# CHAPTER 4

Chapter 4: Experimental Investigation

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of sub-title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Collection of Plant materials</td>
<td>32</td>
</tr>
<tr>
<td>4.2</td>
<td>Extract of plant materials</td>
<td>32</td>
</tr>
<tr>
<td>4.3</td>
<td>Preliminary phytochemical screening of extracts</td>
<td>33</td>
</tr>
<tr>
<td>4.4</td>
<td><em>In-vitro</em> antioxidant screening assays</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>4.4.1 DPPH radical scavenging assay</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>4.4.2 ABTS radical scavenging Activity</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>4.4.3 Metal chelating ability assay</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>4.4.4 Total antioxidant activity</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>4.4.5 Reducing power assay</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>4.4.6 Total Phenol Content</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>4.4.7 Total Flavonoids Content</td>
<td>38</td>
</tr>
<tr>
<td>4.5</td>
<td>Pharmacological studies</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.5.1 Experimental animals</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.5.2 Acute toxicity studies</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.5.3 Alleviatory effects of hydroalcoholic extracts against- Sodium fluoride induced toxicity</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.5.3.1 Preparation of 100ppm fluoride (F) water</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>4.5.3.2 Treatment protocol</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>4.5.3.3 Measurements and sample collection</td>
<td>39</td>
</tr>
</tbody>
</table>
4.5.3.4 Estimation of biochemical parameters and \textit{in vivo} antioxidant markers 40

4.5.4 Protocols for estimation of serum biochemical markers 40

4.5.5 Protocols for estimation of \textit{In vivo} oxidative stress markers of tissue homogenate 64

4.5.6 Protocols for estimation of bone parameters 65
CHAPTER 4
EXPERIMENTAL INVESTIGATIONS

4.1 Collection of Plant Materials

Fresh leaves of *Brassica oleracea* Var. *Botrytis* were collected from local market, Kadapa, YSR district, Andhra Pradesh state and *Arthrospira platensis* freeze dried powder was purchased from Parry Neutraceuticals, Division of EID Parry (India) Ltd, Chennai, Tamil Nadu state, India and authentication was done by Dr. Sunita Garg, Chief scientist, Raw Material Herbarium and Museum, Delhi (RHMD), CSIR-NISCAIR; Voucher specimens were stored in the department of Pharmacology, CMR college of Pharmacy, Hyderabad, Telangana state, India.

4.2 Extract of plant materials

The collected plant material of *Brassica oleracea* Var. *Botrytis* was washed thoroughly in distilled water to remove the dirt and shade dried for three weeks. This material was then made into coarse powder with the help of hand crushing machine. *Arthrospira platensis* was directly available as powder form. Both plant materials were extracted separately with various proportions of hydroalcoholic solvents (50:50, 30:70 and 70:30 respectively) and kept at 25°C for one week with occasional shaking. Thereafter, they were stirred for 20 min and filtered. The filtrates were dried in rotary evaporator (ROTA VAP) apparatus and suitable extract of both plant materials were selected based on the percentage of yield and stored in refrigerator at 4°C for further studies.

![Figure No. 4.1: Selection of plant extraction.](image)
4.3 Preliminary phytochemical screening of extracts

Table No. 4.1: Preliminary phytochemical studies [108].

<table>
<thead>
<tr>
<th>NAME OF THE TEST</th>
<th>OBSERVATION</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tests for Carbohydrates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molish’s Test:</td>
<td>Few drops of alcoholic α-naphthol solution were added to 2 ml of plant extract and then Conc. H₂SO₄ was added.</td>
<td>Formation of violet ring at the junction of two liquids.</td>
</tr>
<tr>
<td>Fehling’s test:</td>
<td>Equal volumes of Fehling’s A and B reagents were added to plant extract solution followed by heating on the water bath.</td>
<td>Appearance of yellow color, followed by brick red precipitate.</td>
</tr>
<tr>
<td>Benedict’s test:</td>
<td>Equal volumes of Benedict’s reagent and plant extract were mixed and heated on water bath.</td>
<td>Formation of green or yellow or red color.</td>
</tr>
<tr>
<td>Barfoed’s test:</td>
<td>Equal volumes of plant extract and Barfoed’s reagent were taken and heated on water bath followed by cooling.</td>
<td>Development of red precipitate.</td>
</tr>
<tr>
<td>Iodine test:</td>
<td>Dilute iodine solution was added to the plant extract solution.</td>
<td>Development of blue color.</td>
</tr>
<tr>
<td><strong>Tests for proteins:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biuret test:</td>
<td>4% of Sodium hydroxide solution and few drops of 1 % of Copper sulphate solution were added to the 3 ml of plant extract.</td>
<td>Formation of violet or pink color.</td>
</tr>
<tr>
<td>Million’s test:</td>
<td>1.5 ml of plant extract was mixed with 2.5 ml of Million’s reagent.</td>
<td>Formation of white precipitate which slowly turns to red color.</td>
</tr>
<tr>
<td><strong>Test for amino acids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ninhydrin test:</td>
<td>5% Ninhydrin solution was added to the plant extract and heated on water bath.</td>
<td>Appearance of purple or bluish color.</td>
</tr>
</tbody>
</table>
### Tests for steroids

**Salkowski reaction:**
Equal volumes of the chloroform and Conc. H\(_2\)SO\(_4\) were added to the plant extract.

**Liebermann-Burchard reaction:**
Chloroform was added to the plant extract. Then acetic anhydride was added and few drops of Conc. H\(_2\)SO\(_4\) from the sides of test tube.

**Liebermann’s reaction:**
An equal volume of plant extract and acetic anhydride were taken and heated. After cooling, few drops of Conc. H\(_2\)SO\(_4\) were added.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Steroids present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of red color in the chloroform layer.</td>
<td></td>
</tr>
<tr>
<td>Development of a transient color from red to blue and finally turns to green color.</td>
<td></td>
</tr>
<tr>
<td>Appearance of blue color.</td>
<td></td>
</tr>
</tbody>
</table>

### Tests For Glycosides:

**Cardiac Glycosides**

**Legal’s Test:**
Equal volumes of pyridine and sodium nitroprusside were added to the plant extract.

**Keller-Killiani Test:**
Few drops of glacial acetic acid, Ferric chloride and Conc. H\(_2\)SO\(_4\) were added to the 2 ml of plant extract.

**Borntrager’s Test:**
To the plant extract, dil. H\(_2\)SO\(_4\) was added then boiled and filtered. To the filtrate benzene was added. Finally, ammonia solution was added to the separated Organic layer.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Glycoside present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of pink or red color.</td>
<td></td>
</tr>
<tr>
<td>Appearance of reddish brown color at the junction of two layers and upper layer turned to bluish green color.</td>
<td></td>
</tr>
<tr>
<td>Development of reddish pink color.</td>
<td></td>
</tr>
</tbody>
</table>

### Tests For Flavonoids:

**Shinoda test:**
To the plant extract, 5 ml of 95% of ethanol, Conc. H\(_2\)SO\(_4\) and 0.5 g of magnesium turnings were added.

**Ferric chloride test:**
Few drops of 10% of basic lead acetate solution were added to the plant extract.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Flavonoids present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of pink color.</td>
<td></td>
</tr>
<tr>
<td>Appearance of yellow color.</td>
<td></td>
</tr>
</tbody>
</table>

### Tests for Alkaloids:

**Dragendorff’s test:**
To the plant extract, few drops of Dragendorff’s reagent were added.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Alkaloids present.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of orange brown precipitation.</td>
<td></td>
</tr>
</tbody>
</table>
Mayer’s test:
Few drops of Mayer’s reagent were added to the plant extract. Formation of cream color precipitation. Alkaloids present.

Hager’s test:
To the plant extract, few drops of Hager’s reagent were added. Formation of orange yellow color precipitation. Alkaloids present.

Wager’s test:
To plant extract, Wagner’s reagent was added. Formation of reddish brown precipitation. Alkaloids present.

Test for Tannins and Phenols:
5 % Ferric chloride solution:
To the plant extract, 5% ferric chloride solution was added. Development of dark blue or dark black color. Tannins present.

Lead acetate solution:
Plant extract was added to the lead acetate. Formation of white precipitation. Tannins present.

4.4 In vitro antioxidant screening assays

4.4.1 DPPH radical scavenging assay [109]

DPPH radical scavenging activity was assessed according to the method of Blois, 1958. Various concentrations of the plant extract or standard (2 ml) were added to 6 ml of methanolic solution of DPPH (33 mg/l) in a test tube. The reaction mixture was kept at 25°C for an hour in an incubator. The absorbance of the residual DPPH solution was determined at 517 nm in a UV-Visible Spectrophotometer. The experiment was performed in triplicate. Ascorbic acid was used as standard. The inhibition was calculated in terms of percentage inhibition (I %) using following formula and lower IC₅₀ value indicates high antioxidant capacity.

\[
I\% = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100
\]

4.4.2 ABTS radical scavenging Activity [110]

ABTS radical scavenging ability was assessed according to the method of Roberta et al., 1999. Initially, ABTS 2 mM (0.0548 gm in 50ml) and potassium per sulphate 70 mM (0.0189 gm in 1ml) were prepared in distilled water. Next, 200 ml of potassium per sulphate and 50 ml of ABTS were mixed and kept aside for 2 hrs. This solution was used for assessing ABTS radical scavenging activity. To the 1 ml of various concentrations of plant extract or standard, 0.6 ml of ABTS radical cation and 3.4 ml of phosphate buffer pH 7.4
were added and the absorbance was measured at 734 nm. The experiment was performed in triplicate. Ascorbic acid was used as standard. The percentage of inhibition (I %) was calculated using following formula and lower IC50 value indicates high antioxidant capacity.

\[
I \% = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100
\]

4.4.3 Metal chelating assay [111]

Metal chelating ability was carried out according to the Dinis et al., 1994. In this assay, 10 ml of plant extract or standard, 0.2 ml of 2 mM ferric chloride and 0.4 ml of ferrozine solution were mixed and kept aside for 10 min at room temperature with continuous shaking. The absorbance was measured at 562 nm. The experiment was performed in triplicate. EDTA was used as standard. The percentage inhibition was calculated using following formula and lower IC50 value indicates high antioxidant capacity.

\[
I \% = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100
\]

4.4.4 Total antioxidant activity [112]

The total antioxidant activity was eluted by using the method described by Prieto et al., 1999. In this process, 0.2 ml of various concentrations of plant extract or standard was added to the 6 ml of reagent solution (0.6 M Sulphuric Acid, 28 mM Sodium Phosphate, 4 mM Ammonium molybdate) and solution was incubated at 95°C for 1 h 30 min. After incubation, solution was cooled to room temperature and then the absorbance of the solution was measured using UV-Visible spectrophotometer at 695 nm. The experiment was performed in triplicate. Ascorbic acid was used as standard. The total antioxidant ability of the plant extract was expressed as ascorbic acid equivalents in microgram per milligram of extract.

4.4.5 Reducing power assay [113]

Reducing power assay was carried out according to the method of Manisha et al., 2009. In this method, 2.5 ml of various concentrations of plant extract were mixed with 2.5 ml of phosphate buffer (0.2 M P H 6.6) and 2.5 ml of 1 % potassium ferricyanide. This solution was incubated at 50°C for 20 min. After incubation, 2.5 ml of 10 % of trichloroacetic acid was added to reaction mixture and centrifuged at 3500 rpm for 10 min. Next, 2.5ml of supernatant was added to 2.5 ml of distilled water and 0.5 ml of freshly prepared 0.1% of ferric chloride. The absorbance of the solution was measured using UV-Visible spectrophotometer at 700 nm. The experiment was performed in triplicate. The total
reducing power ability was calculated using standard ascorbic acid graph. The total reducing ability of the plant extract was expressed as ascorbic acid equivalents in micrograms per milligrams of the extract.

**4.4.6 Total phenol content** [114]

Total phenolic content was determined according to the Folin ciocalteu method. 0.4 ml of plant extract was added to 2 ml of folin ciocalteu reagent and 1.6 ml of 7.5% sodium carbonate. Then the solution was mixed and kept aside for 30 min at room temperature. The absorbance of the solution was measured at 765 nm using UV-Visible spectrophotometer. The experiment was performed in triplicate. The total flavonoid content was calculated using standard gallic acid graph. The total phenol content of the plant extract was expressed as gallic acid equivalents in micrograms per milligrams of the extract.

**4.4.7 Total flavonoid content** [115]

Total flavonoid content was quantified according to the modified method of Zhishen et al., 1999. 1ml of plant extract, 1 ml of distilled water and 0.075 ml of 5% sodium nitrite were added in the test tube. After 5 min 0.075 ml of 10% aluminium chloride was added to it. After 5 min 0.5 ml of 1M NaOH was added. The solution was mixed well and allowed to stand for 15 min. The absorbance was measured at 510 nm. The experiment was performed in triplicate. The total flavonoid content was calculated using standard quercetin graph. The total flavonoid content of the plant extract was expressed as quercetin equivalents in micrograms per milligrams of the extract.

**4.5 Pharmacological studies**

**4.5.1 Experimental animals**

Healthy adult male Wistar rats weighing between 220–250 g were used for this study. The animals were obtained from Sai Tirumala Enterprises Pvt Ltd, Hyderabad, Telangana state, India. The animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24 ± 2°C and maintained under a 12:12 light: dark cycle. They were fed with standard commercial pellet rat chow and water *ad libitum* during the experiment. All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics Committee (IAEC) of CMR College of Pharmacy, Kandlakoya village, Medchal Road,
Hyderabad and were accordance with guidelines of the IAEC (IAEC no: CPCSEA/1657/IAEC/CMRCP/PhD-14/35 & CPCSEA/1657/IAEC/CMRCP/PhD-15/40).

4.5.2 Acute toxicity studies

Acute toxicity study was performed according to the guidelines of OECD 425. Three groups of female Wistar rats (n= 3 in each group) were used for the study. First group received BOB 2000 mg/kg p.o, second group received APP 2000 mg/kg b. wt p.o and third group received vehicle, distilled water. After administration, animals were observed individually at least once every 30 min, periodically during the first 24 h, with special attention given during the first 4 hours and daily. Thereafter, observed continuously for a total of 14 days for the profiles such as lethargy, alertness, irritability, spontaneous activity, changes in skin, fur and eyes, behavior pattern, tremors, convulsions, salivation, defecation and urination, coma and death.

4.5.3 Alleviatory effects of hydroalcoholic extracts against sodium fluoride induced toxicity

4.5.3.1 Preparation of 100ppm fluoride (F⁻) water

The 100 ppm fluoride water was prepared by dissolving 0.221 g of sodium fluoride in 500 ml of drinking water (< 1 ppm F⁻) and the volume was made up to 1 liter.
4.5.3.2 Treatment protocol

After ten days of adaptation period, the experimental animals were divided into ten groups of six animals each as follows:

Table No. 4.2: Treatment protocol.

<table>
<thead>
<tr>
<th>S. NO</th>
<th>NAME OF THE GROUP</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal control</td>
<td>Received normal drinking water for 30 days.</td>
</tr>
<tr>
<td>2.</td>
<td>NaF control</td>
<td>Received sodium fluoride 100 ppm through drinking water for 30 days.</td>
</tr>
<tr>
<td>3.</td>
<td>BOB Control 400 mg/kg</td>
<td>Received normal drinking water + BOB at dose of 400 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>4.</td>
<td>ASP Control 400 mg/kg</td>
<td>Received normal drinking water + ASP at dose of 400 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>5.</td>
<td>BOB 100 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + BOB at dose of 100 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>6.</td>
<td>BOB 200 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + BOB at dose of 200 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>7.</td>
<td>BOB 400 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + BOB at dose of 400 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>8.</td>
<td>ASP 100 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + ASP at dose of 100 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>9.</td>
<td>ASP 200 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + ASP at dose of 200 mg/kg, p.o for 30 days.</td>
</tr>
<tr>
<td>10.</td>
<td>ASP 400 mg/kg</td>
<td>Received sodium fluoride 100 ppm through drinking water + ASP at dose of 400 mg/kg, p.o for 30 days.</td>
</tr>
</tbody>
</table>

4.5.3.3 Measurements and sample collection

Body weights were recorded twice a week throughout the experimental study. At the end of the study, animals were fasted over night and blood samples were collected from the retro orbital plexus. Blood samples were kept aside for approximately 1h at room temperature and centrifuged at 2500 rpm at 4ºC for 15 min to separate the serum from blood. The serum samples were used for estimation of various biochemical parameters.

After blood collection, animals were sacrificed by cervical dislocation and were cut open to isolate heart, liver and kidney and were weighed immediately. Then each organ was divided into two portions. First portion was fixed in 10 % formalin and were used for histopathological study. Second portion of the organ was chopped into fine slices and was chilled in the ice cold potassium chloride (1.15 %). These fine slices were homogenized in ice cold potassium chloride (1.15 %) using tissue homogenizer. It was then centrifuged at
5000 rpm at 4°C for 20 min to separate the homogenate from cellular debris. The supernatant was collected and used to estimate oxidative stress markers.

**4.5.3.4 Estimation of biochemical parameters and In vivo antioxidant markers**

Various biochemical parameters such as Glucose, Total protein, Albumin, T & D. bilirubin, GOT, GPT, Total cholesterol, Triglycerides, HDL-C, Urea, BUN, Uric acid, Creatinine, Iron, Calcium, Phosphorus, Magnesium, Triiodothyronine (T3) and Thyroxine (T4) were estimated in serum by using coral kit and semi auto analyzer (Inkarp ES-100). Complete Blood Profile (Leukocyte count, Erythrocyte count, Haemoglobin, Haematocrit, Mean Corpuscular Volume, Mean Corpuscular Haemoglobin, Mean Corpuscular Haemoglobin Concentration and platelet Count) was estimated using med cell hematology counter. In vivo antioxidant markers including lipidperoxidation were estimated by method of Niehaus, W. G. et al., 1968, reduced glutathione was estimated by method of Jollow, D.J. et al.,1974 and catalase was estimated by method of Greenwald, A.R., 1985 [116-118]. Detailed protocol for estimation of biochemical parameters and in vivo antioxidant markers are specified in section 4.5.4 and 4.5.5.

**4.5.4 Protocols for estimation of serum biochemical markers**

**4.5.4.1 Estimation of serum Glucose level**

Blood serum glucose level was estimated using GOD-POD Method.

**Principle**

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + \text{Hydrogen peroxide}
\]

\[
\text{Hydrogen peroxide} + 4\text{-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Red Quinoneimine dye} + \text{H}_2\text{O}
\]

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Reagent (L₁)</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Glucose standard (S)</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
After the additions, the contents in the test tubes were mixed well and incubated at 37\(^{0}\)C for 10 min. Absorbance of the S and T against B were measured by using semi auto analyzer.

**Formula:**

\[
\text{Glucose level} = \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times \text{Concentration standard (mg/dl)}.
\]

**System parameters used for auto analyzer:**

- **Reaction type**: End point
- **Wavelength**: 505 nm
- **Zero setting**: Reagent blank
- **Incubation temperature**: 37\(^{0}\)C
- **Incubation time**: 10 min
- **Sample volume**: 10 \(\mu\)l
- **Reagent volume**: 1000 \(\mu\)l
- **Standard concentration**: 100 mg/dl
- **Reaction slope**: Increasing
- **Linearity**: 500 mg/dl
- **Units**: mg/dl

### 4.5.4.2 Estimation of serum total Protein

Blood serum total protein level was estimated using Biuret Method.

**Principle**

Protein + Copper ions \(\xrightarrow{\text{alkaline medium}}\) Protein-copper ion complex (Blue-Violet color)

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret reagent</td>
<td>1000 (\mu)l</td>
<td>1000 (\mu)l</td>
<td>1000 (\mu)l</td>
</tr>
<tr>
<td>Total protein standard</td>
<td>------</td>
<td>10 (\mu)l</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>------</td>
<td>10 (\mu)l</td>
</tr>
</tbody>
</table>
After the additions, the contents in the test tubes were mixed well and incubated at 37°C for 10 min. Absorbance of the \( S \) and \( T \) against \( B \) were measured by using semi auto analyzer.

**Formula:**

Total protein level = Absorbance of \( T \) / Absorbance of \( S \) X Concentration standard (g/dl).

**System parameters used for auto analyzer:**

- **Reaction type**: End point
- **Wavelength**: 555 nm
- **Zero setting**: Reagent blank
- **Incubation temperature**: 37°C
- **Incubation time**: 5 min
- **Sample volume**: 10 µl
- **Reagent volume**: 1000 µl
- **Standard concentration**: 5.5 g/dl
- **Reaction slope**: Increasing
- **Linearity**: 10 g/dl
- **Units**: g/dl

### 4.5.4.3 Estimation of serum Albumin level

Blood serum albumin level was estimated using BCG Method.

**Principle**

\[
\text{Albumin + 3', 3'', 5, 5''-tetrabromo-m-cresol sulphonphthalein (Bromocresol green (BCG))}
\]

\[
\text{Albumin-3', 3'', 5, 5''-tetrabromo-m-cresol sulphonphthalein complex}
\]
Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>5 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 5 min. Absorbance of the S and T against B were measured by using semi auto analyzer.

Formula:

\[ \text{Albumin level} = \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times \text{Concentration standard (g/dl)} \]

System parameters used for auto analyzer:

- Reaction type : End point
- Wavelength : 620 nm
- Zero setting : Reagent bank
- Incubation temperature : 37°C
- Incubation time : 5 min
- Sample volume : 10 µl
- Reagent volume : 1000 µl
- Standard concentration : 4 g/dl
- Reaction slope : Increasing
- Linearity : 8 g/dl
- Units : g/dl
4.5.4.4 Estimation of serum Bilirubin level (BIT & BID)

Blood serum bilirubin level was estimated using Diazo Method.

Principle

Direct Bilirubin

Bilirubin + Diazotized sulphanilic acid → Azobilirubin

Total bilirubin

Bilirubin + Diazotized sulphanilic acid + Surfactant → Azobilirubin

Reagent contents

Reagent 1 contains Surfactant, HCl and Sulphanilic acid

Reagent 2 contains HCl and Sulphanilic acid

Reagent 3 contains Sodium Nitrite

Preparation of working reagents:

<table>
<thead>
<tr>
<th>Name of the test</th>
<th>Working reagent</th>
<th>Add</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reagent 1</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Direct bilirubin (DB) and Total bilirubin (TB) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
<th>Direct bilirubin</th>
<th>Total bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>25 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at 37°C for 5 min. Absorbance of the DB and TB against B were measured by using semi auto analyzer.
Formula:

Direct bilirubin = Absorbance of DB X Factor = mg/dl.

Total bilirubin = Absorbance of TB X Factor = mg/dl.

System parameters used for auto analyzer:

<table>
<thead>
<tr>
<th>Name of the parameter</th>
<th>Direct bilirubin</th>
<th>Total bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction type</td>
<td>End point</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength</td>
<td>546 nm</td>
<td>546 nm</td>
</tr>
<tr>
<td>Zero setting</td>
<td>Reagent bank</td>
<td>Reagent bank</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>$37^\circ$C</td>
<td>$37^\circ$C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Sample volume</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Factor</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Linearity</td>
<td>20 mg/dl</td>
<td>20 mg/dl</td>
</tr>
<tr>
<td>Units</td>
<td>mg/dl</td>
<td>mg/dl</td>
</tr>
</tbody>
</table>

4.5.4.5 Estimation of serum Alkaline Phosphatase level

Blood serum Alkaline Phosphatase level was estimated using king & king’s Method.

Principle

Phenyl phosphate $\xrightarrow{\text{alkaline phosphatase}}$ Phenol + disodium hydrogen phosphate (Hydrolysis (pH 10.0))

Phenol + 4-Aminoantipyrine + Potassium ferricyanide $\xrightarrow{\text{alkaline medium}}$ Red colored-

Complex
Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S), Control (C) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer substrate</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Deionized water</td>
<td>3010</td>
<td>3010</td>
<td>3010</td>
<td>3010</td>
</tr>
</tbody>
</table>

**Incubate for 3 min at 37°C**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th>100 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>Phenol Standard</td>
<td>100 µl</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
</tbody>
</table>

**Incubate for 15 min at 37°C**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Color reagent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>------</td>
<td>------</td>
<td>100 µl</td>
<td>------</td>
</tr>
</tbody>
</table>

After each addition all the test tubes were mixed well and absorbances of the B, S, C and T against the deionized water were measured by using semi auto analyzer.

**Formula:**

\[
\text{Absorbance of (T - C) / Absorbance of (S - B) \times Standard concentration = KA Unit/dl.}
\]

**System parameters used for auto analyzer:**

- Reaction type: End point
- Wavelength: 510 nm
- Zero setting: Deionized water
- Incubation temperature: 37°C
- Incubation time: 3 +15 min
- Sample volume: 100 µl
- Reagent volume: 1000 µl
- Standard concentration: 10 KA units
- Reaction slope: Increasing
- Units: KA units
4.5.4.6 Estimation of SGOT level

Blood SGOT level was estimated using 2, 4-DNPH (Reitman & Frankel) Method.

Principle

\[
\text{L-Aspartate} + \alpha-\text{Ketoglutarate} \xrightarrow{\text{SGOT}^\text{pH 7.4}} \text{Oxaloacetate} + \text{L-Glutamate}
\]

\[
\text{Oxaloacetate} + 2, 4\text{-DNPH} \xrightarrow{\text{alkaline medium}} \text{2,4-Dinitrophenyl Hydrazone (Brown colored complex)}
\]

Preparation of working NaOH reagent

Sodium hydroxide (4N, 1 ml) was diluted to 10 ml of distilled water.

Procedure

Non-contaminated dry test tubes were labeled as Blank (B) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate reagent</td>
<td>125 µl</td>
<td>125 µl</td>
</tr>
<tr>
<td><strong>Incubated at 37\textdegree C for 3 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>250 µl</td>
</tr>
<tr>
<td><strong>Incubated at 37\textdegree C for 60 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNPH reagent</td>
<td>125 µl</td>
<td>125 µl</td>
</tr>
<tr>
<td><strong>Shake well and allowed to stand for 20 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>25 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Working NaOH reagent</td>
<td>1250 µl</td>
<td>1250 µl</td>
</tr>
</tbody>
</table>

After each addition all test tubes were mixed well and incubated at 37\textdegree C for 10 min. Absorbance of the T against B was measured using semi auto analyzer.

System parameters used for auto analyzer:

- Reaction type : End point
- Wavelength : 505 nm
- Zero setting : Reagent bank
- Incubation temperature : 37\textdegree C
- Incubation time : 50 min
Sample volume : 100 µl
Reagent volume : 1.5025 ml
Standard concentration : Calibration curve
Reaction slope : Increasing
Linearity : 150 U/ml
Units : U/ml

4.5.4.7. Estimation of SGPT level

Blood SGPT level was estimated using 2, 4-DNPH (Reitman & Frankel) Method.

Principle

\[
\text{L-Aspartate} + \alpha-\text{Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate}
\]

\[
\text{Pyruvate} + 2, 4\text{-DNPH} \xrightarrow{\text{alkaline medium}} 2, 4\text{-Dinitrophenyl Hydrazine (Brown colored complex)}
\]

Preparation of working NaOH reagent

Sodium hydroxide (4N, 1 ml) was diluted to 10 ml of distilled water.

Procedure

Non-contaminated dry test tubes were labeled as Blank (B) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate reagent</td>
<td>125 µl</td>
<td>125 µl</td>
</tr>
<tr>
<td><strong>Incubated at 37°C for 3 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>250 µl</td>
</tr>
<tr>
<td><strong>Incubated at 37°C for 60 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNPH reagent</td>
<td>125 µl</td>
<td>125 µl</td>
</tr>
<tr>
<td><strong>Shake well and allowed to stand for 20 min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>25 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Working NaOH reagent</td>
<td>1250 µl</td>
<td>1250 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at 37°C for 10 min. Absorbance of the T against B was measured by using semi auto analyzer.
System parameters used for auto analyzer:

- **Reaction type**: End point
- **Wavelength**: 505 nm
- **Zero setting**: Reagent bank
- **Incubation temperature**: 37°C
- **Incubation time**: 50 min
- **Sample volume**: 100 µl
- **Reagent volume**: 1.5025 ml
- **Standard concentration**: Calibration curve
- **Reaction slope**: Increasing
- **Linearity**: 150 U/ml
- **Units**: U/ml

### 4.5.4.8 Estimation of serum Cholesterol level

Blood serum cholesterol level was estimated using Chod-Pod Method.

**Principle**

Serum cholesterol esters $\xrightarrow{\text{cholesterol esterase}}$ Cholesterol + Fatty acids.

Cholesterol + O$_2$ $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one + H$_2$O$_2$

$2$ H$_2$O$_2$ + 4-Aminoantipyrine + Phenol $\xrightarrow{\text{peroxidase}}$ Quinoneimine + 4 H$_2$O (Red Color)

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 5 min. Absorbance of the \( S \) and \( T \) against \( B \) was measured by using semi auto analyzer.

Formula:

\[
\text{Cholesterol concentration} = \frac{\text{Absorbance of } T}{\text{Absorbance of } S} \times \text{Standard concentration (mg/dl)}
\]

System parameters used for auto analyzer:

- Reaction type: End point
- Wavelength: 505 nm
- Zero setting: Reagent bank
- Incubation temperature: 37°C
- Incubation time: 10 min
- Sample volume: 10 µl
- Reagent volume: 1000 µl
- Standard concentration: 100 mg/dl
- Reaction slope: Increasing
- Units: mg/dl

4.5.4.9 Estimation of serum Triglyceride level

Blood serum triglyceride level was estimated using God-Pod Method.

**Principle**

\[
\text{Triglycerides} \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol + Fatty acids.}\\
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-Phosphate} + \text{ADP}\\
\text{Glycerol-3-Phosphate} + 4\text{-Aminoantipyrine} + \text{Sodium N-ethyl-N-(3-Sulfopropyl) m-toludine} \\
\xrightarrow{\text{peroxidase}} \text{Quinoneimine dye} + \text{H}_2\text{O}
\]
Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 15 min. Absorbance of the S and T against the B were measured by using semi auto analyzer.

Formula:

Triglyceride concentration = \frac{\text{Absorbance of } T}{\text{Absorbance of } S} \times \text{Standard concentration (mg/dl)}

System parameters used for auto analyzer:

- Reaction type: End point
- Wavelength: 546 nm
- Zero setting: Reagent bank
- Incubation temperature: 37°C
- Incubation time: 15 min
- Sample volume: 10 µl
- Reagent volume: 1000 µl
- Standard concentration: 200 mg/dl
- Reaction slope: Increasing
- Units: mg/dl
4.5.4.10 Estimation of serum HDL-Cholesterol level

Blood serum HDL-C level was estimated using Phosphotungstic acid Method.

**Principle**

Serum sample $\xrightarrow{\text{phosphotungstate}}$ Mg$^{2+}$ HDL+ (LDL+ VLDL+ Chylomicrons).

**Procedure**

**Step-I (Precipitation of LDL, VLDL and Chylomicrons)**

Non-contaminated dry test tube was labeled as Test (T). Sample and reagent were pipetted into the test tube as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>250 µl</td>
</tr>
<tr>
<td>Precipitation reagent</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tube were mixed well and allowed to stand for 10 min at room temperature, centrifuged at 4000 rpm for 10 min to separate the supernatant for estimation of HDL-C level in the serum sample.

**Step-II (Estimation of HDL-C)**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>HDL-C standard</td>
<td>-----</td>
<td>50 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-----</td>
<td>-----</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at $37^0$C for 10 min. Absorbance of the S and T against B were measured by using semi auto analyzer.

**Formula:**

HDL-C concentration=$\frac{\text{Absorbance of } T}{\text{Absorbance of } S} \times $ Standard concentration (mg/dl).
System parameters used for auto analyzer:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction type</td>
<td>End point</td>
</tr>
<tr>
<td>Wavelength</td>
<td>546 nm</td>
</tr>
<tr>
<td>Zero setting</td>
<td>Reagent bank</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>10 min</td>
</tr>
<tr>
<td>Sample volume</td>
<td>50 µl</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard concentration</td>
<td>200 mg/dl</td>
</tr>
<tr>
<td>Reaction slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>Units</td>
<td>mg/dl</td>
</tr>
</tbody>
</table>

### 4.5.4.11 Estimation of serum Creatinine Kinase- Markers B enzyme level

Blood serum CK-MB level was estimated using IFCC (International Federation Clinical Chemistry), UV-Kinetic Method.

**Principle**

Creatinine Phosphate + ADP $\xrightarrow{\text{creatine kinase}}$ Creatinine + ATP

D-Glucose + ATP $\xrightarrow{\text{hexokinase}}$ ADP + Glucose-6-Phosphate

Glucose-6-Phosphate + NADP$^+$ $\xrightarrow{\text{G6PDH}}$ 6-Phospho Gluconate + NADPH + H$^+$

**Procedure**

Non-contaminated dry test tube was labeled as Test (T). Sample and reagent were pipetted into the test tube as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>40 µl</td>
</tr>
</tbody>
</table>
After the additions, the contents in the test tube were mixed well and immediately initial absorbance was recorded. The absorbance was recorded after every 1, 2 and 3 minutes. The mean absorbance change per min (ΔA/min) was calculated.

Formula:

\[ \text{CK-MB} = 8360 \text{A (f) X } \Delta \text{A/min} \]

\( f = \text{Kinetic factor for 340 nm incubated at } 37^0\text{C} \)

System parameters used for auto analyzer:

- Reaction type: Kinetic
- Wavelength: 340 nm
- Zero setting: Reagent bank
- Incubation temperature: 37°C
- Incubation time: 1 min
- Sample volume: 40 µl
- Reagent volume: 1000 µl
- Kinetic interval: 60 sec
- No. of readings: 4
- Reaction slope: Increasing
- Units: IU/L

### 4.5.4.12 Estimation of serum Lactate Dehydrogenase (LDH) level

Blood serum LDH level was estimated using Mod. IFCC (International Federation Clinical-Chemistry), UV- Kinetic Method.

**Principle**

\[ \text{Pyruvate } + \text{NADH } + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate } + \text{NAD}^+ \]
Procedure

Non-contaminated dry test tube was labeled as Test (T) and reagents were pipetted into the test tube as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer reagent</td>
<td>800 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>20 µl</td>
</tr>
<tr>
<td><strong>Incubated at 37°C for 1 min and added</strong></td>
<td></td>
</tr>
<tr>
<td>Starter reagent</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tube were mixed well and immediately initial absorbance was recorded. The absorbance was recorded after every 1, 2 and 3 minutes. The mean absorbance change per min (ΔA/min) was calculated.

**Formula:**

\[
LDH = 8095 \times f \times (\Delta A/\text{min}).
\]

\[
f = \text{Kinetic factor for 340 nm incubated at } 37^\circ\text{C}.
\]

**System parameters used for auto analyzer:**

- **Reaction type**: Kinetic
- **Wavelength**: 340 nm
- **Zero setting**: Reagent bank
- **Incubation temperature**: 37°C
- **Sample volume**: 20 µl
- **Reagent volume**: 1000 µl
- **Kinetic interval**: 60 sec
- **No. of readings**: 4
- **Reaction slope**: Decreasing
- **Units**: IU/L
4.5.4.13 Estimation of serum Calcium level

Blood serum calcium level was estimated using Arsenazo dye Method.

**Principle**

Calcium + Arsenazo III $\xrightarrow{\text{Neutral } P^+}$ Calcium-Arsenazo III dye complex  
(Blue color development)

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Calcium standard</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 3 min. Absorbance of the S and T against B were measured using semi auto analyzer.

**Formula:**

Calcium concentration = Absorbance of T/Absorbance of S X Standard concentration  
(mg/dl).

**System parameters used for auto analyzer:**

- Reaction type : End point
- Wavelength : 630 nm
- Zero setting : Reagent bank
- Incubation temperature : $37^0\text{C}$
- Incubation time : 3 min
- Sample volume : 10 µl
- Reagent volume : 1000 µl
- Standard concentration : 100 mg/dl
- Reaction slope : Increasing
- Linearity : 20 mg/dl
- Units : mg/dl
4.5.4.14 Estimation of serum Phosphorus level

Blood serum Phosphorus level was estimated using Molybdate U.V. Method.

**Principle**

Phosphorus + Ammonium Molybdate → Phosphomolybdate complex.

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Phosphorus standard</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 5 min. Absorbance of the S and T against B were measured by using semi auto analyzer.

**Formula:**

Phosphorus concentration = Absorbance of T/Absorbance of S X Standard concentration (mg/dl).

**System parameters used for auto analyzer:**

Reaction type : U.V. End point  
Wavelength : 340 nm  
Zero setting : Reagent bank  
Incubation temperature : 37°C  
Incubation time : 5 min  
Sample volume : 10 µl  
Reagent volume : 1000 µl  
Standard concentration : 5 mg/dl  
Reaction slope : Increasing
Linearity: 20 mg/dl
Units: mg/dl

**4.5.4.15 Estimation of serum Magnesium level**

Blood serum magnesium level was estimated using Calmagite Method.

**Principle**

Magnesium + Calmagite \( \rightarrow \) Magnesium-Calmagite complex (Red colored complex)

**Procedure**

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer reagent</td>
<td>500 µl</td>
<td>500 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>Color reagent</td>
<td>500 µl</td>
<td>500 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Magnesium Standard</td>
<td>-----</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 5 min. Absorbance of the S and T against the B were measured by using semi auto analyzer.

**Formula:**

Magnesium Level = Absorbance of T / Absorbance of S X Standard concentration (mEq/L).

**System parameters used for auto analyzer:**

- Reaction type: End point
- Wavelength: 510 nm
- Zero setting: Reagent bank
- Incubation temperature: Room temperature
- Incubation time: 10 min
- Sample volume: 10 µl
Reagent volume : 1000 µl
Standard concentration : 2 mEq/L
Reaction slope : Increasing
Linearity : 10 mEq/L
Units : mEq/L

4.5.4.16 Estimation of serum Iron level

Blood serum iron level was estimated using Ferrozine Method.

Principle

Fe (III) \(\rightarrow\) Fe (II) in acidic medium
Fe (II) + Ferrozine \(\rightarrow\) Violet colored complex

Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S), Sample with blank (SB) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition\nsequence</th>
<th>B</th>
<th>S</th>
<th>SB</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>200 µl</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>200 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Color reagent</td>
<td>50 µl</td>
<td>50 µl</td>
<td>-----</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at room temperature for 5 min. Absorbances of the B, S, SB and T against distilled water were measured by using semi auto analyzer.

Formula:

Iron Level = \[ \frac{\text{Absorbance of } T - (\text{Absorbance of } SB + \text{Absorbance of } B)}{\text{Absorbance of } S - \text{Absorbance of } B} \times 100 \text{ (µg/dl)} \]
System parameters used for auto analyzer:

- **Reaction type**: End point
- **Wavelength**: 578 nm
- **Zero setting**: Distilled water
- **Incubation temperature**: Room temperature
- **Incubation time**: 5 min
- **Sample volume**: 200 µl
- **Reagent volume**: 1050 µl
- **Standard concentration**: 100 µg/dl
- **Reaction slope**: Increasing
- **Linearity**: 1000 µg/dl
- **Units**: µg/dl

### 4.5.4.17 Estimation of serum Urea level

Blood serum urea level was estimated using Urease Method.

**Principle**

$$\text{Urea} \xrightarrow{\text{Water + urease}} \text{Ammonia + Carbon dioxide}$$

$$\text{Ammonia + } \alpha\text{- Oxoglutarate + NADH} \xrightarrow{\text{glutamate dehydrogenase}} \text{Glutamate + NAD}$$

**Procedure**

Non-contaminated dry test tubes were labeled as Test (T) and Standard (S) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-----</td>
<td>10 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>10 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well, initial absorbance was measured after 30 sec and timer was started simultaneously to record an absorbance after 60 sec.
**Formula:**

Urea level = \( \frac{\text{Absorbance of } T_2 - T_1}{\text{Absorbance of } S_2 - S_1} \times \text{Standard (mg/dl)} \)

System parameters used for auto analyzer:

- **Reaction type**: Fixed time kinetic
- **Wavelength**: 340 nm
- **Zero setting**: Distilled water
- **Incubation temperature**: 37°C
- **Delay time**: 30 sec
- **Interval time**: 60 sec
- **No. of readings**: 1
- **Sample volume**: 10 µl
- **Reagent volume**: 1000 µl
- **Standard concentration**: 50 mg/dl
- **Reaction slope**: Decreasing
- **Linearity**: 300 mg/dl
- **Units**: mg/dl

### 4.5.4.18 Estimation of serum Uric acid level

Blood serum uric acid level was estimated using Uricase Method.

**Principle**

\[
\text{Uric acid} + \text{O}_2 + 2 \text{H}_2\text{O} \xrightarrow{\text{uricase}} \text{Allantoine} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{ESPAS} + 4- \text{APP} \xrightarrow{\text{POD}} \text{Purple Quinoneimine} + 4\text{H}_2\text{O}_2
\]
Procedure

Non-contaminated dry test tubes were labeled as Blank (B), Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100 µl</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>100 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>-----</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

After the additions, the contents in the test tubes were mixed well and incubated at 37°C for 10 min. Absorbance of the S and T against B were measured by using semi auto analyzer.

Formula:

Uric acid Level = Absorbance of T / Absorbance of S × Standard concentration (mEq/L)

System parameters used for auto analyzer:

- Reaction type: End point
- Wavelength: 546 nm
- Zero setting: Reagent blank
- Incubation temperature: 37°C
- Incubation time: 10 min
- Sample volume: 100 µl
- Reagent volume: 1000 µl
- Standard concentration: 5 mg/dl
- Reaction slope: Increasing
- Linearity: 25 mg/dl
- Units: mg/dl
4.5.4.19 Estimation of serum Creatinine level

Blood serum creatinine level was estimated using Jaffe’s Method.

Principle

Creatinine + Picric acid \( \xrightarrow{\text{alkaline medium}} \) Creatinine-Picrate complex
(Yellow-Orange colored complex)

Procedure

Non-contaminated dry test tubes were labeled as Standard (S) and Test (T) and reagents were pipetted into the test tubes as follows:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>100 µl</td>
<td>-----</td>
</tr>
<tr>
<td>Sample</td>
<td>-----</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

After addition the contents in the test tubes were mixed well and measured initial absorbance of sample and standard after 30 sec and started timer simultaneously to record again after 60 sec.

Formula:

Creatinine Level = Absorbance of \( \Delta T \) / Absorbance of \( \Delta S \) \times Standard concentration (mg/dl).

System parameters used for auto analyzer:

- Reaction type : Fixed time kinetic
- Wavelength : 505 nm
- Zero setting : Reagent blank
- Incubation temperature : 37°C
- Sample volume : 100 µl
- Reagent volume : 1000 µl
- Standard concentration : 1.5 mg/dl
- Reaction slope : Increasing
- Units : mg/dl
4.5.5 Protocols for estimation of *in vivo* oxidative stress markers of tissue homogenates

Tissue was homogenized in chilled 1.15 % potassium chloride using a homogenizer. The homogenate was centrifuged at 800 rpm for 5 min at 4°C (REMI C-24) to separate the molecular debris. The supernatant so obtained was centrifuged at 10,000 rpm for 20 min at 4°C (REMI CM-12) to get the post mitochondrial supernatant (PMS), which was used for estimation of lipidperoxidation, reduced glutathione and catalase levels.

4.5.5.1 Estimation of Lipidperoxidation

0.5 ml of PMS was taken and to it was added with 0.5 ml of Tris-Hcl buffer and incubated at 37°C for 2 h and then 1 ml of 10% w/v ice cold trichloroacetic acid was added and centrifuged at 1000 rpm for 10 min. From the above, 1 ml of supernatant was taken and added 1 ml of 0.67% w/v thiobarbituric acid and the tubes were kept in boiling water bath for 10 min. The tubes were removed and brought up to room temperature and 1 ml of distilled water was added. Absorbance was measured at 532 nm by using a UV-Visible spectrophotometer and the lipidperoxidation was calculated using following formula:

\[
\frac{3 \times \text{Absorbance of sample}}{50.156 \times (\text{mg of tissue taken})} = \mu M / \text{mg tissue}
\]

4.5.5.2 Estimation of Reduced Glutathione (GSH)

In this method, 1 ml of post mitochondrial supernatant (PMS) was added to 1 ml of 10% trichloroacetic acid. The precipitated fraction was centrifuged at 4°C. Supernatant (0.5 ml) was added to 2 ml of DTNB (0.6M) solution. The final volume was made up to 3 ml with phosphate buffer (0.2 M, pH 8.00). Absorbance was read at 412 nm by using UV-Visible spectrophotometer. The amount of glutathione was expressed as µg/mg of tissue and the reduced glutathione level was calculated using following formula:

\[
\frac{3 \times \text{Absorbance of sample}}{13.6 \times (\text{mg of tissue taken})} = \mu M \text{ of GSH / mg tissue}
\]

4.5.5.3 Estimation of Catalase

In this process, PMS was diluted 20 times with phosphate buffer (50 mM, pH 7.0). 1 ml of diluted PMS and 1 ml of H₂O₂ were added and absorbance was recorded at 254 nm
for three times with 1 min interval against blank. Change in absorbance was recorded. The results were expressed as µM/ mg of tissue.

4.5.6 Protocols for estimation of bone parameters

Bone weight, breaking strength and fluoride content were evaluated on the isolated left femur of rat.

4.5.6.1 Estimation of bone weight

The bone weight was calculated by using 1 mg sensitivity balance.

4.5.6.2 Estimation of breaking strength

The breaking strength was performed by breaking strength apparatus (Monsanto). The fresh bone was placed in the hardness compressor, until it fractured. The reading was recorded in Newtons (N).

4.5.6.3 Estimation of bone fluoride

1) Preparation of Ash: After measuring the bone weight and breaking strength, the bone was placed in fused crucibles and kept in muffle furnace dried to a constant temperature at 1000°C for 24 h.

2) Fluoride estimation: Fluoride concentration in bone ash was measured by digital fluoride ion-analyzer (Orion Research Model No. 701A). The final values were represented as µg of fluoride per mg of dry bone.