Chapter 3

Materials and Methods

3.1 Plant Material

*Sphaeranthus indicus* Linn. grows as a weed in the paddy fields and was collected from these rice fields of Manikandam, Tiruchirappalli, Tamil Nadu, India. Herbarium specimens were prepared and the taxonomic identification of the plant was confirmed in Rapinat Herbarium, St. Joseph’s College, Tiruchirappalli, Tamil Nadu, India. A voucher specimen of flowering plant was deposited in that herbarium for future reference (Plate I).

3.2 Botanical Information of the selected plant *Sphaeranthus indicus* (Plate II)

<table>
<thead>
<tr>
<th>Class</th>
<th>Magnoliopsida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subclass</td>
<td>Asteridae</td>
</tr>
<tr>
<td>Order</td>
<td>Asterales</td>
</tr>
<tr>
<td>Family</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Sphaerantus</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>indicus</em></td>
</tr>
<tr>
<td>Common name</td>
<td>East India Globe Thistle</td>
</tr>
<tr>
<td>Tamil Name</td>
<td>Kottaikaranthai</td>
</tr>
</tbody>
</table>

3.3 Preparation of Plant Extract

The collected plant material was washed thoroughly in tap water to remove adhering dirt, shade dried. The leaves, flowers, stem and root of the plant were separated and coarsely powdered by a mechanical grinder.
Plate Ia
Herbarium – *Sphaerantus indicus*
Plate Ib
Herbarium – *Sphaerantus indicus*
Plate II

a. *Sphaeranthus indicus*

b. Exposed view

c. Flower
Each dried powder of the plant was soaked separately in different solvents like methanol, ethanol, chloroform and petroleum ether in conical flask and then subjected to agitation on a Rotary shaker for 3 days at 190 – 220 rpm. After 72 hours, the plant extracts were filtered through Whatman No. 44 paper (125 mm) separately. Solvents present in the resulting filtrate were allowed to be dried by vacuum evaporator. The yields of the different extract as per the solvents used were 6.32% w/w, 4.96% w/w, 3.12% w/w and 5.1% w/w. Concentrated extracts were then preserved in labeled, sterilized, air tight bottles at 4°C in refrigerator until when required for use.

Aqueous extract of the plant was also prepared by soaking dried powder in distilled water and the extracts was collected as described earlier. The yield was found to be 12.2% w/w.

3.4 Phytochemical Screening

Preliminary phytochemical screening was carried out by the method described by Harborne (1998), Kokate et al. (2007) and Trease and Evans (1989).

3.4.1 Test for Alkaloids

i) Dragendorff’s test: To the 1mL of extract, add 1mL of Dragendorff’s reagent (potassium bismuth iodide solution). An orange-red precipitate indicates the presence of alkaloids.

ii) Mayer’s test: To the 1mL of extract, add 1mL of Mayer’s reagent (Potassium mercuric iodide solution). Whitish yellow or cream colored precipitate indicates the presence of alkaloids.
3.4.2 Test for steroids

2mL of acetic anhydride was added to 0.5g ethanolic extract of each sample with 2mL of H₂SO₄. The color changed from violet to blue or green in some samples indicating the presence of steroids.

3.4.3 Test for terpenoids

Salkowski test: To 0.5g of the extract, 2mL of chloroform was added and Concentrated H₂SO₄ (3 ml) was also carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

3.4.5 Test for flavonoids

i) Alkaline reagent test: Few drops of dilute ammonia were added to the portion of extract. Con. HCl was added. A yellow colouration indicates the presence of flavonoids.

ii) Zinc Hydrochloride test: To few drops of extract, zinc dust and con. HCl was added, the presence of red colouration indicates the presence of flavonoids.

iii) Aluminium test: To few drops of extract, 1% Aluminium solution was added, yellow colour indicates the presence of flavonoids.

3.4.6 Test for Saponins

About 2g of the powdered sample was boiled in 20mL of distilled water in a water bath and filtered. 10mL of the filtrate was mixed with 5mL of distilled water and shaked vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and again shaked vigorously, and observed for the formation of emulsion.
3.4.7 Phenolic Compounds

(i) To 1 ml of extract, add few ml of gelatine solution, a white precipitate reveals the presence of tannins and phenolic compounds.

(ii) To 1 ml of extract, add lead tetra acetate, and a precipitate production shows the presence of tannins and phenolic compounds.

3.4.8 Test for tannins

i) **Lead acetate test:** Take the little quantity of test solution and mixed with basic lead acetate solution. Formation of white precipitates indicates the presence of tannins.

ii) To 1mL of the extract, add ferric chloride solution, formation of a blue black or brownish green color product shows the presence of tannins.

iii) The little quantity of the extract is treated with aqueous ammonia solution. A deep green color indicates the presence of tannins.

3.4.9 Test for cardiac glycosides

**Keller-Killiani test:** To 0.5g of extract was diluted to 5mL water and added 2mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1mL of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

3.4.10 Test for Aminoacids

**Ninhydrin Test:** To 3mL of test solution and 3 drops of 5% ninhydrin solution in a test tube and were heated in boiling water bath for 10 minutes. Formation of purple or bluish color indicated the presence of amino acid.
3.4.11 Test for Proteins

**Xanthoproteic test:** To 1 mL of the extract, 1 mL of concentrated nitric acid was added. A white precipitate is formed, which is boiled and cooled. To that 20% of sodium hydroxide or ammonia was added. Orange color indicated the presence of aromatic amino acids.

3.4.12 Test for carbohydrates

To 2 mL of the extract, add 1 mL of Barfoed’s reagent and boil. Reddish brown precipitates indicate the presence of carbohydrates.

3.4.13 Test for reducing sugars

i) **Fehling’s test:** To 1 mL of the extract, add equal quantities of Fehling solution A and B, upon heating formation of a brick red precipitate indicates the presence of sugars.

ii) **Benedict’s test:** To 5 mL of Benedict’s reagent, add 1 mL of extract solution and boil for 2 minutes and cool. Formation of red precipitate shows the presence of sugars.

3.5 Quantitative Estimation of Phytochemicals

3.5.1 Alkaloid determination (Harborne, 1973)

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium
hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

3.5.2 Flavonoid determination (Bohm and Kocipai- Abyazan, 1994)

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed.

3.5.3 Saponin determination (Obadoni and Ochuko, 2001)

The samples were ground and 20 g of each were put into a conical flask and 100 cm$^3$ of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously.

The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

3.5.4 Determination of total phenols (Harborne, 1973)

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml
flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amylalcohol were also added. The samples were made up to mark and left to react for 30 min for colour development. This was measured at 505 nm.

3.5.5 Tannin determination (Van-Burden and Robinson, 1981)

500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M FeCl3 in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.

3.6 Pharmacognostical Studies (Kokate et al., 2006 and Harborne, 1998)

3.6.1 Determination of Total ash

About 2 gm of the powdered drug was accurately weighted in silica crucible, which was previously ignited and weighed. The powdered drug was spread as a fine even layer on the bottom of the crucible. The crucible was incinerated gradually by increasing temperature upto 450 °C to make it dull red until free from carbon. The crucible was cooled and weighted. The percentage of total ash was calculated with reference to the air dried drug.

3.6.2 Determination of Acid Insoluble ash

The resultant ash obtained in the determination of total ash, was boiled with 25 ml of 2N hydrochloric acid for 5 minutes and filtered on ashless filter paper.
The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred to pre-weighed silica crucible, incinerated, cooled and weighted. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

3.6.3 Determination of Water Soluble ash

The resultant ash obtained in the determination of the total ash, was boiled for 5 minutes with 25 ml of water. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a silica crucible and ignited for 15 minutes and weighted. The percentage of water soluble ash was calculated with reference to the air dried drug. The temperature maintained was 450 °C.

3.6.4 Determination of Sulphated ash

A silica crucible was heated to redness for 20 minutes allowed to cool in a desiccator and weighed. 2 gm of the powdered drug material was transferred to the crucible and weighed the crucible and its content accurately. Ignited gently at first until the substance is thoroughly charred, cooled and moistered with 1 ml of concentrated sulphuric acid, heated gently until white fumes are no longer evolved and ignited at 800 °C until all black particles had disappeared. Allow the crucible, to cool, add few drops of sulphuric acid and ignited as before. Allowed to cool and was weighed.

3.6.5 Extractive Values (Usha et al., 1984)

These help in evaluating the constituents of crude drug, which cannot be determined by any other means.
**Alcohol Soluble Extractive Value**

About 5 gm of powdered material was macerated with 100 ml of 90% ethanol in a stopper conical flask for 24 hours with occasional shaking during first 6 hours and the first 5 ml was discarded. Then 25 ml of the filtrate was evaporated on a tarred evaporating dish and the residue was dried at 105 °C, until a constant weight of residue was obtained. The percentage of alcohol soluble extractive was calculated with respect to the air dried material.

**Water Soluble Extractive Value**

About 5 gm of powdered material was macerated with chloroform water in a stopper conical flask for 24 hours with occasional shaking during first 6 hours and the first 5 ml was discarded. Then 25 ml of the filtrate was evaporated on a tarred evaporating dish, and the resultant residue was dried at 105 °C, until a constant weight of residue was obtained. The percentage of water soluble extractive was calculated with respect to the air dried material.

**3.6.6 Fluorescence analysis (Chase and Pratt, 1949)**

The methanolic and aqueous extracts *S. indicus* leaves and flowers were subjected to fluorescence analysis under ultraviolet after treatment with various chemicals and organic reagents like chloroform, hexane, benzene, aqueous NaOH, alcoholic NaOH, 1N HCl, ethanol, ethyl acetate, and 50% H₂SO₄ (Kokate, 1994).

**3.6.7 HPTLC Fingerprinting of methanolic leaves extract**

Plant extracts were dissolved in respective solvents. Sample was applied on 0.22mm thick silica gel plate by making use of Camag automatic TLC sampler. Sample was applied as band and is not spot. Chromatogram was developed with active stationary, mobile and vapor phases. Stationary phase
plate was put into the mobile phase containing organic solvents like hexane: ethyl acetate: methanol in the ratio of 1:2:1. Stepwise automatic procedure was followed at room temperature to run the column. Automatic developing chamber was used to develop the chromatogram. Chemical compounds are quantitatively and evaluated through spectral scanner. Scanning was controlled by Camag software © 1998 available in the instrument. Computerized scanning HPTLC report provided the informations like Rf value, \( \lambda \) max and % of chemical constituents present in the sample. The results were recorded and interpreted.

Stationary Phase : Silica Gel 60 F 254  
Mobile Phase : Dichloromethane:methanol:acetic acid (6.5:3.0:0.5)  
Wave length : 600 nm  
Spraying reagent : Spray the plate with anisaldehyde in sulphuric acid reagent heat at 100ºC till the coloured band appear and observe under visible light.  
Sample Preparation : The methanolic extract is dissolved in 5 ml of Chloroform  
Evaluation : The band so obtained may correspond to Sphaeranthanolide compounds in methanolic extract

3.6.8 Identification of Compounds using GC-MS  
GC-MS analysis was carried out by using Perkin Elmer-Clarus 500 GC-MS Unit. The analysis was carried out to detect the possible compounds present in the active fraction of the column type used was PE-S (equivalent to DB-5) with a column length of 30 m using carrier gas as Helium. The flow rate maintained was 1 ml / min with an initial column temperature of 50 ºC and final temperature of 250 ºC. The rate of temperature change in the column was maintained as 5 ºC / min. 1 M volume of sample was taken for injection. The
identification of compounds was accomplished using computer searches in commercial libraries.

**Instrument Details**

Make : PerkinElmer Clarus 500

Column Type : Capillary Column Elite-5ms (5% Phenyl 95% dimethylpolysiloxane)

Column length : 30m

Column id : 250µm

Oven Program : 50ºC (3min) @ 5 ºC/min to 150 ºC (1 min)

Injector temp. : 250ºC

Carrier gas : He @ 1ml/min

Mass Range : 40-450amu

### 3.7 In vitro Anticancer Activity Studies

#### 3.7.1 Cell Lines and Chemicals

Human liver cell lines (HepG2) were used to evaluate the anticancer properties of the extracts of the plant S. indicus. The cell lines used for the present study was purchased from National Centre for Cell Sciences, Pune, India. Dulbecco’s modified eagle medium (DMEM), Fetal Borine serum (FBS), Phosphate Buffer Saline (PBS), Trypan Blue (0.5%) solution, 0.25% Trypsin and 0.02% EDTA, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Himedia, Mumbai.

#### 3.7.2 Cell Culture Conditions

HepG2 cells were cultured at 37 ºC in 5% CO₂ in growth medium (Dulbecco’s modified eagle medium) supplemented with 10% (v/v) foetal calf serum, 100 µg ml⁻¹ penicilium and 10 µg ml⁻¹ streptomycin cells were passed
every 5 – 7 days. Suspension of HepG2 were produced from confluent cultures using trypsin / EDTA solution and cell concentration determined using a hemacytometer. HepG2 cells were seeded at equal densities either directly into wells of a standard -6-well plate. Growth medium was changed every 3- 4 days or as required.

3.7.3 *In vitro* cytotoxicity by MTT Assay method (Mossman, 1983)

The methanolic and aqueous leaves and flower extracts were subjected to in vitro cytotoxicity by MTT assay method using HEP-G2 cell lines (Human Liver Cancer cell lines).

**Principle**

The ability of the cell to survive a toxic insult has been the basis of most cytotoxicity assays.

**Experimental Protocol**

0.1 ml of diluted cell suspension was added to each well of the microtitre plate and incubated. After 24 hrs when a partial monolayer was formed and the supernatant was flicked off. The drug at a concentration of 2.5 to 25 µg/ml was added to the microtitre plate well and incubated at 37 °C in 5% CO₂ atmosphere for 3 days. After 72 hrs the drug solution in the wells were discarded and 50 µl of MTT was added and incubated for 3 hrs at 37 °C in 5% CO₂ atmosphere. The supernatant was discarded and 5 µl of propanol was added to solubilize the formed formazan. The absorbance was measured using a microtitre reader at a wave length of 540 nm.
**Materials and Methods**

**B. Thamilmaraiselvi and S. Ahmed John**

### Calculation

\[
\text{Control OD} - \text{Treated OD} \times 100
\]

% of growth incubation = \[ \frac{\text{Control OD} - \text{Treated OD}}{\text{Control OD}} \times 100 \]

### 3.7.4 *In vitro* cytotoxicity by short-term dye exclusion method (Freshny, 1994)

The methanolic and aqueous leaf and flower extracts were subjected to tryphan blue exclusion assay on HEP-G2 cell line.

**Principle**

It is based on the principle that, live cells or tissues with intact cell membranes do not take colour (living cells are excluded from staining). The cell membrane of a dead cell loose its permeability and therefore they take stain. The dead cells are shown as a distinctive blue colour cytoplasm under microscope.

**Experimental Protocol**

HEPG2 cell lines were incubated with different concentration (100 – 1000 µg/ml) of plant extracts. Control tubes contain saline, tumor cells and without the drug. All the tubes were incubated at 37 °C for 3 hrs. After incubation 0.1 ml of 0.4% tryphan blue dye was added to each tube. The number of viable (unstained) and dead cells (stained) were counted using Haemocytometer.

**Calculation**

\[
\frac{\text{Total cells counted} - \text{Total viable cells}}{\text{Total cells counted}} \times 100
\]

\[
\text{% of dead cells} = \frac{\text{Total cells counted} - \text{Total viable cells}}{\text{Total cells counted}} \times 100
\]
3.8 \textit{In vivo} anti-cancer activity Studies

3.8.1 Animals

Male Wistar Albino mice (18 – 20 g) were used. The animals were housed in poly propylene cages with sterile, inert husk materials as bedding. The experimental animal were maintained under controlled environment conditions of light and dark cycles (light 12 h : dark 12 h, temperature 23 ± 2 °C and relative humidity 55 ± 10%). Animals were allowed to take standard laboratory feed and tap water.

3.8.2 Chemicals

Diethylnitrosamine (DEN) were obtained from M/s. Sigma Chemcial Company, St. Louis, MO, USA. Other chemicals used in this study were obtained from Himedia Laboratories, Mumbai, India, and were of analytical grade.

3.8.3 Acute toxicity studies

The acute toxicity of methanolic and aqueous extract of \textit{S.indicus leaves and flower heads} were evaluated in mice using the up and down procedure. Mice of either sex (three females and three males, weight: 20-23 g, age: 6-8 weeks) received plant extracts starting from 250, 500, 1000, 1500, 2000 mg/kg body weight intraperitonially. The animals were observed for toxic symptoms continuously for the first 2 h and for mortality upto 24 hrs after injection. The number of survivors were noted after 24 h, and these animals were then maintained and observed daily for next 3 weeks for any further toxicity.
3.8.4 Experimental Design

Prior to start the experiment, all animals were caged and acclimatized for one week period. In total, 42 mice with body weights ranging from 18 ± 2 gms to 20 ± 1.9 gms were used in the experiment and were divided into 7 groups.

<table>
<thead>
<tr>
<th>Group I</th>
<th>Control animals (treated with 0.9% saline throughout the study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>DEN induced animal 200 mg/kg body wt of DEN was given intraperitonially. Two weeks after the administration of DEN, Phenobarbital (0.05%) was given as a promoter of carcinogenesis.</td>
</tr>
<tr>
<td>Group III</td>
<td>Hepacellular carcinoma bearing animals treated with adriomycin 20 mg/kg body wt. interperitonally for three successive weeks.</td>
</tr>
<tr>
<td>Group IV</td>
<td>HCC bearing animals treated with 200 mg/kg body wt. of methanolic leaf extract of S. indicus for 3 successive weeks.</td>
</tr>
<tr>
<td>Group V</td>
<td>HCC bearing animals treated with 200 mg/kg body wt. of aqueous leaf extract of S. indicus for 3 successive weeks.</td>
</tr>
<tr>
<td>Group VI</td>
<td>HCC bearing animals treated with 200 mg/kg body wt. of methanolic flower extract of S. indicus for 3 successive weeks.</td>
</tr>
<tr>
<td>Group VII</td>
<td>HCC bearing animals treated with 200 mg/kg body wt. of aqueous flower extract of S. indicus for 3 successive weeks.</td>
</tr>
</tbody>
</table>

3.8.5 Collection of Sample and Preparation of liver homogenize

During the experimental period, the body weight of the mice were monitored and recorded regularly. At the end of the treatment period the mice were sacrificed and blood was collected and it was centrifuged 10 mts t 3000 rpm to separate the serum. The blood and the serum was used for the estimation of various haematological and biochemical parameters. The liver was removed and washed with ice cold physiological saline and homogenized in 0.1 m Tris-
HCl buffer pH 7.4, to give a 10% homogenate. One portion of the liver was fixed in 10% formalin for histopathological observation.

3.9 Biochemical Estimation

3.9.1 Alanine Aminotransferase (ALT)

Alanine aminotransferase was assayed by the method King 1965

**Reagents**

1. Phosphate buffer: 0.1 M, pH 7.5
2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 ml of buffer, 0.5 ml of 1 N sodium hydroxide was added and made up to 100 ml with distilled water.
3. 2,4-dinitrophenyl hydrazine (DNPH): 0.02% of DNPH in 1 N hydrochloric acid
4. Sodium hydroxide: 0.4 N
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 µmole of pyruvate/ml.

**Procedure**

1.0 ml of substrate was incubated at 37 °C for 10 minutes. Then 0.2 ml of enzyme solution was added. The tubes were incubated at 37 °C for 30 min. To the control tubes enzyme was added after arresting the reaction with 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 20 min. Then 5.0 ml of sodium hydroxide was added and then the colour developed was read at 540 nm using a photohem colorimeter. The enzyme activity was expressed as µmoles of pyruvate liberated/mg protein under incubation conditions.
3.9.2  Aspartate Aminotransferase (AST)

Aspartate aminotransferase was estimated by the method King 1965a.

Reagents

1. Phosphate buffer: 0.1 M, pH 7.5
2. Substrate: 1.33 g of aspartic acid and 1.5 mg of 2-oxoglutarate were dissolved in 20.5 ml of 1 N sodium hydroxide and made up to 100 ml with buffer.
3. 2,4-dinitrophenyl hydrazine (DNPH): 0.02% of DNPH in 1 N hydrochloric acid.
4. Sodium hydroxide: 0.4 N
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1 µmole of pyruvate/ml.

Procedure

1.0 ml of substrate was incubated at 37 °C for 10 minutes. Then 0.2 ml of enzyme was added and mixture was incubated at 37 °C for one hour. To the control tubes enzyme was added after the reaction and it was arrested by the addition of 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 30 minutes. Then 5.0 ml of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The colour developed and read at 540 nm using a photochem colorimeter. The enzyme activity was expressed as µmoles of pyruvate liberated/mg protein under incubation conditions.

3.9.3  Alkaline Phosphatase (ALP)

Alkaline phosphatase was assayed by the method of Moog (1946) as modified by King (1965b).
Reagents

1. Carbonate bicarbonate buffer 0.1 M, pH 10.0. 6.36 g of sodium carbonate and 3.36 g of sodium bicarbonate were dissolved in, 1000 ml of water.
2. Substrate 0.1 M: 254 mg of disodium phenyl phosphate was dissolved in 100 ml of water.
3. Magnesium chloride 0.1 M: 406 mg of magnesium chloride was dissolved in 20 ml of water.
4. Sodium carbonate 15%: 15 g of sodium carbonate was dissolved in 100 ml of water.
5. Folin's phenol reagent: As in section 2.2.7. TCA 10%
6. Standard: 10 mg of phenol was dissolved in 100 ml of water.

Procedure

The mixture containing 1.5 ml buffer, 1.0 ml substrate and 0.1 ml of magnesium chloride were pre-incubated at 37 °C for 10 minutes. Then 0.1 ml of enzyme was added and incubated at 37 °C for 15 minutes. The reaction was arrested by 1.0 ml of 10% TCA. Control without enzyme was also incubated and the enzyme was added after the addition of TCA solution. Then 1.0 ml of sodium carbonate and 0.5 ml of Folin's phenol reagent were added. After 10 minutes the blue colour developed was read at 640 nm using a photochem colorimeter. The enzyme activity was expressed as µ moles of phenol liberated/min/mg protein under incubation conditions.

3.9.4 Lactate Dehydrogenase

Lactate dehydrogenase was assayed by the method of King (1965b).
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**Reagents**

1. Glycine buffer 0. 1 M pH 7.2: 750.5 mg of glycine and 585 mg of NaCl were dissolved in 100 ml water.
2. Buffered substrate : 125 ml of glycine buffer, 75 ml of 0.1 N NaOH and 4.0 g of lithium lactate were added, mixed well and kept in cold room.
3. Nicotinamide adenine dinucleotide (NAD') : 10 mg of NAD' was solved in 2.0 ml of water.
4. 2,4-Dinitrophenyl hydrazine (DNPH) 0.02% in 1 N HCl 20 mg of DNPH was dissolved in 100 ml of 1 N HCL.
5. NaOH : 0.4 N.
6. Standard : 11 mg of sodium pyruvate was dissolved in 100 ml of buffer. This contained one µmole of pyruvate/ml.

**Procedure**

Pipetted out 1.0 ml of the buffered substrate and 0.1 ml of plasma or tissue homogenate into two tubes, and 0.2 ml of distilled water was added to the blank. The tubes were placed in a water bath at 37 °C. Then to the test, 0.2 ml of NAD' solution was added and shaken well. The tubes were incubated at 37 °C for 15 minutes. Exactly after that time the reaction was stopped by adding 1.0 ml of DNPH solution. NAD' was added to the control tubes. It was A. at 37 °C for another 15 min. 5.0 ml of 0.4 N NaOH was added and the colour developed was read at 420 nm using a photochem colorimeter within 5 minutes. A set of standards were also treated in a similar manner. The enzyme activity was expressed as µmoles of pyruvate liberated / min / mg protein.

**3.9.5 γ-glutamyl Transpeptidase**

γ-glutamyl transpeptidase was assayed by the method of Orlowski and Meister (1965) and modified by Rosalki and Rau (1972).
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Reagents

1. Tris-HCl buffer 0.1 M pH 8-5: 1.21 g of Tris was dissolved in 100 ml of water and the pH was adjusted to 8.5 with HCL

2. Glycylglycine 0.013%: 13.2 mg of glycylglycine was dissolved in 10 ml of water.

3. Substrate: 30.37 mg of -γ-glutamyl-p-4itroanilide was added in 10 ml of water. The substrate was sparingly soluble and hence dissolved by heating at 50-60 °C to give a clear solution. It was used with in 2 hours of preparation.

4. Acetic acid 10%.

5. Standard : 13.8 mg of p-nitroaniline was dissolved in 100 ml of water to give a concentration of one µ mole/ml.

Procedure

The incubation mixture contained 0.5 ml of substrate, 1.0 ml of buffer and 2.2 ml of glycylglycine. 0.2 ml of plasma of tissue homogenate was added the above mixture and the total volume was made upto 4.0 ml with distilled water. The mixture was incubated at 37 °C for 30 minutes and heated at 700 °C for 5 minutes and centrifuged. The control tubes received enzyme after incubation. Standard p-nitroaniline solution was also treated in a similar manner.

The amount of liberated p-nitroaniline in the supernatant was measured as the difference in optical density at 410 nm between sample with and without substrate. The substrate incubated in the absence of enzyme under the same condition was used as a reference blank.
The enzyme activity in plasma was expressed as µmol p-nitroaniline tabulated / min / mg protein.

3.9.6 5'-Nucleotidase

5'-Nucleotidase was assayed by the method of Luly et al. (1972).

Reagents

0. Tris-HCl buffer 184 mM, pH 7.5: 2.23 g Tris was dissolved in 100 ml of deionised water and pH was adjusted to 7.5 with HCl.
0. Magnesium sulphate 50 mM: 616.2 mg of MgSO₄ was dissolved in 50 ml of deionised water.
0. Potassium chloride 650 mM: 1.211 g of KCl was dissolved in 25 ml of deionised water.
0. EDTA 1 mM: 37.23 mg of EDTA was dissolved in 100 ml of deionised water. TCA 10%.
0. Substrate : 5'-Adenosine monophosphate 40 mM was prepared by dissolving 69.4 mg in 5.0 ml of deionised water.

Reagents

The reaction mixture contained 1.0 ml of Tris-HCl buffer and 0.1 ml each of magnesium sulphate, potassium chloride, EDTA, substrate and water. The reaction was initiated by the addition of 0.2 ml plasma or tissue homogenate and incubated at 37 °C for 15 minutes. The reaction was arrested by the addition of 2.0 ml of 10% TCA and centrifuged.

The phosphorus liberated in the supernatant was estimated by the method of Fiske and Subbarow as described in section below. The enzyme activity was expressed as nmoles of inorganic phosphorus liberated / min/mg protein.
3.9.7 Estimation of Lipid Peroxidation (LPO)

Lipid peroxide concentration was determined by thiobarbituric acid reaction as described by Ohkawa et al. (1979).

Reagents
1. 20% acetic acid
2. 8.1% sodium dodecyl sulphate (SDS)
3. 0.8% Thiobarbituric acid (TBA)
4. m-Butanol-pyridine mixture (15 : 1 v/v)
5. Standard solution
6. 16 mg of 1,1’, 3,3’-tetramethoxy propane was accurately weighed and dissolved in 100 ml of distilled water. Further dilution was made so that the working standard solution contained 100 n moles per ml.

Procedure
To 0.2 ml of tissue homogenate, 1.5 ml of 20% acetic acid, 0.2 ml SDS of and 1.5 ml of TBA were added. The mixture was made upto 4.0 ml with distilled water and then heated for 30 minutes at 95 °C using glass ball as condenser. After cooling, 4.0 ml of n-butanol-pyridine mixture was added and shaken well. After centrifugation at 4000 g for 10 min, the organic layer was taken and its absorbance was read at 532 nm in a Shimadzu Spectrophotometer. The lipid peroxide concentration was expressed in n moles of MDA nmoles / mg protein.

3.9.8 Catalase

Catalase was assayed according to the method of Takahara et al. (1960).
Reagents
1. 50 mM Phosphate buffer, pH 7.0
2. 30 mM Hydrogen peroxide in phosphate buffer

Procedure
To 1.2 ml of phosphate buffer, 0.05 ml of the tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of the hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm at 30 seconds intervals for 3 minutes. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as n moles hydrogen peroxide consumed minute\(^{-1}\) mg protein\(^{-1}\).

3.9.9 Superoxide Dismutase
Superoxide dismutase was assayed according to the method of Misra and Fridovich (1972).

Reagents
1. 0.1 M Carbonate-bicarbonate buffer pH 10.2, containing 57 mg EDTA/dl
2. 3 mM Epinephrine.

Procedure
To 0.05 ml tissue supernatant, 1.5 ml of the buffer was added. The reaction was initiated by the addition of 0.4 ml of epinephrine and change in optical density per minute was measured at 480 nm in a Shimadzu spectrophotometer.
One unit of superoxide dismutase activity is the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

3.9.10 Glutathione Peroxidase

Glutathione peroxidase was assayed according to the Method of Necheles et al. (1968).

**Reagents**

- 0.1 M Phosphate buffer, pH 7.4 mm
- 8 mM Reduced glutathione
- 30 mM sodium azide
- 9 mM EDTA
- 18 mM Hydrogen peroxide
- 1 mM 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB)
- 10% Trichloro acetic acid

The final incubation mixture consisted of 0.2 ml enzyme, 1.0 ml buffer, 0.5 ml glutathione, 0.5 ml sodium azide, 0.5 ml EDTA and 2.0 ml water. The solution was incubated at 37 °C for 5 minutes and the reaction was started by the addition of 1.0 ml of H₂O₂. 1.0 ml samples was taken exactly at zero minute and one minute after the addition of H₂O₂ and to arrest the reaction, 5.0 ml of 10% TCA was added. Non-enzymatic oxidation of glutathione was measured in a blank containing the above reagents with buffer substituted for enzyme source. Under these conditions, the non-enzymatic oxidation was minimal. The residual glutathione was then measured by the reaction with 1 ml of DTNB at 412 mn in a Shimadzu spectrophotometer. The activity of glutathione peroxidase was expressed as mg of GSH utilized / minute / mg protein.
3.9.11 Na\(^+/\)K\(^+\) ATPase

\(\text{Na}^+ / \text{K}^+\) - ATPase was assayed according to the method of Bonting (1970).

**Reagents**

1. Tris-HCl buffer 184 mM pH 7.5: 2.23 g of Tris was dissolved in 100 ml of deionised water and pH was adjusted to 7.5 with HCl.
2. MgSO\(_4\) 50 mM: 369.9 mg of MgSO\(_4\) was dissolved in 25 ml of deionised H\(_2\)O.
3. Potassium chloride 50 mM: 93.2 mg of KCl was dissolved in 25 ml of deionised water.
4. Sodium chloride 600 mM: 876.5 mg of NaCl was dissolved in 25 ml of deionised water.
5. EDTA 1 mM: 3.72 mg of EDTA was dissolved in 10 ml of deionised water.
6. ATP 40 mM: 44-56 mg of ATP was dissolved in 2.0 ml of deionised H\(_2\)O.

**Procedure**

The incubation mixture contained 1.0 ml of buffer, 0.2 ml each of MgSO\(_4\), KCl, NaCl, EDTA, ATP and enzyme respectively. The mixture was incubated at 37 °C for 15 minutes. The reaction was arrested by addition of 1.0 ml of TCA, mixed well and centrifuged and the supernatant was used for estimation of inorganic phosphorus by the method of Fiske and Subborow. The control without enzyme was also incubated, and after arresting with TCA, the enzyme was added. Then inorganic phosphorus was estimated in the supernatant. The enzyme activity was expressed as \(\mu\)moles of inorganic phosphorus liberated/min/mg protein.
3.9.12 \( \text{Ca}^+ \) ATPase

\( \text{Ca}^+ \) ATPase was assayed according to the method of Hjerken and Pan (1983).

**Reagents**

1. Tris-HCl buffer: 125 mM pH 8.0: 1.514 g of Tris was dissolved in 100 ml of deionised H\(_2\)O and the pH was adjusted to 8.0 C HCl
2. CaCl\(_2\) 50 mM: 273.6 mg of calcium chloride was dissolved in 25 ml of deionised H\(_2\)O.
3. ATP 10 mM: 33 mg of ATP was dissolved in 6.0 ml of deionised H\(_2\)O.

**Procedure**

The incubation mixture contained 0.1 ml each of buffer, CaCl\(_2\) ATP and tissue homogenate. The mixture was incubated at 37\(^\circ\)C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme activity was expressed as \( \mu \) moles of inorganic phosphorus liberated/min/mg protein.

3.9.13 \( \text{Mg}^{2+} \) ATPase

\( \text{Mg}^{2+} \) ATPase was assayed by the method of Ohnishi *et al.* (1982).

**Reagents**

1. Tris-HCl buffer 374 mM pH 7.6: 4.536 g Tris was dissolved in 100 ml of deionised H\(_2\)O and the pH was adjusted to 7.6 with HCl.
2. MgCl\(_2\) 50 mM: 50.8 mg of MgCl\(_2\) was dissolved in 5.0 ml of deionised H\(_2\)O.
3. ATP 10 mm: 33 mg of ATP in 600 ml of deionised H\(_2\)O.
**Procedure**

The incubation mixture contained 0.1 ml each of buffer, MgCl₂, ATP and tissue homogenate was incubated at 37 °C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme activity was expressed as µ moles of inorganic phosphorus liberated/min/mg protein.

**3.9.14 Estimation of α-Fetoprotein (AFP)**

Alpha-fetoprotein (AFP) is measured quantitatively by solid phase enzyme linked immunosorbent assay (ELIZA). The wells are coated with anti-AFP antibodies. The samples, standards and controls were incubated in the wells. During the incubation, AFP is bound to anti-AFP antibodies on the wells. Unbound AFP is removed by washing the wells with tap water. Enzyme conjugate which is a mixture of mouse anti-AFP antibodies with different affinity towards epitopes of AFP molecules and chemically conjugated with horse radish peroxidase is added to each well. After the incubation, unbound enzyme conjugate is washed off and the amount of, bound peroxidase is proportional to the concentration of the AFP present in the each sample. Upon addition of chromogen substrate, the intensity of colour developed is proportional to the concentration of AFP in the sample.

**Materials and Reagents**

1. Microwell strips Anti-AFP antibodies coated -wells.
2. Enzyme conjugate : Anti-AFP antibodies conjugated to horse-radish peroxidase.
3. Sample diluent or zero standard.
4. Reference standard set: Calibrated to 0, 37 10, 25, 100 and 200 IU/ml against WHO IS 12/388.
6. Solution B: Tetramethyl benzidine.
7. Well holder for securing wells.
8. Microwell reader.
9. H₂SO₄ 1N
10. Pippetor with tip for 10 µl and 100 µl.

**Procedure**

The collected blood was allowed to clot and serum was separated. All the reagents and samples were brought to room temperature and mixed. Then 25 µl of serum sample, control and standards were dispensed in to the assigned wells. Then 100 µl of 0 IU/ml of AFP standard solution was dispensed, immediately in to those assigned wells and incubated for 30 minutes at room temperature. The incubation mixture was removed and the wells were rinsed 5 times with running water. Then 100 µl of enzyme conjugate was dispensed in to each well and again incubated for 30 minutes at room temperature. 100 µl of solution A and 100 µl of solution B were added into each well, and incubated for 10 minutes at room temperature. The reaction was stopped by the addition of 1 N H₂SO₄ to each well and optical density at 450 nm was read with microwell eader. The AFP content is expressed in IU/ml.

3.10 **Haematological Studies**

3.10.1 **Estimation of blood haemoglobin (Drabkin and Austing, 1932)**

**Reagents**

1. Drabkins reagent: Dissolved 200 mg of potassium ferricyanide, 50 mg potassium cyanide and 1 g sodium bicarbonate in water and made upto litre. The reagent had a pale yellow colour and a pH of 9.6.
2. Cyanomethernoglobin standard: 16
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0.02 ml of blood was diluted with 5 ml of the reagent. The diluted blood was mixed well and allowed to stand for 10 minutes, to ensure the completion of the reaction. The solution was read at 540 nm together with the standard solution of cyanomethemoglobin. Blood hemoglobin levels were expressed as gl mol.

### 3.10.2 Enumeration of red blood corpuscles (Huxtable, 1990)

**Reagents**

Red blood cell diluting fluid (Hayem's fluid) 5 g of sodium sulfate, 1 g of sodium chloride and 0.5 g of mercuric chloride were dissolved in 200 ml of distilled water.

**Procedure**

Blood was sucked exactly up to the 0.5 ml mark in the RBC pipette and the diluting fluid was drawn immediately up to the mark and mixed thoroughly. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with its cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding the first 3-5 drops.

**Charging of the counting chambers**

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 minutes. The counting chamber was placed under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting areas of 1 nm square. The number of cells in the 4 corner squares and one central square was counted. The results are expressed as number of cells / mm 3 of blood.
3.10.3 Enumeration of white blood corpuscles (Raghuramulu et al., 1983)

Reagents

WBC diluting fluid was prepared by mixing gentian violet (1%) in glacial acetic acid and made up to 109 ml with water. WBC dilution fluid or Turk's fluid was used as the diluant which can destroy RBC's.

Procedure

In a clean test tube, 0.38 ml of WBC diluting fluid was added. Added 0.02 ml of blood with the help of Hb sahli pipette and mixed well. The counting chamber was filled and the cells were counted in 4 large (1 mm) corner squares of the Neubauer counting chamber. The results are expressed as number of cells / mm$^3$ of blood.

3.11 Histopathological Studies

For histopathological studies, liver tissues of 3 mm thickness were fixed in Bouin’s fluid, dehydrated in alcohol series, cleared in methyl benzoate and xylol, embedded in paraffin wax and sectioned at 8 µ. The sections were stained with Ehrlichae’s haematoxylin and counter stained with Ecosine (Humason, 1979).

Microphotography

The stained sections were observed and microphotographed at appropriate magnification using Carl Zeiss microscope model Axiostar Plus (made in Germany).