3. Materials and Methods

3.1 Drugs and chemicals

Metformin, sitagliptin, rosvastatin and coenzyme Q10 were obtained from Zydus Cadila, Ahmedabad, India. Streptozotocin and nicotinamide were purchased from Himedia (Mumbai, India). Serum creatinine (cat no. 85MB100-75), serum urea (cat no. 81MB100-61), serum uric acid (cat no. 82LS200-50), urinary protein (cat no. 86LS100-25) kits used in the study were procured from SPAN Diagnostics, India. TNF-α (cat no. SEA133RA) and TGF-β (cat no. SEA124RA) kits were procured from USCN Life Science Inc. All other chemicals and reagents used in the study were of analytical grade.

3.2 Experimental animals

The experimental protocol was approved by the Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experiment was carried out on healthy adult Wistar rats weighing 200-250 g of either sex. Rats were housed in polypropylene cages, maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and allowed free access to diet (Nav Maharashtra Oil Mills Pvt. Ltd., Pune) and purified drinking water ad libitum.

3.3 Induction of diabetic nephropathy and neuropathy

Type 2 diabetes was induced in overnight fasted adult albino wistar rats weighing 200-250 g by a single intraperitoneal (i.p.) injection of 65 mg/kg streptozotocin (dissolved in citrate buffer, pH 4.5), 15 min after the i.p. administration of 110 mg/kg of nicotinamide (dissolved in normal saline). Hyperglycemia was confirmed by elevated blood glucose levels at 72 h and then on day 7 after injection and only animals with fasting blood glucose level greater than 200 mg/dl were considered as diabetic and were used for diabetic nephropathy and neuropathy studies.
3. Materials and methods

3.4 Experimental design

Diabetic rats were randomly divided into following groups each consisting of six animals.

SET I
Group I: Normal control rats (distilled water 1 ml/kg, p.o.).
Group II: Diabetic control rats.
Group III: Diabetic rats treated with 10 mg/kg Coenzyme Q10 (1% aqueous solution of Tween 80, p.o.).
Group IV: Diabetic rats treated with Metformin (500 mg/kg, p.o.).
Group V: Diabetic rats treated with the combination of Coenzyme Q10 and Metformin.

SET II
Group I: Normal control rats (distilled water 1 ml/kg, p.o.).
Group II: Diabetic control rats.
Group III: Diabetic rats treated with 10 mg/kg Coenzyme Q10 (1% aqueous solution of Tween 80, p.o.).
Group V: Diabetic rats treated with Sitagliptin (10 mg/kg, p.o.).
Group VIII: Diabetic rats treated with the combination of Coenzyme Q10 and Sitagliptin.

SET III
Group I: Diabetic control rats.
Group I: Diabetic rats treated with the combination of Coenzyme Q10 and Metformin.
Group III: Diabetic rats treated with the combination of Coenzyme Q10 and Sitagliptin.
Group IV: Diabetic rats treated with the combination of Coenzyme Q10, Metformin and Sitagliptin.

SET IV
Group I: Normal control rats (distilled water 1 ml/kg, p.o.).
Group II: Diabetic control rats.
Group III: Diabetic rats treated with 10 mg/kg Coenzyme Q10 (1% aqueous solution of Tween 80, p.o.).
Group IV: Diabetic rats treated with Rosuvastatin (10 mg/kg, p.o.\textsuperscript{200}

Group V: Diabetic rats treated with the combination of Coenzyme Q10 and Rosuvastatin.

All the aforementioned treatments were started one week (7 days) after injection of streptozotocin-nicotinamide and treatments continued for 42 days.

At the end of treatment with abovementioned regimen for 42 days, grip strength was evaluated using the Rota-rod apparatus. The test was used to evaluate muscle strength in rodents. The apparatus consists of 3 cm diameter horizontal metal rod coated with rubber and attached to motor with the speed adjusted to 25 rpm/min. the rod is 92 cm in length and is divided into six compartments, thereby allowing simultaneous testing of six rats. Only those animals which showed the ability to remain on the rotating rod for at least 1 min were used for the test. The fall off time was measured. This can be read from the timer which was stopped by the fall of animal.

The sensory function was evaluated by hot plate test and hot immersion test. The hot plate test and hot immersion test were carried out according to the method described by Eddy’s et al. and Sharma et al, respectively.\textsuperscript{201,202} In hot plate test, animals were placed on hot plates maintained at 55±1°C, withdrawal response was measured. The cut-off time for hot plate test was 10 s. In hot immersion test, the tail of the rat was immersed in hot water maintained 55±1°C, tail flick response (tail withdrawal response) was observed. The cut-off time 15 s.

At the end of the experiments, body weight and kidney weight were measured. Blood samples were collected from the retro orbital plexus of rats under light ether anesthesia, using glass capillaries and stored with or without disodium ethylene diamine tetra-acetate for estimation of biochemical parameter. For separation of serum blood was allowed to clot for 15 minutes and it was then centrifuged at 5000 rpm for 20 minutes. The serum was stored at -20°C until further biochemical estimation.

Glycated hemoglobin (Hb\textsubscript{1AC}) was estimated using whole blood. Creatinine, urea, uric acid and lipid profile were estimated from serum using standard diagnostic kit (SPAN Diagnostics, India). Rats were kept in metabolic cages for 24 hours for urine collection. Urine samples were centrifuged at 1400 rpm for 5 minutes after proper
3. Materials and methods

dilution and the supernatant was collected to determine urinary micro protein level using standard kits.

3.5 Estimation of biomarkers of oxidative stress

Kidney and sciatica nerve were removed and kept on autoclaved inverted petridis in cold conditions with ice cubes. The tissues were cross chopped with surgical scalpel into fine slices in chilled 0.25 M sucrose, quickly blotted on a filter paper. They were minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10 %w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 10,000 xg at 0°C using the Remi cooling centrifuge. The clear supernatant obtained was used for assay of lipid peroxidation (MDA content), endogenous antiperoxidative enzymes such as superoxide dismutase (SOD), catalase and GSH. Lipid peroxidation or malondialdehyde (MDA) formation was estimated by the method of Slater and Sawyer. SOD was determined by the method of Mishra and Fridovich. Catalase was estimated by the method given by Aebi Hugo. and GSH was determined by the method of Moron and Depierre.

3.6 Estimation of myeloperoxidation (MPO) assay

Myeloperoxidation (MPO) activity in kidney tissue was determined as described. The kidney tissue was homogenized in 0.5% hexadecyltrimethylammonium bromide containing 50 mM potassium phosphate buffer (pH 6) using a polytron tissue homogenizer. After freeze-thawing three times, the samples were centrifuged at 20,000 x g for 15 min at 40°C, and the resulting supernatant was assayed spectrophotometrically for MPO activity. In brief, 0.1 ml of sample was mixed with 2.9 ml of 50 mM potassium phosphate buffer (pH 6) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was then measured for 5 min using spectrophotometer. Myeloperoxidase activity data are presented as U/g tissue.
3.7 Determination of TNF-α by ELISA

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of TNF-α in rat serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

**Principle:** The microtiter plate provided in this kit has been pre-coated with an antibody specific to TNF-α. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to TNF-α. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain TNF-α, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of TNF-α in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Procedure:**

- Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank.
  - Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37 °C.
- Remove the liquid of each well, don’t wash.
- Add 100μL of Detection Reagent A working solution to each well. Incubate for 1 hour at 37 °C after covering it with the Plate sealer.
- Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
- Add 100μL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
3. Materials and methods

- Repeat the aspiration/wash process for total 5 times as conducted in step 4.
- Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
- Add 50μL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
- Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

3.8 Determination of TGF-β by ELISA

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of TGFβ1 in rat serum, platelet-poor plasma, tissue homogenates, cell culture supernates and other

**Principle:** The microtiter plate provided in this kit has been pre-coated with an antibody specific to TGF-β. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to TGF-β. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain TGF-β, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of TGF-β in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Procedure:**

- Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank.
- Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
Materials and methods

3. Remove the liquid of each well, don’t wash.
4. Add 100μL of Detection Reagent A working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
5. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
6. Add 100μL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
7. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
8. Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
9. Add 50μL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

3.9 Estimation of tissue nitrite content

Nitrite was estimated calorimetrically with the Griess reagent in protein free supernatant of kidney homogenate. Equal volumes of protein free supernatant of kidney homogenate and Griess reagent (sulfanilamide 1% w/v, naphthylenediamine dihydrochloride 0.1 % w/v and orthophosphoric acid 2.5 % v/v) were mixed and incubated at room temperature for 10 min and the absorbance was determined at 540 nm wavelength and compared to those of known concentrations of sodium nitrite.
3. Materials and methods

3.10 Histopathology

After sacrifice, kidney and sciatic nerve tissues of each group were rapidly dissected out and washed immediately with saline and fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 5 μm-thick sections and stained with hematoxylin and eosin (H&E). The sections were examined under the light microscope (Olympus BX10, Tokyo, Japan) for the presence of histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken. The observer performing histopathological evaluation was blinded to the animal treatment group.

3.11 Statistical analysis

All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test as appropriate using computer based fitting program (Prism, Graphpad 5). The significance level was set at $P < 0.05$ for all tests.

3.12 Estimation methods of serum bio-chemical parameters

3.12.1 Estimation of urea

In vitro determination of urea in serum was done using enzymatic kit (Span Diagnostic India Limited).

**Method:** DAM method

**Principle:** Urea reacts with hot acidic Diacetylmonoxime in presence of Thiosemicarbazide and produces a rose purple colored complex which is measured colorimetrically.

**Procedure:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (B)</th>
<th>Test (T)</th>
<th>Standard (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1*</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>0.01ml</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 3 Working Urea Standard, 30 mg%</td>
<td>-</td>
<td>-</td>
<td>0.01ml</td>
</tr>
<tr>
<td>Reagent 2: Diacetylmonoxime (DAM)</td>
<td>0.25ml</td>
<td>0.25ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td></td>
<td>Mix well</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. Materials and methods

*Solution 1:* Dilute 1 ml of Urea reagent to 5 ml with Purified water.

Mix well and keep the tubes in the boiling water exactly for 10 minutes. Cool immediately under running water for 5 minutes, mix by inversion and measure the O.D. at 525 nm within 10 minutes.

**Calculations:** Serum/Plasma: Urea in mg/dl, \((A) = \frac{O.D. \text{ of Test} \times 30}{O.D. \text{ of Std}}\)

### 3.12.2 Estimation of Creatinine

*In vitro* quantitative determination of creatinine in serum was done using enzymatic kit (Span Diagnostic India Limited).

**Method:** Alkaline Picrate method

**Principle:**
Creatinine in a protein free solution reacts with Alkaline Picrate and produces a red colored complex, which is measured colorimetrically.

**Procedure:**

**Step A. Deproteinization of test sample:**

<table>
<thead>
<tr>
<th>Serum/Plasma/Dilute Urine</th>
<th>0.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified water</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Picric Acid : Reagent 1</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

Mix well, keep in a boiling water bath exactly for one minute. Cool immediately under running tap water and centrifuge or filter.

**Step B. Color Development:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (B)</th>
<th>Test (T)</th>
<th>Standard (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate/Supernant (From Step A.)</td>
<td>-</td>
<td>-</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Working Standard</td>
<td>-</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Purified water</td>
<td>0.5 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1 : Picric Acid</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 2 : Sodium Hydroxide, 0.75 N</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>
3. Materials and methods

Mix well and allow standing at room temperature exactly for 20 minutes and measuring immediately the optical density of Blank (B), Standard (S) and Test (T) against Purified water at 520 nm.

**Calculations:** Serum Creatinine in mg/100mL = \( \frac{\text{O.D. test} - \text{O.D. Blank}}{\text{O.D. Std} - \text{O.D. Blank}} \times 3.0 \)

3.12.3 Estimation of urinary protein

*In vitro* quantitative determination of Urinary Protein was done using enzymatic kit (Span Diagnostic India Limited).

**Method:** Pyrogallol Red Method

**Principle:**

A protein, in acidic medium, combines with Pyrogallol red and Molybdate to form a blue purple colored complex. Intensity of the color formed is directly proportional to the amount of proteins presents in the sample.

**Reagent preparation:**

Reagents are ready to use. Protect from bright light.

For doing the High Sensitivity Assay dilute the micro-protein Standard (S) 1+4 with normal saline before use. Prepare fresh each time.

**Procedure:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (ml)</th>
<th>Standard(ml)</th>
<th>Test(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Reagent (L1)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diluted Standard (S)</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mix well and incubate at R.T. for 5 min. Measure the absorbance of the Standard (Abs.S) and Test sample (Abs.T) against Blank, within 30 min.

**Calculation:** Micro protein in mg/24hr = \( \frac{\text{Abs.T}}{\text{Abs.S}} \times 200 \)
3. Materials and methods

3.12.4 Estimation of uric acid

**Method:** Uricase/POD Method

**Principle:**

Uricase converts uric acid to Allenton and hydrogen peroxidase. The hydrogen peroxide formed further reacts with a phenolic compound and aminoantipyrine by the catalytic action of peroxidase. The to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of uric acid present in sample.

**Procedure:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (ml)</th>
<th>Standard (ml)</th>
<th>Test (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Reagent (L1)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Enzyme Reagent(L2)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uric Acid standard(S)</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Mix well and incubate at R.T. for 15 min. Measure the absorbance of the standard (Abs. S), and Test sample (Abs. T) against the blank at 520 nm, within 30 min.

**Calculation:** Uric acid in mg/dl = \( \frac{\text{Abs. T}}{\text{Abs. S}} \times \text{concentration of standard} \)

3.12.5 Estimation of serum cholesterol

Span cogent diagnostics kit was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method.

**Method:** Enzymatic, (Cholesterol Oxidase – Peroxidase), Endpoint colorimetry, Single Reagent Chemistry, with LCF (Lipid clearing factor)

**Principle:** The enzyme, cholesterol esterase catalyzed hydrolysis of cholesterol esters to free cholesterol and fatty acid molecules. Then free cholesterol gets oxidized in the presence of cholesterol to form cholesten-3-one and \( \text{H}_2\text{O}_2 \). Liberated \( \text{H}_2\text{O}_2 \) reacts with phenol and 4 AAP in presence of peroxidase to form red colored quinoneimine complex the intensity of which was measured at 505 nm and is proportional to amount of
3. Materials and methods

total cholesterol concentration in the sample.

Procedure:

<table>
<thead>
<tr>
<th>Pipette into tubes Marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum or Plasma</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

The above were mixed well, incubated at 37 °C for 10 minutes, the absorbance of standard and sample were read against reagent blank at 505 nm within 60 minutes.

Calculation: Cholesterol concentration (mg/dl) = \( \frac{\text{Absorbance of test} \times 200}{\text{Absorbance of standard}} \)

3.12.6 Estimation of serum triglyceride

Span diagnostic kit was used for estimation of triglycerides, which followed endpoint colorimetry, enzymatic test using glycerol-3-phosphate oxidase.

Method: Enzymatic (GPO/Trinder), Endpoint colorimetry, Single reagent chemistry with LCF (Lipid Clearing Factor)

Principle: Triglycerides are determined after enzymatic hydrolysis with lipases. The quinonemine indicator is formed from hydrogen peroxide 4-aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidise.

Procedure:

<table>
<thead>
<tr>
<th>Pipette into tubes Marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum or Plasma</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
<tr>
<td>Triglyceride standard</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Triglycerides reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

The above were mixed well, incubated at 37 °C for 5 minutes, the absorbance of standard and sample were read against blank at 505 nm.

Calculation: Triglycerides (mg/dl) = \( \frac{\text{Absorbance of test} \times 200}{\text{Absorbance of standard}} \)
3. Materials and methods

3.12.7 Estimation of serum high-density lipoprotein cholesterol (HDL-C)

Span cogent diagnostic kit was used for estimation of HDL cholesterol, which followed Cholesterol oxidase / peroxidase (CHOD-POD) method.

**Method:** Polyethylene glycol – CHOD – POD, Endpoint colorimetry, Two – Reagent Chemistry with LCF (Lipid clearing Factor)

**Principle:** HDL-C is measured in the supernatant after the precipitation of the lipoproteins including chylomicrons, very low-density lipoproteins, low-density lipoproteins, intermediate-density lipoproteins directly from serum polyanions like phosphotungstic acid and along with MgCl₂ are added to an aliquot of serum an immediate heavy precipitation is formed. The precipitate then is sedimented by centrifugation and HDL cholesterol is measured in the clear supernatant, which is estimated by enzymatic method as described earlier in estimation serum of TC.

Low and very low density lipoproteins (VLDL) are precipitated by a solution containing PEG 6000, leaving behind the high density Lipoproteins in solution. HDL Cholesterol is estimated in the supernatant by a series of enzymatic reactions which are initiated by the oxidation of cholesterol to cholestenone by cholesterol oxidase accompanied by the formation of hydrogen peroxide to form a red colored quinoneimine.

**Procedure:**

**Step – A: HDL – Cholesterol separation**

<table>
<thead>
<tr>
<th>Pipette into Centrifuge tube</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>200µl</td>
</tr>
<tr>
<td>Precipitating reagent</td>
<td>200µl</td>
</tr>
</tbody>
</table>

The above were mixed well, kept at R. T. for 10 min and then centrifuged at 2000 rpm for 15 minutes to obtain a clear supernatant and proceed to step - B.
3. Materials and methods

Step – B Colour Development:

<table>
<thead>
<tr>
<th>Pipette into tubes Marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant from step A</td>
<td>-</td>
<td>-</td>
<td>100 μl</td>
</tr>
<tr>
<td>HDL Cholesterol standard</td>
<td>-</td>
<td>100 μl</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

The above were mixed well, incubated at 37 °C for 10 minutes or at R.T. for 30 minutes and absorbance were read against reagent blank at 505 nm within 60 min.

**Calculation:** HDL Cholesterol (mg/dl) = \( \text{Absorbance of Test} \times 50 \times 2 \)

Absorbance of Standard

### 3.13 Oxidative stress parameters

#### 3.13.1 Estimation of MDA

**Method:** Slater and Sawyer method.

**Principle:** The method estimates Malondialdehyde (MDA), a product of lipid per oxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink colored chromogen, whose intensity was measured calorimetrically at 535 nm.

**Reagents:**

1. Thiobarbituric acid (0.067% in Tris hydrochloride, pH 7):
   
   0.067 gm of thiobarbituric acid was dissolved in 100 ml of Tris hydrochloride buffer pH 7.

2. Trichloroacetic acid (20%):
   
   20 gm of trichloroacetic acid was dissolved in distilled water.

**Procedure:**

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml of D.W.</td>
<td>2 ml of Homogenate</td>
</tr>
<tr>
<td>1.5 ml of TCA</td>
<td>2 ml of TCA</td>
</tr>
<tr>
<td>Cool for 15 min and centrifuged, take the supernatant</td>
<td></td>
</tr>
</tbody>
</table>
3. Materials and methods

<table>
<thead>
<tr>
<th>3 ml of ST</th>
<th>2 ml of ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ml TBA</td>
<td>2 ml TBA</td>
</tr>
</tbody>
</table>

Keep in boiling water bath for 10 min. Read the absorbance of test against blank at 535 nm using spectrophotometer.
Units: nmol of MDA / gm of tissue.

3.13.2 Estimation of GSH

**Method:** Moron and Depierre method

**Principle:** Glutathione present in RBC consist of sulphhydryl groups. 5,5 dithiobis 2-nitro benzoic acid (DTNB), a disulphide compound, gets readily attacked by these sulphhydryl groups and forms a yellow colored anion which measured calorimetrically at 412 nm.

**Reagents:**
1. Trichloroacetic acid (10%):
   Dissolve 10 gm of TCA in 100 ml of distill water.
2. Dithiobis nitro benzoic acid (DTNB):
   Dissolve 60 gm of DTNB in 1% Sodium citrate solution.
3. Phosphate buffer (0.2 M, pH 8.0):
   Dissolve 1.36 gm of KH2PO4 in 100 ml of distill water and dissolve in 0.8 gm NaOH in 100 ml distill water.
4. Reduced of glutathione standard:
   Dissolve 10 gm of GSH standard in 100 ml of distill water (100µg/ml).

**Procedure:**

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml of D.W.</td>
<td>1 ml of Homogenate</td>
</tr>
<tr>
<td>1 ml of TCA (10%)</td>
<td>1 ml of TCA (10%)</td>
</tr>
<tr>
<td>Cool for 10 min and centrifuged at 2000 rpm take 0.5 ml of supernatant</td>
<td></td>
</tr>
<tr>
<td>0.5 ml of ST</td>
<td>0.5 ml of ST</td>
</tr>
<tr>
<td>4 ml DTNB</td>
<td>4 ml DTNB</td>
</tr>
<tr>
<td>1.5 ml Phosphate buffer</td>
<td>1.5 ml Phosphate buffer</td>
</tr>
</tbody>
</table>
3. Materials and methods

Mix well keep for at RT read the absorbance against blank at 412 nm using spectrophotometer.
Unit: µg/gm of tissue

3.13.3 Estimation of SOD

**Method:** Mishra and Fridovich method

**Principle:** Rate of auto oxidation of epinephrine & the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8 – 10.2, O₂, generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome & the yield of adrenochrome produced per O₂ introduced. The auto oxidation of epinephrine proceeds by least two distinct pathways only one of which is free radical chain reaction involving O₂ & hence inhabitable by SOD.

**Reagent:**
1. Carbonate buffer (0.05 M pH 10.2):
   16.8 gm of NaCO₃ was dissolved in 500 ml of distill water & the final volume was made up to 1000 ml with distill water.
2. EDTA 0.49 M:
   1.82 gm of EDTA was dissolved in 1000 ml of distill water.
3. Epinephrine (3 mM):
   0.99 gm of epinephrine bitartarate was dissolved in 1000 ml of distill water.
4. SOD standard:
   Dissolve 1 mg (1000 units /mg) of SOD from bovine liver in 100 ml of carbonate buffer.

**Procedure:**
Before starting the estimation, see all the reagents required must be kept in freeze and add in cold condition.

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml of Diluted Homogenate</td>
<td>0.5 ml of distill water</td>
</tr>
<tr>
<td>0.5 ml of distill water</td>
<td>-</td>
</tr>
<tr>
<td>0.25 ml of ethanol</td>
<td>0.38 ml ethanol</td>
</tr>
<tr>
<td>0.15 ml Chloroform</td>
<td>0.15 ml Chloroform.</td>
</tr>
</tbody>
</table>
3. Materials and methods

<table>
<thead>
<tr>
<th>Shake for 1 min, Centrifuge at 2000 rpm, and separate supernatant.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml Supernatant</td>
</tr>
<tr>
<td>1.5 ml carbonate buffer</td>
</tr>
<tr>
<td>0.5 ml EDTA</td>
</tr>
</tbody>
</table>

The reaction was initiated by the addition of epinephrine and the change in optical density / min was measured at 480 nm, take for 3 min with 30 second interval.

Units = Unit/ gm of tissue.

3.13.4 Estimation of Catalase

**Method:** Hugo E. Aebi method

**Principle:** In the ultra-violet range $\text{H}_2\text{O}_2$ shows a continuous increase in absorption with decreasing wavelength. The décòr position of $\text{H}_2\text{O}_2$ can be followed directly by the decrease in absorbance at 240 nm. The difference in the absorbance per unit time is a measure of the Catalase activity.

**Reagent:**

1. Phosphate buffer (50 m mol/L pH 7)
   a. Dissolve 6.81 gm of KH$_2$PO$_4$ in distill water and make up volume to 1000 ml with distill water.
   b. Dissolve 8.9gm of Na$_2$HPO$_4$ in distill water and make up volume to 1000 ml with distill water.

2. Hydrogen peroxide (30 nmol/L)

**Procedure:**

Dilute the homogenate 20 times with phosphate buffer.

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml of Diluted Homogenate</td>
<td>2 ml of Diluted Homogenate</td>
</tr>
<tr>
<td>1 ml of phosphate buffer pH 7</td>
<td>1 ml of phosphate buffer pH 7</td>
</tr>
</tbody>
</table>

Add $\text{H}_2\text{O}_2$ just before taking OD at 240 nm; take the reading for 3 min. with 15 second interval.

Units = $\mu$ mol of $\text{H}_2\text{O}_2$ consumed/ gm of tissue.