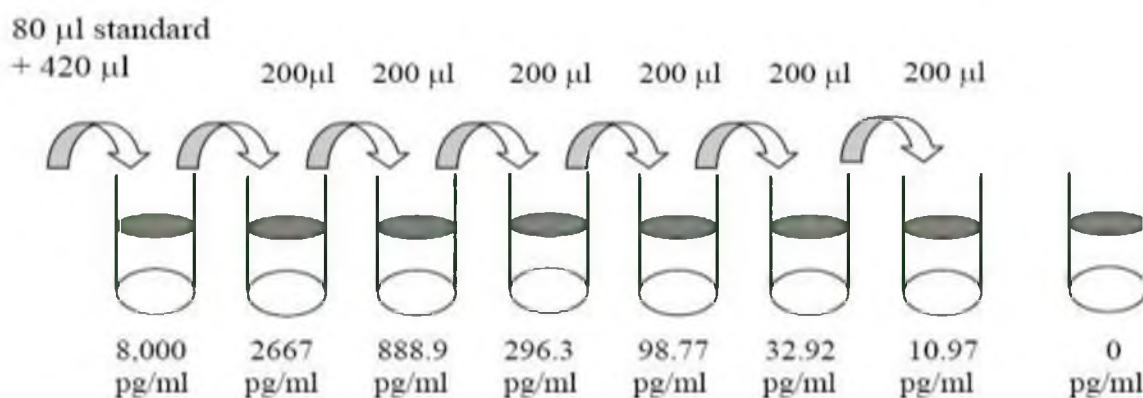
Additional materials required

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Precision pipettes to deliver 2  $\mu$ l to 1 ml volumes.

3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Log-log graph paper or computer and software for ELISA data analysis.
8. Tubes to prepare standard or sample dilutions.

#### Preparations of reagents

1. All reagents and samples were brought to room temperature (18 - 25°C) before use.
2. Sample dilution: If samples needed to be diluted, 1x Assay Diluent D (Item K) was used for dilution of serum.
3. Assay Diluent D (Item K) and Assay Diluent B (Item E) were diluted 5-fold with deionized or distilled water before use.
4. Preparation of standard: **the vial of Item C was briefly spinned**. 400  $\mu$ l 1x Assay Diluent D (Item K) was added into Item C vial to prepare a 50 ng/ml standard solution. **The powder was dissolved thoroughly by a gentle mix**. 80  $\mu$ l Leptin standard from the vial of Item C, was added into a tube with 420  $\mu$ l 1x Assay Diluent D to prepare a 8,000 pg/ml standard solution. 400 $\mu$ l 1x Assay Diluent D was pipette into each tube. The stock standard solution was used to produce a dilution series (shown below). Each tube was mixed thoroughly before the next transfer and gently vortexed to mix. 1x Assay Diluent D served as the zero standard (0 pg/ml).



5. If the Wash Concentrate (20x) (Item B) contained visible crystals, warmed to room temperature and mixed gently until dissolved. 20 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to yield 400 ml of 1x Wash Buffer.



6. The Detection Antibody vial (Item F) was briefly spinned before use. 100  $\mu$ l of 1x Assay Diluent B (Item E) was added into the vial to prepare a detection antibody concentrate. Pipetted up and down to mix gently (the concentrate could be stored at 4°C for 5 days). The detection antibody concentrate was diluted 80-fold with 1x Assay
7. Diluent Band used in step 4 of Part VI Assay Procedure. The HRP-Streptavidin concentrate vial (Item G) was briefly spinned and pipette up and down to mix gently before use. HRP-Streptavidin concentrate was diluted 10,000-fold with 1x Assay Diluent B (Item E).

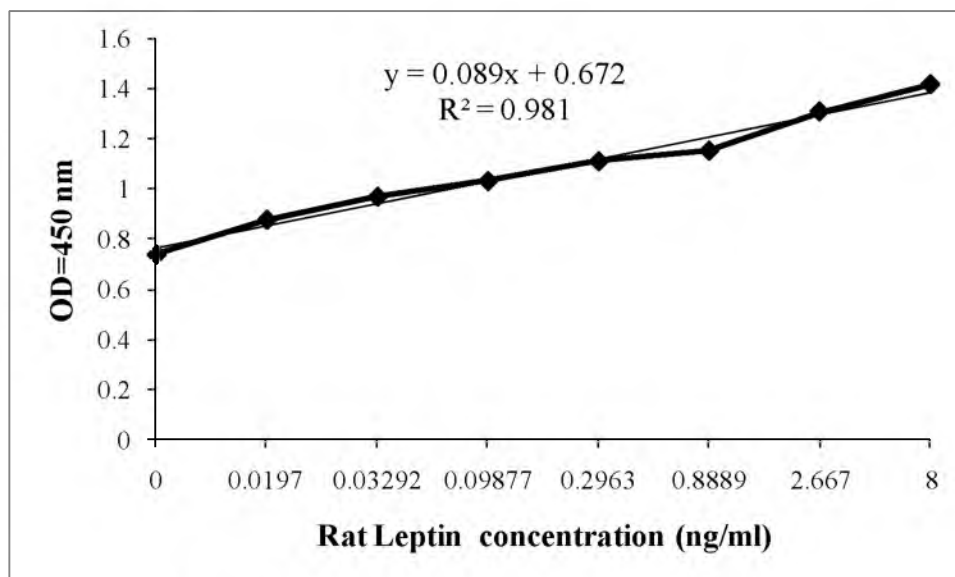
#### Assay Procedure

1. All reagents and samples were brought to room temperature (18-25°C) before use. It was recommended that all standards and samples be run at least in duplicate.
2. 100  $\mu$ l of each standard (see Reagent Preparation step 2) and sample were added into appropriate wells. Covered well and incubated for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
3. The solution was discarded and washed 4 times with 1x Wash Solution. Each well was washed by filling Wash Buffer (300  $\mu$ l) using autowasher. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted it against clean paper towels.
4. 100  $\mu$ l of 1x prepared biotinylated antibody (Reagent Preparation step 6) was added to each well. Incubated for 1 hour at room temperature with gentle shaking.
5. The solution was discarded. Repeated the wash as in step 3.
6. 100  $\mu$ l of prepared Streptavidin solution (see Reagent Preparation step 7) was added to each well. Incubated for 45 minutes at room temperature with gentle shaking.
7. The solution was discarded. Repeated the wash as in step 3.
8. 100  $\mu$ l of TMB One-Step Substrate Reagent (Item H) was added to each well. Incubated for 30 minutes at room temperature in the dark with gentle shaking.
9. 50  $\mu$ l of Stop Solution (Item I) was added to each well. Read at 450 nm immediately.

#### Calculation

The mean absorbance for each set of duplicate standards, controls and samples were calculated and subtracted the average zero standard optical density. The standard curve was plotted on log-log graph paper or using Sigma plot software, with standard concentration on

the x-axis and absorbance on the y-axis. The best-fit straight line was drawn through the standard points.



**Figure 9.** Standard calibration curve for leptin

#### 4.5.4.8. Serum adiponectin (Ray Biotech Inc., GA, USA)

##### Assay Principle

Adiponectin Enzyme Immunoassay (EIA) Kit is an *in vitro* quantitative assay for detecting Adiponectin peptide based on the principle of Competitive Enzyme Immunoassay. The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Adiponectin antibody, both biotinylated Adiponectin peptide and peptide standard or targeted peptide in samples interacts competitively with the Adiponectin antibody. Uncompeted (bound) biotinylated Adiponectin peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP) which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Adiponectin peptide in the standard or samples.

##### Reagents Supplied

Kit Components	Quality
Adiponectin Microplate coated with secondary	96 wells

antibody (Item A)	
Wash Buffer Concentrate (20X) (Item B)	25 ml
Standards Adiponectin Peptide (Item C)	2 vials
Anti-Adiponectin polyclonal antibody (Item N)	2 vials
Assay Diluent A (Item D); contains 0.09% sodium azide as preservative. For Standard/Sample (serum/plasma) diluent.	30 ml
Assay Diluent B (Item E); 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.	15 ml
Biotinylated adiponectin peptide (Item F)	2 vials
HRP-Streptavidin concentrate (Item G); 4,000x concentrated HRP-conjugated streptavidin	8 $\mu$ l
Positive control (Item M)	1 vials
TMB One-Step Substrate Reagent (Item H); 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution	12 ml
Stop Solution (Item I); 2 M sulfuric acid	8 ml

#### Storage

The kit was stored for up to 6 months at  $-20^{\circ}\text{C}$  from the date of shipment. Standard, Biotinylated Adiponectin peptide, and positive control were stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  (recommended at  $-80^{\circ}\text{C}$ ) after arrival. Opened microplate wells and antibody were stored for up to 1 month at  $2^{\circ}$  to  $8^{\circ}\text{C}$ . Unused wells were returned to the pouch containing desiccant pack, reseal along entire edge. Multiple freeze-thaws were avoided for Standard, Biotinylated Adiponectin peptide and positive control.

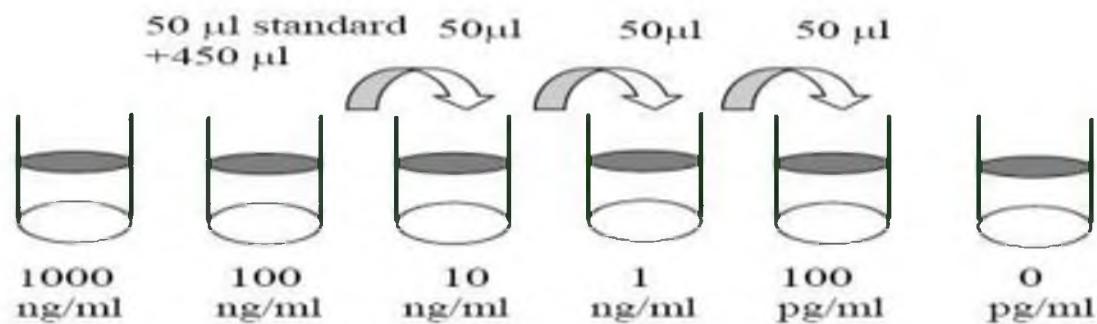
#### Additional materials required

1. Microplate reader capable of measuring absorbance at 450 nm.

2. Precision pipettes to deliver 2  $\mu$ l to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. SigmaPlot software (or other software which can perform four parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker
10. Aluminum foil
11. Saran Wrap

#### Reagent preparation

1. All reagents and samples were brought to room temperature (18-25°C) before use.
2. The Anti-Adiponectin Antibody vial (Item N) was briefly centrifuged before use. 50  $\mu$ l of 1x Assay Diluent B was added into the vial to prepare a detection antibody concentrate. Pipetted up and down to mix gently. The Anti-adiponectin antibody concentrate was diluted 100-fold with 1x Assay Diluent B. This was anti-adiponectin antibody working solution.
3. The vial of Biotinylated Adiponectin (Item F) was briefly centrifuged before use. 10  $\mu$ l of biotinylated Adiponectin (Item F) was added to 5 ml of Assay Diluent A. Pipetted up and down to mix gently. The final concentration of biotinylated Adiponectin was 20 ng/ml.
4. Preparation of standard: standard Adiponectin vial (Item C) was briefly centrifuged. In a separate tube, 10  $\mu$ l of standard Adiponectin Peptide (Item C) was pipette into 990  $\mu$ l of biotinylated Adiponectin solution (prepared in step 3 above) to prepare a 1000 ng/ml standard. Pipetted up and down to mix gently. 50  $\mu$ l of 1000 ng/ml Adiponectin standard was added into a tube with 450  $\mu$ l of biotinylated Adiponectin solution. This was stock standard solution (100 ng/ml Adiponectin, 20 ng/ml biotinylated Adiponectin). 450  $\mu$ l of biotinylated Adiponectin solution was pipette into 6 tubes. The stock standard was used to produce a dilution series (shown below). Mixed each tube thoroughly before the next transfer. Biotinylated Adiponectin served as the zero standard (0 pg/ml), or total binding.



5. Sample dilution: If samples needed to be diluted, Assay Diluent A + biotinylated Adiponectin was used for serum/plasma samples. The final concentration of the biotinylated Adiponectin was 20 ng/ml in all diluted samples.
6. Positive control dilution: the positive control vial (Item M) was briefly centrifuged. To the tube of Item M, 101 µl Assay Diluent B (Item E) and 4 µl of 10-fold diluted biotinylated Adiponectin peptide (item F) were added.
7. Assay Diluent B (Item E) was diluted 5-fold with deionized or distilled water.
8. If the 20X Wash Concentrate (Item B) contained visible crystals, warmed to room temperature and mix gently until dissolved. 20 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
9. The HRP-Streptavidin concentrate vial (Item G) was briefly spinned before use. HRP-Streptavidin concentrate was diluted 4,000-fold with 1X Assay Diluent B.

#### Procedure

1. All reagents and samples were brought to room temperature (18-25°C) before use.
2. 100 µl anti-Adiponectin antibody was added to each well and incubated for 1.5 hours.
3. The solution was discarded and wells were washed 5 times with 1x Wash Solution (200 µl each).
4. 100 µl of each standard, positive control and sample were added into appropriate wells. Covered well and incubate for 2.5 hours at room temperature or overnight at 4°C.
5. The solution was discarded and washed 4 times with 1x Wash Solution (200 µl each).
6. 100 µl of prepared HRP-Streptavidin solution was added to each well. Incubated for 45 minutes at room temperature.
7. The solution was discarded and washed 5 times with 1x Wash Solution (200 µl each).
8. 100 µl of TMB One-Step Substrate Reagent (Item H) was added to each well. Incubate for 30 minutes at room temperature in the dark.

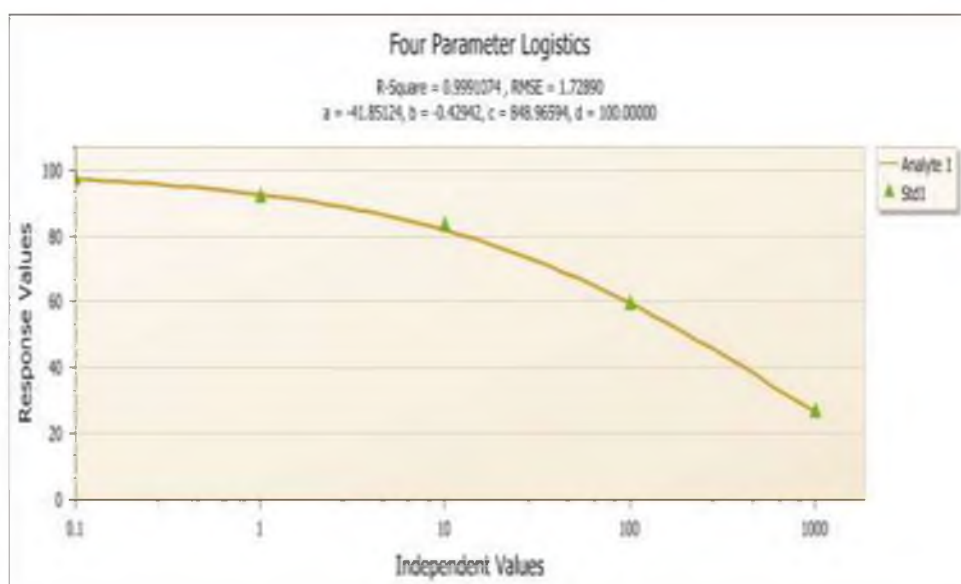
9. 50  $\mu$ l of Stop Solution (Item I) was added to each well. Read at 450 nm immediately.

#### Calculation

The mean absorbance for each set of duplicate standards, controls and samples, were calculated and subtracted the blank optical density. The standard curve, with standard concentration on the x-axis and percentage of absorbance on the y-axis were plotted. The best-fit straight line was drawn through the standard points.

$$\text{Percentage absorbance} = \frac{(B - \text{blank OD})}{(B_0 - \text{blank OD})}$$

Where, B = OD of sample or standard, B<sub>0</sub> = OD of zero standard (total binding)



**Figure 10.** Standard calibration curve for adiponectin

#### Formula for calculation

$$Y = \frac{a - d}{[1 + (\frac{X}{c})^b]} + d$$

Where a = -41.85, b = -0.42942, c = 848.97, d = 1000

X = conc. of adiponectin (ng/ml).

#### **4.5.4.9. Lipase activity (BioAssay Systems, CA, USA)**

##### Assay Principle

Lipase catalyzes the hydrolysis of ester bonds on the glycerol backbone of a lipid substrate. Lipase Assay is based on an improved dimercaptopropanol tributyrate (BALB) method, in which SH groups formed from lipase cleavage of BALB react with 5,5'-dithiobis (2-

nitrobenzoic acid) (DTNB) to form a yellow colored product. The color intensity, measured at 412 nm, is proportionate to the enzyme activity in the sample.

#### Reagent preparation

1. Sample Preparation: Lipase inhibitors (EDTA, and certain detergents Tween-20, NP-40), mercaptoethanol and dithiothreitol interfere with this assay and were avoided in sample preparation. Samples were stored frozen for at least one month, if not assayed immediately.
2. Preparation of Working Reagent: Color Reagent was mixed into Assay Buffer and shake vial to mix. 0.8 ml BALB Reagent (sufficient for 100 assays) was added. Alternatively for partial reconstitution: for each well of reaction, 5 mg Color Reagent, 140  $\mu$ l Assay Buffer and 8  $\mu$ l BALB Reagent were mixed. The Working Reagent was prepared freshly and used within one hour.
3. 150  $\mu$ l H<sub>2</sub>O and 150  $\mu$ l Calibrator were transferred into wells of a clearbottom 96-well plate. 10  $\mu$ l samples were added into separate wells. 140  $\mu$ l Working Reagent was added to each sample well. Plate was briefly tapped to mix reaction mixture.
4. Read OD 412 nm on a plate reader at 10 min (OD<sub>10min</sub>) and at 20 min (OD<sub>20min</sub>).

#### Calculation

Lipase activity (U/L) was calculated as follows:

$$\text{Lipase Activity} = \frac{\text{OD}_{20\text{ min}} - \text{OD}_{10\text{ min}}}{\text{OD}_{\text{Calibrator}} - \text{OD}_{\text{H}_2\text{O}}} \times 735$$

Where, OD<sub>20min</sub> and OD<sub>10min</sub> were the OD<sub>412nm</sub> values of the sample at 20 min and 10 min, respectively. OD<sub>Calibrator</sub> and OD<sub>H<sub>2</sub>O</sub> are the OD<sub>412nm</sub> values of the Calibrator and water at 20 min. The number “735” was the equivalent activity (U/L) of the calibrator under the assay conditions.

#### **4.5.4.10. Lactate dehydrogenase (Crest Biosystems, Goa, India)**

##### Assay Principle

Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the LDH activity in the sample.

Reagents

Reagent No.	Reagent	Pack Size
L1	Buffer Reagent	20 ml
L2	Starter Reagent	5 ml

Working reagent preparation

Reagents were ready to use.

Working reagent: the contents of 1 bottle of L2 (Starter Reagent) was poured into 1 bottle of L1 (Buffer Reagent). This working reagent was stable for at least 1 week when stored at 2-8°C. Alternatively desired amount of working reagent may be made by mixing together 4 parts of L1 (Buffer Reagent) and 1 part of L2 (Starter Reagent). Alternatively 0.8 ml of L1 and 0.2 ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Reaction parameter

Reaction	U.V.Kinetic
Wavelength	340 nm
Zero Setting	Distilled water
Incubation Temperature	37°C
Delay Time	60 seconds
Read Time	180 seconds
Number of readings	4
Interval	60 seconds
Sample volume	0.02 ml
Reagent volume	1 ml



Factor	8095
Reaction Slope	Decreasing
Linearity	2000 U/L
Units	U/L

Procedure

Pipette in to test tubes	Test
Working reagent	1 ml
Incubate at the assay temperature for 1 minute and add	
Sample	0.05 ml

Mixed well and read the initial absorbance  $A_0$  after 1 minute and repeated the absorbance reading after every 1, 2, and 3 minutes. The mean absorbance change per minute ( $\Delta A/\text{minute}$ ) was calculated.

Calculation

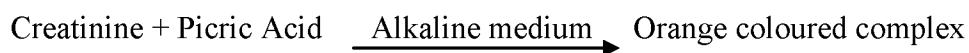
LDH activity (IU/L) was calculated as

$$\text{LDH activity} = \Delta A/\text{min} \times 8095$$

Where  $\Delta A$  = mean change in absorbance per minute

**4.5.4.11. Creatinine (Span Diagnostics Ltd., Surat, India)**Assay Principle

Creatinine reacts with Picric Acid in an alkaline medium to form an orange coloured complex. The rate of formation of this complex is measured by reading the change in absorbance at 505 nm in a selective interval of time and is proportional to the concentration of Creatinine. The reaction time and the concentration of Picric Acid and Sodium Hydroxide have been optimized to avoid interference from ketoacids.

Reagents Composition

Reagent	Reagent	Pack Size	Composition	Concentration
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No.				
1.	Picrate Reagent	1×50 ml	Picric Acid Preservative	40 mM/L qs
2.	Sodium Hydroxide	1×50 ml	Sodium Hydroxide	200 mM/L
3.	Creatinine Standard	1×1 ml	Creatinine Stabiliser	2 mg/dL qs

#### Working Reagent preparation

Working Reagent was prepared by mixing equal volume of Reagent 1 (Picrate Reagent) with Reagent 2 (Sodium Hydroxide) to make up the desired volume and mixed gently for 2 minutes. Reagent 3 was ready-to-use.

#### Reagent Storage and Stability

##### *Prior to reconstitution*

Unopened Reagents 1 and 2 were stable at room temperature (15-30°C) and Reagent 3 was stable at 2-8° C until the expiry date mentioned on the container label.

##### *After reconstitution*

The 'Working Reagent' was stable for 7 days at 2-8°C.

#### Assay Parameters

Mode	Initial rate
Flow-cell temperature	37° C
Wavelength	505 nm (490-530 nm)
Reaction direction	Increasing
Optical path length	1 cm
Blanking	Purified water
Sample volume	100 µl
Working Reagent volume	1000 µl

Concentration of standard	2 mg/dl
Delay	30 seconds
Interval	120 seconds
Number of readings	1
Permissible Reagent Blank absorbance	< 0.35 AU
Linearity	Upto 20 mg/dl
Units	mg/dl

### Procedure

Pipette into tubes marked	Standard	Test
Serum	-	100 µl
Reagent 3	100 µl	-
Working Creatinine Reagent	1000 µl	1000 µl

Mixed well and programmed the analyser as per assay parameters.

1. The analyser was blanked with Purified Water.
2. Initial absorbance of the Standard (AS1) was measured after 30 seconds and final absorbance (AS2) after an interval of another 120 seconds
3. After Standard reading were noted, the reading of Test (AT1) and AT2 were taken accordingly (follow same procedure as for Standard)

### Calculation

$$\text{Serum Creatinine } \left( \frac{\text{mg}}{\text{dl}} \right) = \frac{\text{AT2} - \text{AT1}}{\text{AS2} - \text{AS1}} \times 2$$

Where, AT1= Initial O.D. of Test

AT2= Final O.D. of Test

AS1= Initial O.D. of Standard

AS2= Final O.D. of Standard

**4.5.4.12. Alkaline Phosphatase (Span Diagnostics Ltd., Surat, India)**Assay Principle

At pH 10.3, Alkaline Phosphatase (ALP) catalyses the hydrolysis of colourless p-Nitrophenyl Phosphate (pNPP) to yellow coloured p-Nitrophenol Phosphate. Change in absorbance due to Yellow colour formation is measured kinetically at 405 nm and is proportional to ALP activity in the Sample.

Reagents Composition

Reagent No.	Reagent	Pack Size	Composition	Concentration
1.	AMP (2-Amino-2-Methyl-1 Propanol) buffer	1×24 ml	AMP Magnesium acetate Zinc sulphate Chelator	300 mM 2 mM 0.8 mM qs
2.	pNPP substrate	20 vials	pNPP Stabiliser	10 mM qs

Working Reagent preparation

‘Working Reagent’ was prepared by reconstituting one vial of Reagent 2 (pNPP Substrate) with Reagent 1 (AMP Buffer) as mentioned below, then dissolved properly by gentle swirling: 1 vial of Reagent 2 + 1.2 ml of Reagent 1.

Reagent Storage and Stability*Prior to reconstitution*

Unopened Reagents were stable at 2-8°C until the expiry date mentioned on the container label.

*After reconstitution*

‘Working Reagent’ was stable at 2-8°C for 30 days and room temperature (15-30°C) for 2 days, when stored in dark.

*Assay Parameters*

Mode	Kinetic
Flow-cell temperature	37° C
Wavelength	405 nm
Reaction direction	Increasing
Optical path length	1 cm
Blanking	Purified water
Sample volume	20 µl
Reagent volume	1000 µl
Delay	30 seconds
Interval	30 seconds
Number of readings	4
Permissible Reagent Blank absorbance	< 1 AU
Kinetic factor	2712
Maximum $\Delta A$ /minute	0.36
Linearity	Upto 1000 IU/L
Units	IU/L

*Procedure*

<b>Pipette into tube marked</b>	<b>Test</b>
Serum	20 µl
Working ALP Reagent	1000 µl

Mixed well and aspirated immediately for measurement.

1. The spectrophotometer was blanked with purified water.
2. The absorbance was read after 30 seconds. The reading was repeated after every 30 seconds, upto 120 seconds at 405 nm wavelength.

3. The mean absorbance change per minute ( $\Delta A/\text{minute}$ ) was determined.

#### Calculation

ALP activity (IU/L) =  $\Delta A/\text{minute} \times \text{Kinetic factor}$

Where,  $\Delta A/\text{minute}$  = Change in absorbance per minute

Kinetic factor (K) = 2712

Kinetic factor is calculated using following formula:

$$K = \frac{1}{M} \times \frac{TV}{SV} \times \frac{1}{P} \times 10^6$$

Where, M = Molar extinction coefficient of p- Nitrophenol and was equal to  $18.8 \times 10^3$  lit/mol/cm at 405 nm

TV = Sample volume + Working Reagent volume

SV = Sample volume

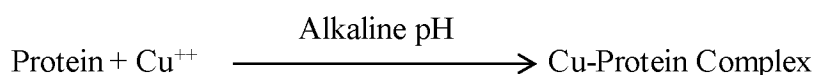
P = Optical path length

$10^6$  = Constant

#### **4.5.4.13. Total Protein (Span Diagnostics Ltd., Surat, India)**

##### Assay Principle

The peptide bonds of Protein react with Cupric ions in alkaline solution to form a coloured chelate, the absorbance of which is measured at 578 nm. The Biuret Reagent contains Sodium-Potassium Tartrate, which helps in maintaining solubility of this complex at alkaline pH. The absorbance of final colour is proportional to the concentration of Total Protein in the Sample.



##### Reagents Composition

Reagent No.	Reagent	Pack Size	Composition	Concentration
1.	Biuret Reagent	1×100 ml	Copper Sulphate Sodium Hydroxide Sodium-Potassium Tartrate Surfactant	7 mM/l 200 mM/l 20 mM/l qs
2.	Protein standard	1×3 ml	BSA Preservative	6.5 g/dl qs

Working Reagent Preparation

Reagents were ready-to-use.

Reagent Storage and Stability

Reagent 1 was stable at room temperature (15-30°C) and Reagent 2 was stable at 2-8°C until the expiry date mentioned on the container label.

Assay Parameters

Mode	End Point
Flow-cell temperature	37° C
Wavelength	578 nm
Optical path length	1 cm
Blanking	Reagent Blank
Sample volume	10 µl
Reagent volume	1000 µl
Incubation time	5 minutes
Concentration of Standard	6.5 g/dl
Stability of final colour	2 hours
Permissible Reagent Blank absorbance	< 0.2 AU
Linearity	Upto 1000 IU/L
Units	IU/L

Procedure

<b>Pipette into tube marked</b>	<b>Blank</b>	<b>Standard</b>	<b>Test</b>
Serum	-	-	10 µl
Reagent 2	-	10 µl	-
Reagent 1	1000 µl	1000 µl	1000 µl

Mixed well and incubated at 37°C for 5 minutes.

1. The spectrophotometer was blanked with Reagent Blank.
2. Absorbance of the Standard followed by the Test was measured.
3. Results were calculated as per the given calculation formula.

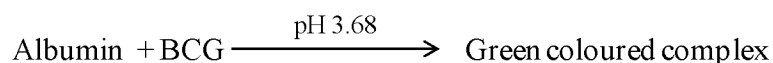
Calculation

$$\text{Total Protein concentration } \left( \frac{\text{g}}{\text{dl}} \right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 6.5$$

**4.5.4.14. Albumin (Span Diagnostics Ltd., Surat, India)**

Assay Principle

At pH 3.68, albumin acts as a cation and binds to the anionic dye Bromocresol Green [BCG], forming a green coloured complex. The absorbance of final colour is measured at 630 nm. The colour intensity of the complex is proportional to Albumin concentrations in the Sample.



Reagent Composition

Reagent No.	Reagent	Pack Size	Composition	Concentration
1.	Albumin Reagent	1×100 ml	Succinic acid Bromocresol Green Sodium Hydroxide Buffer pH 3.68	37 mM/l 0.15 mM/l 1 mM/l qs
2.	Albumin standard	1×3 ml	BSA Preservative	4 g/dl qs

Working Reagent Preparation

Reagents were ready-to-use.

Reagent Storage and Stability

Reagent 1 was stable at room temperature (15-30°C) and Reagent 2 was stable at 2-8°C until the expiry date mentioned on the container label.



Assay Parameters

Mode	End Point
Flow-cell temperature	37° C
Wavelength	630 nm (600-630 nm)
Optical path length	1 cm
Blanking	Reagent Blank
Sample volume	10 µl
Reagent volume	1000 µl
Incubation time	1 minute
Concentration of Standard	4 g/dl
Stability of final colour	2 hours
Permissible Reagent Blank absorbance	< 0.1 AU
Linearity	Upto 6 g/dl
Units	g/dl

Procedure

<b>Pipette into tube marked</b>	<b>Blank</b>	<b>Standard</b>	<b>Test</b>
Serum	-	-	10 µl
Reagent 2	-	10 µl	-
Reagent 1	1000 µl	1000 µl	1000 µl

Mixed well and incubated at room temperature (15-30°C) for 1 minute.

1. The spectrophotometer was blanked with Reagent Blank.
2. Absorbance of the Standard followed by the Test was measured.
3. Results were calculated as per the given calculation formula.

Calculation

$$\text{Albumin } \left(\frac{\text{g}}{\text{dl}}\right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 4$$

#### **4.5.5. Biochemical parameters (tissue)**

##### ***4.5.5.1. $Na^+/K^+$ ATPase activity in hepatic and cardiac tissue (Fiske and SubbaRow, 1925)***

###### **Assay Principle**

The liberation of organic phosphorous on incubation of tissue extract in medium containing Tris-HCl buffer, NaCl, KCl and ATP was measured spectrophotometrically.

###### **Reagents**

- (i) Tris-HCl Buffer (75 mM; pH-7.5)
- (ii) Magnesium sulphate ( $MgSO_4$ ) (5 mM)
- (iii) Potassium chloride (KCl) (50mM)
- (iv) Sodium Chloride (NaCl) (600 mM)
- (v) Ethylene diamine tetra acetic acid (EDTA) (1 mM)
- (vi) Adenosine Triphosphate substrate (ATP) (3 mM)
- (vii) Trichloroacetic acid (TCA) (10%)
- (viii) Ammonium Molybdate (2.5%)
- (ix) Sulphuric acid ( $H_2SO_4$ ) (5 N)
- (x) Ascorbic acid (10%)

###### **Reagents preparation**

- (i) Tris-HCl Buffer (75 mM; pH-7.5): 178.2 mg of Tris-HCl was dissolved in 10 ml of distilled water and its pH was adjusted to 7.5 using 1 N NaOH.
- (ii)  $MgSO_4$  (5 mM): 3.07 mg of  $MgSO_4$  was dissolved in 0.5 ml of distilled water.
- (iii) KCl (50mM): 1.86 mg of KCl was dissolved in 0.5 ml of distilled water.
- (iv) NaCl (600 mM): 17.53 mg of NaCl was dissolved in 0.5 ml of distilled water.
- (v) EDTA (1 mM): 1.46 mg of EDTA was dissolved in 5 ml of distilled water.
- (vi) ATP (3 mM): 1.65 mg of ATP was dissolved in 1 ml of distilled water.
- (vii) TCA (10%): 1 gm of TCA was dissolved and made upto 10 ml with distilled water.
- (viii) Ammonium Molybdate (2.5%): 125 mg of ammonium molybdate was dissolved in 5 ml of 5 N  $H_2SO_4$ .
- (ix)  $H_2SO_4$  (5 N): 1.38 ml of Conc.  $H_2SO_4$  was made upto 10 ml with distilled water.
- (x) Ascorbic acid (10%): It was prepared by dissolving 0.15 gm of ascorbic acid in 1.5 ml of distilled water.

Procedure

1. 50 mg of tissue was homogenized in 0.5 ml 50 mM potassium phosphate buffer.
2. The homogenate was centrifuged at 10,000 RPM at 4<sup>0</sup>C for 20 minutes in (C24, REMI Cooling Centrifuge).

<b>Reagents</b>	<b>Blank</b>	<b>Test</b>
Tris-HCl buffer	1.8 ml	1.5 ml
MgSO <sub>4</sub>	-	0.1 ml
KCl	-	0.1 ml
NaCl	-	0.1 ml
EDTA	-	0.1 ml
ATP	-	0.1 ml
Homogenate	-	0.1 ml
	Incubate at 37 <sup>0</sup> C for 30 minutes	
10% TCA	1 ml	1 ml
	Centrifuge and transfer 0.5 ml supernatant	
Ammonium molybdate	0.5 ml in both Blank and Test	
Ascorbic acid	0.2 ml in both Blank and Test	
	Incubate for 10 minutes and measured the absorbance at 620 nm in spectrophotometer	

Calculation

Na<sup>+</sup>/ K<sup>+</sup> ATPase activity was expressed as  $\mu$ moles of phosphorous liberated/h/gm tissue.

Calculated from regression equation:  $Y = 0.444x + 0.1808$

Where, Y = Absorbance at 620 nm

X = Na<sup>+</sup>/ K<sup>+</sup> ATPase activity

#### 4.5.5.2. Determination of hepatic Glc-6-Pase activity

##### (A). Preparation of liver microsomes (Pushparaj et al., 2007)

Rat liver (1 g) was homogenized by a polytron homogenizer in 10 ml ice-cold sucrose solution (0.25 M). The homogenate obtained was centrifuged at  $11,000 \times g$  for 30 min at  $4^\circ$  C after which the supernatant was re-centrifuged at  $70,000 \times g$  for 75 min at  $4^\circ$  C. The microsomal pellet was suspended in 1ml ice-cold sucrose (0.25 M) and then further homogenized for 10 s. Aliquots of the microsomal suspension were stored at  $-70^\circ$  C.

##### (B). Determination of microsomal protein content (Lowry et al., 1951)

###### Assay Principle

Protein reacts with Folin's ciocalteau phenol reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein as in the Biurette test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

###### Reagents

- (i) Alkaline sodium carbonates (2%  $\text{Na}_2\text{CO}_3$  in 0.10 N NaOH)
- (ii) Copper sulphate in sodium or potassium tartrate (0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium or potassium tartrate)
- (iii) Alkaline copper solution
- (iv) Folin Reagent
- (v) Bovine serum albumin (BSA) solution (0.5 mg/ml)
- (vi) Potassium Chloride (KCl) (0.15 M)

###### Preparation of reagents

- (i). 2%  $\text{Na}_2\text{CO}_3$  in 0.10 N NaOH: 0.10 N NaOH was prepared by dissolving 100 mg of NaOH in distilled water and the volume was made upto 25 ml with distilled water. Then 0.5 gm of  $\text{Na}_2\text{CO}_3$  was added in 25 ml of 0.10 N NaOH solution.
- (ii). 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium or potassium tartrate: 30 mg of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was dissolved in 5 ml of distilled water to prepare 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  solution. 50 mg of sodium potassium tartrate was dissolved in 5 ml distilled water to prepare 1% sodium potassium tartrate solution. Then the  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  solution was added to the sodium potassium tartrate solution with thorough mixing.
- (iii). Alkaline copper solution: It was prepared by mixing 25 ml of reagent (i) and 0.5 ml of reagent (ii).

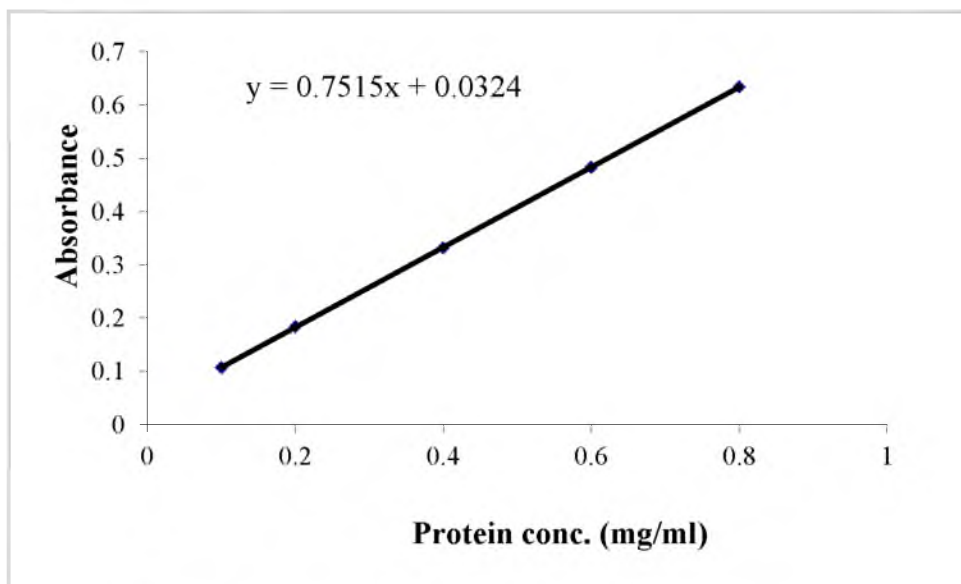
- (iv). Folin reagent: 1 ml of Folin reagent was diluted with 2 ml distilled water (1:2).
- (v). BSA solution (0.5 mg/ml): 2.5 mg of BSA was dissolved in 5 ml of distilled water to get a solution of 0.5 mg/ ml of protein.
- (vi). 0.15 M KCl: 57 mg of KCl was dissolved in 5 ml of distilled water.

#### Procedure

1. 100 mg of hepatic tissue was homogenized in 1 ml 0.15 M KCl and centrifuged at 10,000 RPM for 10 minutes (REMI centrifuge).
2. 1 ml of supernatant was mixed with 5 ml of alkaline copper solution and allowed to stand for 10 minutes at room temperature.
3. 0.5 ml diluted Folin reagent was then added and shaken well to mix the solution, incubate for 30 minutes at room temperature.
4. After 30 minutes the absorbance of sample was read in UV-visible spectrophotometer (UV-1601, SHIMADZU) at 750 nm against blank.
5. Blank was prepared by adding 5 ml alkaline copper solution, 0.5 ml folin's reagent to 1 ml of distilled water.
6. The protein content was expressed in mg/ml.

#### Preparation of standard curve

1. 5 ml of BSA solution (0.5 mg/ml) was prepared and different volumes taken in 6 tubes.
2. To all tubes distilled water was added to make up final volume of 1 ml.
3. The protein concentration in the above 6 tubes was estimated as procedure mentioned above.
4. A graph was plotted between concentrations of protein and optical density (OD).
5. This standard plot was used to calculate the concentration of protein in each ml suspension of the sample.



**Figure 11.** Standard calibration curve for protein

#### Calculation

Amount of protein in particular tissue was calculated using Protein standard curve:

$$Y = 0.751x + 0.0324 \times D.F$$

Where, Y = Absorbance

X = Protein concentration (mg/ml); D.F.= Dilution Factor

#### **(C). Assay of Glc-6-Pase activity (Baginski et al., 1967)**

##### Reagents

1. Sucrose solution (0.25 M): 8.56 gm of sucrose was dissolved in 100 ml distilled water.
2. Citrate Buffer (0.1 M, pH 6.5): 294.12 mg of sodium citrate was dissolved in 10 ml distilled water; 210.14 mg of citric acid was dissolved in 10 ml distilled water. 10 ml of sodium citrate and 10 ml of citric acid were mixed to give 0.1 M citrate buffer. pH 6.5 was adjusted using 1 N NaOH. Citrate buffer was used as stabilizers for the phosphomolybdate reduced by ascorbic acid.
3. ATP (0.1 M): 50.71 mg of ATP was dissolved in 1 ml of distilled water. It is common substrate for various enzymatic analysis.
4. 2% ascorbic acid in 10% trichloroacetic acid (A-TCA): 0.25 gm of ascorbic acid was dissolved in 12.5 ml of 10% ascorbic acid. It was used for hydrolysis of organophosphate compounds.

5. Ammonium molybdate (1%): 0.125 gm of ammonium molybdate was dissolved in 12.5 ml of distilled water.
6. 2% sodium citrate and 2% sodium arsenite in 2% acetic acid was added. It binds with excess molybdate and prevent it from reacting with inorganic phosphate.

#### Procedure

1. Hepatic Glc-6-Pase activity was determined by pipetting 0.2 ml portion of suspension into test tube containing 0.1 ml citrate buffer and 0.1 ml ATP (0.1 M)
2. The above mixture was incubated for 15 min at 37 °C and reaction was terminated by addition of 2 ml of ascorbic acid-trichloroacetic acid (A-TCA)
3. Add 0.5 ml of ammonium molybdate solution and 1 ml arsenite-citrate reagent
4. Control was prepared in similar manner except that suspension was pipetted after A-TCA addition.
5. Enzyme activity was determined by measuring optical density at 700 nm in spectrophotometer.
6. Unit of enzyme activity was  $\mu\text{g}$  of P liberated/ml/min

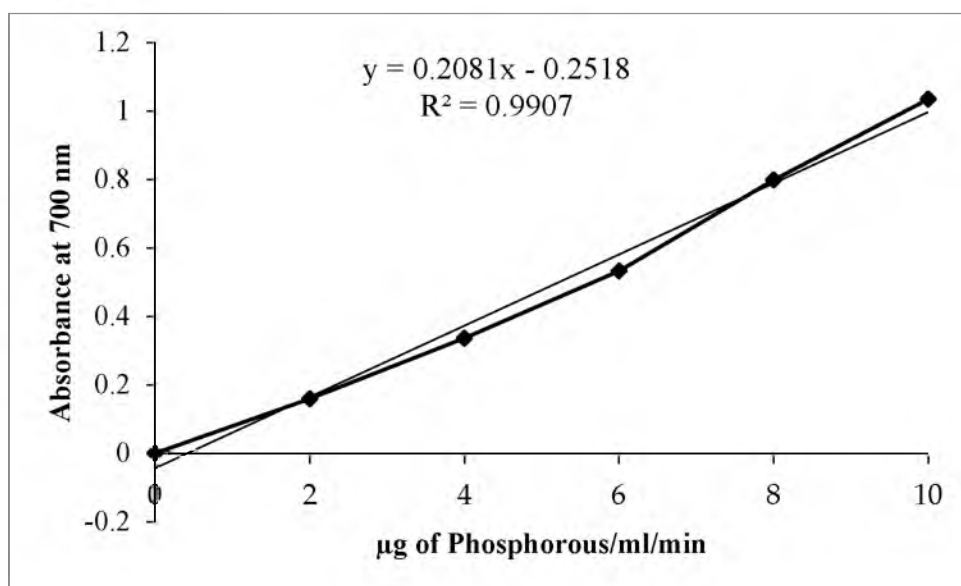
#### Preparation of standard curve

##### *Reagents*

1. Phosphorous stock standard: 21.9 mg of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) was dissolved in 5 ml distilled water.
2. Phosphorous working standard: 0.25 ml of the stock standard was diluted to 25 ml with 2.5 ml of 100% trichloroacetic acid.
3. Trichloroacetic acid (TCA; 10%): 5 gm of TCA was dissolved in 50 ml of distilled water.
4. Ascorbic acid (10%): 1 gm of ascorbic acid was dissolved in 10 ml of distilled water.
5. Ammonium molybdate solution: 0.5 gm of ammonium molybdate was dissolved in 50 ml of distilled water.
6. Arsenite-citrate reagent: 1 gm of sodium citrate and 1 gm of sodium arsenite were dissolved in 40 ml of distilled water, 1 ml of glacial acetic acid was added and diluted the volume upto 50 ml with distilled water.

*Procedure*

1. 0.0 ml, 0.5 ml, 1.0 ml, 1.5 ml, and 2.0 ml of the working phosphorous standard were diluted to 2.0 ml with 10% trichloroacetic acid, mixed well and kept for 10 min (1 ml aliquot)
2. 0.1 ml of distilled water was added to each tube
3. 0.2 ml of 10% ascorbic acid was added to each tube
4. 0.5 ml of ammonium molybdate was added to each tube
5. 1 ml of arsenite-citrate reagent was added
6. After 15 minutes, the absorbance of well mixed solution at 700 nm against reagent blank was read.
7. Blank: Instead of 1 ml aliquots 1 ml distilled water was used and rest of procedure was same.



**Figure 12.** Standard calibration curve for Glc-6-pase

#### 4.5.5.3. Determination of hepatic glycogen content (Yadav et al., 2007)

##### Assay Principle

Carbohydrates are first hydrolysed into simple sugars using acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 650 nm.



*Reagents*

1. Anthrone reagent: 0.5 g of anthrone was dissolved in 250 ml of 95% (w/v) H<sub>2</sub>SO<sub>4</sub>. Anthrone formed a green color product with hydroxyl methyl furfural (dehydrated product of glucose) with an absorption maximum at 650 nm.
2. KOH (10 N): 70.14 g of KOH was dissolved in 125 ml of distilled water. KOH was used for hydrolysis of the liver tissue for determination of glycogen content.
3. Trichloroacetic acid (5%): 5 g of TCA was dissolved in 100 ml distilled water. TCA was used to extract glycogen from liver tissue.
4. Acetic acid: Acetic acid was used to neutralize excess of alkali (KOH).

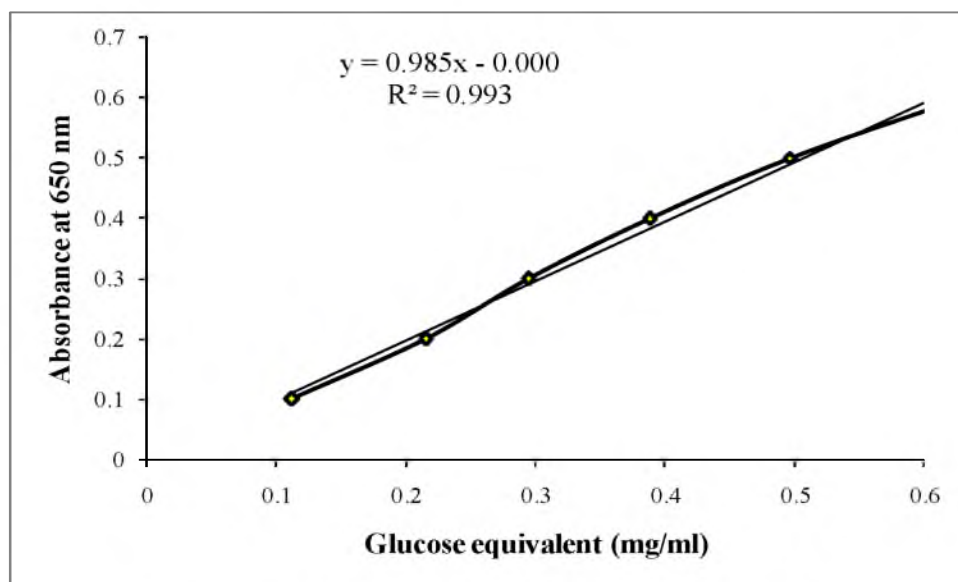
*Procedure*

1. Liver tissue (200 mg) was finely grounded with 20 ml of 5% trichloroacetic acid in a homogenizer. The protein precipitate was filtered and clear filtrate was taken for analysis.
2. Liver filtrate (2 ml) was pipetted into a 20 ml calibrated test tube and then 2 ml of 10 N KOH was added before placing it in a boiling water bath for 1 h.
3. After cooling, 1 ml of acetic acid was added to neutralize the excess of alkali and fluid was brought up to the mark 20 ml with distilled water
4. Slowly, 2 mL of solution from the previous step was added to 4 mL of anthrone reagent in a separate test tube, which was placed in cold water to prevent excessive heating.
5. After thorough shaking, the test tube was placed in a boiling water bath for exactly 10 min for the development of color and cooled with running tap water.
6. The OD was read within 2 h at 650 nm.

*Preparation of standard curve*

1. 2 ml of TCA was heated to 100°C with 2 ml of 10 N KOH for 1 h.
2. After cooling, 1 ml of acetic acid was added to neutralize the excess of alkali.
3. Add 4 ml of glucose solution containing (0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg, 0.5 mg, 0.6 mg) in distilled water and make final volume up to 20 ml with distilled water.
4. 2 mL of solution from the previous step was added to 4 mL of anthrone reagent in a separate test tube, which was placed in cold water to prevent excessive heating.
5. After thorough shaking, the test tube was placed in a boiling water bath for exactly 10 min for the development of color and cooled with running tap water.

6. The OD was read within 2 h at 650 nm.



**Figure 13.** Standard calibration curve for glycogen

#### 4.5.5.4. Oxidative stress parameters in hepatic, cardiac and kidney tissues

##### (A). Thiobarbituric acid reactive substances (TBARS) estimation (Ohkawa et al., 1979)

###### Assay Principle

Lipid peroxidation is a free radical mediated event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of thiobarbituric acid to give a pink colour, the absorbance of which is determined at 540 nm. The colorimetric reaction of TBA (Thiobarbituric acid) with MDA, a secondary product of lipid peroxidation has been widely accepted for measuring lipid peroxidation. It is also known as TBARS (Thiobarbituric acid reactive substance) estimation.

###### Reagents

- (i) Potassium Chloride (KCl) (0.15 M)
- (ii) 0.8% Thiobarbituric acid (TBA) solution
- (iii) 30% Trichloro acetic acid (TCA) solution

###### Preparation of reagents

- (i) KCl (0.15 M): 57.5 mg KCl was dissolved in 10 ml of distilled water.

(ii) TBA (0.8%): 40 mg of TBA was dissolved in 4.95 ml of distilled water and 50  $\mu$ l of glacial acetic acid.

(iii) TCA (30%): 0.30 gm of TCA was dissolved in 3 ml distilled water.

#### Procedure

1. 100 mg of tissue was homogenized in 1 ml 0.15 M KCl and centrifuged at 10,000 RPM for 10 minutes (REMI centrifuge).
2. 1 ml of suspension medium was taken from the above tissue homogenate. 0.5 ml of 30% TCA was added to it, followed by 0.5 ml of 0.8% TBA reagent. The tubes were then covered with aluminium foil and kept in water bath for 30 minutes at 80°C.
3. After 30 minutes tubes were taken out and kept in ice-cold water for 30 minutes and centrifuged at 3000 rpm for 15 minutes (R-BC DX REMI centrifuge).
4. The absorbance of the supernatant was read in spectrophotometer (UV-1601, SHIMADZU) at 540 nm against appropriate blank.
5. Blank consisted of 1 ml distilled water, 0.5 ml of 30% TCA and 0.5 ml of 0.8% TBA.

#### Calculation

The amount of MDA present in a sample was calculated according to the following equation and the result was expressed as nanomole of MDA/mg of protein.

$$\text{Nanomole of MDA/mg protein} = \frac{V \times OD_{540}}{0.156 \times \text{mg protein}}$$

Where, V = Final volume of the test solution

OD<sub>540</sub> = Optical density at 540 nm

#### **(B). Assay for glutathione (GSH) (Sedlak and Lindsay, 1968)**

##### Assay Principle

This is spectrophotometric procedure is based on the method of Ellman's reagent -5, 5' dithiobis-2-nitrobenzoic acid (DTNB) is reduced by SH group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH.

##### Reagents

- (i) Ethylene diamine tetra acetic acid (EDTA) (0.2 M)
- (ii) Tris Buffer (0.4 M, pH 8.9)
- (iii) 5, 5' dithiobis-2-nitrobenzoic acid (DTNB) (0.01 M)
- (iv) Trichloroacetic acid (TCA) (50%)

Preparation of reagents

- (i) EDTA (0.2 M): 0.37 gm EDTA was dissolved in 5 ml of warm distilled water.
- (ii) EDTA (0.02 M): 1 ml of above solution was diluted to 10 ml with distilled water.
- (iii) Tris Buffer (0.4 M, pH 8.9): 0.48 gm of Tris buffer was dissolved in 2 ml of distilled water, 2 ml of 0.2 M EDTA was added to it and final volume of solution was made up to 20 ml with distilled water. The pH was adjusted to 8.9 with 1 N HCl.
- (iv) DTNB (0.01 M): 1.98 mg of DTNB was dissolved in 0.5 ml of absolute methanol.
- (v) Trichloroacetic acid (50%): 2.5 gm of TCA was dissolved in 5 ml distilled water.

Procedure

1. 100 mg of tissue was homogenized in 1 ml of 0.02 M EDTA.
2. 1 ml of the homogenate was mixed with 0.8 ml of distilled water and 0.2 ml 50% TCA.
3. The tubes were then shaken intermittently for 10-15 minutes and then centrifuged at 3000 RPM for 15 minutes (R-BC DX, REMI centrifuge).
4. 2 ml of supernatant was mixed with 4 ml of 0.4 M Tris buffer and 0.1 ml DTNB.
5. The absorbance was read within 5 minutes of addition of DTNB at 410 nm against reagent blank with no homogenate in spectrophotometer (UV-1601, SHIMADZU).
6. Reagent Blank consisted of 4 ml Tris buffer + 0.1 ml DTNB.
7. Standard: Method same as that of test except std. glutathione solution (50 µg/ml) was used.

Calculation

The amount of GSH in the tissue was calculated from the following equation and expressed as µg/ml of protein.

$$C_o = A_o/A \times C \times D.F.$$

Where  $C_o$  = Concentration of GSH in tissue

$A_o$  = O.D of sample at 412 nm

$A$  = O.D. of standard at 412 nm

$C$  = Concentration of GSH;  $D.F$  = Dilution Factor

***(C). Superoxide dismutase (SOD) estimation (Marklund and Marklund, 1974)******Assay Principle***

Pyrogallol (1,2,3- benzenetriol) autooxidizes rapidly in aqueous solution, higher the pH faster is the autoxidation and several intermediate products are formed. Thus, the solution first becomes yellow brown with a spectrum showing absorbance between 400-425 nm. After few minutes the colour begins to turn green and finally after a few hours, yellow colour appears. Superoxide anion radical catalyses the autoxidation of pyrogallol. A simple and rapid method for assay of SOD is described, based on the ability of the enzyme to inhibit the autoxidation of pyrogallol.

***Reagents***

- (i) NaOH (1N)
- (ii) Tris HCl buffer (pH 8.5)
- (iii) Phosphate buffer (50 mM/L, pH 7.4)
- (iv) Pyrogallol (24 mM)

***Reagents preparation***

- (i) 1N NaOH: It was prepared by dissolving 400 mg NaOH in 10 ml distilled water.
- (ii) Tris HCl buffer (pH 8.5): 157.6 mg of Tris HCl buffer and 37.2 mg of EDTA was dissolved in 20 ml distilled water and pH will be adjusted to 8.5 using 1 N NaOH.
- (iii) Phosphate buffer (50 mM/L, pH 7.4): It was prepared by mixing potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (6.81 gm in 1000 ml distilled water) and disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) (8.90 gm in 1000 ml distilled water) in ration of 1:1.55
- (iv) Pyrogallol (24 mM): 1.5 mg of pyrogallol was dissolved in 0.5 ml of 10 mM HCl.

***Procedure***

1. 20 mg of tissue was homogenized in 2 ml of potassium phosphate buffer.
2. The homogenate were centrifuged at 10,000 RPM at 4 °C for 20 minutes in REMI centrifuged (C24 REMI Cooling centrifuge).
3. 100  $\mu\text{l}$  of supernatant was added to 3 ml Tris HCl buffer, followed by 25  $\mu\text{l}$  of pyrogallol and then mixed thoroughly.

4. The change in absorbance at 420 nm was recorded at 1 min. interval for 3 minutes in spectrophotometer (UV-1601, SHIMADZU)

#### Calculation

1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture and given by formula

$$\text{Unit of SOD / ml of sample} = \frac{\frac{\Delta A}{\text{min}} \times 8116}{\text{mg of protein}}$$

#### **(D). Catalase (CAT) estimation (Claiborne, 1985)**

##### Assay Principle

In the UV range H<sub>2</sub>O<sub>2</sub> shows continuous increase in absorption with decreasing wavelength. The decomposition of H<sub>2</sub>O<sub>2</sub> can be followed directly by increase in absorbance at 240 nm. The difference in absorbance ( $\Delta A$ ) per unit time is measure of catalase activity.

##### Reagents

- (i) Potassium phosphate buffer (50 mM, pH 7.4)
- (ii) Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) (30%, 19 mM/ L)

##### Reagents preparation

- (i) Potassium phosphate buffer (50 mM, pH 7.4): It was prepared by mixing potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) (6.81 gm in 1000 ml distilled water) and disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O) (8.90 gm in 1000 ml distilled water) in ration of 1:1.55
- (ii) H<sub>2</sub>O<sub>2</sub> (30%, 19 mM/ L): It was prepared by mixing 28  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> in 15 ml phosphate buffer.

##### Procedure

1. 50 mg of tissue was homogenized in 0.5 ml 50 mM potassium phosphate buffer.
2. The homogenate was centrifuged at 10,000 RPM at 4<sup>0</sup>C for 20 minutes in (C24, REMI Cooling Centrifuge).
3. 50  $\mu$ l of supernatant was then added to cuvette containing 2.95 ml 19 mM/ L H<sub>2</sub>O<sub>2</sub>.
4. The disappearance of H<sub>2</sub>O<sub>2</sub> was monitored at 240 nm wavelength at 1 minute interval for 3 minutes in spectrophotometer (UV-1601, SHIMADZU).

### Calculation

Catalase activity was calculated by following equation and the results were expressed as nanomoles of H<sub>2</sub>O<sub>2</sub> consumed/ minutes/mg protein.

$$\text{Catalase activity} = \frac{\frac{\Delta A}{\text{min}} \times \text{Total volume of assay}}{0.81 \times \text{sample volume} \times \text{mg of protein}}$$

### ***(E). Glutathione-S-transferase (GST) estimation (Habig et al., 1974)***

#### Assay Principle

The glutathione S-transferases, a group of enzymes active in the initial step of mercapturic acid synthesis, catalyze the formation of thioethers by the addition of GSH to a large number of compounds bearing an electrophilic carbon. This pattern suggested that the transferrase might be effective in reactions wherein GSH participates at electrophilic centres other than carbon.

#### Reagents

- (i) Phosphate Buffer (0.1 M, pH 7.0)
- (ii) Reduced Glutathione (1 mM)
- (iii) 1-chloro-2,4 dinitrobenzene (CDNB) (1 mM)

#### Reagent preparation

- (i) Phosphate Buffer (0.1 M, pH 7.0): It was prepared by mixing Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) (6.81 gm in 1000 ml distilled water) and disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O) (8.90 gm in 1000 ml distilled water) in ratio of 1:1.55.
- (ii) Reduced glutathione (1 mM): It was prepared by dissolving 9 mg of reduced glutathione in 1 ml distilled water.
- (iii) CDNB (1 mM): It was prepared dissolving 4 mg of CDNB in 0.25 ml distilled water.

#### Procedure

1. 50 mg of tissue was homogenized in 0.5 ml of Phosphate buffer and the homogenate was centrifuged at 10,000 RPM at 4<sup>0</sup>C for 20 minutes in cooling centrifuge (C24 REMI Cooling centrifuge).
2. For GST activity, the reaction mixture consisted of 1.47 ml phosphate buffer, 0.2 ml reduced glutathione, 0.025 ml CDNB and 0.3 ml post mitochondrial supernatant (PMS) (10%) in total volume of 2 ml.

3. The changes in absorbance was recorded at 340 nm in spectrophotometer at 1 min. interval for 3 minutes (UV-1601, SHIMADZU).

#### Calculation

The enzymatic activity was calculated as nanomol CDNB conjugate formed /min/mg protein using a molar extinction coefficient of  $9.6 \times 10^3 \text{ mM}^{-1} \text{ cm}^{-1}$

Nanomol CDNB conjugate formed/min/mg protein

$$= \frac{\frac{\Delta A}{\text{min}} \times \text{volume of assay} \times 1000}{9.6 \times \text{volume of PMS} \times \text{mg protein}}$$

#### ***(F). Glutathione peroxidase (GPx) estimation (Mohandas et al., 1984)***

##### Assay Principle

The enzyme GPx has been reported to reduce hydroperoxides to water by using GSH as a hydrogen donor. Spectrophotometric determination of NADPH oxidized per minute reflects enzymatic activity.

##### Reagents

- (i) Ethylene diamine tetra acetic acid (EDTA) (10 mM)
- (ii) Sodium azide ( $\text{NaN}_3$ ) (10 mM)
- (iii) NADPH (0.2 mM)
- (iv) Reduced Glutathione (GSH), (1 mM)
- (v) Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (30%)
- (vi) Phosphate buffer (0.1M, PH 7.4)

##### Preparation of reagents

- (i) EDTA (10 mM): It was prepared by dissolving 1.86 mg EDTA in 0.5 ml distilled water.
- (ii)  $\text{NaN}_3$  (10 mM): It was prepared by dissolving 0.325 mg  $\text{NaN}_3$  in 0.5 ml distilled water.
- (iii) NADPH (0.2 mM): It was prepared by dissolving 1.66 mg NADPH in 1 ml distilled water.
- (iv) GSH (1 mM): It was prepared by dissolving 3.72 mg GSH in 1ml distilled water.
- (v) 30%  $\text{H}_2\text{O}_2$ : It was prepared by mixing 1.5 ml  $\text{H}_2\text{O}_2$  and 3.5 ml distilled water.
- (vi) Phosphate buffer (0.1M, pH 7.4): It was prepared by mixing  $\text{KH}_2\text{PO}_4$  (6.81 gm in 1000 ml distilled water) and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (8.90 gm in 1000 ml distilled water) in ration of 1:1.55.



Procedure

1. 50 mg of tissue was homogenized in 0.5 ml of phosphate buffer (0.1M, pH 7.4).
2. The homogenate was centrifuged at 10,000 RPM at 4 °C for 20 minutes in REMI centrifuge (C24 REMI Cooling centrifuge).
3. The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml NaN<sub>3</sub> (1 mM), 0.05 ml GSH (1 mM), 0.1 NADPH (0.2 mM), 0.01 ml H<sub>2</sub>O<sub>2</sub> (0.25 mM) and 0.1 ml Post mitochondrial supernatant (PMS) (10% w/v) in a total volume of 2 ml.
4. The disappearance of NADPH at 340 nm is recorded at 25 °C in spectrophotometer at 1 min interval for 3 minutes (UV-1601, SHIMADZU)

Calculation

GPx activity is defined as nanomol NADPH oxidized/min/mg protein, using a molar extinction coefficient of  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

$$\text{Nanomol NADPH oxidized/min/mg protein} = \frac{\frac{\Delta A}{\text{min}} \times \text{Volume of assay} \times 1000}{6.22 \times \text{volume of PMS} \times \text{mg protein}}$$

***(G). Glutathione reductase (GR) estimation (Carlberg and Mannervik, 1985)***Assay Principle

Glutathione reductase (Glutathione:NADP<sup>+</sup> oxidoreductase; GR) is the key enzyme of glutathione metabolism and is widespread in all tissues and blood cells. This enzyme catalyses reduction of oxidized glutathione (GSSG) to glutathione (GSH) in the presence of NADPH. Functions of GSH in reductive processes are essential for protein synthesis, regulation of enzyme, and protection of the cells against reactive oxygen species and free radicals generated in cellular metabolism.

Reagents

- (i) Phosphate buffer (0.1 M, pH 7.0)
- (ii) Ethylene diamine tetra acetic acid (EDTA) (0.5 mM)
- (iii) NADPH (0.1 mM)
- (iv) Oxidized glutathione (GSSG) (1 mM)

Reagents preparation

- (i) Phosphate buffer (0.1 M, pH 7.0): It was prepared by mixing potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (6.81 gm in 1000 ml distilled water) and disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) (8.90 gm in 1000 ml distilled water) in ratio of 1:1.55
- (ii) EDTA (0.5 mM): It was prepared by dissolving 6.12 mg of EDTA in 0.5 ml distilled water.
- (iii) NADPH (0.1 mM): It was prepared by dissolving 1.6 mg of NADPH in 1 ml distilled water.
- (iv) GSSG (1 mM): It was prepared by dissolving 1.01 mg of GSSG in 0.25 ml distilled water.

Procedure

1. 50 mg of tissue was homogenized in 0.5 ml of Phosphate buffer and the homogenate was centrifuged at 10,000 RPM at 4°C for 20 minutes in cooling centrifuge (C24 REMI Cooling centrifuge).
2. The reaction mixture consisted of 1.65 ml phosphate buffer, 0.1 ml EDTA, 0.05 ml GSSG, 0.1 ml NADPH and 0.1 ml post mitochondrial supernatant (PMS) in total volume of 2.0 ml.
3. The enzymatic activity was quantified at 25°C by measuring disappearance of NADPH at 340 nm in spectrophotometer (UV-1601, SHIMADZU).

Calculation

Enzymatic activity is calculated as nanomol NADPH oxidized/min/mg protein, using a molar extinction coefficient of  $6.22 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$

$$\text{Nanomol NADPH oxidized/min/mg protein} = \frac{\frac{\Delta A}{\text{min}} \times \text{volume of assay} \times 1000}{6.22 \times \text{volume of PMS} \times \text{mg protein}}$$

**4.5.6. Histopathological analysis**

Cardiac and hepatic tissues (Plan-I), cardiac, hepatic and kidney tissues (Plan-II), pancreatic, hepatic, cardiac and kidney tissues (Plan-III) samples were fixed in 10% neutral buffered formalin, and then dehydrated by successively passing through a gradient of mixtures of ethyl alcohol and water. The samples were rinsed by xylene and embedded in paraffin. Tissue sections (5µm thickness) were cut, stained with hematoxylin and eosin dye

(H&E), and examined under a light microscopy by a pathologist blinded to the group studied

#### **4.5.7. Statistical analysis**

Data were represented as mean  $\pm$  standard error of the mean (SEM). The unpaired Student's *t*-test was used for analyzing the data between two groups, whereas one-way ANOVA (analysis of variance) followed by Dunnett's *t*-test was employed, if there were more than two groups using Graph pad InStat<sup>®</sup> version 3.06 (Graph Pad Software, San Diego, CA, USA). A value of  $p < 0.05$  was considered statistically significant.