Materials and Methods

Chemicals

Following analytical grade chemicals were procured from the respective companies.

**Sigma-Aldrich Co. St. Louis, MO, USA**

- Acarbose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Nicotinamide (NA), Streptozotocin (STZ).

**HiMedia Laboratories Pvt. Ltd. Mumbai, Maharashtra, India**

- Ascorbic acid, ATP, Activated alumina, Activated charcoal, Chloroform, Cholesterol, Citric acid, Dichromate acetic acid, Disodium phenyl phosphate, DL-alanine, DL-aspartic acid, Ethylene diamine tetra acetic acid (EDTA), Ferric chloride, Ferrous chloride, Ferrozine, Fiske Subbarow reagent, Fructose-1,6-bisphosphate, Glacial acetic acid, Glucose, Glucose-6-phosphate, Glutathione, Hydrogen peroxide, Magnesium chloride, NADH, NADP⁺, NBT, n-butanol, Phosphotungstic acid, Potassium chloride, Potassium dichromate, Potassium dihydrogen phosphate, Potassium ferricyanide, Potassium hydroxide, Potassium (meta) bisulphite (PMS), Pyruvate, Sodium azide, Sodium citrate, Sodium fluoride, Sodium hydroxide, Sodium (meta) periodate, Thiourea, Trichloroacetic acid, Triolein, Tris, α-Tocopherol, 2,4-Dinitrophenylhydrazine (DNPH), 2,2’-dipyridyl, 4-aminoantipyrine, 5,5’-dithiobis 2-nitrobenzoic acid (DTNB).

**SRL Pvt. Ltd. Mumbai, India**

- α-Amylase, α-Glucosidase, Soluble starch, Maltose, 3,5-dinitrosalicylic acid (DNSA).

Glucose assay kit was purchased from Agappe Diagnostic Pvt Ltd, Kerala, India and insulin assay kit was obtained from BARC, Mumbai, Maharashtra, India. All other chemicals and reagents used were of highest analytical grade.
Plant material

The raw fruits of *Cucumis prophetarum* were collected during July and August 2011 from Western Ghats of Coorg region (Karnataka, India). The plant was botanically authenticated by taxonomist. The herbarium with voucher no KU/BL/MK/105 has been deposited in Applied Botany Department, Kuvempu University, Shimoga, India.

Extract preparation

Fresh fruits were washed, cleaned and air dried. Whole fruits were homogenized in a commercial blender. The fresh juice was filtered using muslin cloth and filtrate was centrifuged at 23000 x g for 10 min, the supernatant was lyophilized and stored at -20°C until use. The extract was used at the required concentrations.

Qualitative preliminary phytochemical group test

The tests for carbohydrates, reducing sugars, proteins, amino acids, saponins, sterols, alkaloids, tannins, glycosides and flavonoids contents of aqueous fruit extract were performed by the standard methods (Brain and Turner, 1975; Evans, 1996).

In vitro antidiabetic activity determination

Pancreatic α-amylase inhibition assay

The inhibition assay was performed by the chromogenic DNSA method as described by Miller (1959), with slight modification. The assay mixture composed of 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.25 units of PPA solution and test samples at 0.02 to 0.1 mg/mL (w/v) concentrations were pre-incubated at 37°C for 10 min. To the above buffer, 500 µL of 1% (w/v) starch solution was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 mL DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature, diluted and the absorbance measured at 540 nm. The control without test sample represented 100% enzyme activity. The absorbance produced by test sample was eliminated by including appropriate sample control with the sample in the reaction mixture except for the enzyme and starch. The known PPA inhibitor acarbose was used as positive control.
**α-Glucosidase inhibition assay**

The inhibition assay was performed according to the method of Andrade-Cetto with slight modifications (Andrade-Cetto et al., 2008). The assay was carried out by incubating a solution of starch substrate (2% w/v maltose) 1 mL of 0.2 M Tris buffer pH 8.0 and various concentrations of test samples for 5 min at 37°C. The reaction was initiated by adding 1 mL of α-glucosidase enzyme (1U/mL) followed by incubation for 10 min at 37°C. The reaction mixture was heated for 2 min in boiling-water bath to stop the reaction. The quantity of glucose liberated was measured using GOD-POD kit. The control represented 100% enzyme activity without any test sample. The absorbance produced by test sample was eliminated by including appropriate sample controls with the sample in the reaction mixture except for the enzyme and substrate. The α-glucosidase inhibitor, acarbose was used as positive control.

**Calculation of IC₅₀ value of antidiabetic assay**

The IC₅₀ value is defined as that concentration of the extract containing the enzyme inhibitor which inhibits the activity by 50%.

The % inhibition was calculated as follows:

% Inhibition = EC – (ET – TC) / EC

Where, EC is enzyme activity of control, ET is enzyme activity of test and TC is test control.

‘One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of product from substrate per min under the assay conditions’.

**In vitro Antioxidant activity determination**

**DPPH assay**

2,2-Diphenyl-1-picrylhydrazyl is a stable free radical and widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution due to the formation of the non-radical form DPPH-H in the presence of a hydrogen donating antioxidant (Blois, 1958). This
transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm.

The DPPH free radical scavenging activity of each sample was determined (Lai et al., 2001). An aliquot of 200 µL of each sample with different concentrations (0.02-0.1 mg/mL) were mixed with 100 mM Tris-HCl buffer (800 µL, pH 7.4) and then added to 1 mL of 500 mM freshly prepared DPPH solution in methanol (final concentration of 250 µM). The mixture after shaking vigorously was allowed to stand for 20 min at room temperature in dark and absorbance was measured at 517 nm. Ascorbic acid was used as standard.

**Superoxide anion radical scavenging assay**

Superoxide anion, a weak oxidant gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaken, 1995). In the PMS/NADH-NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease in absorbance at 560 nm by antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski (1988). The superoxide anion radicals were generated in 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 mL NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL test sample and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). The reaction was initiated by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and the absorbance was read at 560 nm against a blank. Ascorbic acid was used as a positive control.

**Metal chelation assay**

Ferrozine can quantitatively chelate with Fe²⁺ forming a red color complex. This reaction is limited in the presence of other chelating agents. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000).
The effect of chelation of ferrous ions on each sample was estimated using the method of Dinis (Dinis et al., 1994). To 0.1 mL of the test sample, was added a solution of 0.5 mL ferrous chloride (0.2 mM). The reaction was initiated by adding 0.2 mL of ferrozine (5 mM), incubated at room temperature for 10 min and the absorbance measured at 562 nm. Citric acid was used as a positive control.

Calculation of IC$_{50}$ value of antioxidant assay

The concentration of sample required for scavenging 50% of the radicals/to chelate metal ions was calculated as follows.

\[
\% \text{ Inhibition} = \left( \frac{(A_{\text{cont}} - A_{\text{ samp}})}{A_{\text{cont}}} \right) \times 100
\]

Where, ‘Acont’ is the absorbance of control and ‘Asamp’ is the absorbance of sample. IC$_{50}$ value denotes the concentration of a sample required to decrease the absorbance by 50%.

Animals

Male albino wistar rats aged 4 months (150-180g) were used for the study. The rats were maintained at 22 ± 2°C with 12 h light and dark cycles, fed on standard pellet diet supplied by Lipton India Ltd. Animals had free access to diet and water. Animal studies conducted were approved by the Institutional Animal Ethics Committee, University of Mysore (Approval No. UOM/IAEC/4/2012), Mysore, as stated by prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Streptozotocin-Nicotinamide induced type 2 diabetic model

Development of experimental NIDDM (type 2 diabetes) was achieved according to the method described by Masiello et al. (1998).

After initial determination of 12 h fasting blood glucose levels (end tail puncture), experimental type 2 diabetes was induced in rats by intraperitoneal administration of Nicotinamide (NA)-230 mg/kg dissolved in saline 15 min before Streptozotocin (STZ)-65 mg/kg dissolved in 0.1 M citrate buffer (pH 4.5) immediately before use. Eight hours after STZ-NA administration, the rats were kept for next 24 h on 15% glucose solution.
bottles in their cages to prevent hypoglycemia. Animals having marked hyperglycemia (fasting blood glucose $\geq 250$ mg/dL), 48 h after STZ-NA treatment was used in the study.

**Experimental design**

A total of 36 rats were divided into six groups as follows:

- **Group I**: normal untreated rats
- **Group II**: normal rats treated with $50$ mg N-Trisaccharide/kg.b.w/day
- **Group III**: diabetic untreated rats
- **Group IV**: diabetic rats treated with $25$ mg N-Trisaccharide/kg.b.w/day
- **Group V**: diabetic rats treated with $50$ mg N-Trisaccharide/kg.b.w/day
- **Group VI**: diabetic rats treated with $25$ mg glibenclamide/kg.b.w/day

N-Trisaccharide/glibenclamide was administered by gastric intubation with a force feeding needle into the animals of the respective groups every day morning for 28 days. Body weight, blood glucose and plasma insulin were measured at weekly interval during the experimental period.

Rats of all the six groups were sacrificed on 29th day after an overnight fasting under anesthesia. Blood was drawn from the heart. The liver and kidney were removed, washed with chilled saline and small weighed portion of the tissues were immediately processed for determination of glycogen. Homogenates (w/v) of liver and kidney (10%) were prepared in 150 mM KCl using homogenizer at 4°C. The homogenates were centrifuged at 3000 x g for 15 min at 4°C. The supernatants were frozen at $-20\degree$C until assayed for different enzymes. Blood plasma was recovered by centrifugation at 1000 x g for 10 min at 4°C. Pancreas, liver and kidney in small sections were collected in 10% formalin solution and immediately processed for histological studies by the paraffin technique.

**Blood Glucose**

Estimation of blood glucose was carried out by using dextro stix (Glucose oxidase method) with one touch glucometer (Johnson and Baker, 1998; ADA, 2008).
Plasma Insulin

Insulin was determined by using the modified method of Herbert et al. (1965) using Insulin RIA (radio immune assay) kit obtained from BARC, Mumbai, India. Blood samples were collected in EDTA coated vials and plasma was separated immediately and kept frozen at -20°C till the assay. The procedure followed for the assay is shown in Table 2.1. The method is based on the competition of unlabeled insulin in the standard or samples and labeled insulin (I^{125}-Insulin) for the limited binding sites on insulin specific antibody. At the end of incubation, secondary antibody and polyethylene glycol were used to precipitate antibody and free insulin. Radioactivity of bound fractions of samples and standards were measured and the insulin levels of samples were obtained by interpolating from the graph plotted using the known concentration of standard insulin.

Calculations

Per cent B/BO was calculated as follows where, B is sample or standard binding and BO is zero standard binding.

\[
\% = \frac{\text{Corrected average counts of standard or sample}}{\text{Corrected average counts of zero standard}} \times 100
\]

A standard graph was plotted on a logit log graph with % B/BO on the logit scale and standard insulin concentration on the logarithmic scale. The B/BO% values for the samples were extrapolated from the standard curve as µU insulin/mL. The lowest detection limit of the assay was 2 µU/mL. Inter assay coefficients of variation was 5.6-7%. An Intra assay coefficient of variation was 7-8%. Control sera showed 92 to 108% recovery.

Glycosylated hemoglobin (HbA1c)

Glycosylated hemoglobin in the blood was estimated by the method of Sudhakar and Pattabiraman (1981).
Table 2.1: Insulin assay flow chart

<table>
<thead>
<tr>
<th>Tube No</th>
<th>Assay Buffer (mL)</th>
<th>Insulin Standard (mL)</th>
<th>Serum Sample (mL)</th>
<th>Insulin Free Serum (mL)</th>
<th>Insulin Anti-Serum (mL)</th>
<th>I-^{125S} Insulin (mL)</th>
<th>Second antibody (mL)</th>
<th>PEG (mL)</th>
<th>Vortex and keep all the tubes at room temp. for 20 min.</th>
<th>Centrifuge the tubes at 1500 x g for 20 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>Mix gently &amp; incubate all the tubes for 2 h at 4°C over night</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>Mix gently &amp; incubate all the tubes for 3 h at room temp.</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7,8</td>
<td>0.2</td>
<td>0.1F</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9,10</td>
<td>0.2</td>
<td>0.1E</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>11,12</td>
<td>0.2</td>
<td>0.1D</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>13,14</td>
<td>0.2</td>
<td>0.1C</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>15,16</td>
<td>0.2</td>
<td>0.1B</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>17,18</td>
<td>0.2</td>
<td>0.1A</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
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<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>19,20</td>
<td>0.3</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>21,22</td>
<td>0.3</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

After centrifugation, decant and count radioactivity in the precipitate.
Reagents

1. 1 M potassium oxalate in 2 M hydrochloric acid (Oxalate-hydrochloric acid).
2. Concentrated H₂SO₄
3. Phenol: 80%
4. Saline
5. TCA: 40%
6. Working standard: stock standard was prepared by dissolving 100 mg of fructose in 100 mL of water and diluted to get a concentration of 100 µg/mL.

Procedure

Five mL of blood were collected with EDTA and plasma was separated. Saline washed erythrocytes (0.5 mL) was lysed with 5 mL of water, mixed and incubated at 37°C for 15 min. The contents were centrifuged and the supernatant was discarded, then 0.5 mL of saline was added, mixed and processed for estimation. To 2 mL of aliquot, 4 mL of oxalate hydrochloride solution were added and mixed. The contents were heated at 100°C for 4 h, cooled and precipitated with 2 mL of 40% TCA. The mixture was centrifuged and to 0.5 mL of supernatant, 0.5 mL of 80% phenol and 3 mL of concentrated sulphuric acid were added.

Working standard in the range of 10-50 µg were taken and made up to 1 mL; 0.05 mL of 80% phenol and 3 mL of concentrated sulphuric acid were added to this. The color developed was read at 480 nm after 30 min. The concentration of glycosylated hemoglobin is expressed as mg/g of hemoglobin.

Glycogen

Glycogen in the liver was estimated by the method of Kemp and Hejnigen (1954).

Reagents

1. Concentrated sulphuric acid
2. Glucose standard
3. Methanol 90%
4. Trichloroacetic acid 15%
Procedure

Five mL of 10% tissue homogenate were prepared in 90% methanol and the contents were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and to the pellet, 5 mL of deproteinising solution (TCA) was added and the levels were marked on the tubes. The tubes was capped and kept in boiling water bath for 15 min. After boiling, the tubes were cooled and the contents were made up to the mark and centrifuged at 3000 rpm for 15 min. One mL of the supernatant was taken and to this 3 mL of H₂SO₄ was added. The tubes were shaken well and kept in boiling water bath for 6.5 min, cooled and the color developed was read at 520 nm. A blank and a standard were run similarly taking 1 mL of distilled water and 1mL of glucose standard respectively.

Glycogen content was calculated from the amount of glucose present in the sample and is expressed as mg/g tissue.

Protein

Protein determination was carried out after trichloroacetic acid precipitation by the method of Lowry et al. (1951).

Reagents

1. Alkaline copper reagent:
   - Reagent A: 2% sodium carbonate in 0.1 N NaOH
   - Reagent B: 0.5% copper sulphate in 1% sodium potassium tartrate.
   - Reagent C: 50 mL of reagent A was mixed with 0.5 mL of reagent B just before use.
2. Bovine serum albumin (BSA) was used as standard (0.1 mg/mL)
3. Folin’s phenol reagent: commercially available Folin ciocalteau’s reagent was diluted with distilled water in 1:1 proportion.

Procedure

Tissue homogenate (0.5 mL) was mixed with 0.5 mL of 10% TCA and centrifuged for 10 min. The precipitate was dissolved in 1 mL of 0.1 N NaOH. From this, an aliquot was taken, added 4.5 mL of alkaline copper reagent and allowed to stand at room temperature for 10 min. Folin’s phenol reagent (0.5 mL) was added and the blue color developed was read after 20 min at 640 nm. A standard curve was obtained with
standard BSA and was used to assay the tissue protein level for enzyme activity. Values are expressed as mg/g of tissue.

**Carbohydrate metabolizing enzymes**

**Hexokinase**

The activity of Hexokinase was assayed according to the method of Branstrup et al. (1957).

**Reagents**

1. ATP: 0.72 M
2. Glucose: 0.005 M
3. KCl: 0.1 M
4. KH$_2$PO$_4$: 0.0125 M
5. MgCl$_2$: 0.05 M
6. O-Toluidine reagents for glucose estimation
7. Sodium fluoride: 0.15 M
8. Tris-HCL buffer: 0.10 M, pH 8

**Procedure**

The test incubation mixture (5 mL) contained 2.5 mL of buffer, 1mL of substrate, 0.5 mL of ATP, 0.1 mL of MgCl$_2$, 0.1 mL of NaF, 0.4 mL of KH$_2$PO$_4$ and 0.4 mL of KCl. The mixture was pre-incubated at 37°C for 5 min. Reaction was initiated by the adding 0.2 mL of tissue homogenate. The mixture was incubated for 30 min at 37°C and the reaction was arrested by adding 1.0 mL of 10% TCA. The control was run in the same manner but, TCA was added at zero time i.e., before incubation with enzyme. The reaction mixture was centrifuged at 3000 rpm for 15 min and 0.5 mL of the supernatant was used for the estimation of residual glucose by o-toluidine method. The activity of enzyme is expressed as micromoles of glucose phosphorylated/min/mg protein.

**Glucose-6-phosphatase**

The activity of glucose-6-phosphatase was assayed by the method of King (1965).

**Reagents**

1. Citrate buffer: 0.1 M, pH 6.5
2. Fiske Subbarow reagents for inorganic phosphate estimation
3. Glucose-6-phosphate: 0.01 M
4. TCA: 10%

Procedure

The test incubation mixture in a total volume of 1.0 mL contained 0.5 mL of substrate, 0.4 mL of buffer and 0.1 mL of homogenate. The reaction mixture was incubated at 37°C for 60 min. The enzyme action was terminated by the addition of 1.0 mL of 10% TCA. A control was also run similarly but, TCA was added prior to the addition of enzyme. The supernatant obtained after centrifugation was used for estimating inorganic phosphate by Fiske and Subbarow (1925) method. The enzyme activity is expressed as micromoles of inorganic phosphate liberated/min/mg protein.

Fructose-1,6-bisphosphate

The activity of fructose-1,6-bisphosphate was assayed according to the method of Gancedo and Gancedo (1971).

Reagents
1. Ethylenediamine tetra acetic acid: 0.001 M
2. Fructose-1,6-bisphosphate: 0.05 M
3. KCl: 0.1 M
4. MgCl₂: 0.1 M
5. Trichloro acetic acid: 10%
6. Tris-HCl buffer: 0.1 M, pH 7.

Procedure

The assay mixture of test (2.5 mL) contained 1.7 mL of Tris-HCl buffer, 0.1 mL of KCl, 0.1 mL of fructose-1,6-bisphosphate, 0.25 mL of EDTA, 0.25 mL of MgCl₂ and 0.1 mL of tissue homogenate. The reagent mixture was incubated at 37°C for 15 min and the enzyme reaction was terminated by adding 1.0 mL of 10% TCA. The control was also run similarly but, TCA was added at zero time i.e. before incubation with enzyme. The contents were centrifuged and supernatant was used for the estimation of inorganic phosphate by Fiske and Subbarow (1925) method. The activity is expressed in micromoles of inorganic phosphate liberated/min/mg protein.
**Glucose-6-phosphate dehydrogenase**

The activity of glucose-6-phosphate dehydrogenase was assayed by the method of Langdon (1966).

**Reagents**
1. MgCl₂: 0.2 M
2. Tris-HCl buffer: 1 M, pH 7.5
3. Glucose-6-phosphate: $2.5 \times 10^{-2}$ M
4. NADP⁺: $2 \times 10^{-3}$ M

**Procedure**

The assay mixture consisted of 2.5 mL of buffer, 0.1 mL of glucose-6-phosphate, 0.2 mL of NADP⁺, 0.1 mL of MgCl₂ and 0.1 mL of homogenate. Immediately after the addition of the homogenate, the rate of change in the absorbance was measured at 340 nm for 2 min with the time interval of 30 sec in a spectrophotometer against a blank without the enzyme. One unit of enzyme activity is defined as the quantity required catalyzing the reduction of 1 micromole of NADP⁺ per minute under the above assay conditions.

**Estimation of Lipid Peroxidation**

**Thiobarbituric Acid Reactive Substances (TBARS)**

The level of TBARS in tissue and plasma was estimated by the method of Nichans and Samuelson (1968).

**Reagents**
1. Hydrochloric acid (HCl): 0.25 N
2. TBA-TCA-HCl reagent: 1:1:1 (v/v)
3. Thiobarbituric acid (TBA): 0.375%
4. Trichloro acetic acid; 15%
5. Tris-HCl buffer: 0.025 M, pH 7.5
6. Working standard: 4.8 molar solution of stock was prepared from standard (1,1’,3,3’ tetramethoxy propane). This was diluted to get a concentration of 48 nmol/mL.
Chapter II – Methodology

Procedure

Tissue homogenate (10%) was prepared in Tris-HCl buffer (pH 7.5). Plasma (0.5 mL) or 1.0 mL of tissue homogenate was treated with 2.0 mL of TBA-TCA-HCl reagent and mixed. The mixture was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged at 1000 x g for 10 min and the supernatant was estimated. Series of standard solutions in 2-10 nmole concentrations were treated similarly. The absorbance of the chromophore (pink color) generated due to the reaction between malondialdehyde and other thiobarbituric acid reactive substances (TBARS) and thiobarbituric acid under acidic condition was read at 535 nm against the reagent blank.

Serum lipids and lipoprotein profiles

Cholesterol

Estimation of cholesterol was carried out by the method of Zlatkis et al. (1953).

Reagents

1. Concentrated sulphuric acid
2. Ferric chloride and acetic acid reagent: 0.05% in aldehyde free acetic acid.
3. Standard cholesterol in aldehyde free acetic acid (2 mg/mL)

Procedure

Ferric chloride-acetic acid reagent (9.9 mL) was added to serum (0.1 mL) for deproteinization. The contents were centrifuged at 3000 rpm for 15 min. Five mL of the supernatant were taken and to this, 3.0 mL of concentrated sulphuric acid was added and kept for 20 min at room temperature. The pink color formed was read at 540 nm against a blank containing 5.0 mL of ferric chloride-acetic acid reagent. A set of standards were also performed similarly.

Triglycerides

Serum triglycerides were measured by the method of Foster and Dunn (1973).

Reagents

1. Acetyl acetone reagent: to 200 mL of isopropanol added 7.5 mL of acetyl acetone and the contents were made up to 1 L with distilled water
2. Activated alumina
3. Alcoholic KOH: 50 g of KOH in 1 L of mixture of isopropanol and water (2:3).

4. Isopropanol

5. Sodium (meta) periodate: 60 mL of acetic acid and 77 g of anhydrous ammonium acetate were added to 700 mL of water; 650 mg of sodium (meta) periodate were dissolved in this solution and the final volume was made up to 1 L with distilled water.

6. Standard Triolein in isopropanol was used as standard (0.1 mg/mL)

Procedure

Serum (0.1 mL) was taken in a glass stoppered centrifuge tube and to this added 4.0 mL of isopropanol and 400 mg of alumina. The tubes were tightly capped and shaken vigorously for 10 min. The tubes were centrifuged at 3000 rpm for 15 min and 2 mL of the supernatant were pipetted into clean, dry test tubes. To these was added 0.6 mL of alcoholic KOH and kept at 70°C for 15 min and cooled to room temperature. To this were added 0.5 mL of acetyl acetone reagent and 1.0 mL of meta-periodate reagent and incubated at 50°C for 30 min. Standard was also run similarly with triolein instead of serum. The color developed was read at 405 nm against the reagent blank.

**HDL-Cholesterol (HDL-C)**

Determination of serum HDL-cholesterol was carried out by the method of Burstein *et al.* (1970).

Reagents

1. Magnesium chloride solution: 101.7g of MgCl₂ was dissolved in 250 mL of distilled water.

2. Phosphotungstic acid reagent: To 200 mL of distilled water were added 22.5g of phosphotungstic acid and 80 mL of 1 M sodium hydroxide and the volume was made up to 500 mL with distilled water.

Procedure

Serum (0.5 mL) was taken in a centrifuge tube and to this was added 0.25 mL of phosphotungstic acid reagent, 0.25 mL of MgCl₂ and centrifuged at 1500 x g for 30 min in a refrigerated centrifuge. The amount of cholesterol was determined in the supernatant by the method of Zlatkis *et al.* (1953).
**VLDL and LDL cholesterol (VLDL-C & LDL-C)**

By employing Freidwald formula, the concentrations of VLDL and LDL cholesterol in serum were calculated (Freidwald *et al.*, 1972).

\[
\text{VLDL-C} = \frac{\text{Triglycerides}}{5}
\]

\[
\text{LDL-C} = (\text{Total Cholesterol} - \text{VLDL-C}) + (\text{HDL-C})
\]

**Estimation of Enzymatic antioxidants**

**Catalase**

The activity of catalase was determined by the method of Sinha (1972).

**Reagents**

1. Dichromate acetic acid: 1:3 ratio of potassium dichromate mixed with glacial acetic acid. From this 1.0 mL was diluted with 4.0 mL acetic acid.
2. Hydrogen peroxide: 0.2 M
3. Phosphate buffer: 0.01 M, pH 7.0
4. Potassium dichromate: 5%
5. Standard \( \text{H}_2\text{O}_2 \): 0.1 mL of 0.2 \( \text{H}_2\text{O}_2 \) was diluted to 100 mL using distilled water.

**Procedure**

To 0.9 mL of phosphate buffer, 0.1 mL of tissue homogenate and 0.4 mL of hydrogen peroxide were added. After 60 sec, 2.0 mL of dichromate-acetic acid mixture was added. The tubes were kept in boiling water bath for 10 min and the color developed was read at 620 nm. Standards in the range of 2-10 \( \mu \)mol were taken and preceded as test with blank containing reagent alone. The activities are expressed as \( \mu \)moles of \( \text{H}_2\text{O}_2 \) consumed/min/mg protein.

**Glutathione peroxidase**

Glutathione peroxidase activity was determined by the method of Rotruck *et al.* (1973).

**Reagents**

1. Ellman’s reagent: 19.8 mg of 5,5’-dithiobis (2-nitrobenzoic acid) in 100 mL of 1% sodium citrate solution.
2. Ethylene diamine tetra acetic acid: 0.4 mM
3. Glutathione solution: 2 mM
4. Hydrogen peroxide solution: 20 mM
5. Sodium azide: 10 mM
6. TCA: 10%
7. Tris buffer: 0.4 M, pH 7.0

Procedure
To 0.2 mL of Tris buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide and 0.5 mL of tissue homogenate were added. To the mixture, 0.2 mL of glutathione followed by 0.1 mL of hydrogen peroxide was added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except the sample. After 10 min, the reaction was arrested by the addition of 0.5 mL of 10% TCA, centrifuged and the supernatant was assayed for glutathione by the method of Ellman (1959). The activities are expressed as µg of GSH consumed/min/mg protein.

Superoxide dismutase
Superoxide dismutase was estimated by the method of Kakkar et al. (1984).

Reagents
1. Absolute ethanol
2. Chloroform
3. Glacial acetic acid
4. NADH: 780 µM
5. n-butanol
6. Nitroblue tetrazolium: 300 µM
7. Phenazine methosulphate: 186 µM
8. Sodium pyrophosphate buffer: 0.025 M, pH 8.3

Procedure
Tissue homogenate (0.5 mL) was diluted to 1.0 mL with water. Then 2.5 mL of ethanol and 1.5 mL of chloroform (all reagents chilled) were added. This mixture was shaken for 1 min at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.
The assay mixture contained 1.2 mL of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 mL of 186 µM phenazine methosulphate, 0.3 mL of 300 µM nitroblue tetrazolium, 0.2 mL of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3.0 mL. Reaction was started by adding NADH. After incubation at 30°C for 90 sec, the reaction was stopped by adding 1.0 mL of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 mL of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control.

‘One unit of enzyme activity is defined as the enzyme reaction which gives 50% inhibition of NBT reduction in one min under the assay condition’ and expressed as specific activity in units/mg protein.

**Estimation of non-enzymatic antioxidants**

**α-Tocopherol**

α-Tocopherol was estimated by the method of Baker et al. (1980).

**Reagents**

1. 0.2% of 2,2’-dipyridyl in ethanol
2. 0.5% ferric chloride in ethanol
3. Double distilled ethanol
4. Petroleum ether 60-80°C
5. Stock standard: 100 mg of α-tocopherol in 100 mL of distilled ethanol
6. Working standard: stock solution was diluted to a concentration of 10 µg/mL.

**Procedure**

To 0.1 mL of tissue homogenate, 1.5 mL of ethanol and 2.0 mL of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C; 0.2 mL of 2,2’-dipyridyl solution and 0.2 mL of ferric chloride solution were added, mixed well and kept in dark for 5 min along with 2.0 mL of butanol. The intense red color developed was read at 520 nm. Standard tocopherol in the range of 10-100 µg were taken and treated similarly along with blank containing only the reagent.
Ascorbic acid

Ascorbic acid was estimated by the method of Omaye et al. (1979).

Reagents

1. 2,4-DNPH reagent: 2.0 g of DNPH was dissolved in 100 mL of 9 N sulphuric acid. To this 4.0 g thiourea was added and mixed.
2. Activated charcoal
3. Standard ascorbic acid: 10 mg of L-ascorbic acid was dissolved in 100 mL of 4% TCA. This was diluted to prepare a working standard of concentration 100 µg/ml.
4. Sulphuric acid: 85%
5. Trichloroacetic acid: 6%

Procedure

Tissue homogenate (0.5 mL) was mixed thoroughly with 1.5 mL of 6% TCA and centrifuged for 20 min at 3500 x g. To 0.5 mL of the supernatant, 0.5 mL of DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3h, after which, removed, placed in ice-cold water and added 2.5 mL of 85% sulphuric acid and allowed to stand for 30 min. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 mL of 4% TCA. The color developed was read at 530 nm.

Reduced glutathione

Total glutathione (reduced) was estimated by the method of Ellman (1959).

Reagents

1. 5, 5’-dithiobis, 2-nitrobenzoic acid (DTNB): 19.8 mg of DTNB in100 mL of 1% sodium citrate solution.
2. Phosphate buffer: 0.2 M, pH 8
3. Standard glutathione (10 mg/mL)
4. Trichloroacetic acid: 5%

Procedure

To 0.5 mL of tissue homogenate was added 2.0 mL of 5% TCA and the protein was precipitated. The contents were centrifuged at 3000 rpm for 15 min and the precipitate was discarded. To 1.0 mL of the supernatant, 0.5 mL of DTNB reagent and
3.0 mL of phosphate buffer were added. The yellow color developed was read at 412 nm against a blank containing TCA instead of sample. A series of standards were treated similarly.

**Toxicity Evaluation Studies**

*Serum glutamic pyruvate transaminase (SGPT) or Alanine aminotransferase (ALT)*

SGPT or ALT activity was determined by the method of Reitman and Frankel (1957).

Transamination is a process in which an amino group of an amino acid (L-alanine) is transferred to a α-keto acid (ketoglutaric acid) by transaminases. The products formed by enzyme action are glutamate and pyruvate. Addition of 2,4-dinitrophenyl hydrazine results in the formation of hydrozone complex with the ketoacids. A red color produced after the addition of sodium hydroxide is read at 510 nm. The intensity of color is related to enzyme activity. One unit/L of AST or ALT is defined as the liberation of 1 mmol of pyruvate per minute at 37°C incubation per liter of serum.

**Reagents**

1. ALT Substrate: DL-alanine
2. Color reagent: dissolved 200 mg of 2,4-dinitrophenyl hydrazine in hot 1 M HCl and made upto 1 liter with 1 M HCl.
3. Phosphate buffer pH 7.4
4. Pyruvate standard: 2 mM/mL
5. Sodium hydroxide: 0.4 M.

**Procedure**

Two test tubes were separately labeled as control and test respectively. Substrate (0.5 mL) was added to both the test tubes and 0.1 mL of serum was added only to the tube marked as test. The sample was mixed and incubated in a water bath at 37°C for 1 h. The tubes were removed and 0.5 mL of 2,4-DNPH was added to each of the test tubes. Serum (0.1 mL) was now added to the control test tube. Mixed well and left the tubes at room temperature for 20 min. Then 5.0 mL of 0.4 M NaOH was added to each of the tubes. Mixed well and left the tubes at room temperature for 5 min. The
spectrophotometer was adjusted to zero using distilled water at 510 nm and measured the absorbance of control and test in the order.

**Serum glutamic pyruvate oxaloacetate (SGOT) or Aspartate aminotransferase (AST)**

SGOT or AST activity was determined by the method of Reitman and Frankel (1957).

**Reagents**

1. AST Substrate: DL-aspartic acid
2. Color reagent: dissolved 200 mg of 2,4-dinitrophenyl hydrazine in hot 1 M HCl and made upto 1 liter with 1 M HCl.
3. Phosphate buffer pH 7.4
4. Pyruvate standard: 2 mM/mL
5. Sodium hydroxide: 0.4 M.

**Procedure**

Procedure followed was same as that used for the assay of Alanine aminotransferase.

**Construction of calibration curve**

In the measurement of both serum AST and ALT, only pyruvate was used as the standard. Theoretically, oxaloacetate should be used as the standard for AST assay and pyruvate as the standard for ALT assay. Oxaloacetate formed in the AST assay is unstable and immediately gets converted into pyruvate; hence the use of pyruvate standard for AST assay. One unit/L of AST or ALT is defined as the liberation of 1 mmol of pyruvate per minute at 37°C incubation per liter of serum.

Different volumes of pyruvate (0.1 to 0.4 mL) were pipetted into approximately labeled test tubes. The final volume in each tube was adjusted to 1.0 mL by addition of corresponding volume of ALT/AST substrate. Then 0.2 mL of water was added to each test tube followed by 1.0 mL of 2,4-DNPH. The tubes were left for 20 min at room temperature and 10 mL of 0.4 M NaOH were added to each test tube.

Calibration curve was constructed by plotting the corresponding absorbance of standards with concentrations.
**Assay of alkaline phosphatase (ALP)**

Plasma alkaline phosphatase was estimated by using the diagnostic kit based on Kind and King’s method (1954). ALP catalyzes disodium phenyl phosphate into phenol and disodium hydrogen phosphate at pH 10.0. Phenol so formed reacts with 4-aminoantipyrine in alkaline medium in the presence of oxidizing agent potassium ferricyanide to form a red colored complex whose absorbance is proportional to the enzyme activity.

**Reagents**

1. Buffered substrate: 0.01 M Disodium phenyl phosphate dissolved in carbonate-bicarbonate buffer (0.1 M, pH 10.0)
2. Color reagent: 4-Aminoantipyrine, sodium hydroxide and potassium ferricyanide
3. Phenol standard: 10 mg/mL

**Procedure**

The incubation mixture containing 1.0 mL of buffered substrate, 3.1 mL of deionized water and 0.1 mL of serum was incubated at 37°C. Exactly after 15 min, 2.0 mL of color reagent was added to all the tubes. The control tube received the enzyme after the addition of color reagent; 0.1 mL of standard and 0.1 mL of distilled water (blank) were also treated simultaneously and the color developed was read at 510 nm. The enzyme activity is expressed as IU/L of serum.

**Estimation of serum urea and creatinine**

Serum urea was determined by urease-GLDH enzymatic UV test described by Talke and Schubert (1965) using DIASYS UREA-FS kit.

**Reagents**

1. 2-Oxoglutarate: 9 mM/L
2. Adenosine-5’ diphosphate: 0.75 mM/L
3. Glutamate dehydrogenase: ≥ 1 kU/L
4. NADH: 1.3 mM/L
5. Tris buffer: 150 mM/L (pH 7.8)
6. Urease: ≥ 7 kU/L
Urea in the sample is hydrolysed by urease to produce ammonia and carbon dioxide. The ammonia so formed, reacts with 2-oxoglutarate to form L-glutamate. While, NADH is simultaneously oxidized to NAD\(^+\). The resulting decrease in absorbance due to oxidation of NADH is measured bichromatically at 340/380 nm and the rate of decrease in absorbance is proportional to the concentration of urea (mg/dL) in the sample.

Serum creatinine was measured by alkaline picrate method. (Bartels and Bohmer, 1971) using IDENTI kit.

**Reagents**

1. Picric acid: 25 mM/L
2. Sodium hydroxide: 0.4 mM/L

Creatinine reacts with picric acid in alkaline condition to form a yellow complex. The rate of color formation is measured bichromatically at 520/800 which is proportional to the creatinine concentration (mg/dL) in the sample.

**Histological Examinations**

Immediately after the sacrifice of the rats, the organs/part of organs was fixed in 10% formalin. Embedding was done in paraffin. Embedding is the orientation of the tissue in melted paraffin which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut. Melted, filtered paraffin was poured in properly labeled metal blocks of size 1-2 square inches and the piece of tissue was placed in position into the paraffin. When the tissues were oriented and are properly placed, the paraffin was hardened by cooling. The hardened paraffin blocks with tissues (tissue blocks) were separated from the metal blocks. Sections having 3 microns thickness were cut from the tissue blocks by microtome. Using a heated tissue separator (water bath), the sections were uniformly separated from each other and carefully placed on a clean glass slide. Sections were properly drained at approximately 60\(^\circ\)C for 30 min. A small drop of Mayer’s egg albumin was used to attach sections to the glass slides.

Harris’ Hematoxylin and Eosin stain was used for staining the tissue sections following the procedure from the manual of Histologic Staining Methods of the Armed
Forces Institute of Pathology (Third Edition), American Registry of Pathology (Luna and Lee) Progressive stain. Harris’ Hematoxylin stain contains hematoxylin crystals in 100% alcohol, ammonium alum and mercuric oxide (as oxidizing agent to hasten the oxidation of hematoxylin to hematein).

**Statistical analysis**

All the values are expressed as mean ± SD. The statistical analysis was carried out by One Way Analysis of Variance (ANOVA) followed by Student’s t-test to determine the significant differences between treatments at $p \leq 0.05$. The number of samples used in each experiment is indicated in the respective results section or along with the figure legends.