CHAPTER 3

MATERIALS
&
METHODS
3.1. Plant material and preparation of aqueous extract (aqWC)

Fruit of *W. coagulans* was purchased from the local market of Delhi and was identified and authenticated by National Institute of Science Communication and Information Resources, Pusa, New Delhi (Voucher Number, NISCAIR/RHMD/Consult/-2008-09/979/10).

Whole fruit of *W. coagulans* was taken to prepare the extract. After removal of calyx and pedicle, 10.0 kg of fruit were ground and soaked in distilled water (2.0 L) and kept overnight in cold at 4-8°C. After 24 hours of incubation, the extract was vortexed and filtered through a filter paper/sterile muslin cloth to get water extract of fruit of *W. coagulans* (aqWC). Extract was lyophilized and get brown gum (yield 16% w/w) and store at 4°C and used for further experiment.

3.2. Phytochemical screening of aqWC extract

3.2.1. Detection of carbohydrates (Ronsenthaler 1930)

1.0 gm of extract was dissolved in 10.0 mL of distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

3.2.1.1. Molisch’s test

1.0 gm of α-Napthol was dissolved in 10 mL of 95% alcohol to prepare Molisch’s reagent. 0.1 mL of filtrate, 2 drops of Molisch’s reagent was added in a test tube and 0.2 mL of concentrated sulphuric acid was added carefully along the side of the test tube wall. Formation of violet ring at the junction indicates the presence of carbohydrates.

3.2.1.2. Fehling’s test

(a) 34.6 gm of copper sulphate was dissolved in distilled water and made up to 500 mL (b) 173.0 gm of potassium sodium tartrate and 50 gm of sodium hydroxide were dissolved in distilled water and volume was made up to 500 mL. (a) and (b) solutions were mixed in equal volume to give Fehling’s solution. 1.0 mL of filtrate and 4.0 mL of Fehling’s reagent was added in a test tube and heated for 10 minutes in a water bath. Formation of red precipitate indicated the presence of reducing sugar.
3.2.2. Detection of glycosides (Ronsenthaler 1930, Middeltone 1956)

3.2.2.1. Modified Borntrager’s Test

1.0 mL of extract filtrate and 2 mL of 1% ferric chloride solution was added in a test tube and heated for 10 minutes in boiling water bath. The mixture was cooled and shaken with equal volume of benzene and separated the benzene layer and treated with half of its volume of ammonia solution. Formation of rose pink or cherry color in the ammoniacal layer indicated the presence of anthranol glycoside.

3.2.2.2. Legal’s Test

1.0 mL of filtrate, 3.0 mL of sodium nitropruside in pyridine and methanolic alkali (KOH) was added in a test tube. Formation of pink to blood red color indicated the presence of cardiac glycoside.

3.2.3. Detection of alkaloids (Ronsenthaler 1930, Peach & Tracey 1955)

0.5 gm of extract was dissolved in 10 mL of dilute hydrochloric acid (0.1 N) and filtered. The filtrate was used to test the presence of alkaloids.

3.2.3.1. Mayer’s test

(a) Dissolve 1.36 gm of mercuric chloride in 60 mL of distilled water (b) Dissolve 5.0 gm of potassium iodide in 20 mL distilled water, mix (a) and (b) and adjust the volume to 100 mL with distilled water. Filtrates were treated with Mayer’s reagent; formation of yellow cream colored precipitate indicates the presence of alkaloids.

3.2.3.2. Dragendorff’s test

(a) Dissolve 8.0 gm of bismuth nitrate in 20 mL of nitric acid (b) Dissolve 27.2 gm of potassium iodide in 50 mL distilled water, mix (a) and (b) and adjust the volume to 100 mL with distilled water (Dragendorff’s reagent). Filtrate were treated with Dragendorff’s reagent, formation of red colored precipitate indicates the presence of alkaloids.

3.2.3.3. Hager’s test

Saturated solution of picric acid in distilled water. Filtrates were treated with Hager’s reagent; formation of yellow colored precipitate indicates the presence of alkaloids.
3.2.4. Detection of phytosterols and triterpenoids (Paech & Tracey 1955, Finar 1959)

0.5 gm of extract was treated with 10 mL chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids.

3.2.4.1. Libermanns test

To 2.0 mL filtrate in hot alcohol, few drops of acetic anhydride were added. Formation of brown precipitate indicates presence of sterols.

3.2.4.2. Libermanns Burchard test

To 100 mg of extract was treated with 2 mL of chloroform and filtered. To the filtrate few drops of acetic anhydride was added, boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. Formation of brown ring at the junction indicates the presence of steroidal saponins.

3.2.5. Detection of phenolics and tannins (Kokate 2001)

100.0 mg of each extract was boiled with 1.0 mL of distilled water and filtered and used for following tests:

3.2.5.1. Ferric chloride test

To 2.0 mL of filtrate, 2.0 mL of 1% ferric chloride solution was added in a test tube. Formation of bluish black color indicates the presence of phenolic nucleus.

3.2.5.2. Lead acetate test

To 2.0 mL of filtrate and few drops lead acetate solution was added in a test tube. Formation of yellow precipitate indicates the presence of tannins.

3.2.6. Detection of flavonoids (Shellard 1957)

3.2.6.1. Shinoda test

To 100.0 mg of extract and few fragments of magnesium metal were added in a test tube followed by drop wise addition of concentrated hydrochloric acid. Formation of magenta color indicates the presence of flavonoids.
3.2.6.2. **Alkaline reagent test**

To 100.0 mg of extract, few drops of sodium hydroxide solution were added in a test tube. Formation of intense yellow color that becomes colorless on addition of few drops of dilute acid (HCl), indicates the presence of flavonoids.

3.2.7. **Detection of saponins** (Kokate 2001)

3.2.7.1. **Foam test**: 1 mL of extract was diluted with distilled water and made up to 20 mL and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

3.3. **Induction of diabetes and hypercholesterolemia**

3.3.1. **Induction of diabetes**

Male wistar rats weighing 150 ± 10 gm were housed in an air-conditioned room at 22 ± 1°C, relative humidity 50 ± 10% and 12 hr light and 12 hr dark cycle throughout the duration of experiment. Overnight fasted animals were made diabetic by intraperitoneal injection of nicotinamide (230 mg/kg bw) followed by freshly prepared streptozotocin in citrate buffer (0.1M, pH 4.5) at a dose of 55 mg/kg bw after 15 minutes interval. It is considered a better model of experimental diabetes mellitus in animals, which is similar to type 2 DM in humans (Maisello et al. 1998). After 96 hours of injections, animals having plasma glucose (FPG) ≥ 126 mg/dL were considered as diabetic. Since the FPG of STZ-treated rats varied from 126 to 300 mg/dL, therefore to minimize the variation in the degree of hyperglycemia and the effect of treatment, STZ-treated rats were further categorized into two groups; mild diabetic (MD) having FPG = 126-200 mg/dL and severe diabetic (SD) having FPG > 200 mg/dL. No mortality has been observed in this diabetic model. These rats were treated with different doses of extract and the active component to standardize the most effective dose. The treatment regime has been described individually in each chapter.

3.3.2. **Induction of hypercholesterolemia**

Experimental rabbits weighing 1.5 ± 0.10 kg were used. These animals were fed with cholesterol suspended in groundnut oil at a dose of 100 mg/kg bw daily orally for 6 weeks (Shukla et al. 2004). The beneficial effect of aqWC treatment was studied in
these animals by simultaneous administration of aqWC followed by high cholesterol orally for 6 weeks. Rabbits were divided into three major groups and treated as follows: group 1; healthy control, group 2; high cholesterol-fed and group 3; high cholesterol + aqWC-fed.

3.4. Collection of blood and tissues
Whole blood was collected by retro-orbital venipuncture with the help of heparinized capillary from rat and marginal ear vein from rabbit. Blood for the estimation of plasma glucose was taken in vials containing sodium fluoride and potassium oxalate and in EDTA vials for the estimation of HbA\textsubscript{1c}, insulin and C-peptide, lipid profile and other parameters.

To study the effect of single treatment of aqWC on FPG, the blood was drawn after overnight fast and animals were fed immediately with drugs and blood was drawn after 2 hrs and FPG were estimated. Similarly, to study the effect of single treatment on PPPG, overnight fasted, animals were fed with glucose at 2.0 gm/kg bw followed by aqWC and blood was drawn after 2 hrs and the PPPG were estimated.

FPG, PPPG and other parameters were also determined after 7, 15 and 30 days of treatment. After 30 days of treatment, animals were anesthetized by single ip injection of pentabarbitone at a dose of 150 mg/kg bw. Tissues (pancreas, liver, heart and muscle) were removed, washed with cold saline and stored at -80\degree C for tissue constituents and enzyme assays.

3.5. Biochemical estimations
3.5.1. Estimation of plasma glucose
Plasma glucose was estimated by glucose oxidase-peroxidase method using kits from Accurex Biomedical Pvt Ltd.

**Principle:**
Glucose oxidase (GOD) converts glucose to gluconic acid, hydrogen peroxide formed in this reaction, in the presence of peroxidase (POD) is oxidatively coupled with 4- aminoantipyrine and phenol to produce red quinoneimine dye. This dye has
absorbance maximum at 505nm. The intensity of the colour complex is directly proportional to the concentration of glucose in the specimen.

\[ \beta-D \text{ glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluoconic acid} + H_2O_2 \]

\[ H_2O_2 + 4\text{-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red dye} + H_2O_2 \]

**Reagents:**

1. **Enzyme reagent:**
   - Phosphate buffer (pH 7.0) (120 mmol/L)
   - 4-aminophenazone (0.20 mmol/L)
   - Phenol (11.0 mmol/L)
   - Glucose oxidase (GOD) (≥ 5000 IU/L)
   - Peroxidase (1050 IU/L)
   - Stabilizers and inactive ingredients

2. **Standard**
   - Glucose (100 mg/dL)

**Procedure:**

1.0 mL of enzyme reagent was mixed with 10µl of test serum/standard and incubated for 15 min at 37°C or 30 min at room temperature. After incubation, the absorbance of the obtained product was measured at 505 nm against reagent blank. The final colour was stable for two hours and do not exposed to direct light.

**Calculation:**

Glucose (mg/dL) = Absorbance of test sample / Absorbance of standard x 100.

**3.5.2. Postprandial plasma glucose (PPPG)**

After collection of blood for FPG, fasted animals were fed to glucose at a dose of 2.0 gm/kg bw orally by gastric intubation. Blood were collected after 2 hrs of glucose load for PPPG (Shukla et al. 2004).
3.5.3. Glycosylated hemoglobin (HbA\textsubscript{1c})

**Principle:**
Throughout the circulatory life of red cell, glycohemoglobin is formed continuously by the addition of glucose to the N-terminal of the hemoglobin β-chain. This non-enzymatic process, reflects the average exposure of hemoglobin to glucose over an extended period. Glycohemoglobin has been defined operationally as the “fast fraction” hemoglobin (HbA\textsubscript{1a}, A\textsubscript{1c}) which elutes first during column chromatography with cation-exchange resins. The non-glycosylated fraction, which consists of the bulk of the hemoglobin has been designated as HbA\textsubscript{0}. The present glycohemoglobin procedure employs a weak binding cation-exchange resin for the rapid separation of glycohemoglobin (Fast fraction) from non-glycosylated hemoglobin. GHb in blood was estimated by ion-exchange resin method (Goldstein et al. 1986).

The whole blood was mixed with lysing reagent containing detergent and borate ions. Hemolysate was further mixed with anion exchange resin. All Hb’s are retained by the resin and glycated hemoglobin was eluted. The % of HbA\textsubscript{1c} was determined by measuring the ratio of absorbance of the glycohemoglobin (GHb) fraction and total hemoglobin (THb) fraction at 415 nm and results were compared with a standard GHb preparation, carried through out the test.

\[
\text{Hemoglobin (whole blood preparation)} + \text{Cation exchange resin} \rightarrow \text{Fast fractions} (\text{HbA1a, HbA1b, HbA1c})
\]

**Reagent:**
1. Cation exchange resin (8.0 mg/mL, pH 6.9)
2. Lysing reagent (10 mM)
3. Glycohemoglobin calibrator (10%)

**Procedure:**
1. **Hemolysate preparation:**
100µl of whole blood was mixed with 500µl of lysing reagent, mix well and allowed it to stand for 5 minutes till lysis was complete.
2. **Separation of glycohemoglobin:**
   - Added 100µl of the hemolysate into the appropriate marked ion-exchange resin tube.
   - Positioned the filter separator approximately 2 cm above the liquid level in the tube.
   - Placed the tubes on the shaker and allowed to mix continuously for 5 minutes.
   - Removed the tube from shaker and pushed the filter separator until the resin is firmly packed.
   - Pour the supernatant of each tube into appropriately marked tubes.
   - Read and record absorbance of each tube at 415nm against deionised water blank.

3. **Total hemoglobin fraction:**

   20µl sample hemolysate / calibrator were added to 5.0 mL deionised water, mixed well and take absorbance at 415nm for total hemoglobin reading (THb).

**Calculations:**

The ratio of the glycosylated hemoglobin absorbance / total hemoglobin absorbance.

\[ R_c = \frac{\text{Absorbance of Calibrator (Glyco)}}{\text{Absorbance of Calibrator (THb)}} \]

\[ R_u = \frac{\text{Absorbance of Unknown (Glyco)}}{\text{Absorbance of Unknown (THb)}} \]

Glycohemoglobin (%) = \( \frac{R_u}{R_c} \times 10 \) (value of calibrator)

**3.5.4. Lipid profile parameters**

**3.5.4.1. Total cholesterol (TC)**

Total serum cholesterol was estimated by enzymatic method as described by Allain et al (1974) using kit from Accurex Biomedical Pvt Ltd.

**Principle:**

Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction, cholesterol oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of peroxide, hydrogen peroxide oxidatively coupled with 4-aminoantipyrine and phenol to produce red
quinoneimine dye which had absorbance maximum at 510nm. The intensity of
colour is proportional to the amount of total cholesterol in the specimen.

\[
\begin{align*}
\text{Cholesterol esterase} \quad & \quad \text{Cholesterol + Fatty acids} \\
\text{Cholesterol + O}_2 \quad & \quad \text{H}_2\text{O}_2 + \text{Cholest-4-en-3-one} \\
\text{Peroxidase} \quad & \quad \text{Red quinoneimine Dye + H}_2\text{O}
\end{align*}
\]

**Reagents:**
- Buffer, pH 6.8 = 50.0 mmol/L
- Cholesterol oxidase ≥ 100 IU/L
- Cholesterol esterase ≥ 150 IU/L
- Peroxidase ≥ 500 IU/L
- 4-amino antipyrine = 0.5 mmol/L
- Stabilizers / Surfactant ≥ 10.0 mmol/L

**Procedure:**

10.0 µl of sample/standard was mixed with 1.0 mL of working reagent and incubated
for 15 minutes at 37°C or 30 minutes at room temperature (25-30°C). Absorbance was
taken at 510 nm within 10 min of colour development.

**Calculation:**

\[
\text{TAG (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{absorbance of standard}} \times 200
\]

3.5.4.2. **High density lipoprotein (HDL-C)**

HDL-C was estimated by method of Burstein et al. (1970) by using kit Accurex
Biomedical Pvt Ltd.

**Principle:**

Phosphotungstate/ Mg\(^{2+}\) precipitate chylomicrons, low-density lipoprotein (LDL) and
very low-density lipoprotein (VLDL) fractions. High-density lipoprotein (HDL) fraction
remains unaffected in supernatant, which was then separated by centrifugation. Cholesterol content of HDL fraction was assayed by enzymatic method.

**Reagent 1 (Buffer)**

- PIPES buffer, pH 6.9 (90 mmol/L)
- Phenol (26.0 mmol/L)

**Reagent 2 (Enzymes)**

- Cholesterol esterase (CHE) (300 U/L)
- Cholesterol oxidase (CHOD) (300 U/L)
- Peroxidase (POD) (1250 U/L)
- 4-aminophenazone (0.4 mmol/L)

**Precipitating reagent:**

- Phosphotungstic acid (14.0 mmol/L)
- Magnesium chloride (2.0 mol/L)

**Standard**

- HDL (50.0 mg/dL)

**Working reagent:** The content of reagent 2 was dissolved in reagent 1.

**Procedure:**

100.0 µl of precipitating reagent was mixed with 10.0 µl of sample. It was allowed to stand for 10 min at room temperature and then centrifuged at 4000rpm for 10min. The supernatant was collected to test HDL. 10.0 µl of supernatant/standard was mixed with 1.0 mL of working reagent and incubated from 15 min at 37°C. The absorbance of the coloured product was taken at 505nm against reagent blank.

**Calculation:**

\[
HDL-C \text{ (mg/dL)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times 100
\]
3.5.4.3. *Triacylglycerol (TAG)*

TAG was estimated by an enzymatic method by using kit from Accurex Biomedical Pvt. Ltd (Fossati and Prencipe 1982).

**Principle:**

Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate which is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red coloured compound.

\[
\text{Lipoprotein lipase} \\
\text{TAG} \rightarrow \text{Fatty acids + glycerol} \\
\text{Glycerol kinase} \\
\text{Glycerol + ATP} \rightarrow \text{Glycerol-3-phosphate + ADP} \\
\text{Glycerol phosphate oxidase} \\
\text{Glycerol-3-phosphate + O}_2 \rightarrow \text{Dihydroxyacetone phosphate + H}_2\text{O}_2
\]

**Reagents:**

- **Buffer** = 50.0 mmol/L
- **Lipase** $\geq$ 2000 IU/L
- **Glycerol kinase** $\geq$ 300 IU/L
- **Glycerol phosphate oxidase** $\geq$ 1000 IU/L
- **Peroxidase** $\geq$ 500 IU/L
- **ATP** = 1.0 mmol/L
- **Activators & stabilizers** = 2.0 mmol/L

**Procedure:**

10.0 µl of sample/standard was mixed with 1.0 mL of working reagent and incubated for 10 minutes at 37°C or 20 minutes at room temperature (25-30°C). Absorbance was taken at 510 nm after colour development.

**Calculation:**

\[
\text{LDL-C (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200
\]
3.5.4.4. **Low density lipoprotein (LDL)**

Serum LDL had been calculated by Friedwald’s and Fredricson’s formula (1972).

\[ \text{LDL} = \text{TC} - (\text{HDL-C} + \text{VLDL}); \text{VLDL} = \frac{\text{TG}}{5} \]

3.5.5. **Liver and kidney functions tests**

3.5.5.1. **Serum glutamate pyruvate transaminase (SGPT)**

The estimation is based on the method of Reitman and Frankal (1957). In this method the amount of oxaloacetate or pyruvate produced by transamination is reacted with 2,4 dinitrophenyl hydrazine (DNPH) to form a brown coloured hydrazone, the colour of which in alkaline solution is read at 520 nm.

**Reagents:**

1. *Phosphate buffer (pH 7.4):* 11.3 gm of anhydrous Na$_2$HPO$_4$ and 2.7 g of anhydrous KH$_2$PO$_4$ was dissolved in distilled water and made up to 1.0 L after adjusting the pH 7.4.

2. *Substrate:* 18.0 gm of L-alanine and 0.146 g of alpha-keto glutaric acid were dissolved in 90 mL water; pH was adjusted to 7.4 with NaOH and made up to 500 mL with phosphate buffer.

3. *Pyruvate standard:* 44.0 mg of sodium pyruvate / 100 mL of phosphate buffer.

4. *2,4-dinitrophenyl hydrazine:* 19.8 mg DNPH was dissolved in 10 mL conc. HCl and made up to 100 mL with water.

**Procedure:** The assay tubes were prepared in the following way:

**Test:** 0.5 mL of substrate and 0.1 mL serum were mixed and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 0.5 mL of DNPH.

**Control:** To 0.5 mL of substrate was added first 0.5 mL DNPH and then 0.1 mL of serum.

**Standard:** 0.1 mL of pyruvate standard, 0.4 mL substrate, 0.1 mL water and 0.5 mL DNPH were mixed together.
Blank: To 0.5 mL of substrate, 0.1 mL of water and 0.5 mL DNPH were added and mixed. The reaction with DNPH was allowed to take place for 20 minutes at room temperature and then 5 mL of 0.4 N NaOH was added. After 10 minutes the absorbance was measured at 510 nm. The activity was expressed as IU/litre.

3.5.5.2. Serum glutamate oxaloacetate transminase (SGOT)

The estimation was based on the method of Reitman and Frankal (1957). In this method the amount of oxaloacetate or pyruvate produced by transamination is reacted with 2,4 dinitrophenyl hydrazine (DNPH) to form a brown coloured hydrazone, the colour of which in alkaline solution is read at 520nm. The only difference from SGPT procedure is in the substrate which is prepared as follows for SGOT.

Substrate : 13.3 gm of L- aspartic acid was dissolved in about 90 mL of 1N NaOH, adjust the pH to 7.4, added 146 mg of 2-ketoglutaric acid and dissolved it by adding a little more of 1N NaOH. Again adjust the pH 7.4, made to a final volume of 500 mL with buffer, and stored at 4 °C.

3.5.5.3. Serum alkaline phosphatase (ALP)

The estimation of alkaline phosphatase (ALP) was based on the method of Bowers and Me-Comb (1966). 4-nitrophenyl phosphate formed phosphate and 4-nitrophenolate in the presence of alkaline phosphatase. The rate of increase in 4-nitrophenolate is determined photometrically and is directly propotional to the ALP is the activity in the sample.

\[
\text{ALP} + 4\text{-nitrophenylphosphate} + \text{H}_2\text{O} \leftrightarrow \text{Phosphate} + 4\text{-nitrophenolate}
\]

Reagent:

1. Reagent (1): Diethanolamine HCl 1.0 mmol /L, pH 9.8

2. Reagent (2): Magnesium chloride 0.5 mmol/L, 4-nitrophenyl phosphate 10mmol/L

Preparation of reaction solution: 8 mL of reagent 1 and 2 mL of reagent 2
**Procedure:**

20µl of serum was added to 1.0 mL of reaction solution in a 1 mL cuvette, mixed and after a minute measured increase in absorbance every minute for 3 minutes at 405 nm.

**Calculation:**

Enzyme activity (U/L) = (ΔA min) x TV x 1000/18.45 x SV x LP

Where:

ΔA min= Change in absorbance/min.

TV = Total volume (1.02 mL)

SV = Sample volume (0.02 mL)

18.45 = Millimolar absorvtivity of p-nitrophenol

LP= Light path

1000 = Conversion of units per mL to units per litre

ALP (U/L) = (ΔA min) x 1.02 x 1000/18.45 x 0.02 x 1

ALP (U/L) = A per min x 2764

**Precautions:** EDTA, Citrate and oxalate which inhibit ALP activity were avoided.

**3.5.5.4. Blood urea / blood urea nitrogen (BUN)**

The estimation of blood urea was done by kinetic method of Martinek 1969.

**Principle**

The enzymatic reactions involved in the BUN assay are as follows:

Urea + H₂O → 2NH₃ + CO₂

NH₃ + α-ketoglutarate + NADH → Glutamate + NAD

GLDH: Glutamate dehydrogenase
Urea is hydrolyzed to ammonia and carbon dioxide by urease. Ammonia produced reacts with α-ketoglutarate to form glutamate in the presence of glutamate dehydrogenase. NADH is oxidized to NAD$^+$ in this reaction, which is measured as decrease in absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to BUN concentration in the specimen. The BUN concentration in the specimen is determined by comparing the sample reaction rate to that obtained with a BUN standard.

**Components and concentration of working solutions**

- Tris Buffer, pH 7.7 ......................... 50 mmol/L
- α-ketoglutarate ............................. 10 mmol/L
- NADH ........................................ 25 mmol/L
- Urease ....................................... 10 KU/L
- Glutamate dehydrogenase .............. 900 U/L

**Stabilizers and inactive ingredients**

**Procedure**

- Reaction type ............................... UV - Kinetic
- Reaction time ............................... Down
- Wavelength ................................. 340
- Flowcell temperature ...................... 30°C
- Zero setting with ........................... Distilled water
- Delay time ................................. 30 seconds
- No. of readings ............................. 2
- Interval ..................................... 30 seconds
- Blank absorbance limit ................... ≥ 1.000 Abs.
- Sample volume .............................. 0.01 mL (10 μL)
- Reagent volume ............................. 1.0 mL
- Factor ....................................... 20 (Δ Abs. of Std)
- Linearity .................................... 250 mg/dL BUN
Manual assay procedure
Pre warmed at 30°C the required amount of working solution before use.

Performed the assay as given below:

1.0 mL procedure
Standard/Specimen 0.01 mL (10 μL)
Working solution 1.0 mL

First carried out the assay of standard, mixed and started stopwatch simultaneously. The absorbance of assay was recorded at exactly 30 seconds after standard addition and then again at 60 seconds. Subsequently, carried out the assay of the specimen, following exactly the same procedure mentioned above.

Calculations:
Calculated the change in absorbance (Δ Abs.) of standard and specimen (s).

Factor = Concentration of standard/ Δ Abs. of standard

= 20 / ΔAbs. of Standard

BUN mg/dL = ΔAbs. of Specimen × Factor

Blood urea = BUN × 2.14

3.5.5.5. Serum creatinine
The estimation of serum creatinine was done by picrate method, described by Owen et al. (1954).

Principle:
Creatinine in alkaline medium reacts with picrate to produce orange colour. This colour absorbs light at 492 nm. (490 - 510 nm). The rate of increase in absorbance is directly proportional to the concentration of creatinine in the specimen.

Creatinine + Picrate \[\xrightarrow{Alkaline~medium}\] Orange colour
Preparation of working solution

Prepared working solution by mixing equal volume of picrate reagent and diluents reagent.

Components and concentration of working solutions

Sodium Picrate..........................7.7 mmol/L
Sodium Hydroxide.......................500 mmol/L

Procedure

Reaction type.............................Initial rate
Reaction time.............................Up
Wavelength..................................492 nm (490-510 nm.)
Flowcell temperature......................37°C
Zero setting with.........................Distilled water
Delay time.................................30 seconds
No. of readings............................2
Interval......................................60 seconds
Sample volume.............................0.05 mL (50 μL)
Reagent Volume.............................1.0mL
Standard concentration..................2mg/dL
Factor......................................2 ÷ Δ Abs. of Standard
Linearity...................................30 mg/dL

Manual assay procedure

Pre warmed the required amount of working solution to 37°C before use. Performed the assay as given below

Standard / Sample ....................... 0.05 mL (50 μL)
Working Solution........................1.0 mL

Mixed and started stopwatch simultaneously. The absorbance of assay mixture was recorded at exactly 30 seconds after standard / specimen addition and then again at 90 seconds.
**Calculation:**
Calculated the average change in absorbance per minute (Δ Abs) of standard & specimen(s).

\[ \Delta \text{Abs.} = \text{Abs. at 90 sec.} - \text{Abs. at 30 sec.} \]

Serum creatinine (mg/dL) = \( \Delta \text{Abs. of specimen} \times 2 / \Delta \text{Abs. of standard} \).

**3.6. Hematological estimations**

**3.6.1. Total leukocyte count (TLC)**

**Principle:**
A sample of whole blood is mixed with a weak acid solution that lyses non-nucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume were counted.

**Reagent:**
White-count diluting fluid. Either of the following diluting fluids may be used:

1. **2% acetic acid:** Add 2.0 mL glacial acetic acid to a 100 mL volumetric flask. Dilute to the mark with distilled water.

2. **1% hydrochloric acid:** Add 1.0 mL hydrochloric acid to a 100 mL volumetric flask. Dilute to the mark with distilled water.

**Procedure**

1. Draw well-mixed capillary or venous blood exactly to the 0.5 mark in a white blood cell diluting pipette. This blood column must be free of air bubbles.

2. Wiped the excess blood from the outside of the pipette to avoid transfer of cells to the diluting fluid. Take care not to touch the tip of the pipette with the gauze.

3. Immediately draw diluting fluid to the "11" mark while rotating the pipette between the thumb and forefinger to mix the specimen and diluent. Hold the pipette upright to prevent air bubbles in the bulb.
(4) Mix the contents of the pipette for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipette (usually 4 drops).

(5) Place the forefinger over the top (short end) of the pipette, hold the pipette at a 450 angle, and touch the pipette tip to the junction of the cover glass and the counting chamber.

(6) Allow the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemacytometer.

**NOTE** If the mixture overflows into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipette, and refill both chambers.

(7) Allow the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution of cells.

(8) Count the white cells in the four 1 sq mm corner areas.

(9) Count all the white cells lying within the square and those touching the upper and right-hand center lines. The white cells that touch the left-hand and bottom lines were not to be counted. A variation of more than 10 cells between any of the four areas counted or a variation of more than 20 cells between sides of the hemacytometer indicate uneven distribution and require that the procedure be repeated.

**Calculations:**

(1) Routinely, blood is drawn to the 0.5 mark and diluted to the 11 mark with WBC diluting fluid. All the blood is washed into the bulb of the pipette (which has a volume of 10). Therefore, 0.5 volumes of blood were contained in 10 volumes of diluting fluid. The resulting dilution is 1:20. (These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.)

(2) The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area of 1.0 sq mm therefore, 4 x 1.0 sq mm = a total of 4 sq mm). The volume counted is: area x depth = volume. Four sq mm x 0.1 mm = 0.4 cu mm.
(3) The formula is as follows:

$$WBCs\ per\ cu\ mm = \frac{Average\ number\ of\ chambers\ (2)\ WBCs\ counted \times\ dilution\ (20)}{Volume\ (0.4)}$$

3.6.2. Differential leukocyte count (DLC)

Principle:

A total white blood cell count is not necessarily indicative of the severity of a disease, since some serious ailments may show a low white cell count. For this reason, a differential white cell count is performed. A differential white cell count consists of an examination of blood to determine the presence and the number of different types of white blood cells. This study often provides helpful information in determining the severity and extent of an infection, more than any other single procedure used in the examination of the blood. The role of white blood cells, or leukocytes, is to control various disease conditions. Although these cells do most of their work outside the circulatory system, they use the blood for transportation to sites of infection.

Five types of white cells are normally found in the circulating blood. They are as follows:-

**Cell identification:** To perform a differential white cell count, identified the different types of white cells through the staining and morphology. Use blood smear to obtain a differential white cell count. To prepare a blood smear, a blood specimen is spread across a glass slide, stained to enhance leukocyte identification and examined microscopically.

**Neutrophils:** Account for the largest percentage of leukocytes found in a normal blood sample, and function by ingesting invading bacteria. On a stained blood smear, the cytoplasm of a neutrophil has numerous fine, barely visible lilac-colored granules and a dark purple or reddish purple nucleus. The nucleus may be oval, horseshoe, or "S"-shaped, or segmented (lobulated). Neutrophils are subclassified according to their age or maturity, which is indicated by changes in the nucleus. The subclassifications for neutrophilic cells are metamyelocyte, band, segmented, and hyper-segmented.

**Neutrophilic metamyelocyte:** A neutrophilic metamyelocyte, also called a "juvenile" cell, is the youngest neutrophil generally reported. The nucleus is fat, indented, and is usually "bean"-shaped or "cashew nut"-shaped.
**Neutrophilic Band:** A neutrophilic band, sometimes called a "stab" cell, is an older or intermediate neutrophil. The nucleus has started to elongate and has curved itself into a horseshoe or S-shape. As the band ages, it matures into a segmented neutrophil.

**Segmented Neutrophil:** A segmented neutrophil is a mature neutrophil. The nucleus of a segmented neutrophil is separated into two, three, four, or five segments or lobes.

**Hypersegmented Neutrophil:** A hyper-segmented neutrophil is a mature neutrophil. The nucleus of a hyper-segmented neutrophil is divided into six or more segments or lobes.

**Eosinophil:** The cytoplasm of an eosinophil contains numerous coarse, reddish-orange granules, which are lighter colored than the nucleus.

**Basophil:** The function of basophilic cells is unknown. It is believed, however, that basophilic cell keep the blood from clotting in inflamed tissue. Scattered large, dark-blue granules that are darker than the nucleus, characterize the cell as a basophil. Granules may overlay the nucleus as well as the cytoplasm.

**Lymphocyte:** The function of lymphocytes is also unknown, but it is believed that they produce antibodies and destroy the toxic products of protein metabolism. The cytoplasm of a lymphocyte is clear sky blue, scanty, with few unevenly distributed, azurophilic granules with a halo around them. The nucleus is generally round, oval, or slightly indented, and the chromatin (a network of fibers within the nucleus) is lumpy and condensed at the periphery.

**Monocyte:** The monocyte, the largest of the normal white blood cells, destroys bacteria, foreign particles, and protozoa. Its color resembles that of a lymphocyte.

### 3.7. Insulin

Insulin was estimated by ELISA using commercial kits (DRG International, Germany).

**Principle:**

Insulin ELISA is a solid phase two-site immunoassay. It was based on direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the
sample reacts with peroxidase-conjugated anti-insulin antibodies and is bound to microtiter plate wells. Unbound enzymes labeled antibodies are removed by washing. The bound conjugate was detected by reaction with 3,3’,5,5’-tetramethylbenzidine. The reaction was stopped by the addition of stop solution to give a colorimetric end point and absorbance taken at 450 nm.

**Reagents:**

1. Anti-insulin microtitration plates (mouse monoclonal)

2. Insulin standard

3. Anti-insulin conjugate stock solution (peroxidase conjugated mouse monoclonal anti-insulin)

4. Conjugate buffer

5. Washing solution (20X)

6. Peroxidase substrate (TMB)

7. Stop solution (1M, Sulphuric acids)

**Procedure:**

- 25 µl test sample / standards were added in appropriate well
- 100 µl reconstituted conjugate solution and incubated for 1 hr at room temperature.
- Reaction volume was then aspirated and wells were washed with 300 µl of washing solution 4 times to each wells.
- 200 µl of peroxidase substrate was added and incubated for 15 min at room temperature.
- 50 µl of stop solution added into each well and sake on metabolic shaker for 10 min and mix properly.
- Absorbance taken at 450 nm.

**Calculation:** Concentration was calculated from standard curve plotted on log paper.
3.8. C-peptide

C-peptide was estimated by ELISA by using commercially available kits (DRG International, Germany).

**Principle:**

C-peptide was estimated by direct sandwich ELISA in which two monoclonal antibodies was directed against antigenic determinants on the C-peptide molecule. C-peptide in test sample react with anti-C-peptide antibodies bound to the microtitration plates during incubation. After incubation, plate wells were washed trice with washing solution and peroxidase-conjugated anti-C-peptide antibodies were added and incubated at room temperature. Plates were again washed with wash buffer to remove unbound enzymes labeled anti-bodies. TMB was added into each well to detect unbound conjugate. The stop solution was added to end the reaction. Absorbance was measured at 450 nm by colorimetry.

**Reagent:**

1. Anti-C-peptide microtitration plates
2. Standards
3. Anti-C-peptide conjugate (peroxidase-conjugated mouse monoclonal anti-C-peptide, 10X), diluted to 1X with conjugate buffer
4. Conjugate buffer
5. Assay buffer
6. Washing solution
7. Peroxidase substrate (TMB)
8. Stop solution: H$_2$SO$_4$

**Procedure:**

1. 25 µl of standards / test sample were taken in individual well in duplicate and assay buffer added in each
2. Incubated 1hr at room temperature and wash with washing solution 3 times

3. Added 100 µl of conjugate solution and incubated for 1hr at room temperature

4. Wells were washed with washing solution trice

5. Added 200 µl of substrate solution into each wells and incubated for 15 min at room temperature.

6. 50 µl of stop solution was added into each well to stop the reactions and mix well.

7. Absorbance was taken at 450nm by spectrophotometer.

**Calculation:**

1. Absorbance value obtained for the standards were plotted against the C-peptide concentration on log paper and constructed a standard curve.

2. Concentration of unknown test samples was calculated from the standard curve.

**Conversion factor:**

1.0 µg/L corresponds to 331.0 pmol/L.


*In vitro* release of insulin was also studied in isolated perfused pancreatic islets from diabetic and diabetic-treated animals under glucose stimulation. After 15 days treatment, animals were anaesthetized by intraperitoneal injection of pentobarbitone (100mg) and dissected. After laparotomy and exsanguination, the whole abdomen was cooled with sterile ice cooled saline. Before the removal of pancreas from abdomen, 7-10 mL of cold Hank’s bicarbonate buffered salt (HBSS) of pH 7.4 were injected into the central duct to distend the pancreas. Fat and blood vessels were removed and clean the pancreas. Whole pancreas were removed from cavity and kept in HBSS containing clean petridis and pancreas was chopped as finely as possible into very small pieces with scissors. The pieces were transferred into a HBSS containing centrifuge tube and any floating material should remove from tube. The pancreatic islets pieces were centrifuged at 5000rpm for 20 min in cooling
centrifuge. Supernatant was discarded and remain residue were transferred into broad bottom conical flask and incubated with 2.0 mL of collagenase (10.0mg/mL of HBSS) for half an hour at 37\(^\circ\)C in a metabolic shaker (Tager et al. 1975). The unwanted acinar debris was further separated by repeated differential layering by using Ficoll (Kemp et al. 1973). The individual islets were picked up with a glass loop under dissecting microscope and kept in small vials. Selected islets were further incubated into incubation buffer i.e. glucose-Krebs-Ringer bicarbonate buffer (KRBB) contain sodium bicarbonate (0.2%), Hepes (0.4%), insulin free bovine serum albumin (BSA) (0.1%). Two different concentration of glucose i.e 3mM and 10mM in KRBB has been used in incubation buffer in this experiment. Further, in all the experimental groups, 10 islets were per-incubated at 37\(^\circ\)C with 0.5mL incubation buffer containing glucose 3 and 10mM separately followed by addition of active component for 1hr and 2 hrs. Mixture was centrifuged at 12,000 rpm and supernatant was used for insulin estimation by ELISA.

3.10. Histopathology of tissues

Animals were sacrificed and pancreas, liver, heart and kidneys were removed, washed with cold saline and preserved in 10 % formalin in buffered form. Blocks from tissues were routinely processed and embedded in paraffin cassette. 5µ thick slide sections were cut using rotary microtome (A.O. Spencer) with 45\(^\circ\) angles. These sections were melted down at 60\(^\circ\)C for 10 min and dipped 10-12 times in xylene in different bowls for 15 min each then it again dipped in different concentration alcohol (100, 80 & 60% ) for 3 min each. It kept in tap water and stained with heamatoxylin and eosin for 10 min for histology.

3.11. Tyrosine kinase activity of insulin receptors

The tyrosine kinase (TK) of insulin receptors was assayed by using the commercially available ELISA kits from Sigma-Aldrich, USA (TK 101). This non-radioactive assay kit was intended for the measurement of tyrosine kinase activity in cell and tissue extracts (Rijksen et al. 1989 & 1991).
Principle:
The TK kit assay system for the in vitro determination of tyrosine kinase activity was based on an ELISA assay using a TK-specific polymer substrate-coated multiwell plate:

1. Multiwell plates was pre-coated with a synthetic random polymer substrate poly-Glu-Tyr (PGT) containing multiple tyrosine residues. PGT could be phosphorylated by a wide range of TKs.

2. The phosphorylation reaction was initiated by the addition of a tyrosine kinase (tissue and cell extracts or a purified tyrosine kinase control such as EGFR) in the tyrosine kinase reaction buffer that contains Mg$^{2+}$, Mn$^{2+}$, and ATP. The phosphorylated polymer substrate was probed with a purified phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase (HRP).

3. Color was developed with HRP chromogenic substrate (OPD).

4. The color was quantitated by spectrophotometry (ELISA reader) and reflected the relative amount of tyrosine kinase activity in the sample (qualitative).

5. Tyrosine kinase activity in the sample (quantitative) was derived from the EGFR control or extrapolated from the EGFR standard curve (absorbance at 492 nm vs. units of EGFR activity).

Advantages of the TK assay kit:
Radioactive methods using $^{32}$P-labeled ATP are labor intensive, produce large amounts of hazardous radioactive waste, and require a constant supply of radioactive labeled ATP. In addition, the half-life of the $^{32}$P isotope is relatively short (14.3 days).

The tyrosine kinase assay kit totally avoids the use of radioactive reagents and has several advantages over conventional radioactive techniques:

High specificity:

1. No cross-reactivity with tyrosine, phosphothreonine, or phosphoserine.

2. Synthetic polymeric peptide substrate has no Ser or Thr residues.

3. Monoclonal antibody-peroxidase conjugate allows a direct and specific detection of only phosphorylated tyrosine residues
4. High Sensitivity comparable to radioactive methods
5. Easy and reliable calibration
6. Fast performance (1.5-2.0 hours hands-on assay)
7. Convenient component sizes for easy handling

**Components:**

**Reagents and materials supplied in the TK assay kit:**
1. TK substrate, poly-Glu-Tyr (PGT),
2. 96-Well multiwell plate with a strip holder
3. Adenosine-5’-triphosphate (ATP) disodium salt, MW = 551.1
4. Tyrosine kinase buffer (10x): 500 mM HEPES buffer, pH 7.4, 200 mM MgCl$_2$, 1 mM MnCl$_2$, and 2 mM Na$_3$VO$_4$
5. Epidermal growth factor receptor (EGFR): Positive control for TK. Lyophilized with trehalose as cryoprotectant
7. Washing Buffer (PBS-TWEEN 20), 10 mM Phosphate Buffered Saline, pH 7.4, and 0.05% TWEEN
8. SIGMAFAST (OPD) Tablets, o-Phenylenediamine dihydrochloride,
9. SIGMAFAST Tablets, 5 tablets in gold foil phosphate-citrate buffer with urea hydrogen peroxide.

**Reagents and materials not supplied with the TK assay kit:**
1. *PBS 10 mM pH 7.2-7.4*
2. *Protein assay reagent and BSA standards.*
3. *Glycerol*
4. *0.1 M activated sodium vanadate solution:* 0.64 gm of sodium orthovanadate was dissolved into 35 mL of dH$_2$O on magnetic stirrer. pH adjusted to 10 with 1n HCl. If the solution turned yellow, it was boiled (~ 5 min) until it become colourless.
5. **Lysis buffer**: prepared 50mM HEPES buffer, pH 7.4 contained 0.1-1% triton x-100, 10% glycerol, 1.0 mM dithiothreitol, 1.0 mM activated Na$_3$VO$_4$. Protease inhibitors were added: 1.0 mM benzamidine, 10.0 µg/mL pepstatin A.

6. **Homogenization buffer**: 20.0 mM tris-HCl buffer, pH 7.5, containing 0.25 M protease inhibitors: 1mM PMSF, 50µg/mL leupeptin, 25 mg/mL aprotinin, 10 µg/pepstatin A and 2 mM dithiothretiol were added.

7. **ELISA reader**

8. **2.5N H2SO4 in ultrapure water**

9. **Precision pipettors**

10. **37°C incubator**

11. **Graduated cylinder**

12. **Microcentrifuge and tubes**

3.11.1. **Preparation of sample and purification of insulin receptors**

**Preparation of cell lysate:**

1. Cells were growing in appropriate culture medium.

2. Cells were washed or rinsed plates with ice-cold PBS (2 x 10 mL). For cells in suspension, centrifuged at 800 × g for 5 minutes at room temperature. Supernatant removed

3. For adherent cells, added freshly prepared lysis buffer at 4°C. For cells in suspension, added lysis buffer to wash cell pellet in a 15 mL conical tube.

4. Transferred cell lysate to microcentrifuge tube and leave for 10-20 minutes at 4°C.

5. Centrifuged lysate at 10,000 × g for 15 minutes at 4°C.

6. Collected supernatant containing lysate fraction. Pellet containing nuclear and cell debris were discarded.
7. 0.2 mL aliquot of lysate for protein determination using protein standard (e.g., BSA) were removed.

8. Sample of the lysate might be used in the TK assay. It was recommended to test different lysate dilutions to obtain an optimal signal.

9. Aliquoted 0.1 mL samples of the cell lysate and stored at -70 °C. Tyrosine kinases from cell lysate or tissue homogenates could not be recovered after freezing and thawing. To enhance stability of TKs, glycerol had been added to the lysate up to a final concentration 50% (w/v) and the samples had been stored at -70 °C.

**Preparation of tissue extracts:**

1. Rapidly dissected required tissues were collected into ice-cold homogenization buffer. Weigh tissue.

2. Homogenize tissue in 2-5 volumes of ice-cold homogenization buffer using a PTFE/glass or mechanical homogenizer at maximum speed (3 times with 1 minute rest in between).

3. Centrifuge homogenate at 1,000 × g for 10 minutes at 4 °C to remove nuclei and cell debris. Collect supernatant (S1).

4. Centrifuge supernatant at 10,000 × g for 20 minutes at 4 °C. Collect clear supernatant (S2). Retain the pellet (P2), representing the crude membrane fraction. The S2 fraction contains cytosolic proteins and may be used for assay of TK activity or further purification by chromatography.

5. If the activity is presumed to be present in the membrane fraction (P2), solubilize the pellet in 1-2 volumes of lysis buffer for 10 minutes on ice.

6. Centrifuge lysate at 10,000 × g for 10 minutes at 4 °C. Collect the clear supernatant representing the lysate of the crude membrane fraction. This fraction contains the solubilized membrane proteins and may be used for assay of TK activity or further purification by chromatography.
Assay reagents:
Prepare the reagent solutions as close as possible to the time of use. Store reagent solutions (where applicable) at the appropriate temperature until used.

1. **PBS**: 10 mM Phosphate Buffered Saline, pH 7.2-7.4

2. **10% Glycerol**: in ultrapure water

3. **TK Substrate-reconstitute contents**: TK substrate, with 1 mL PBS (TK Substrate Stock Solution). Add 1 mL of the TK substrate stock solution to 19 mL of PBS in an appropriate tube (50 mL). Mix well. The TK substrate diluted solution can be stored in aliquot samples (e.g., 1.0 mL) at -20 °C. Avoid repeated freeze and thaw of this reagent.

4. **Epidermal Growth Factor Receptor (EGFR)**: Reconstitute contents, EGFR, with 125 mL of 10% glycerol in ultrapure water. Mix gently until dissolved. The EGFR stock solution can be stored in small aliquots (e.g., 5 mL, 20 units) at -70 °C. Avoid repeated freeze and thaw of this reagent.

5. **Adenosine-5'-Triphosphate (ATP)**: Reconstitute contents of A6714, ATP, with 1.0 mL of ultrapure water. Mix gently until dissolved. Final concentration of stock solution is ~5 mg/mL (9 mM). The ATP solution can be stored in small aliquots (e.g., 50 mL) at -20 °C. Avoid repeated freeze and thaw of this reagent.

6. **Monoclonal Anti-Phosphotyrosine-Peroxidase Conjugate**: Reconstitute contents of A5964, Anti-Phosphotyrosine-Peroxidase, in 0.2 mL of ultrapure water. Mix gently until dissolved. The antibodyperoxidase conjugate solution can be stored frozen in aliquots at -20°C. Dilute reconstituted A5964, Monoclonal Anti-Phosphotyrosine-Peroxidase conjugate, washing buffer.

7. **Washing Buffer (PBS-TWEEN 20)**: Dissolve contents, PBS-TWEEN 20, powder in 1 liter of ultrapure water to give 10 mM sodium phosphate buffer, 138 mM NaCl, 27 mM KCl and 0.05% TWEEN 20, pH 7.4. Stored at 2-8°C.
3.11.2. Assay procedure

Coating of 96-well plate:
1. Remove the plate from the plastic bag. Place the required number of wells or strips in the well holder. Return any unused strips to the plastic bag, seal the zip lock and store at room temperature.

2. Thaw a sample of the TK substrate diluted solution.

3. Add 125 mL of TK Substrate diluted solution to each well. Cover multiwell plate.

4. Incubate plate overnight at 37°C. If required, the coating time may be shortened to 4 hours. However, this may result in a lower signal.

5. Remove coating solution and wash each well with 200 mL of washing buffer (PBS-TWEEN 20).

6. Remove washing buffer and dry wells for 2 hours at 37°C.

Tyrosine kinase assay:
1. Prepared 1× TK buffer by adding 1 mL supplied 10× concentrate into 9 mL of ultrapure water. Mixed well.

2. Thaw a 5 µl sample (20 units) of the EGFR (see assay Reagents). 95 mL of 1× TK buffer were added. Mixed gently and placed on ice.

3. Thaw a sample of the ATP stock solution (see Assay Reagents). Added 40 mL of ATP solution to 1 mL of 1× tyrosine kinase Buffer. Mixed well and placed on ice.

4. Add 90 mL of 1× TK buffer containing ATP to each well.

5. Blank (control without EGFR): Added 20 mL of 1× TK buffer.

6. Added to the appropriate wells, 20 mL (4.0 units) of EGFR or test samples (cell or tissue lysates), diluted 1:1 or 1:2, etc. in 1× TK buffer. Final concentration of ATP in the TK reaction is 0.3 mM.

7. Covered wells with plate cover. Incubated wells at room temperature for 30 minutes.
8. Reaction mixture removed from each well.

9. Each well was washed with 200 mL of washing Buffer. After each wash, blot the plate on a paper towel until all residual moisture is removed. Repeat this step 5 times.

10. Added to each well 100 mL of the appropriate dilution of antibody conjugate.

11. Covered wells with plate cover. Incubated wells for 30 minutes at room temperature.

12. Freshly prepare peroxidase substrate solution: dissolved one OPD tablet (silver foil) and one Urea Hydrogen Peroxide tablet (gold foil) in 20 mL of ultrapure water. Mixed well until dissolved. Protect from light until used. Do not store this solution.

13. Removed antibody solution from wells.

14. Wash each well with 200 mL of Washing Buffer. After each wash, blot the plate on a paper towel until all residual moisture is removed. Repeat this step 5 times.

15. Added 100 mL of freshly prepared OPD substrate solution to each well and incubate for exactly 7 minutes, in the dark, at room temperature. An orange-yellow color should develop in positive wells.

16. 100 mL of 2.5 N H₂SO₄ was added to each well to stop reaction.

17. Absorbance taken at 492nm.

Calculation of results:

TK standard curve (EGFR control): TK standard curve was prepared by plotting the curve of absorbance of EGFR control in serial dilutions at 492 nm (A492) versus the EGFR activity (units). To determine the activity of the test sample (e.g., cell lysate), the linear part of the TK standard curve have been used and extrapolating the units of TK activity in the sample from the absorbance obtained at 492 nm.
3.12. Glucose transporter 4 (GLUT4)

3.12.1. Immunohistochemistry

Immunohistostaining was performed by using GLUT4 primary antibody (Sigma-Aldrich Pvt Ltd, USA) and detection kit i.e. Ultravision one HRP polymer detection system (Thermo Fisher Scientific, USA). The detection kit detects rabbit or mouse immunoglobulins bound to antigen in tissue sections. The specific primary antibody is located by a universal secondary antibody polymer formulation. The amino acid polymer is conjugated to horseradish peroxidase (HRP) and the Fab fragment of goat anti-rabbit or anti-mouse, the polymer complex is then visualized with an appropriate chromogen / substrate.

The tissue sections were deparffinized and rehydrated. Antigen retrieval was done by incubating the slide in citrate buffer (pH 6.0) for 20 min in a microwave oven set a temperature of 98°C following by cooling to room temperature. Therefore, to reduce nonspecific background staining due to endogenous peroxidase, the slide were incubated in hydrogen peroxide block for 10-15 min at room temperature and washed four times in tris buffer (pH 7.6). Then applied Ultra V block for 5 min at room temperature to reduce background staining and washed 4 times in buffer. Therefore, the slides were exposed to primary antibody i.e. mouse anti-rat GLUT4 antibody and incubated for overnight at 4°C followed by washing for 4 times in buffer. Slides were exposed to Ultra Vision One HRP polymer and incubated for 30 min at room temperature followed by washing step. Diaminobenzidine (DAB) was used as a substrate for colour development, applied to the slides and left for 10 min at room temperature in dark. Slides were then washed 4 times in distilled water. Slides were counterstained with haematoxylin and covered with cover slip using an aqueous mounting media. Slides were examined microscopically.

3.12.2. Western Blot

The GLUT4 content was evaluated in crude membranes of myocytes which reflects plasma membrane GLUT4 concentrations (Duehlmeier et al. 2007).
Preparation of Reagent:

1. **Homogenizing buffer:** 20mM HEPES, 250 mM sucrose, 5mM sodium azide (NaN₃), 1µM leupeptin, 1µM antipain, 2µg/mL aprotinin, 100 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4.

2. **Trichloroacetic acid:** 10mM (TCA)

3. **Laemmli buffer:** 63mM Tris/HCl pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 5% (v/v) 2-mercaptoethanol, 30 mM bromophenol blue to final dilution of 30µg/µL.

4. **Membrane buffer:** 25mM Tris, 192mM glycine, 10% methanol (v/v), pH 8.6

5. **10% (w/v) non-fat dry milk in Tris buffer saline:** 20mM Tris base, 137mM NaCl, 0.05% Tween 20, pH 7.6.

6. **Hybond nitrocellulose membrane**

7. **Anti-GLUT4 antibodies (1:200)**

Procedure:

**Preparation of membrane fractions:**

Crude membranes from muscles were prepared according to the method of Guma et al. 1995. To isolate crude membrane, about 100 mg of muscle tissue was homogenized in 2.5 mL ice cold homogenizing buffer. The homogenate were centrifuged at 1200 × g at 4°C for 10 min and supernatant (SN1) retained. The pellet (P1) was resuspended in 2.5 mL of homogenization buffer, re-homogenized and centrifuged in the same way. The new pellet (P2) was discarded and supernatant (SN2) pooled with SN1 and centrifuged at 9000 × g at 4°C for 10 min to remove mitochondria. The pellet (P3) was discarded and supernatant (SN3) centrifuged at 190,000 × g at 4°C (ultracentrifugation). The resulting pellet (P4) i.e. crude membrane was collected and re-suspended in 500 µl ice cold homogenizing buffer. Protein concentrations of the crude membrane preparation were determined using Lowry assay. Membrane proteins were precipitated with 10 mM TCA at 4°C for 30 min. Then crude membrane proteins were resuspended in Laemmili buffer to final dilution of 30µg/15µl.
Electrophoresis:
Crude membrane proteins were separated by SDS-PAGE electrophoresis (9%). Proteins were transferred to a hybond nitrocellulose membrane in buffer at pH 8.6. After the transfer, the membranes were blocked with 10% (w/v) non-fat dry milk in Tris-buffered saline solution for 1 hr at room temperature and then incubated overnight at 4°C with antibodies directed against 12-amino acid carboxy terminus of rat GLUT4 and rat whole GLUT4 protein. Primary antibodies were diluted according to the manufacturers recommendations (GLUT4; 1:200) in Tris-buffered saline solution as described above containing 5% (w/v) non-fat dry milk and 0.05% NaN₃. The immune complex was visualized by using an enhanced chemiluminescence system.

3.13. Assessment of the activity of regulatory enzymes of carbohydrate metabolism

3.13.1. Glucokinase (GK)
Glucokinase activity was determined by the standard method of Porter and Chassy (1982). Glucose-6-phosphate (G-6-PO₄) formation was measured by following the reduction of NADP⁺ in the presence of glucose-6-phosphate dehydrogenase (G-6-PD). Since the phosphorylation of glucose was equivalent to the rate of NADP⁺ reduction, which was measured by the increased absorbance at 340nm.

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{Glucokinase}} \text{Glucose-6-phosphate} + \text{ADP}
\]

\[
\text{Glucose-6-phosphate} + \text{NADP}⁺ \xrightarrow{\text{G-6-PD}} \text{Glucose} + \text{NADPH} + \text{H}⁺
\]

Reagents:
1. *Tris Buffer (1M, pH 7.5)*: 12.1 gm of Tris was dissolved in 50 mL of distilled water, pH was adjusted to 7.5 with 1N HCL and makeup to 100 mL with distilled water.
2. *MgCl₂ (0.1M)*: 0.95gm MgCl₂ dissolved into 100 mL of distilled water
3. *ATP (0.1M, pH 7.0)*: 275.5 mg ATP disodium salt was dissolved in 2 mL of distilled water and pH adjusted to 7.0 with dilute NaOH solution. The volume was made upto 5 mL with distilled water.
4. **Nicotinamide adenine dinucleotide phosphate (NADP+) (0.01M, pH 7.0):** 78.7 mg disodium salt of NADP was dissolved in 4 mL of distilled water and pH adjusted to 7.0 with dilute NaOH solution. The volume was made up to 10 mL with distilled water.

5. **Glucose-6-phosphate dehydrogenase (G-6-PD) (Sigma-Aldrich, USA)**

6. **Homogenizing buffer:** (0.1M tris buffer, pH 7.0 containing 1 mM dithiothreitol and 5 mM MgCl₂)

**Procedure:**

Tissues were homogenized with homogenizing buffer (10% w/v) and centrifuged at 1000 rpm at 4°C for 10 min. Supernatant was taken and kept in ice bath for the enzyme assays. The assay mixture containing 100 µl of tris buffer, 50 µl of ATP, 50 µl MgCl₂, 50 µl glucose solution, 50 µl NADP⁺, G-6-PD (5 IU), 20 µl supernatant and 500 µl of distilled water in a 1.0 mL cuvette. The control contained all reagents except ATP. The addition of tissue homogenate in the reaction mixture initiated the reactions. Absorbance (ΔA) was taken at 340 nm for 14 minutes.

**Calculations:**

One unit of enzyme activity was taken as the amount of glucokinase that catalyses the formation of 1µmole of glucose-6-phosphate / min, which is equivalent to 1 µmol of NADPH formed per minute.

Activity of glucokinase was calculated by taking the molar extinction coefficient of NAD(P)H / NAD(P)⁺ as 6.22 x 10⁶ cm²/M. The formula for calculating the enzyme activity is as follows:

\[
\text{GK Activity (U/g)} = \frac{\Delta A \times \text{Total volume} \times \text{Dilution factor}}{\text{Sample volume} \times 6.22 \times \text{Time}}
\]

Whereas, ΔA is change in absorbance per min.
3.13.2. Phosphofructokinase (PFK)

**Principle:**
The assay for PFK is based on the method of Racker et al 1947. PFK is an allosteric enzymes that catalyses the phosphorylation of fructose-6-phosphate to yield fructose-1,6-bisphosphate.

\[
\text{Fructose-6-phosphate} + \text{ATP} \xrightarrow{\text{PFK}} \text{Fructose-1,6-bisphosphate} + \text{ADP}
\]

The assay is based on conversion of fructose-1,6-bisphosphate via dihydroxyacetone phosphate to glycerol phosphate. The rate of NADH oxidation in the glycerol-3-phosphate dehydrogenase reaction is twice the rate of the phosphorylation of fructose-5-phosphate.

**Reagent:**
1. *Assay mixture:* 100 mM Tris HCl (pH 8.0), 6 mM MgCl₂, 0.5 mM ATP, 1 mM AMP, 30mM fructose-6-phosphate, 40mM (NH₄)₂SO₄ and 0.25 mM NADH, made up as a single solution and store at -20°C

2. *Auxillary enzyme solution:* Aldolase (6 mg/mL), α-glycerol phosphate dehydrogenase (3 mg/mL) and triosphosphate isomerase (1 mg/mL) in 25 mM triethanolamine-HCl, pH 7.6 and 1mM dithiothreitol (DTT).

3. *Homogenizing solution:* 50 mM tris-potassium phosphate, pH 8.0, 0.2 M KCl, 10 mM KF, 1 mM EDTA, 1mM ATP and 5 mM dithiothreitol

**Procedure:**
Tissue homogenate were prepared as mentioned in above enzymatic assay. 1 mL of assay mixture was taken in a cuvette in addition to 0.01 mL of auxiliary enzyme solution. The reaction was started by the addition of 0.01 mL of homogenate after equilibrating the reaction mixture to 30°C. After 4 min, the change in absorbance was read at 340 nm for 10 min against blank containing mixture except tissue homogenate.

**Calculation:**
One unit of PFK is that amount of enzyme required to convert 1 µmol of fructose-6-phosphate to fructose-1,6-bisphosphate per min at 30°C and activity was represented in U/mg protein.
3.13.3. Glucose-6-phosphatase (G6Pase)

Principle:
Glucose-6-phosphatase was estimated by method of Harper (1965). G6Pase activity was determined by measuring the amount of inorganic phosphate (Pi) formed on incubation with glucose-6-phosphate.

\[
\text{Glucose-6-phosphate + H}_2\text{O} \rightarrow \text{Glucose} + \text{Pi}
\]

The rate of reaction was measured by the increased inorganic phosphate with time duration by the method of Fiske and Subbarow (1925).

Reagents:
1. *Citrate buffer (0.1M, pH 6.5):* 2.1 gm citric acid dissolved in 75 mL dH₂O and pH adjusted to 6.5 with 30% NaOH and volume makeup to 100 mL with dH₂O
2. *Glucose-6-phosphate (0.08 M, G-6-P):* 417 mg G-6-P was dissolved in 3 mL dH₂O and pH was adjusted to 6.5 with 30% NaOH and volume make up to 10 mL with dH₂O
3. *Trichloroacetic acid (TCA; 10% w/v):* 10 gm TCA was dissolved into dH₂O and makeup to 100 mL
4. *Ammonium molybdate (2 × 10⁻³ M):* 25 gm ammonium molybdate was dissolved into 500 mL dH₂O. 14 mL conc. H₂SO₄ was added into 200 mL dH₂O. The dilute acid was poured into the molybdate solution and makeup to 1000 mL with dH₂O.
5. *Reducing agent (4.2 × 10⁻² M) (1-amino-2-nephthol-4-sulphuric acids):* 5.7 gm sodium meta bisulfate and 0.2 gm sodium sulfite were dissolved in 50 mL dH₂O. 0.1 gm of reducing agent was dissolved into this mixture and volume makeup to 1000mL with dH₂O
6. *Phosphate standard solutions (5 × 10⁻²M):* 68 mg potassium phosphate was dissolved in 60 mL distilled water. 10 mL of conc. H₂SO₄ was added and volume makeup to100 mL with dH₂O.
Procedure:
250 mg of tissues were homogenized with 9.75 mL citrate buffer in homogenizer. The tissue homogenate contains approximately 2.5 mg tissue / 0.1 mL. Centrifuged at 1000rpm at 4°C for 10 minutes. Supernatant were taken for further estimation. The centrifuge tubes were labeled as test, control and reagent control appropriately. 0.1 mL of supernatant was added to the test and control tubes and 0.1 mL of buffer pipette into reagent control tube and placed it in a water bath at 37°C. After 5 min incubation, 0.1 mL of glucose-6-phosphate solution was added into the each tube except control and 0.1 mL buffer to the control tube. All the tubes were incubated at 37°C for 15 min. After incubation, 2 mL TCA were added into each tube, centrifuged and the clear supernatant was used for phosphate determination.

Precaution:
The activity was measured at pH 6.5 to minimize the interference from alkaline phosphate.

Phosphate determination:
The phosphate content of supernatant was determined colorimetrically by method of Fisk and Subbarow (1925).

Procedure:
5.0 mL molybdate solution was added into 1.0 mL of supernatant from each experimental tube. 1.0 mL reducing agent was added into all tubes. The tubes were incubated and allowed to stand at room temperature for 20 min. Absorbance was taken at 680 nm against blank.

Calculation:
\[ E_E - E_{C1} \times [P] \times 2.2 = \mu \text{moles phosphate liberated in enzymatic reaction} \]

Where,

\( E_E = \) Absorbance of the experimental tube

\( E_{C1} = \) Absorbance of the control tube 1
\[ E_S = \text{Absorbance of the standard} \]

\[ [P] = \mu \text{moles phosphate in the standard tube (0.5 \mu \text{moles})} \]

\[ 2.2 = \text{Volume into \mu \text{moles phosphate / min / gm tissue, and multiplied by 1000/15 x 2.5} } \]

Where,

\[ 15 = \text{Period of enzymatic reaction (min)} \]

\[ 2.5 = \text{mg tissue in the enzymatic reaction mixture} \]

\[ 1000 = \text{conversion from mg to gm} \]

### 3.14. Estimation of key enzymes of lipid biosynthesis

#### 3.14.1. 3-hydroxy 3-methyl glutaryl CoA reductase (HMG CoA reductase)

HMG CoA reductase activity was assayed by standard method of Rao and Ramakrishnan (1975). \(^{14}\text{C}\)- labeled HMG-CoA should be used and follow the reductase activity by measuring the radioactivity as indicated for conversion to mevalonate. However we have used alternative method to determined HMG CoA reductase activity in order to avoid radioactivity. In this methodology, HMG CoA and mevalonate levels in the tissue were determined colorimetrically and ratio of HMG CoA / mevalonate was taken as an index of the activity of the enzyme.

**Principle:**

HMG CoA activity was determined by the reaction with hydroxylamine at pH 6.5 and reducing hydroxylamine acid was colorimetrically measured by the formation of complexes with ferric salt. Alkaline hydroxylamine was used to eliminate the interference of mevalonate, Mevalonate was estimated with the same reagent but at pH 2.1, where the lactone form of mevalonate readily reacts with hydroxylamine to form hydroxamate.

**Reagent:**

1. *Saline arsenate solution:* 0.1% sodium arsenate (w/v) solution in normal saline.
2. *Dilute perchloric acid:* 5 mL of 70% perchloric acid was diluted to 1 litre with dH\(_2\)O.
3. *Hydroxylamine hydrochloride solutions (2M)*: 3.47 gm hydroxylamine hydrochloride was dissolved in 25 mL of dH₂O.

4. *Hydroxylamine hydrochloride reagent for mevalonate*: Equal volume of 2M hydroxylamine hydrochloride solution and dH₂O.

5. *Hydroxylamine hydrochloride reagent for mevalonate*: Equal volume of 2M hydroxylamine hydrochloride solution and 4.5M NaOH were mixed freshly before use.

6. *Ferric chloride reagent*: 5.2 gm TCA and 10 gm ferric chloride were dissolved in 50 mL of 0.65 M HCl and volume upto 1000 mL with 0.65 M HCl.

**Procedure:**

Liver tissue (1 gm) was homogenized with saline arsenate to make 10 mL of homogenate. 2 mL of the homogenate was mixed with equal volume of diluted perchloric acid, mixed well, allowed to stand for 5 min and centrifuged at 5000g at 4°C for 10 min. In two test tubes, 1 mL supernatant was taken in each test tube. 0.5 mL freshly prepared hydroxylamine reagent was added in one tube to determine HMG CoA. After 5 min, 1.5 mL of ferric chloride reagent was added in each tube and allowed to stand at room temperature for 10 min. Absorbance was taken at 540 nm.

**3.14.2. Acetyl CoA carboxylase (ACC)**

ACC activity was assayed by standard method of Numa et al (1960).

**Principle:**

ACC catalyzes the ATP-dependent carboxylation of acetyl CoA from malonyl CoA, ADP and orthophosphate.

\[
\text{Acetyl Co-A + CO}_2 + \text{ATP} \xrightarrow{\text{Carboxylase}} \text{Malonyl Co-A + ADP + Pi}
\]

In this assay, the formation of ADP is followed spectrophotometrically by coupling the carboxylase reaction with the reactions catalyzed by pyruvate kinase (PK) & lactate dehydrogenase (LDH).
Phosphoenolpyruvate + ADP → Pyruvate kinase → ATP

DPNH + H⁺ → (NADH)

LDH

Lactate + DPN⁺ → (NAD⁺)

NAD⁺ formed in this reaction was measured colorimetrically at 340 nm.

One unit of enzyme is defined as that amount which catalyzes the carboxylation of 1 µmol of acetyl CoA per minute under conditions described. With an excess of PK & LDH, the rate of oxidation of NADH is proportional to the rate of formation of ADP.

**Reagents:**

1. *Tris-HCL buffer (1M, pH 8.0):* 1.21 gm Tris was dissolved in 7 mL of dH₂O, pH adjusted to 8 with HCL and volume makeup to 10 mL with dH₂O.
2. *MgCl₂ (40 mM):* 38 mg MgCl₂ was dissolved in 10 mL of dH₂O.
3. *Adenoside triphosphate (20 mM):* 11.02 mg ATP was dissolved in 1 mL dH₂O.
4. *KHCO₃ (0.5 M):* 10 mg potassium bicarbonate was dissolved in 1 mL of dH₂O.
5. *Potassium chloride (1 M):* 7.45 gm KCl in 100 mL dH₂O.
6. *Phosphoenolpyruvate (PEP) (10 mM):* 2.67 mg PEP was dissolved in 1 mL of dH₂O.
7. *Pyruvate Kinase (PK)*
8. *Lactate dehydrogenase*
9. *Acetyl CoA carboxylase* (i.e. tissue homogenate)
10. *Acetyl CoA (10 mM):* 8.27 mg of acetyl CoA was dissolved in 10 mL of dH₂O.

**Assay Procedure:**

Reaction was started in quartz cell at 30°C. Each cell contained (in µmoles) tris buffer, pH 8.0, 100 MgCl₂, 4 GSH, 2 ATP, 2 KHCO₃, 50 KCl, 100 PEP, 1 NADH, 0.6; excess of PK and LDH flowed by addition of tissue homogenate in total volume of 1.0 mL. The reaction was started by addition of 0.5µmolen of acetyl CoA and the
rate of decrease of absorbance at 340 nm was taken for 5 min.

Calculations:

\[
\text{Acetyl CoA carboxylase} = \frac{\Delta A/\text{min} \times \text{total volume}}{6.22 \times \text{enzyme volume} \times \text{protein (mg)}}
\]

3.15. Estimation of glycogen content

Glycogen was isolated from tissue by the standardized method of Good et al. (1933). Glycogen was extracted from tissue and precipitated with ethanol. Glycogen was estimated by the method of Carroll et al. (1956) using conc. \( \text{H}_2\text{SO}_4 \). It hydrolyzes glycosidic bonds to give monosaccharide which are taken dehydrated to furfural and its derivatives. These products then combined with anthron to give a blue green complex.

Reagents:

1. \( \text{KOH (50\%)} \)
2. \( \text{Ethanol (95\%)} \)
3. \( \text{Ethanol (80\%)} \)
4. \( \text{Anthron reagent:} 50\text{mg anthrone and 1 gm thiourea were dissolved in 100 mL of 72\% H}_2\text{SO}_4 \)

Procedure:

To a pre-weighed amount of tissue (liver and muscle) in the test tube, 50\% KOH (2 mL/gm tissue) was added and kept in a boiling water bath till homogeneous solution was obtained (10-20min). To this extract, 1.1 to 1.2 times its volume of 95\% ethanol was added and the mixture was boiled in water bath. The tubes were left overnight at room temperature and allow for complete precipitation of glycogen. Centrifuge the tube and supernatant were discarded. Precipitated glycogen was washed with 80\% ethanol. After washing, the precipitate was re-dissolved in a suitable volume of water and aliquots of precipitate was mixed with 5 mL of anthron reagent and kept in boiling water bath for 15 min. After cooling, the absorbance was taken at 620 nm. Glucose solution at different concentration (10, 20, 40, 80, 100 mg/mL) was used to
plot the standard graph. The value of the samples obtained from the graph was multiplied by 0.9 for conversion of glucose to glycogen.

3.16. Estimation of lipid content

Total lipid was extracted from the tissue by the method of Folch et al. (1957) as modified by Radin (1969). Weight portion of tissue was homogenized with about 17 volumes (v/w) of chloroform: methanol (2:1 v/v). The fine suspension was then filtered through Whatman No. 1 filter paper and homogenizer and funnel were washed with again 2 volumes of chloroform : methanol. The filtrate obtained were shaken well with 1/5th its volume of water in a glass stopped tube. The tubes were centrifuged to make the separation of phases completely and to avoid contamination by microdroplets. The upper aqueous phase and interphase were removed carefully by a Pasteur pipettes. The lower phase was taken and washed with “Folch upper phase” consisting of chloroform : methanol : water (3:48:47), trice. The organic phase was evaporated in rotator evaporator. For this lipid extract, approximately 1-2 mL of benzene was added, mixed and the solvent was evaporated under vacuum. The traces of moisture were removed by drying under a steam of nitrogen. The extract was dissolved in a known volume of chloroform and stored in air tight container at -20°C for further analysis.

An aliquot of the above lipid extract (about 100 µl) was taken in a pre-weight petridish and dried at 60°C to constant weight. The amount of total lipid was calculated and expressed as mg / g wet tissue.

3.17. Protein estimation

Protein was estimated by the Folin Ciocalteau reagent (Lowry et al. 1951). The blue color developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartrate was measured by the Lowry’s method.

Reagent:

_Reagent A_: 2% Na₂CO₃ in 0.10 N NaOH

_Reagent B_: 0.5% CuSO₄.5H₂O in 1% Sodium tartrate.

_Reagent C_: Alkaline Cu solution (fresh)
Reagent D: Carbonate-copper solution

Reagent E: Dilute Folin reagent

Refluxed gently for 10 hours a mixture consisted of 100g sodium tungstate (Na$_2$WO$_4$·2H$_2$O), 25g sodium molybdate, 700 mL water, 50 mL of 85% phosphoric acid, and 100 mL of concentrated hydrochloric acid in a 1.5L flask. Added 150g lithium sulfate, 50 mL water and a few drops of bromine water. Boiled the mixture for 15 min without condenser to remove excess bromine. Allowed to cool, dilute and make up to 1L and filter. The reagent did not have greenish tint. (Determine the acid concentration of the reagent by titration with 1N NaOH to a phenolphthalein end-point).

Protein solution (Stock standard)

Weigh accurately 50mg of bovine serum albumin (Fraction V) and dissolve in distilled water and made up to 50 mL in a flask.

Working standard

Diluted 10 mL of the stock solution to 50 mL with distilled water in a standard flask. One mL of this solution contains 200mg protein.

Procedure:

Extraction of protein from sample

Extraction was carried out with buffers used for the enzyme assay. Weigh 500.0 mg of the sample was grind well with a pestle and mortar with 5-10 mL of the buffer. Centrifuged and used the supernatant for protein estimation.

Estimation of protein

1. Pipette 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard into a series of test tube.
2. Pipette 0.1 mL and 0.2 mL of the homogenate in two other test tubes.
3. Made up the volume to 1 mL in all the test tubes. A tube with 1 mL of water served as the blank.
4. Added 5 mL of reagent C to each tube including the blank. Mixed well and allowed to stand for 10min.
5. Then added 0.5 mL of reagent D, mixed well and incubated at room temperature in the dark for 30 min. Blue color is developed.

6. Taken the reading at 660 nm.

7. Drawn a standard graph and calculate the amount of protein in the sample.

**Calculation**

Amount of protein is expressed in mg/g wet tissue.

**3.18. Estimation of parameters of oxidative stress**

**3.18.1. Lipid peroxidation (malondialdehyde; MDA)**

**3.18.1.1. In serum**

Serum MDA levels were measured as an index of lipid peroxidation using the colorimetric method as described by Satoh (1978).

**Principle:**

Lipid peroxide is precipitated from serum with trichloroacetic acid (TCA) and heated with thiobarbituric acid (TBA). The reaction results in formation of a pink coloured chromogen, which is extracted with n-butyl alcohol. Absorbance of organic phase is determined at 530nm.

**Reagents:**

1. *Trichloroacetic acid (TCA) 20%*

2. *Sulphuric acid (0.05M)*

3. *Sodium sulfate solution (2M)*

4. *Thiobarbituric acid (TBA) 0.22% w/v in 2M sodium sulfate*

5. *n-butanol*

**Procedure:**

1. 2.5 mL of TCA was added to 0.5 mL of serum in a test tube and left for 10 min at room temperature.
2. The tube was centrifuged at 3500rpm for 10 min. After centrifugation, the supernatant was discarded and precipitate was washed twice with sulphuric acid.

3. 2.0 mL of sulphuric acid and 3.0 mL of TBA were added to the precipitate and the mixture was kept in boiling water for 30 min to allow coupling of lipid peroxides with TBA.

4. Therefore, the tube was kept in cold water. After cooling, the result chromogen was extracted with 4.0 mL of n-butyl alcohol by vigorous shaking.

5. Organic phase was separated by centrifugation at 3000rpm for 10 min and absorbance was taken at 530 nm. The value was expressed in terms of MDA concentration in nmol/mL using standard graph.

3.18.1.2. In tissues

Reagents:
1. 0.1 M Tris-HCl buffer, pH 7.4: 1.21 gm of Tris dissolved in 90 mL of distilled water, pH adjusted to 7.4 with dilute HCl and volume made upto 100 mL with distilled water.

2. 5% TCA: 5 gm TCA dissolved in 100 mL distilled water

3. 0.67% TBA: 0.67 gm TBA in 100 mL distilled water

Procedure:
Tissue homogenate (5% w/v) were prepared in ice cold 0.1 M tis HCl buffer (pH 7.4), using a mechanically driven Teflon fitted potter-Elvejhem homogenates (1.0 mL) were incubated at 37°C for 2 hrs with consistent shaking. After incubation, 1.5 mL ice cold TCA (5%) was added and centrifuged at 2500 rpm for 10 min 1.5 mL of supernatant and 1.5 mL of 0.67% TBA mixed and coloured was developed at 100°C for 10 min. Sample was cooled and absorbance taken at 532 nm.

 Calculation: The amount of MDA formed was calculated on the basis of molar
extinction coefficient of MDA ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and the results were expressed as nmol / mg wet tissue.

### 3.18.2. Superoxide dismutase (SOD)

Activity of SOD in erythrocytes was assayed by the method as described by Murklund and Marklund (1974) and modified by Nandi and Chatterjee (1988).

**Principle:**
The method is based on the ability of the enzyme SOD to inhibit the autooxidation of pyrogallol.

**Reagent:**
1. *Disodium EDTA* (30 mM)
2. *Tris HCl buffer* (50 mM, pH 8.5)
3. *Pyrogallol solution* (2.6 mM), prepared fresh in 10 mM HCl

**Procedure:**

#### 3.18.2.1. Preparation of hemolysate

Red blood cells (RBCs) were washed twice with normal saline and hemolysed with three volumes of cold distilled water. Hemoglobin concentration was measured in the hemolysate by cyanmethaemoglobin method using Drabkin’s reagent. For Hb estimation, 20 µl of hemolysate was mixed with 5.0 mL of Drabkin’s reagent and incubated for 5 min at room temperature. The absorbance was taken at 540 nm.

Tsuchihasi extract was prepared by adding 3.5 mL of ice cold water, 1 mL of ethanol and 0.6 mL of chloroform to 0.5 mL of hemolysate and vortexed and mixed well for 10 min. The tubes were centrifuged for 10 min at 3000 rpm. The clear supernatant was used for the enzyme assay.

#### 3.18.2.2. Preparation of tissue homogenate

After dissection of tissue, tissue were washed with ice cold saline, blotted dry and weighed. A 10% homogenate (w/v) of the liver was prepared using a potter homogenizer fitted with Teflon plunger. The homogenizing medium was 10mM
phosphate buffer, pH 7.4 supplemented with 30 mM KCl. The homogenate were centrifuged at 1500 rpm for 10 min to remove nuclei and cell debris. The clear supernatant was used for the enzyme assay.

**Assay procedure:**
Firstly, to adjust the concentration of pyrogallol required to get the rate of change of absorbance per minute approximately 0.020-0.023, the reaction was initiated by addition of 100 µl (variable) of freshly prepared 2.6 mM pyrogallol solution in assay mixture containing 100 µl of EDTA followed by addition of 2.8 mL tris buffer (variable to get final volume of 3 mL). As a blank, a mixture of 2.9 mL of tris buffer and 100 µl of EDTA was used. The rate of increase in absorbance at 420 nm was recorded for 2 min from 1 min to 3 min. The lag of 1 min was allowed for steady state auto-oxidation of pyrogallol to be attained.

The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the addition of SOD. To get the volume of supernatant (containing enzyme) required for 50% inhibition, the reaction was carried out by taking different amounts of supernatant (50-300 µl). The reaction was initiated by addition of pyrogallol solution as amount set by prior reaction in an assay mixture containing supernatant (variable, 50-300 µl), 100 µl of EDTA and 2.7 mL tris buffer (variable to get final volume of 3 mL). One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation per 3 mL of assay mixture. 50% inhibition was considered when change in absorbance per min from 1 min to 3 min at 420 nm was 0.010-0.012. Finally, the results were expressed as unit per gm of hemolysate Hb (U/g Hb).

**3.18.3. Reduced glutathione (GSH)**

**3.18.3.1. In RBCs**

GSH in erythrocytes was estimated by the method of Beutler et al. 1963.

**Principle:**
The method is based on the development of yellow colour, when 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) is added to sulphydryl compounds.
Reagents:
1. *Precipitating reagent*: Metaphosphoric acid (1.67 gm), Disodium-EDTA (0.2 gm) and sodium chloride (30 gm) were dissolved in dH₂O and makeup to 100 mL.
2. *Na₂HPO₄ (0.3M)*
3. *Na₂EDTA (1 gm/L)*
4. *Sodium citrate (10 gm/L)*
5. *DTNB*: 40 mg DTNB dissolved into 10 gm/L sodium citrate
6. *Glutathione standard (GSH)*

Procedure:
1. 0.2 mL blood was lysed by addition of 1.8 mL of EDTA.
2. 3.0 mL of precipitating reagent was added to the hemolysate and mixed well. The mixture was allowed to stand for 5 min and then filtered.
3. 4.0 mL of Na₂HPO₄ and 1.0 mL of DTNB reagent were added to 2.0 mL of filtrate. DTNB was added just before taking the absorbance.
4. A blank was prepared containing 1.2 mL of precipitating reagent, 0.8 mL EDTA solution, 4.0 mL of Na₂HPO₄ and 1 mL of DTNB reagent.

The absorbance was measured at a wavelength of 412 nm. Results were expressed as mg/g Hb.

**Calculation:** The GSH content can be calculated by using standard graph for glutathione.

3.18.3.2. *In tissue*

GSH in tissue was assayed by the method of Ellamn 1959.

Reagents:
1. *0.1 M Phosphate buffer, pH 8.0*: (a). 1.56 gm NaH₂PO₄ in 100 mL dH₂O (b). 1.42 mg Na₂HPO₄ in 100 mL dH₂O (3.3 mL of solution (a) and 94.7 mL of solution (b) was taken to make 0.1 M phosphate buffer of pH 8.0).
2. *4% Sulfosalicylic acid*: 4 gm sulfosalicyclic acid was dissolved in 100 mL dH₂O.

3. *0.1 mM DTNB*: 3.96 mg DTNB was dissolved in 100 mL, 0.1 M phosphate buffer, pH 8.0

**Procedure:**

Tissue homogenate prepared for enzyme assays were used for glutathione estimation. 1.0 mL of tissue homogenate was precipitated with 1.0 mL of sulfosalicyclic acid (4 %) and centrifuged at 1200 rpm for 5 min. To 0.5 mL of supernatant, 4.5 mL of Ellman’s reagent (DTNB) was added and absorbance was taken at 412 nm after 2 min. GSH was calculated as µmol/g wet tissue using standard GSH graph. Ellman’s reagent consisted of 0.1mM DTNB (5-5’-dithiobis (2-nitrobenzoic acid) in 0.1 M phosphate buffer, pH 8.0.

### 3.18.4. Ferric reducing ability of plasma (FRAP)

**Principle:**

Ferric reducing ability of plasma (FRAP) is a novel method for assessing “antioxidant power” by ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripryidyltriazine complex (Benzie and Strain 1996 & 1999).

**Reagent:**

1. 300 mmol/L acetate buffer pH 3.6

2. 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine)

3. 40 mmol/L HCl

4. 20 mmol/L FeCl₃.6H₂O

**Working solution:** Added 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL FeCl₃.6H₂O.

**Procedure:**

300µl freshly prepared FRAP reagent was warmed to 37⁰C and a reagent blank reading was taken at 593nm; 10µl of sample was then added, along with 30µl H₂O. Absorbance (ΔA) reading was taken after 0.5sec and every 15sec upto 4 minutes during the monitoring period and expressed in µmol/L.
Calculated:

\[
\text{FRAP activity} = \frac{\Delta A \text{ of test sample}}{\Delta A \text{ of test standard}} \times \text{FRAP volume of standard}
\]

3.19. Statistical analysis

The results are expressed as mean ± SEM for six animals in each group. The data were analyzed by repeated measure analysis of variance (ANOVA) followed by Dunett’s multiple comparison test for more than one time-point studies and one way analysis of variance followed by Tukey’s multiple comparison test for one time-point studies. SPSS software version 17.0 was used. P value of <0.05 was considered significant.