MATERIALS AND METHODS
MATERIALS AND METHODS

3.1, Ambient Air Quality Sampling and analysis:

The quarterly analysis of air quality was carried out at residential areas near Bus Station and Lalpulia region at Wani.

Sampling was carried out simultaneously at both the places for 24 hrs, with a frequency of 8 hrs. At the end of every 8 hrs, filter papers, absorbing solutions were replaced and the samples so collected were refrigerated till further analysis. The analysis of all the samples were carried out in the laboratory. The results are expressed as 24 hrs average and are compared with standards prescribed by Central Pollution Control Board (CPCB).

3.1.1, Apparatus:

The ambient air quality sampling was carried out at different places at Wani area, Dist. Yeotmal, (M.S.), with the High Volume Sampler (HVS) and analysis was carried out by means of mass spectrophotometry.

3.1.2, High Volume Sampler:

High Volume Sampler is used for sampling the suspended particulate matter/ Respirable suspended particulate matter (SPM/ RSPM). It also has provisions to collect samples of gaseous pollutants such as \( \text{SO}_x \), \( \text{NO}_x \), \( \text{CO} \), \( \text{H}_2\text{S} \), \( \text{F} \), \( \text{Cl} \) etc from ambient air by absorbing them in appropriate reagents kept in impinger tubes followed by further analysis in the laboratory.
Materials and Methods

3.1.3, Principle:

Air is drawn in to a covered housing through a filter by a high flow rate blower at 1.1 to 1.5 M³/min. that allows total suspended particulates with diameters of < 100mm are ordinarily collected on glass fibre papers. The mass concentration in the ambient air is computed by measuring the mass of SPM collected and the volume of air sampled. The size of sample collected is usually adequate for further analysis for trace elements.

3.2, Measurement of Respirable Suspended Particulate Matter (RSPM):

3.2.1, Requirement:

i) High Volume Sampler

ii) Electronic balance

iii) Glass fibre filters Whatman

3.2.2, Procedure:

i) Installed a fresh preweighed filter sheet in the filter holder and the dust collection bottle below the cyclone has been cleaned.

ii) Checked all the program pins of the timer. They were reset and the coloured outer ring was visible continuously round the front face of the timer.

iii) Checked the mains ON/OFF toggle switch and it was brought in the OFF position.

iv) Plugged in the mains chord. The neon light wired to come ON as soon as the chord was connected indicating that the instrument is connected to a live mains outlet.

v) Switched ON the toggle switch. The voltage stabilizer after receiving
power increased the input voltage. It was within the operation range (220V+/−10%) of the instrument it would feed power to the blower.

vi) Checked the time totalizer and recorded the initial totalizer reading. Noted the time totalizer digits read the true operating time in hrs upto two places of decimal. The last two digits DO NOT denote minutes.

vii) Allowed the flow of stabilizer for a few minutes and recorded the rate of sampling in M³/min.

viii) When the sampling time was over, switched OFF the toggle switch to stop the blower. Recorded the time totalizer reading. Loosen the wing nut and carefully removed the top cover to expose the filter sheet. Removed the filter sheet and stored it away in a clean envelope. Gradually pulled down the dust collection bottle at the bottom of the cyclone. Used a small brush to dislodge any dust that might be sticking to the bottom portion of the cyclone. Recovered the dust from the sampling bottle and cleaned it with a fresh tissue before reinstalling below the cyclone.

3.2.3, Calculation of Mass Concentration of RSPM:

The mass concentration of suspended particulate matter was be calculated as follows and reported to the nearest microgram/ m³.

\[
\text{RSPM as mg/m}^3 = \frac{(W_2 - W_1)}{V} \times 10^6
\]

Where,

- \( W_2 \) = final weight of exposed filter in gram
- \( W_1 \) = initial weight of the filter in gram
- \( V \) = total volume of air sampled in m³
- \( 10^6 \) = conversion of g to mg
3.3, Measurement of Gaseous Pollutants:

3.3.1, Method of Analysis of Sulphur dioxide (SO\textsubscript{2}):

The sulphur dioxide concentration in the ambient air was detected by Pararosaniline method.

3.3.1.1, Principle:

SO\textsubscript{2} is absorbed from air in a solution of potassium tetra-hloromercurate (TCM), a di-chlorosulphathomercurate complex, stable to strong oxidants (for eg. Ozone, oxides or nitrogen). The complex reacts with pararosanaline and formaldehyde to form intensely coloured pararosanaline methyl sulphuric acid. The absorbancy of the solution is measured spectrophotometrically.

3.3.1.2, Reagents Required:

i) Absorbing reagent 0.04N TCM:

10.86 g HgCl\textsubscript{2}, 0.066 g EDTA and 6.0 g KCL were dissolved in water and brought to make in a 1000 ml volumetric flask. The pH of this reagent was maintained over the range of pH 3 to pH 5.

ii) Pararosaniline Reagent:

To a 250 ml volumetric flask, added 20 ml of 2 % pararosaniline solution. Then added 25 ml 3M H\textsubscript{3}PO\textsubscript{4} and diluted to 250 ml with the distilled water.

iii) Sulfamic acid (0.6 %):

0.6 g H\textsubscript{2}NSO\textsubscript{3}H was dissolved in 100 ml of distilled water.

iv) Formaldehyde (0.2 %):

5 ml of Formaldehyde was diluted to 1000 ml with distilled water.

3.3.1.3, Procedure:

i) 10 ml of exposed TCM solution was taken in a 50 ml volumetric flask.
Materials and Methods

ii) To this added 1 ml of 0.6% sulfamic acid and allowed to react for 10 min to destroy the nitrate from the oxides of nitrogen.

iii) To this added exactly 2 ml formaldehyde and then added 5 ml of pararosaniline solution.

iv) Then the laboratory timer was started simultaneously and allowed for 30 min for the colour development.

v) After 30 min and before 60 min, the absorbency of the sample was determined using a spectrophotometer at 548 nm.

3.3.1.4, Calculation:

The concentration of \( SO_2 \) in the sample is determined from the standard graph.

3.3.2, Method of Analysis of Nitrogen dioxide (\( NO_2 \)):

The concentration of \( NO_2 \) in the ambient air was detected by Jacobs & Hochheiser method.

3.3.2.1, Principle:

Nitrogen dioxide is collected by bubbling air to a sodium hydroxide solution to form a stable compound of sodium Nitrate. The nitrate ion produced during sampling is determined calorimetrically by reaction of sulphanilamide and NEDA.

3.3.2.2, Reagents Required:

i) Sodium Hydroxide as Absorbing reagent (0.1 N):

4.0 g of NaOH was dissolved in the water.

ii) Sulfanilamide Solution (Diazotizing reagent):

4.0 g of sulfanilamide (melting point 165-167 °C, pharmaceutical grade) was dissolved in about 500 ml water containing 100 ml orthophosphoric
Materials and Methods.

acid (sp.gr. 1.75) and diluted to 1 lit. with distilled water. This solution can be stored for 1 month.

iii) Coupling reagent (NEDA solution):

0.1 g of N-1napthyl ethylene diamide dihydrochlorine was dissolved in the distilled and diluted to 100 ml.

iv) Hydrogen Peroxide solution (0.5 %):

1 ml of hydrogen peroxide (30 %) was diluted to 60 ml with distilled water.

3.2.2.3, Procedure:

i) 10 ml of exposed NaOH was taken in a volumetric flask. To this added 1 ml of $H_2O_2$ and well shaked.

ii) Then added 10 ml of sulfanilamide solution and 1.4 ml of NEDA solution and thoroughly mixed after addition of each reagent.

iii) Then allowed to stand for 30 min for the colour development and then the absorbance was measured at 540 nm against the blank.

iv) Distilled water is used as blank.

3.3.2.4, Calculation:

The conc. of NO$_2$ in the sample is determined from a standard graph.

3.4, Measurement of Particulate Size:

The size of airborne dust particles was measured by using oculometer scale.

3.5, Measurement of Trace metals in airborne dust:

The trace metals like Iron, Copper, Managnese, Zinc and lead are estimated with the help of atomic absorption spectrophotometer model perkin
Elmer Analyst 100/300 in the central instrumentation cell, Sant Gadge Baba Amravati University, Amravati. The concentration of trace metals is represented in μg/m³.

3.6, Physico-chemical studies of water:

Following physico-chemical parameters were analyzed by using standard methods for water analysis suggested by APHA (1998).

Physico-chemical parameters analyzed are water temperature, pH, Dissolved Oxygen (DO), BOD, COD, Alkalinity, Hardness, TDS, Chloride, Fluoride, Nitrite, Nitrate, Sulphate, Iron, Lead, Copper, Zinc and Manganese.

Water temperature, pH and Dissolved Oxygen were estimated on the spot and rest of the parameters were estimated in the laboratory.

3.6.1, pH:

pH is negative logarithm of the concentration of free hydrogen ions in a solution. It can be measured by electrometric method.

Activity of hydrogen ions in a solution is measured as the difference in potential (e.m.v.) of glass electrode with that of calomel reference electrode over a scale callibration directly in pH units.

3.6.1.1, Requirements:

a) pH meter
b) Glass and calomel or combined electrode
c) Buffer solution of pH 4.0 and 9.2
d) beakers
3.6.1.2, Method:

pH was recorded at the sampling site by BDH colour chart later on it was confirmed in the laboratory by electric pH meter maintained at the room temperature.

pH meter was set with a buffer solution of pH 4.0 and 9.2. Then the electrode was washed in distilled water. Suitable quantity of water sample was taken in a beaker, then the electrode was dipped in the sample and operated the pH meter accordingly and noted the pH value of sample.

3.6.2, Dissolved oxygen (D.O.):

Manganous sulphate react with the alkali to form a white precipitate of manganous hydroxide which in the presence of oxygen get oxidized to a brown colour compound. In the strong acid medium, manganic ions are reduced by iodide ions which get converted into iodide equivalent to the original concentration of thiosulphate using starch as an indicator.

\[ \text{MnSO}_4 + 2 \text{KOH} = \text{Mn(OH)}_2 + \text{K}_2\text{SO}_4 \]

\[ \text{Mn(OH)}_2 + \text{O} = \text{MnO(OH)}_2 \]

\[ \text{MnO(OH)}_2 + \text{H}_2\text{SO}_4 + 2\text{KI} = \text{MnSO}_4 + \text{K}_2\text{SO}_4 + 2\text{H}_2\text{O} + \text{I}_2 \]

3.6.2.1, Requirement:

a) Sodium thiosulphate solution (0.025N):

Dissolved 6.205 gm sodium thiosulphate (AR grade) in freshly boiled and cooled distilled water and diluted to 1 lit. Added one pellet of NaOH as preservative.
Materials and Methods

b) Manganous sulphate solution (Winkler A):

Dissolved 182 gm MnSO₄·H₂O in distilled water, filtered and diluted to 500 ml.

c) Alkaline iodide azide solution (Winkler B):

350 gm KOH and 75 gm KI were dissolved separately in distilled water. Mixed them and made the volume up to 500 ml. 5gm sodium azide (Na₃N) in 20 ml distilled water was dissolved separately and then added this azide solution to the alkaline iodide reagent.

d) Starch indicator:

1 gm starch (soluble) was dissolved in 200 ml distilled water and added few drops of toluene as preservative.

e) Concentrated sulphuric acid (Sp.Gr. 1.84; 18M)

f) BOD bottles (150 ml)

g) Titration assembly

3.6.2.2, Method:

Water sample was collected in a glass stoppered bottles (BOD) carefully avoiding any type of bubbling. Then added 2ml each of manganous sulphate and alkaline iodide azide solution one after other, right at the bottom of the bottle with separate pipettes and replaced the stopper. After that shaken the contents well by inverting the bottles repeatedly and allowed the brown ppt to settle. Then precipitate was dissolved by adding 2 ml concentrated H₂SO₄ and shaked the stoppered bottle till the complete precipitate is dissolved. After that 50ml solution was taken in a conical flask and titrated with thiosulphate solution (0.025N) till the colour changed to pale blue colour.
3.6.2.3, Calculation:

\[
\text{D.O. in mg/L} = \frac{(8 \times 1000 \times N)}{V} \times V
\]

Where,

- \( V \) = Volume of sample (ml)
- \( V \) = Volume of titrant used (ml)
- \( N \) = Normality of titrant (0.025 N)
- \( 8 \) = 1 ml of 1N sodium thiosulphate is equivalent to 8mg of oxygen

3.6.3, Bio Chemical Oxygen Demand (BOD):

Biochemical oxygen demand is the measure of the degradable organic material present in water sample and can be defined as the amount of oxygen required by micro-organisms in stabilizing the biologically degradable organic matter under aerobic conditions.

The principle of the method involves, measuring the difference of the oxygen concentration between the sample and after incubation in the dark at 20°C for 5 days (BOD).

Nitrification consumes oxygen significantly, thereby resulting in overestimation of BOD and must be checked by adding 1 ml of 0.05% solution of allylthiourea. If more than 70% of the initial oxygen is consumed, it is necessary to aerate / oxygenate and or dilute the sample with BOD free water to avoid oxygen stress.

3.6.3.1, Requirement:

1) BOD free water:

It is obtained by passing deionized glass distilled water through a column packed with aquated carbon followed by redistillation. This \( \text{H}_2\text{O} \) is used for dilution of sample.
Materials and Methods

2) MgSO₄ 7H₂O (8.252%) unhydrous, CaCl₂ (2.75%) and FeCl₃ 6H₂O : (0.0025%) all prepared in BOD free water. Stabilized pH to 7.2 by addition of 1 ml of phosphate buffer solution.

3) Allythiourea solution (0.05N) :
   - Dissolved 500 mg allythiourea in a liter of BOD free water.

4) Phosphate buffer solution :
   - Dissolved 8.59mg K₂HPO₄, 334g Na₂HPO₄ 7 H₂O and 107m MH₄Cl in 800ml of BOD free water and make up to 1 litre.

5) H₂SO₄ (1N) :
   - Added 2.8 ml of concentrated H₂SO₄ to 100ml distilled water.

6) NaOH (1N) :
   - Added 4gm NaOH to 100 ml distilled water.

3.6.3.2, Method :

   The sample neutralized to pH around 7.0 by using 1N acid (H₂SO₄) alkali (NaOH) solution then filled the sample in 6 BOD bottles without bubbling. After that 1 ml of allythiourea (0.05%) was added to each bottle and then incubated the remaining 3 bottles in BOD incubator at 20°C. After 5 days of incubation, the oxygen concentration was estimated by Winkler method.

3.6.3.3, Calculation :

   BOD of sample in mg/L = D₀ - D₅

Where,

   D₀ = initial D.O. in sample (mg/L)
   D₅ = D.O. after 5 days incubation (mg/L)
3.6.4, Total Alkalinity:

In most natural waters bicarbonate and some times carbonates are present in appreciable amounts. Their salts get hydrolysed in solution and produce hydroxyl ions, consequently raising the pH.

Alkalinity can be estimated by titrating the sample with a standard solution of strong acid first to pH 8.3 using phenolphthaleine as indicator and then further to pH 4.5 with methyl orange indicator. In first case, value is called as phenolphthaleine alkalinity and in second case, it is called total alkalinity.

3.6.4.1, Chemicals:

a) Sulphuric acid titrant (0.02N):

Prepared stock solution 0.1N by diluting 2.8 ml concentrated sulphuric acid to 1 litre to obtain 0.02 N acid titrant. Standardized it.

b) Phenolphthaleine Indicator:

Dissolved 1.25gm Phenolphthaleine in 125 ml ethyl alcohol and added 125ml distilled water. Added 0.02N NaOH dropwise until a faint pink colour appeared.

c) Sodium hydroxide solution (0.02N):

Dissolved 0.909gm NaOH in CO2 free distilled water (freshly boiled and cooled) and raised the volume to 1 litre.

d) Methyl orange indicator:

Dissolved 0.1gm methyl orange in 200 ml of distilled water.

3.6.4.2, Method:

50 ml water sample was taken in a conical flask and added 2 drops of Phenolphthaleine indicator. Slight pink colour appeared and then it was titrated with acid titrant (0.02N H₂SO₄) to a colourless end point and noted the reading as "P".
Materials and Methods

Then added 2 drops of methyl orange indicator in the same flask and continued titration further, till the colour changed from yellow to orange.

3.6.4.3, Calculation:

Total alkalinity (T) as mg/L CaCO\(_3\) = \(\frac{\text{ml of titrant } 't' \times 1000}{\text{ml of sample}}\)

3.6.5, Total Hardness:

Total hardness is generally caused by the calcium and magnesium ions present in water. Polyvalent ions of some other metals like Sr, Fe, Al, Zn and Mn etc. are also capable of precipitating the soap and thus contribute to hardness. However, concentration of these ions is very low in natural water, therefore, hardness is generally measured as concentration of only calcium and magnesium which are far higher in quantities over other hardness producing ions.

Erichrome black T form wine red complex with metal ions (Ca and Mg). The disodium salt of EDTA extracts the metal ions from the dye metal ions complex as coloured chelate complex, leaving blue colour aqueous solution.

3.6.5.1, Requirement:

a) Standard EDTA titrant (0.01M):

Dissolved 3.723 of disodium salt of EDTA in distilled water to prepare 1 litre titrant. Stored in polythene bottles.

b) Ammonia buffer solution:

Added 114 ml concentrated NH\(_4\)OH to 13.5 gm of NH\(_4\)Cl and made the volume up to 200 ml.

C) Erichrome Black T Indicator:

Dissolved 0.5 gm dye in 100 ml of 80 % alcohol.

D) Titration assembly.
3.6.5.2, Method:

Total hardness was determined titrimetrically by EDTA method (APHA, 1998). To 50ml of water sample taken in flask, added 1 ml of ammonia buffer and 5 drops of indicator (Erichrome black T) solution by which colour of the sample turned wine red. After that titrated it with EDTA solution (0.01M) until a clear blue colour appeared.

3.6.5.3, Calculation:

\[
\text{Total Hardness mg/L} = \frac{A \times 1000}{\text{ml of sample}}
\]

Where,

\( A = \text{ml of titrant used.} \)

3.6.6, Total dissolved solids:

In the preweighted dried dish of suitable size, 100 ml of filtered water sample was taken and evaporated on water bath. The weight of the dish was noted after cooling it in a desicator. The total dissolved solids (TDS) are expressed in mg/L.

3.6.6.1, Calculations:

\[
\text{TDS mg/L} = \frac{(A-B) \times 10^6}{V}
\]

Where,

\( A = \text{Final weight of the dish (gm)} \)
\( B = \text{Initial weight of dish (gm)} \)
\( V = \text{Volume of sample taken (ml)} \).

3.6.7, Chloride, Fluoride, Nitrite, Nitrate Sulphate:

Chloride, Fluoride, Nitrite, Nitrate and Sulphate were determined by Mohr's agentometry method (APHA, 1998).
3.6.8, Heavy Metals:

The same collected water samples were also used for the analysis of heavy metals like Iron, lead, copper, Zn and Manganese. The analysis of heavy metals in water was done by using Atomic Absorption Spectrophotometer Model Perkin Elmer 100/300.

3.6.9, ANIMAL EXPERIMENTS:

3.6.9.1, Experimental Animal Model:

In the present investigation the Wistar Albino male and female rats (Rattus norvegicus) weighing about 85 ± 5 g were used as test animals. The rats were procured from National institute of Nutrition (NIN), Hyderabad, India. Animal experimentations were conducted according to “INSA-Ethical guidelines for use of animals for scientific research after getting permission from Ethical Committee”.

The animals were kept in vivarium throughout the period of experiment. They were regularly fed on standard pellet diet with recommended daily dose and provided by National Institute of Nutrition, Hyderabad and water ad-libitum. The remaining food and waste matter was removed from the cages daily and proper care was taken to avoid any infection. The food was given to the rats daily at 9.30 am. Only healthy rats were used for the present experiments. Experimental animals were acclimatized for fortnight. After recording their initial body weight, they are divided into two main groups:

1) Control and 2) Experimental.

The animals were observed daily for mortality and signs of intoxication up to 90 days (the period of experimentation).

3.6.9.2, Experimental set up:

Experiments were carried out by dividing male and female albino rats into two groups:
Materials and Methods

**Group I**: Control male and female albino rats kept at normal atmospheric conditions in the animal house.

**Group II**: Experimental 18 male and 18 female albino rats exposed to coalfield atmosphere for 90 days at Wani colliery.

**Sub-group I**: Experimental 6 male and 6 female albino rats exposed to coalfield atmosphere for 30 days.

**Sub-group II**: Experimental 6 male and 6 female albino rats exposed to coalfield atmosphere for 60 days.

**Sub-group III**: Experimental 6 male and 6 female albino rats exposed to coalfield atmosphere for 90 days.

3.6.9.3, Collection of Blood sample:

The venous blood was obtained from the orbital sinus of the control and experimental albino rats prior to sacrifice to study blood/serum biochemical parameters. The blood was collected into vacutainer tubes (5 ml) and were kept for separation of serum. Then the separated serum was centrifuged at 3000 rpm in ultracooling centrifuge (Remi) for 10 minutes. The serum was stored at -20°C until it was assayed for biochemical parameters.

3.6.9.4, Haematological studies:

<table>
<thead>
<tr>
<th>Sr.no.</th>
<th>Parameters</th>
<th>Methods</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Haemoglobin gm%</td>
<td>Sahil's acid haematin</td>
<td>Dacie and Lewis (1982).</td>
</tr>
</tbody>
</table>
Materials and Methods

3.6.9.5, Biochemical estimations:

The tissues were used for biochemical estimations like total tissue protein, GOT, GPT, Acid phosphatase, Alkaline phosphatase, Lactate dehydrogenase (LDH) and Catalase. Part of the same tissue was used to study histological alterations.

Table: Biochemical methods used:

Following standard methods were used during the present investigation of various aspects.

<table>
<thead>
<tr>
<th>Sr.no</th>
<th>Biochemical Parameters</th>
<th>Tissue/Serum</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Protein</td>
<td>Serum</td>
<td>Biuret.</td>
</tr>
<tr>
<td>2.</td>
<td>SGPT</td>
<td>Serum</td>
<td>Reitman and Frankel (1957).</td>
</tr>
<tr>
<td>3.</td>
<td>SGOT</td>
<td>Serum</td>
<td>Reitman and Frankel (1957).</td>
</tr>
<tr>
<td>5.</td>
<td>Acid phosphatase</td>
<td>Serum</td>
<td>King's (Varley, 1980).</td>
</tr>
<tr>
<td>6.</td>
<td>LDH</td>
<td>Serum</td>
<td>Modified IFCC.</td>
</tr>
<tr>
<td>7.</td>
<td>GPT</td>
<td>Tissue</td>
<td>Reitman and Frankel (1957).</td>
</tr>
<tr>
<td>8.</td>
<td>GOT</td>
<td>Tissue</td>
<td>Reitman and Frankel (1957).</td>
</tr>
<tr>
<td>10.</td>
<td>Acid phosphatase</td>
<td>Tissue</td>
<td>King's (Varley, 1980).</td>
</tr>
<tr>
<td>11.</td>
<td>LDH</td>
<td>Tissue</td>
<td>Modified IFCC.</td>
</tr>
<tr>
<td>12.</td>
<td>Catalase</td>
<td>Tissue</td>
<td>$\text{H}_2\text{O}_2$ Oxidoreductase, E.C. 1.11.1.6</td>
</tr>
<tr>
<td>15.</td>
<td>Sodium/Potassium</td>
<td>Serum</td>
<td>Flame Photometry</td>
</tr>
</tbody>
</table>
3.6.9.5.1, Estimation of total Protein in Serum :

Protein in the serum of control as well as experimental male and female albino rats was estimated by Biuret Method.

3.6.9.5.1.1, Principle :

Proteins react with cupric ions in alkaline medium to form violet coloured complex. The intensity of the colour produced is directly proportional to proteins present in the specimen and can be measured on a photometer at 530 nm (or by using a green filter).

3.6.9.5.1.2, Reagents :

i) Stock Biuret Reagent.

ii) Working Biuret Reagent

iii) Protein Standard (6.0 gm/dl).

3.6.9.5.1.3, Preparation of the regents :

i. Stock Biuret reagent :

45 gm of Rochelle salt was dissolved in about 400 ml of 0.2 N sodium hydroxide and to it was added 15 gm of copper sulfate by stirring continuously until the solution is complete. To it was added 5 g of potassium iodide and made up to a liter with 0.2 N sodium hydroxide.

ii. Working Biuret reagent :

200 ml of Stock reagent was diluted to a litre with 0.2 N Sodium hydroxide which contains 5 g of potassium iodide per litre.

iii. Protein standard :

6.0 g/dl: 6 g of bovine albumin dissolved in 100 ml of normal saline, containing 0.1 g/dl, sodium azide.
Materials and Methods

3.6.9.5.1.4, Procedure:

i) Three test tubes Blank (B), Standard (S), Test (T) were taken.

ii) To them working Biuret reagent 5.0 ml was added.

iii) 0.05 ml of serum was added to the test tube (T).

iv) 0.05 ml protein standard was added to the test tube (S).

v) 0.05 ml distilled water was added to the test tube (B).

Mixed thoroughly and was kept at room temperature (25 °C ± 5°C) for exactly 10 minutes. The intensities of the test and standard were measured by setting blank at 100 % T, at 530 nm (Green filter) wavelength.

3.6.9.5.1.5, Calculations:

\[
\text{Serum Proteins g/dl} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 6
\]

3.6.9.5.2, Electrophoresis (SDS-PAGE):

Serum proteins (albumin and immunoglobulin) from control, and experimental male and female albino rats were separated by SDS-PAGE (Laemmli, 1970) and were run on the densitometer for finding out the fractions of immunological proteins. The principle of the method is briefed here as under.

Electrophoresis is migration of charged molecular ions under the influence of electric field. Molecules masked with overall negative charges begin to separate due to their different electrophoretic mobility based on molecular weight. In SDS-PAGE, the Sodium Dodecyl Sulphate used is an anionic detergent, which binds to proteins and masks their molecules to make them all negatively charged. The negatively charged molecules move to anode. By
Materials and Methods

...doing so, they separate as per their size (or) molecular weight due to frictional forces through a constant pore size. The composition of the buffers used in this study was as below:

3.6.9.5.2.1, Reagents and Buffers:

1.5M Tris (pH 8.8) as separating gel buffer:

Tris (18.2g) was dissolved in 100 ml distilled water and pH was set to 8.8 with HCL.

1.0M Tris (pH 6.8) as stacking gel buffer:

Tris (12.1g) was dissolved in 100 ml distilled water and pH was set to 6.8 with HCL.

30% Acrylamide mix:

29.2g of acrylamide and 0.8g of NN' Bis acrylamide were dissolved in 100 ml distilled water and the prepared solution was kept in dark.

10% SDS:

SDS (10g) was dissolved in 100 ml distilled water.

TEMED:

TEMED is the catalyst for acrylamide polymerization, it is a commercially available solution.

10% APS:

APS (100 mg) was dissolved in 1 ml distilled water

Sample solubilising buffer (4 x strength) - 10 ml

Stacking buffer (0.5 M Tris) - 2.5 ml

Sucrose - 3.0 g
Materials and Methods

\[ \beta\text{-mercapto ethanol} - 1.0 \text{ ml} \]
\[ \text{Bromophenol blue} - 1.0 \text{ mg} \]

Above solution was made up to 10 ml by adding double distilled water.

Running Gel Buffer:

\[(\text{Tris – Glycine buffer – 2 litres}) (RGB) : \]

Tris 6.2 g and 28.8 g glycine were dissolved in 2 litres distilled water and pH was set to 8.3 (with glycine). SDS (0.1 %) was added to it.

Staining solution:

\[
\begin{align*}
\text{Distilled water} & \quad - \quad 43 \text{ ml} \\
\text{Methanol} & \quad - \quad 50 \text{ ml} \\
\text{Acetic Acid} & \quad - \quad 7 \text{ ml}
\end{align*}
\]

Then in 100 ml of staining solution, 250 mg of Coomassie brilliant blue was added.

Destaining solution:

Destaining solution was prepared by mixing distilled water, methanol and acetic acid in the ratio of 7:2:1 respectively.

3.6.9.5.2.2, Casting SDS-PAGE Gel:

Two clean glass plates were taken, wiped with alcohol and dried. Then the spacer was selected according to the thickness of the gel. Further, the required comb with definite wells were also selected by considering it's thickness which matches with the thickness of the spacers. After pouring water, the inner portion of the plates were cleaned by using a blotting paper. A 10 % gel was used. The percentage strength depended on acrylamide mix and its ratio with distilled water.
3.6.9.5.2.3, Separating Gel Buffer:

The separating gel buffer was prepared with the following composition:

- Distilled water: 11.9 ml
- 30% Acrylamide mix: 10.0 ml
- 1.5 M Tris (pH 8.8): 7.5 ml
- 10% SDS: 300 ml
- 10% APS: 300 ml
- TEMED: 12 ml

After the preparation of the separating gel, it was poured into the cast. APS and TEMED were added just before pouring. About 0.2 ml of 2-butanol was added over the surface of the separating gel to help quicker polymerization. After 20 minutes, when the polymerization was completed, the gel was washed gently with distilled water. Further, 2-butanol and water if any were removed by using a Whatmann paper.

3.6.9.5.2.4, Stacking Gel Buffer:

The stacking gel was prepared with the following composition:

- Distilled water: 6.80 ml
- 30% Acrylamide mix: 1.70 ml
- 1.0 M Tris (pH 6.8): 1.25 ml
- 10% SDS: 100 ml
- 10% APS: 100 ml
- TEMED: 10 ml

After the preparation of the stacking gel, it was poured over the separating gel. The comb was placed by inserting between the two plates. However, the
Materials and Methods...

Comb was removed after polymerization and the wells were filled with water just to remove the air bubbles. Before loading the samples, the water was removed from the wells by forcing it out. The bottom spacer was also removed to establish continuity of gel with buffer system. After this, by using filter paper, the grease was removed.

3.6.9.5.2.5, Sample preparation:

In each well, 20 ml of serum sample was loaded. Prior to loading, the serum was diluted to five times with glysine buffer and then sample loading buffer was added and kept in boiling water for 1 minute.

3.6.9.5.2.6, Running Gel Electrophoresis:

Tris glycine was used as the buffer system. RGB was poured in buffer reservoir of electrophoretic apparatus both at the top and bottom, when the buffer system established continuity in flow of current. Prior to this, the gel was placed with well facing the top reservoir. Then the sample was loaded in each well as per the plan of work. After this the apparatus was put on with the current setting at 50 mA.

3.6.9.5.2.7, Staining and Destaining:

Once the dye front reached the edges of the gel at the anode end, the plates were removed and the gel was placed in Coomassie blue staining solution for 6h. After completion of staining, the gel was kept in the destainer. Then the destainer was replaced frequently until clear protein bands were visible. The gel was stored in 8 % acetic acid with 0.1% sodium azide.

3.6.9.5.3, Estimation of SGPT and GPT:

Serum Glutamate Pyruvate Transaminase (SGPT) and GPT from serum and tissue homogenate of experimental as well as control male and female albino rats were estimated spectrophotometrically by 2,4 -DNPH method of Reitman and Frankel (1957). For tissue GPT, tissue homogenate was used.
3.6.9.5.3.1, Principle:

SGPT catalyses the following reaction:

\[ \text{a-Ketoglutarate + L-Alanine} \underset{\text{SGPT}}{\longrightarrow} \text{L-Glutamate + Pyruvate.} \]

Pyruvate, so formed was coupled with 2,4-dinitrophenylhydrazine (2,4-DNPH) to give the corresponding hydrazone, which gave brown colour in alkaline medium. This coloured compound was measured spectrophotometrically.

3.6.9.5.3.2, Reagents required:

i) Buffered alanine – a-Ketoglutarate substrate, (pH 7.4).

ii) DNPH colour reagent

iii) Sodium hydroxide (NaOH), 4N

iv) Working pyruvate Standard, 2mM.

3.6.9.5.3.3, Preparation of working solution:

i) Solution I:

1 ml of reagent III (NaOH) was diluted to 10 ml with distilled water.

3.6.9.5.3.4, Procedure:

a) For standard curve.

i) 5 test tubes were labelled as 1, 2, 3, 4 & 5

ii) In the above test tubes 0.25 ml, 0.225 ml, 0.2 ml, 0.175 ml of and 0.15 ml of reagent 1, i.e. buffered alanine – a-ketoglutarate substrate in tubes 1, 2, 3, 4 & 5 respectively was added.

iii) 0 ml, 0.025 ml, 0.05 ml, 0.075 ml and 0.1 ml of working pyruvate standard was added to the tubes 1, 2, 3, 4 and 5 respectively.

v) 0.05 ml of distilled water was poured in all the 5 test tubes.
vi) 0.25 ml of DNPH colour reagent was added to all 5 tubes; mixed well and allowed to stand at room temperature for 20 minutes.

vii) 2.5 ml of NaOH solution was poured in all 5 tubes mixed well by inversion, allowed to stand at room temp for 10 minutes and measured the O.D. of all 5 tubes against distilled water blank on spectrophotometer at 505 nm.

b) For Test:

i) 0.25 ml of buffered substrate was taken in a test tube, labelled test and incubated at 37°C for 5 minutes.

ii) 0.05 ml of serum/tissue homogenate was added to the above tube and incubated at 37°C for 30 minutes.

iii) 0.25 ml of coloured reagent was poured, mixed well and allowed to stand of room temperature for 20 minutes.

iv) 2.5 ml of NaOH solution was added, mixed well allowed to stand at room temperature for 10 minutes and measured the O.D on spectrophotometer at 505 nm against distilled water.

3.6.9.5.3.5, Calculations:

i) A standard graph by taking enzyme activity on X-axis and O.D. on Y-axis of tubes 1,2,3,4, and 5 respectively was plotted.

ii) The O.D. of test (T) on the Y axis of the standard curve were marked and extrapolated it to the corresponding enzyme activity on X – axis.

3.6.9.5.4, Estimation of SGOT and GOT:

Serum Glutamate Oxaloacetate Transaminase (SGOT) and GOT from serum and tissue homogenate of experimental as well as control male and female albino rat was estimated spectrophotometrically by 2,4-DNPH method of Reitman & Frankel (1957).
Materials and Methods.

SGOT (SAST) catalyses the following reaction:

\[
a \text{Ketoglutarate} + \text{L-aspartate} \rightarrow \text{L-Glutamate} + \text{Oxaloacetate}
\]

Oxaloacetate so formed couples with 2,4 dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which give brown colour in alkaline medium. This coloured compound was measured at 505 nm.

3.6.9.5.4.1, Reagents:

i) Buffered aspartate a – Ketoglutarate substrate, pH 7.4

ii) DNPH colour reagent.

iii) Sodium hydroxide (NaOH), 4N.

iv) Working pyruvate standard 2 mM.

3.6.9.5.4.2, Preparation of working solutions:

i) Solution I:

1 ml of reagent III (NaOH) was diluted to 10 ml with distilled water.

3.6.9.5.4.3, Procedure:

a) For standard curve:

i) 5 test tubes labelled 1, 2, 3, 4 and 5 were taken.

ii) 0.25 ml, 0.225 ml, 0.2 ml, 0.175 ml and 0.15 ml of reagent 1 i.e. buffered aspartate a – Ketoglutarate substrate in tubes 1, 2, 3, 4 and 5 respectively was poured.

iii) 0 ml, 0.025 ml, 0.05 ml, 0.075 ml and 0.1 ml of working pyruvate standard was added to the tubes 1, 2, 3, 4 and 5 respectively.

iv) 0.05 ml of distilled water was transferred to all the 5 tubes.

v) 0.25 ml of DNPH colour reagent was added to all 5 tubes, mixed well and allowed to stand at room temperature for 20 minutes.
vi) 2.5 ml of NaOH solution was poured to all 5 tubes, mixed well by inversion, and was allowed to stand at room temperature for 10 minutes and the O.D. of all 5 tubes was measured against distilled water on spectrophotometer at 505 nm.

b) Test:

i) 0.25 ml of buffered substrate was taken in a test tube, labelled test and was incubated at 37°C for 5 minutes.

ii) 0.05 ml of serum/tissue homogenate was added to the above tube and was incubated at 37°C for 60 minutes.

iii) 0.25 ml of coloured reagent was poured, mixed well and allowed to stand at room temperature for 20 minutes.

iv) 2.5 ml of NaOH solution was added, mixed well and allowed to stand at room temperature for 10 minutes and the O.D. was measured on spectrophotometer at 505 nm against distilled water blank.

3.6.9.5.4.4, Calculations:

i) A standard graph was plotted by taking enzyme activity on X-axis and O.D. on Y-axis of tubes 1, 2, 3, 4 and 5 respectively.

ii) The O.D. of test (T) was marked on the Y-axis of the standard curve and extrapolated it to the corresponding enzyme activity on X-axis.

3.6.9.5.5, Estimation of Alkaline phosphatase:

Alkaline phosphatase from serum/tissue of the experimental and the control male and female albino rats was estimated spectrophotometrically as given below.
3.6.9.5.5.1, Principle:

Alkaline phosphatase from the serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in the presence of oxidizing agent potassium ferricyanide and forms an orange red coloured complex, which is measured spectrophotometrically. The intensity of colour is proportional to the enzyme activity.

The reaction can be represented as:

i. Phenyl phosphate $\xrightarrow{\text{Alkaline phosphatase}}$ Phenol + Phosphate
   $\text{pH 10}$

ii. Phenol + 4-Aminoantipyrine $\xrightarrow{\text{Pot. ferricyanide}}$ orange red coloured complex
   $\text{OH}^-$

3.6.9.5.5.2, Reagents:

i) Buffered substrate pH 10.0

ii) Chromogen reagent.

iii) Phenol standard, 10 mg%

3.6.9.5.5.3, Preparation of working Standard:

i) Each vial of reagent 1 (buffered substrate) was reconstituted with 2.2 ml of distilled water.

ii) Working buffered substrate solution was prepared fresh just before use.

3.6.9.5.5.4, Procedure:

i) Four tubes labeled blank, standard, control and test were set.

ii) 0.5ml of working buffered substrate in all four tubes respectively was taken.
iii) 1.5 ml of distilled water in each of blank, standard, control and test tubes respectively was added, mixed well and incubated for 3 minutes at 37°C.

iv) 0.05 ml of phenol standard and 0.05 ml of serum was added to standard tubes and the test tubes respectively, mixed well and incubated at 37°C of 15 minutes.

v) There after 1.0 ml chromogen reagent was added to each of the four tubes.

vi) 0.05 ml of serum was added to control tube.

vii) Mixed well after each addition and the O.D. of Blank, Standard, Control and test were read spectrophotometerically at 510 nm.

3.6.9.5.5.5, Calculation:

\[
\text{Alkaline Phosphatase Activity (in KA Units) } = \frac{\text{O.D. of Test} - \text{O.D. of Control}}{\text{O.D. of Std.} - \text{O.D. of Blank}} \times 10
\]

3.6.9.5.6, Estimation of Acid phosphatase:

Acid phosphatase in serum/tissue of the experimental as well as control male and female albino rats was estimated spectrophotometrically as given below.

3.6.9.5.6.1, Principle:

Acid phosphatase from the serum converts phenyl phosphate to inorganic phosphate and phenol, at pH 4.9. Phenol so formed reacts in acid medium with aminoantipyrine in the presence of oxidizing agent potassium ferricyanide and forms an orange red colour complex, which is measured spectrophotometrically. The intensity of the colour is proportional to the enzyme activity.

\[
i. \quad \text{Phenyl phosphate} \quad \xrightarrow{\text{Acid phosphatase}} \quad \text{Phenol + Phosphate}
\]
Materials and Methods

**ii. Phenol + 4 Aminoantipyrine**

\[
\text{Phenol} + 4\text{ Aminoantipyrine} \rightarrow \text{orange red coloured complex}
\]

**3.6.9.5.6.2. Reagents:**

i) Buffered substrate pH 4.9
ii) Sodium hydroxide 0.5 N
iii) Sodium bicarbonate 0.5 N
iv) 4- Aminoantipyrine 0.6 %
v) Potassium ferricyanide 2.4 %
vi) Stock Phenol standard 10 mg %

**3.6.9.5.6.3. Preparation of Working Solutions:**

i) Solution I: Substrate was reconstituted with 2.0 ml water and mixed well. This solution was prepared fresh just before use.

ii) Solution II: 4 - aminoantipyrine (0.6%) was dissolved in 25 ml of distilled water.

iii) Solution III: Potassium ferricyanide (2.4%) was dissolved in 25 ml of distilled water.

**3.6.9.5.6.4. Procedure:**

i) Four tubes labelled blank, standard, control and test were set.

ii) 0.5 ml of solution I was transferred to test and control tubes.

iii) 1.1, 0.6, 0.5 and 0.5 ml of distilled water was added to tubes of blank, standard, control and test respectively.

iv) The contents of all tubes were mixed and incubated at 37°C for 3 minutes.
v) 0.5 ml of working standard was added to standard tube and 0.1 ml of serum to test tube and was mixed well and further incubated at 37 °C for 60 minutes.

vi) 0.5 ml of sodium hydroxide was added to all four tubes and mixed well.

vii) 0.1 ml of serum/tissue homogenate was transferred to control tube.

viii) 0.5 ml of sodium bicarbonate was added to each tube.

ix) 0.5 ml of solution II (4- aminoantipyrine) and 0.5 ml of solution iii (potassium ferricyanide) was added to all the four tubes sequentially and mixed well after each addition.

x) The O.D. were observed at 510 nm.

3.6.9.5.6.5, Calculation :

\[
\text{Acid Phosphatase Activity (in KA Units)} = \frac{\text{O.D. of Test} - \text{O.D. of Control}}{\text{O.D. of Std.} - \text{O.D. of Blank}} \times 5
\]

3.6.9.5.7, Estimation of Serum/tissue homogenate Lactate dehydrogenase (LDH) :

Lactate dehydrogenase from the serum/tissue homogenate of control as well as experimental male and female albino rats was estimated by modified IFCC method (Kinetic method).

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

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ 
\]
Materials and Methods

3.6.9.5.7.1, Reagents:
   i) Buffer reagent
   ii) Starter reagent

3.6.9.5.7.2, Preparation of Reagent:
   i) Working reagent:
      Content of 1 bottle of starter reagent were poured into 1 bottle of buffer reagent.

3.6.9.5.7.3, Procedure:
   i) Two tubes labelled "blank" and "test" were set.
   ii) Distilled water in the blank tube and 0.1 ml of working reagent in the "test" tube was taken.
   iii) The "test" tube were incubated for 1 min at 37 °C.
   iv) 20 ml i.e. 0.02 ml of serum/tissue homogenate was added in test tube.
   v) Mixed well and the initial absorbance $A_0$ after 1 minute and thereafter every 1, 2 and 3 minutes (Four readings) at 340 nm were read with Clinical Analyzer.
   vi) D $A$/minute was determined.

3.6.9.5.7.4, Calculations:

   $LDH$ activity in U/L = $D A$/minute $\times 8025$

Where,

$F = 8025$ which was calculated on the basis of millimolar absorption of NADH at 340 nm.
3.6.9.5.8, Estimation of Super Oxide Dismutase (SOD):

3.6.9.5.8.1, Principle:

SOD is coenzyme in mammalian tissues, primarily occurs in a manner of co-ordinated by cytokines, rather than as a response of individual cells to oxidants.

The activity of superoxide dismutase (SOD) in erythrocyte ghost was determined by the method of Marklund and Marklund (1974). The assay of SOD is based upon the ability of this enzyme to inhibit the auto-oxidation of pyrogallol in the presence of EDTA.

3.6.9.5.8.2, Preparation of Reagents:

I. Pyrogallol 0.6mM:

7.56 mg of pyrogallol was dissolved in 100 ml of water and stored in the brown bottle. This solution was prepared freshly at the time of estimation.

II. EDTA 6mM:

275 mg of disodium salt of EDTA was dissolved in 100 ml of water.

III. Tris HCL buffer, 100mM:

1.214g of Tris was dissolved in 80 ml of water. The pH was adjusted to 8.2 with 10 mM HCL and the volume was made to 100 ml.

3.6.9.5.8.3, Isolation and Purification of red cells:

Plasma was centrifuged in refrigerated centrifuge at 750 g for 7 min to separate erythrocytes (Nelson and Freeman, 1959; Goodman and Rosmussan, 1980).

The separated cells were repeatedly washed to completely remove leukocytes and platelets. Erythrocytes were washed in isotonic saline solution and centrifuged two times at 4°C for 15 min each and at 2500 rpm. After each centrifugation, buffy layer was discarded to insure complete removal of leukocytes and platelets.
3.6.9.5.8.4, Preparation of Red Cell Ghost:

Red cell ghost was prepared according to the method of Dodge et al. 1963 in which cold hypotonic phosphate buffer 25 mosm having 7.4 pH was mixed 14:1 (v/v) with wells were centrifuged at 20,000 xg for 30 min. The supernatant decnated carefully and the process was repeated 3 times. The milky white ghosts were used for further process.

3.6.9.5.8.5, Procedure:

1. To 3 ml cuvette, 1.5 ml of 100mM Tris- HCL buffer, 0.5 ml of 6mM EDTA, 0.05 ml of erythrocyte ghost and 1 ml of 0.6mM pyrogallol solution were added.

2. The rate of oxidation of pyrogallol was read as an increase in absorbance at 420 nm in a spectrophotometer, every minute after a lag of 30 sec., upto 3 hrs.

3. For the test sample appropriate amount of enzyme was added to inhibit the autooxidation of pyrogallol by about 50 to 70 %. The reaction starts here.

4. The blank was prepared without homogenate.

5. The assay was performed in duplicate in a two-fold concentration range.

3.6.9.5.8.6, Calculations:

One unit of enzyme activity is the 50 % inhibition of the rate of autooxidation of pyrogallol as determined by the change in absorbance at 420 nm / min. The activity of SOD is expressed as Unit / mg protein.

3.6.9.5.9, Estimation of Serum Potassium:

Clinical Significance:

Hyponatremia:

Low serum sodium levels are observed in the condition such as (1) severe prolonged diarrhea and vomiting (2) salt losing nephritis.
Materials and Methods.

Hypernatremia:

Increased serum sodium values are observed in the conditions such as (1) severe dehydration (2) diabetes insipidus etc.

Hypokalemia:

It is observed in the conditions such as (1) Cushing’s syndrome (2) renal tubular damaged (3) metabolic alkalosis and in (4) malnutrition.

3.6.9.5.9.1, Reagents:

1) Stock std. for sodium.

2) Stock std. for potassium.

3.6.9.5.9.2, Preparation of Standards:

Mixed standards were prepared by using following two stock standards.

1) Stock std. for Sodium:

1000 mEq/l: It was prepared by dissolving 5.85 g of analar grade sodium chloride in glass distilled water and was diluted to 100 ml by using a volumetric flask.

2) Stock std. for Potassium (100 mEq/l):

It was prepared by dissolving 0.740 g of potassium chloride (AR) in glass distilled water and was diluted to 100 ml by using a volumetric flask.

Mixed working standards were prepared as follows:

1) Sodium/potassium (120 / 2.0 mEq/l):

It contains 120 mEq of sodium and 2.0 mEq of potassium per liter of distilled water. It was prepared by mixing 12 ml of stock standard 1 and 2.0 ml of stock standard, 2 in 82 ml glass distilled water.
2) Sodium/potassium (140/4.0 mEq/L):

It was prepared by mixing 14 ml of stock standard, 1 and 4.0 ml of stock standard 2, in 82 ml of glass distilled water.

3) Sodium/potassium (160/6.0 mEq/L):

It was prepared by mixing 16 ml stock standard 1 and 6.0 ml of stock standard 2, in 78 ml of distilled water.

3.6.9.5.9.3, Procedure:

1) The tubes labelled Test (T), Std.1, Std.2 and Std.3 were taken.
2) In all the test tubes, 10 ml of glass distilled water was added.
3) To the test tube (T), 0.1 ml of serum was added.
4) To the test tube Std.1, 0.1 ml of Std: 120/2.0 was added.
5) To the test tube Std.2, 0.1 ml of Std: 140/4.0 was added.
6) To the test tube Std.3, 0.1 ml of Std: 160/6.0 was added. Mixed and transferred to the beakers (bulbs) for the flame photometric determination.
7) Flame photometer was operated according to the guidelines provided with the manual.

3.6.9.5.9.4, Calculations:

The digital display of flame photometer indicate exact concentration for both sodium and potassium. It was expressed in mol/kg.

3.6.9.5.10, Estimation of Catalase:

Catalase activity in tissue and serum of the control and experimental male and female albino rats were estimated by the method of E.C. 1.11.1.6. as described below.
3.6.9.5.10.1, Principle:

Catalase has a double function as it catalyses the following reactions:

1. Decomposition of hydrogen peroxide to give water and oxygen.

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

2. Oxidation of H donors with the consumption of one mole of peroxide.

\[ \text{ROOH} + \text{AH}_2 \rightarrow \text{H}_2\text{O} + \text{ROH} + \text{A} \]

The UV light absorbance of the hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

3.6.9.5.10.2, Reagents:

i) Phosphate Buffer, 0.067M (pH 7.0):

3.522g \( \text{KH}_2\text{PO}_4 \) and 7.268g \( \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} \) were dissolved in distilled water and the volume was made to 1 lit.

ii) Hydrogen Peroxide-Phosphate Buffer:

Dilute 0.16mL of \( \text{H}_2\text{O}_2 \) (10% w/v) to 100mL with phosphate buffer. Prepare fresh. The absorbance of the solution should be about 0.5 at 240nm with a 1 cm light path.

iii) Enzyme Extract:

The tissue was homogenized with M/150 phosphate buffer (assay buffer diluted 10 times) at 1-4 °C and centrifuged. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then the extraction was repeated once or twice. The extraction did not last longer than 24hr. The combined supernatants were used for assay.
3.6.9.5.10.3, Procedure:

i) 3mL H$_2$O$_2$.PO$_4$ buffer was taken in the experimental cuvette

ii) 0.01-0.04mL enzyme extract was mixed with it.

iii) A decrease in absorbance from 0.45 to 0.4 was read against a control cuvette containing enzyme solution as in experimental cuvette, but without H$_2$O$_2$.PO$_4$ at 240nm at room temperature.

iv) The time was noted which is required for the decrease in the absorbance.

3.6.9.5.10.4, Calculations:

One g of tissue is homogenized in a total volume of 20mL, diluted 1 to 10 volume with water and taken 0.01 mL for assay. It was expressed as m/ mg protein.

3.6.9.5.11, Estimation of Na- K-ATPase:

The Na-K-ATPase was estimated in tissue of control as well as experimental male and female albino rat.

3.6.9.5.11.1, Principle:

Electrolytes play an important role in maintenance of structure of living cell. Cell membrane Na+ and K+ activated adenosine triphosphatase (Na- K-ATPase) activity forms the enzymatic basis of the cation pump activity.

3.6.9.5.11.2, Reagents:

1) Tris-HCL(pH 7.5)

2) NaCl

3) MgCl2

4) ATP

5) EDTA
3.6.9.5.11.3, Procedure:

i) The tissues were separated, blotted and were made free of water and blood, homogenized in 10 volumes of ice-cold isolation medium (0.25M sucrose, 5mM ethylenediamine tetra acetic acid).

ii) After homogenization of the tissue, blood cells, cell debris and cartilage etc. were removed by centrifugation at 4000 rpm for 5 min and the supernant was then freeze-dried, dried and stored at -15°C until assayed.

iii) Each freeze-dried homogenate was reconstituted with cold isolation medium to a concentration of 7 to 8 mg/ml.

iv) The reconstituted homogenate (0.2 ml) was then added to 1.1 ml of concentrated assay medium (preincubated for 10 minutes). The reaction was initiated by adding 0.2 ml of ATP, magnesium chloride solution (L30 mM) disodium ATP, and 45 mM MgCl$_2$.

The final assay medium contained the following final concentrations (in mM) sodium chloride, 100; potassium chloride, 10; magnesium chloride, 6; ATP, 4; EDTA, 0.67; tris, 92; at pH 7.7.

The reaction was terminated after 15 minutes of incubation at 30°C by adding 4.0 ml ice-cold colour reagent (1% ammonium molybdate in 1.15N sulphuric acid made 4% with ferrous sulphate just prior to use). The absorbancy at 700nm was a measure of the inorganic phosphate produced. The Na-K-ATPase activity was determined by the difference in the amount of inorganic phosphate liberated from ATP when all the necessary other factors were present and when potassium was omitted and 0.1 mM quabain was added.

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard. The enzyme activity was expressed as nanomoles of inorganic phosphate liberated per milligram of protein in the
sample per minute. The total activity was calculated as the product of Na-K-ATPase specific activity and total protein in supernatant.

3.6.9.5.12, Estimation of serum Immunoglobins:

Blood was collected from orbital sinus of both control and experimental male and female albino rats after 30 days, 60 days and 90 days of exposure. The IgG and IgM levels were measured in serum using Enzyme Linked Immunosorbant Assay (ELISA).

3.6.9.5.12.1, Principle:

The assay for antigen depends on being able to couple highly specific antibody to enzyme and a solid substrate such as a plastic beads or plates (the inner walls of the wells where assay is done).

The antigen is immobilized on a solid surface. The antibody specific to this antigen is added to the plate which binds to the antigen. Then an antibody conjugation is done with the antigen again. This binds to antigen portion of the first or primary antibody. This complex can be detected by enzyme substrate reaction. The intensity of this chromogenic reaction is measured.

3.6.9.5.12.1.1, Estimation of IgG:

3.6.9.5.12.1.1.1, Requirement

i) Rat serum:

Blood was collected from orbital sinus of both control and experimental male and female albino rats after 30 days, 60 days and 90 days of exposure. It was centrifuged to obtain the serum.

ii) Goat anti-rat IgG as primary antibody:

Goat anti-rat IgG antibody was obtained from the Bangalore genei company. This goat anti-rat IgG was used as primary antibody.
iii) Goat anti-rat IgG peroxidase conjugate as secondary antibody:

Goat anti-rat IgG conjugate was obtained from the Bangalore Genei Company. It was used as secondary antibody.

iv) Phosphate buffer saline (PBS):

Added 19 ml NaH$_2$PO$_4$ (0.2M) to 81 ml NaHPO$_4$ and volume was made up to 200 ml with 0.9% NaCl solution.

v) Substrate Solution (Ortho phenylene diamine):

5 mg of Ortho phenylene diamine (OPD) was added in 12.5 ml of carbonate buffer and 5 ml of 30% hydrogen peroxide was added to it.

vi) Microtitre plate (96 welled)

3.6.9.5.12.1.1.2, Procedure:

i) Added serial dilutions of primary antibody (100 ml) to each well of the microtitre plate.

ii) Incubated for 2 hrs at 37 °C and transferred to 4 °C overnight.

iii) Next day the plate was washed with PBS.

iv) Then the wells were filled with 200 ml of blocking reagent (3% skimmed milk in PBS) to block the exposed area.

v) Then added the 100 ml of rat serum as antigen.

vi) Incubated for 1 hr at 37 °C.

vii) The plate was again washed with PBS and then 100 ml of conjugated secondary antibody was added to each well.

viii) Again the plate was incubated for 1 hr at 37 °C and washed with PBS.

ix) The substrate solution (H$_2$O$_2$ + OPD) was added to each well.
Materials and Methods

x) The reaction was stopped by adding 2N Sulphuric acid after 15-20 min.

xi) The O.D. of each well was checked at 492nm.

xii) The first two columns of the plate were left as blank. To this, added every thing except primary antibody. This acted as “zero intensity” well while taking the O.D.

3.6.9.5.12.1.1.3, Calculation:

A graph was plotted with O.D vs Concentration of primary antibody on a semilog graph paper. O.D of peak was multiplied by diluting factor to get the antibody concentration.

3.6.9.5.12.1.2, Estimation of IgM:

3.6.9.5.12.1.2.1, Requirements:

i) Rat Serum:

Blood was collected from orbital sinus of both control and experimental male and female albino rats after 30 days, 60 days and 90 days of exposure. It was centrifuged to obtain the serum.

ii) Goat anti-rat IgM as primary antibody.

Goat anti-rat IgM antibody was obtained from the Banglore genei company. This goat anti-rat IgG was used as primary antibody.

iii) Goat anti-rat IgM peroxidase conjugate as secondary antibody.

Goat anti-rat IgM conjugate was obtained from the Banglore Gene iCompany. It was used as secondary antibody.

iv) Phosphate buffer saline (PBS).

Added 19 ml NaH$_2$PO$_4$ (0.2M) to 81 ml NaHPO$_4$ and volume was made up to 200 ml with 0.9% NaCl solution.
Materials and Methods

v) Substrate Solution (Ortho phenylene diamine):

5 mg of Ortho phenylene diamine (OPD) was added in 12.5 ml of carbonate buffer and 5ml of 30 % hydrogen peroxide was added to it.

vi) Microtitre plate (96 welled).

3.6.9.5.12.1.2.2, Procedure:

i) Added serial dilutions of primary antibody (100ml) to each well of the microtitre plate.

ii) Incubated for 2 hrs at 37 °C and transferred to 4 °C overnight.

iii) Next day the plate was washed with PBS.

iv) Then the wells were filled with 200ml of blocking reagent (3% skimmed milk in PBS) to block the exposed area.

v) Then added the 100ml of rat serum as antigen.

vi) Incubated for 1 hr at 37 °C.

vii) The plate was again washed with PBS and then 100ml of conjugated secondary antibody was added to each well.

viii) Again the plate was incubated for 1hr at 37 °C and washed with PBS.

ix) The substrate solution ($H_2O_2 +$ OPD) was added to each well.

x) The reaction was stopped by adding 2N Sulphuric acid after 15-20 min.

xi) The O.D. of each well was checked at 492nm.

xii) The first two columns of the plate were left as blank. To this, added every thing except primary antibody. This act as “zero intensity” well while taking the O.D.

3.6.9.5.12.1.2.3, Calculation:

A graph was plotted with O.D vs Concentration of primary antibody on
Materials and Methods

a semilog graph paper. O.D of peak was multiplied by diluting factor to get the antibody concentration.

3.6.9.5.13, Histological Studies:

The histological studies of liver, kidney and lung of the control and experimental male and female albino rats were carried out. The tissues were first washed with saline water to remove adhering particles and blood stains and then fixed in Bouin's fixative for 24 hrs. Then the tissues were washed thoroughly with water for 24 hours, dehydrated with graded series of alcohols and embedded in paraffin wax with (melting point 58 °C ± 2°C) and sections were cut at 4 to 5 microns. The sections were processed and stained with Haematoxylin–Eosin by standard methods as described by Weissman (1978).

3.6.9.5.14, Statistical Analysis:

Student's 't' test was used, p < 0.05 was regarded as moderately significant and p < 0.01 as significant (Fischer, 1950).