CHAPTER 3
METHODOLOGY RELATED TO PLANT DRUG AND CANCER

3.1 EXPERIMENTAL ANIMALS

Inbred Swiss Albino male mice of two months age, weighing 20 ± 5 g, purchased from Veterinary College, Mannuthy, Thrissur, were used for the study. The mice were obtained from the stock in breed colony, which was maintained by mating brothers and sisters. They were housed in ventilated cages maintained on dry pellets of rat feed (Gold Mohur mouse chow, Hindustan Lever, Ltd.) and water ad libitum.

3.2 TUMOUR CELL LINE AND THEIR MAINTENANCE

Dalton’s lymphoma ascites tumour cell line and Ehrlich Ascites tumour cell line kindly provided by Amala Cancer Research Institute, Thrissur, was used for the study. The tumour cell line was maintained by serial intraperitoneal (IP) transplantation in mice. Full-grown tumour cell-line were aspirated from the mouse peritoneum, washed thrice with 0.9% saline and suspended in saline. About 1x10^5 cells were injected intraperitoneally into a new healthy mouse.

3.3 SOLID TUMOUR INDUCTION

Solid tumour growth was obtained by injecting 5x10^5 cells subcutaneously under an area of shaved skin on the flank region. All operations were done asceptically.
3.4 PREPARATION OF THE DRUG FOR THE EXPERIMENTAL STUDY

_Centella asiatica_ of 6-7 weeks age was collected and the whole plant was dried in shade and ground to a fine powder.

20.0 g of the dried powder was extracted in Soxhlet apparatus for 24-48 h using different solvents separately namely Methanol, Acetone, Chloroform and Petroleum ether. These extracts were evaporated completely to dryness so as to get the extract as dry solid (powder) and the dry weight was taken.

Different amounts of this crude extract were taken for experimental study.

3.5 PARTIAL PURIFICATION OF THE CRUDE DRUG

As the methanol extract of _Centella asiatica_ showed best cytotoxic effects, it was subjected to further purification.

About 2 ml of the methanol extract was subjected to column chromatography, where column was packed with silica gel (100-200 mesh). Fractions were collected by eluting with different solvents continuously with increasing polarity namely petroleum ether, chloroform, acetone, and methanol. These fractions were evaporated to dryness and then used for further investigations.

3.6 DRUG ADMINISTRATION

Crude drug (crude extract) was given orally along with the normal feed and the partially purified drug was given as intraperitoneal injections. For intraperitoneal injections the drug was always prepared in normal saline
(0.9% NaCl). The appropriate drug concentration for the study was detected by cytotoxic studies (refer Chapter 2).

2 g of crude powder of Centella asiatica/g body wt/day was mixed with 240-300 mg of normal lab feed/g body wt/day and fed to animals till the end of the experiment. 6 g of the prepared diet was found to be consumed by each mice and the same was given to each mice.

2 mg of crude drug/gm bocyweight of mice was mixed with normal feed (240-300 mg/day/g body wt) fed to animals till the end of the experiment.

0.075 mg of partially purified drug/gm of body wt of mice was given to mice as intraperitoneal injections, prepared in normal saline (0.9% NaCl) on alternate days.

3.7 STUDY OF SURVIVAL PERIOD

3.7.1 Survival period in Dalton's Ascites lymphoma and Solid tumour bearing mice

(a) Cell line induced lymphoma treated with crude drug and partially purified drug of Centella asiatica

Oral treatment with crude drug

Mice of average weight 21±1.5 g were grouped into six groups consisting of six mice in each group.

Normal: Fed with normal lab feed.

Group I: Control (Diseased) - Each mice received intraperitoneal injections of $1 \times 10^7$ Dalton's lymphoma ascites cells and not treated.
Group II: Each mice received intraperitoneal injections of $1 \times 10^5$ Dalton's lymphoma ascites cells and treated with Crude powder of *Centella asiatica*.

Group III: Each mice received intraperitoneal injections of $1 \times 10^5$ Dalton's lymphoma ascites cells and treated with Petroleum ether extract of *Centella asiatica*.

Group IV: Each mice received intraperitoneal injections of $1 \times 10^5$ Dalton's lymphoma ascites cells and treated with Chloroform extract of *Centella asiatica*.

Group V: Each mice received intraperitoneal injections of $1 \times 10^5$ Dalton's lymphoma ascites cells and treated with Acetone extract of *Centella asiatica*.

Group VI: Each mice received intraperitoneal injections of $1 \times 10^5$ Dalton's lymphoma ascites cells and treated with Methanol extract of *Centella asiatica*.

**Intraperitoneal treatment with partially purified drug**

Mice of average weight $2 \pm 1.5$ gm were grouped into six groups consisting of six mice in each group.

Normal: Fed with normal lab feed.

Group I: Control (Diseased)- Each mice received intraperitoneal injections of $1 \times 10^5$ cells of Dalton's lymphoma ascites cells and normal saline.

Group II: Each mice received intraperitoneal injections of $1 \times 10^5$ cells of Dalton's lymphoma ascites cells and treated with Petroleum
ether fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

**Group III:** Each mice received intraperitoneal injections of $1 \times 10^5$ cells of Dalton’s lymphoma ascites cells and treated with Chloroform fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

**Group IV:** Each mice received intraperitoneal injections of $1 \times 10^5$ cells of Dalton’s lymphoma ascites cells and treated with Acetone fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

**Group V:** Each mice received intraperitoneal injections of $1 \times 10^5$ cells of Dalton’s lymphoma ascites cells and treated with Methanol fraction of methanol extract of *Centella asiatica* in normal saline (0.075 mg/gm body weight).

(b) **Solid tumour treated with crude drug and partially purified drug of *Centella asiatica***

**Oral treatment with the crude drug**

Mice of average weight 21±1.5 g were grouped into six groups consisting of six mice in each group.

**Normal:** Fed with normal lab feed.

**Group I:** Control (Diseased)- Each mice received subcutaneous injections of $5 \times 10^5$ Dalton’s lymphoma ascites cells and not treated.
Group II: Each mice received subcutaneous injections of $5 \times 10^4$ Dalton's lymphoma ascites cells and treated with Crude powder of *Centella asiatica*.

Group III: Each mice received subcutaneous injections of $5 \times 10^4$ Dalton's lymphoma ascites cells and treated with Petroleum extract of *Centella asiatica*.

Group IV: Each mice received subcutaneous injections of $5 \times 10^4$ Dalton's lymphoma ascites cells and treated with Chloroform extract of *Centella asiatica*.

Group V: Each mice received subcutaneous injections of $5 \times 10^4$ Dalton's lymphoma ascites cells and treated with Acetone extract of *Centella asiatica*.

Group VI: Each mice received subcutaneous injections of $5 \times 10^4$ Dalton's lymphoma ascites cells and treated with Methanol extract of *Centella asiatica*.

**Intraperitoneal treatment with partially purified drug**

Mice of average weight $21 \pm 1.5$ g were grouped into six containing 6 mice in each group.

**Normal:** Fed with normal laboratory feed.

**Group I:** Control (Diseased): Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's lymphoma ascites cells and treated with intraperitoneal injections of normal saline.

**Group II:** Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's lymphoma ascites cells and treated with Petroleum
ether fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

Group III: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s lymphoma ascites cells and treated with Chloroform fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

Group IV: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s lymphoma ascites cells and treated with Acetone fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

Group V: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s lymphoma ascites cells and treated with Methanol fraction of methanol extract of *Centella asiatica* in normal saline. (0.075 mg/gm body weight)

3.7.2 Survival period in Ehrlich Ascites lymphoma

The studies were carried out same as in 3.6.1 where Ehrlich’s Ascites Lymphoma Cells were used for the induction of Ehrlich’s Ascites Lymphoma and Solid tumour instead of Dalton’s Ascites Lymphoma.

3.8 SOLID TUMOUR REDUCTION STUDIES

3.8.1 Solid tumour induced by Dalton’s Lymphoma Ascites.

Oral treatment with the crude drug

Mice of average weight $21 \pm 1.5$ g were grouped into six groups consisting of six mice in each group.
Normal: Fed with normal \textit{laboratory feed}.

Group I: \textit{Control(Diseased)} - Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and not treated.

Group II: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and treated with Crude powder of \textit{Centella asiatica}.

Group III: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and treated with Petroleum ether extract of \textit{Centella asiatica}.

Group IV: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and treated with Chloroform extract of \textit{Centella asiatica}.

Group V: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and treated with Acetone extract of \textit{Centella asiatica}.

Group VI: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton's Ascites lymphoma cells and treated with Methanol extract of \textit{Centella asiatica}.

\textbf{Intraperitoneal treatment with partially purified drug}

Mice of average weight 21±1.5 g were grouped into six groups consisting of six mice in each group.
Normal: Fed with normal laboratory feed.

Group I: Control (Diseased) Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s Ascites lymphoma cells and normal saline intraperitoneally.

Group II: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s Ascites lymphoma cells and Petroleum ether fraction of methanol extract of Centella asiatica in normal saline. (0.075 mg/gm body weight)

Group III: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s Ascites lymphoma cells and Chloroform fraction of methanol extract of Centella asiatica in normal saline. (0.075 mg/gm body weight)

Group IV: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s Ascites lymphoma cells and Acetone fraction of methanol extract of Centella asiatica in normal saline. (0.075 mg/gm body weight)

Group V: Each mice received subcutaneous injections of $5 \times 10^4$ cells of Dalton’s Ascites lymphoma cells and Methanol fraction of methanol extract of Centella asiatica in normal saline (0.075 mg/g body weight).

3.8.2 Solid tumour induced by Ehrlich’s Lymphoma Ascites

In this case solid tumour was induced by Ehrlich’s ascites cells and the Group Iing was done as in the above case (3.8.1)

In all the above cases the size of tumour developed was determined by measuring the diameter of the tumour at right angles. Solid tumour
reduction studies were noted by the diminution of the tumour size after the treatment. Survival period was also noted in all groups.

3.9 STUDY OF CHANGES IN BODY WEIGHT

In all the above cases the body weight of each mice before and after the experiments was noted.

3.10 GROUPING OF ANIMALS FOR DALTON'S LYMPHOMA AND EHRlich ASCITES LYMPHOMA

3.10.1 Crude drug studies
(a) On Dalton's Lymphoma bearing mice

Mice of average weight of 20 ± 1.5gm were divided into six groups containing 6 mice in each group.

N - Normal control: Maintained with normal laboratory feed.

G1 - Disease control: For inducing solid tumour about 5x10^4 tumour cells of Dalton's lymphoma anti cells were injected intraperitoneally as a single dose. These mice were not treated and six mice were sacrificed on 14th day of tumour implantation.

G2 - Tumour implanted mice, each consumed 2gm of crude dry powder/gm body wt/day along with the normal lab feed. Each mice was sacrificed on the 14th day of tumour implantation.

G3 - Tumour implanted mice, each consumed 2 mg of Petroleum ether extract/day/ g of body wt along with the normal lab feed. Each mice was sacrificed on 14th day of tumour implantation.
G2 - Tumour implanted mice, each consumed 2 mg of Chloroform extract/day/gm body wt along with normal lab feed. Each mice was sacrificed on 14th day of tumour implantation.

G3 - Tumour implanted mice, each consumed 2 mg of Acetone extract/day/gm body weight along with normal lab feed. Each mice was sacrificed on 14th day of tumour implantation.

G4 - Tumour implanted mice, each consumed 2 mg of methanol extract/day/gm body weight along with normal lab feed. Each mice was sacrificed on 14th day of tumour implantation.

(b) On Ehrlich's Lymphoma bearing mice

The grouping of animals for the study on Ehrlich lymphoma Ascites was the same as that for Dalton’s lymphoma except that here Ehrlich Ascites cells were used for tumour implantation instead of Dalton’s Ascites Lymphoma cells.

3.10.2 Partially purified drug studies

(a) On Dalton's Lymphoma bearing mice

N - Normal control: Maintained with normal laboratory feed.

G1 - Disease control: For inducing Dalton’s lymphoma about $1 \times 10^5$ tumour cells were injected into the peritoneal cavity of each mouse as a single dose. These mice were given normal saline (0.9% NaCl) as intraperitoneal injections on alternate days and were sacrificed at the 14th day of tumour implantation.

G2 - Tumour implanted mice, each received 0.075 mg of petroleum ether fraction/gm of body weight in normal saline as intraperitoneal
injections on alternate days. All the mice were sacrificed on 14th day of tumour implantation.

G₅ - Tumour implanted mice, each received 0.075 mg of chloroform fraction/g of body weight in normal saline as intraperitoneal injections on alternate days. All mice were sacrificed on 14th day of tumour implantation.

G₆ - Tumour implanted mice, each received 0.075 mg of Acetone fraction/g body weight in normal saline as intraperitoneal injections on alternate days. All the mice were sacrificed on 14th of tumour implantation.

G₇ - Tumour implanted mice, each received 0.075 mg of methanol fraction/gm body weight in normal saline as intraperitoneal injections on alternate days. All the mice were sacrificed on 14th day of tumour implantation.

(b) On Ehrlich's Lymphoma bearing mice

The Grouping of animals for the study on Ehrlich Ascite lymphoma was the same as that for Dalton's lymphoma except here Ehrlich Ascites cells were used in tumour implantation instead of Dalton's Ascites cells.

3.10.3 Preparation of serum and tissue samples for the biochemical studies

After the experimental period the mice were fasted overnight. Sacrificed by cervical dislocation. Blood was collected and tissues were transferred to ice cold containers and serum was separated from blood cells by centrifugation at about 2000 rpm for 30 min. Tissues like Liver and Kidney were removed from the mouse body, wiped thoroughly using blotting
paper to remove blood and other body fluids. Then they were washed in normal saline, again wiped and desired amounts of the dried tissues were used for various Biochemical Analysis.

3.11 METHODS USED FOR THE BIOCHEMICAL ANALYSIS
The following were the parameters studied.

3.11.1 Antioxidants such as Ascorbic acid and glutathione (GSH)
(a) Estimation of Ascorbic acid

Ascorbic acid level in tissues was determined by using the method of Roe.

Reagents:

- Trichloro acetic acid (TCA) : 6%
- Thiourea agent : 50% in alcohol
- 2,4-dinitrophenyl hydrazine : 2% in 9NH₄SO₄
- Con. H₂SO₄ : 85%
- Ascorbic acid standard
  - Stock: 100 mg ascorbic acid in 100 ml of 6% TCA.
  - Working standard: 100 mg ascorbic acid in 1 ml 6% TCA.
- Activated charcoal

Procedure

Immediately after sacrificing the animals a weighed sample of tissue was homogenised in 5 ml ice cold 6% TCA in a pre-chilled mortar. The extract was shaken well in a test tube, added activated charcoal and allowed to stand for 15 min. The clear supernatant was filtered through Whatman
filter paper. To 4 ml of supernatant, added a drop of Thiourea reagent (50% in alcohol) and 1 ml of 2%, 2, 4-dinitrophenyl hydrazine in 9N H$_2$SO$_4$ and incubated for 3 h at 37°C in a water bath. At the end of the incubation, place the test tubes in an ice bath and added carefully 4 ml of 85% Conc. H$_2$SO$_4$. Kept for 30 min in refrigerator. Centrifuged and OD measured at 540 nm in a spectrophotometer. The values are expressed in mg/100g tissue.

(b) Estimation of Glutathione (GSH)

Glutathione level in tissue was determined using the method of Patterson and Lazarrow.  

Reagents

- Alloxan: 0.1 M
- Phosphate buffer: 0.5 M (pH 7.5)
- NaOH: 0.5 N
- NaOH: 1 N
- GSH Standard: 3 mg GSH in 5%, 25 ml metaphosphoric acid

Procedure

Weighed sample of tissue was homogenized in phosphate buffer. The reaction mixture containing 50 µl tissue extract, 50 µl alloxan, 50 µl phosphate buffer and 50 µl NaOH (0.5N) was incubated at 25°C for 6 minutes. The reaction was stopped by the addition of 50 µl 1N NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1cm length path in a spectrophotometer. A control tube was maintained with phosphate buffer instead of extract. The values are expressed in mg/100 g tissue.
3.11.2 Antioxidant enzymes such as superoxide dismutase (SOD), catalase, Glutathione-peroxidase (GSH-Px)

(a) Estimation of Superoxide Dismutase

Superoxide dismutase in tissues was determined using the method of Kakkar et al.\textsuperscript{116}

Reagents

- Sodium pyrophosphate buffer: 0.052 M (pH 8.3)
- Tris-HCl buffer: 0.0025 M (pH 7.4)
- Sucrose: 0.25 M
- Phenazine methosulphate: 186 µM
- Nitro blue tetrazolium: 300 µM
- NADH: 780 µM
- Glacial acetic acid
- n-butanol

Procedure

100 mg tissue was homogenized in 2 ml 0.25M sucrose and differentially centrifuged to get cytosol fraction. This fraction was then dialysed against 0.0025 M Tris HCl buffer (pH 7.4) overnight before using for enzyme assay. Assay mixture contained 1.2 ml of the sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml nitroblue tetrazolium, 0.2 ml NADH and 1.2 ml of the enzyme source. Reaction was initiated by the addition of NADH. Incubated at 30°C for 90 sec. and stopped the reaction by the addition of 1 ml glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min. and was centrifuged. The upper butanol layer was taken out. Colour intensity of the chromogen in butanol was measured at 560 nm, against n-butanol blank. A system devoid of enzyme served as control. Protein estimation was carried out on the same enzyme source by
The values are expressed as 50% inhibition of nitroblue tetrazolium /min/mg protein.

(b) Estimation of Catalase

Catalase level in tissues was determined using the method of Cohen et al.\textsuperscript{120}

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>0.067 M (pH 7.0)</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>30 ( \mu )l/100 ul buffer</td>
</tr>
</tbody>
</table>

Procedure

100 mg of tissue was homogenized in 2 ml phosphate buffer and centrifuged. To 30 \( \mu \)l of the supernatant, added 3 ml of buffer and 0.75 ml of \( \text{H}_2\text{O}_2 \). Change in OD was measured at 240 nm at 0 sec, 30 sec, 60 sec respectively. The control system devoid of tissue extract was used as blank. Values are expressed in \( \mu \) moles of \( \text{H}_2\text{O}_2 \) consumed /min/mg protein.

(c) Estimation of Glutathione peroxidase (GSH-Px)

Glutathione peroxidase was estimated by the method of Rotruck.\textsuperscript{121}

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>0.4 M, pH 7.0</td>
</tr>
<tr>
<td>Sodium azide solution</td>
<td>10 mM</td>
</tr>
<tr>
<td>Trichloro acetic acid (TCA)</td>
<td>10%</td>
</tr>
<tr>
<td>Ethylene diamine tetraacetic acid (EDTA)</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Hydrogen peroxide (( \text{H}_2\text{O}_2 ))</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Glutathione solution (GSH)</td>
<td>2 mM</td>
</tr>
</tbody>
</table>
Procedure

Weighed sample of tissue was homogenised in a known volume of Tris buffer. To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml Sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2 ml of GSH followed by 0.1 ml H_2O_2 solution were added. The contents were mixed well and incubated at 37°C for 10 min along with a control containing all reagents except tissue homogenate. After 10 min the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH by the method of Beutler and Kelley.

**GSH estimation by the method of Beutler and Kelley**

**Reagents**

- Phosphate buffer: 0.2 M, pH 8
- T.C.A: 5%
- Ellman's reagent: 19.8 mg DTNB/100 ml of 0.1% sodium citrate
- Standard glutathione solution: 10 mg GSH/100 ml H_2O.

**Procedure**

1 ml of the supernatent from the above test tubes was taken, 0.5 ml of Ellman's reagent and 3 ml of phosphate buffer were added. The yellow colour developed was read at 412 nm with a blank containing 3.5 ml of phosphate buffer. A series of standards were also treated similarly. The amount of GSH was expressed in mg/100 g tissue.
3.11.3 Detoxifying enzymes such as Glutathione-S-transferase (GST), Glutathione reductase (GR)

(a) Estimation of Glutathione-S-transferase (GST)

Glutathione-S-transferase in tissues was determined using the method of Beutler et al.\textsuperscript{123}

Reagents

- Phosphate buffer : 0.5 M (pH 6.5)
- CDNB : 25 mM in 95% ethanol.
- GSH : 20 mM

Procedure

Tissues were homogenized in phosphate buffer. The reaction mixture containing 200 µl phosphate buffer, 20 µl CDNB, and 730 µl distilled water were taken in the control tubes and 200 µl phosphate buffer, 20 µl CDNB and 680 µl distilled water were taken in the test sample tubes. Then the tubes were incubated at 37°C for 10 min. After the incubation added 50 µl of GSH in both the set of tubes. After mixing well, added 50 µl of tissue extract in the test sample tubes. Absorbance was noted at 340 nm for 5 minutes in a quartz cuvette of 1 cm length path in a spectrophotometer. Values are expressed in n moles of CDNB complexed/min/mg protein.

(b) Estimation of Glutathione reductase (GR)

Glutathione reductase was estimated by the method of David, M. Goldberg.\textsuperscript{124}
Reagents

- Phosphate buffer: 0.12 mol/l, pH-7.2
- EDTA: 8.25 mmol/l
- NADPH: 9.6 mmol/l
- GSSG: 65.3 mmol/l

Procedure

Weighed sample of tissue was homogenised in a known volume of phosphate buffer. 2.6 ml of buffer, 0.1 ml EDTA, and 0.1 ml GSSG acts as reagent mixture to which was added 0.1 ml of the homogenised sample and waited for 5 min. Then 0.05 ml of NADPH was added, mixed thoroughly and read at 340 nm for 5 min against buffer blank.

3.11.4 Lipid peroxidation products such as Malondialdehyde (MDA) and conjugated dienes (CD)

(a) Estimation of Malondialdehyde

Malondialdehyde was estimated by the method of Ohkawa.105

Reagents

- Sodium phosphate buffer: 50 mM, pH 7.4.
- SDS: 8.1%
- Glacial acetic acid: 20% pH-3.5 adjusted with 1N NaOH
- Thiobarbituric acid (TBA): 0.8%
- n-butanol and pyridine mixture: 15:1 (v/v)

Procedure

Weighed sample of the tissue was homogenised in a known volume of the buffer. 0.1 ml homogenate was taken and mixed with 0.2 ml SDS,
1.5 ml glacial acetic acid and 1.5 ml thiobarbituric acid. The mixture was heated at 95°C for 1 hr on a water bath and cooled under tapwater. Then added 1 ml distilled water and 5 ml mixture of n-butanol and pyridine. The mixture was shaken vigorously and centrifuged at 2000 rpm for 5 min. The upper organic layer was read at 532 nm. The blank contained all the reagents except the sample. The result was expressed as moles of MDA/mg protein.

(b) Estimation of Conjugated dienes

Conjugated dienes were estimated by the method of Beuge J.A.126.

Reagents

Tris HCl buffer : 0.025 M, pH 7.5
Chloroform : Methanol : 2:1 (v/v)
Cyclohexane AR

Procedure

Weighed sample of tissue was homogenised in a known volume of the buffer. An aliquot of the homogenate was shaken with Chloroform : Methanol (2:1) and the lower layer was recovered. Then evaporated this layer to dryness and re-dissolved in a known volume of cyclohexane. Read the absorbance at 233 nm against cyclohexane as blank. The amount of conjugated dienes was expressed in terms of millimoles/100 g tissue.
3.11.5 Serum enzymes such as $\gamma$-glutamyl transpeptidase (GGT), Lactate dehydrogenase (LDH), Transaminases [Aspartate transaminase (AST) and Alanine transaminase (ALT)], Phosphatases [Alkaline phosphatase (ALP), Acid Phosphatase (ALP)]

(a) Estimation of $\gamma$-glutamyl transpeptidase (GGT)

GGT in serum was assayed by the method of Szasz.127

Reagents

Reagent Buffer (pH 7.8) contained
- Tris 120 nmol
- MgCl$_2$ 12 mmol
- Glycyl Glycine 90 mmol

Substrate
Dissolved 1.28 g L-$\gamma$-glutamyl 4-nitro anilide (anhydrous) in 0.15 mol/l HCl made up to 100 ml with acid. Constant stirring was required to dissolve the substrate. Stored at -20°C and it was stable for several weeks (The reagent was stable for only a few days at room temperature).

Procedure

100 µl serum and 1 ml buffer were incubated at 37°C. Started the reaction by adding 0.1 ml substrate, mixed and monitored the reaction continuously at 405 nm, so as to obtain the change in absorbance per minute. Values are expected in µg/min/100 ml serum.

(b) Estimation of serum Lactate dehydrogenase

The serum lactate dehydrogenase was determined by the method of Wroblewski et al.126
Reagents

Phosphate Buffer : 0.1 M (pH 7.4)
NADH₂ : 2.5 mg/ml of Phosphate Buffer (Freshly prepared before use)
Sodium pyruvate : 2.5 mg/ml. Refrigerate.

Procedure

Measured 2.7 ml of the phosphate buffer into a cuvette and added 0.1 ml of serum and 0.1 ml of NADH₂. Allowed to stand for 20 min at 37°C to reduce any keto acids already present in the serum. Then added 0.1 ml of sodium pyruvate. Read the extinction for 5 min at intervals of 15-30 sec at 340 nm.

(c) Estimation of Serum Transaminases

Serum levels of alanine and aspartate transaminases were determined by the method of Mohun and Cook.¹²⁹

Reagents

Potassium phosphate buffer : 0.075 M (pH 7.5)
Buffered Substrates
Aspartate transaminase: 300 mg, L-aspartic acid (Sigma) and 50 mg alpha Ketoglutaric acid (sigma) in 100 ml phosphate buffer and the pH was adjusted to 7.5 with sodium hydroxide.

Alanine transaminase: 5 g DL-alanine (Sigma) and 20 mg alpha Ketoglutaric acid (Sigma) in 100 ml phosphate buffer and the pH was adjusted to 7.5 with sodium hydroxide.

Aniline-citrate reagent: Dissolved 50 g citric acid (SRL) in 50 ml distilled water and to this an equal volume of redistilled aniline (Merck) was added.
Dinitrophenyl hydrazine reagent: Dissolved 200 mg of 2,4-dinitrophenyl hydrazine (Merck) in 85 ml of con. Hydrochloric acid and made up to one litre with distilled water.

Sodium hydroxide: 4 N

Procedure

Pipetted out 1 ml of substrate solution into two tubes and placed in water bath at 37°C for few minutes. To one of the tubes (test), added 0.2 ml of serum, shaken gently to mix. Exactly after one hour, in the case of aspartate transaminase and half an hour in the case of alanine transaminase, with the tubes still in the bath added two drops of aniline-citrate reagent to both and added 0.2 ml of serum in the second tube (control). Tubes were left undisturbed for 20 minutes. Then into both the tubes added 1 ml of dinitrophenyl hydrazine reagent. Left the tubes for another 20 minutes in room temperature and then added 10 ml of 0.4 N sodium hydroxide and read at 520 nm after 10 min. A solution of sodium pyruvate containing 10 mg pyruvate per ml was used as standard. Values are expressed in IU/100 ml serum.

(d) Estimation of Serum Phosphatases

Serum phosphatases were assayed following the method of King and Jagatheesan using 4-amino antipyrene.[30]

- Alkaline phosphatase

Reagents

Disodium phenyl phosphate (Merck): 0.01 M.

Sodium carbonate and bicarbonate Buffer.
(0.1M) 3.18 g anhydrous sodium carbonate and 1.68 g Sodium bicarbonate dissolved in 500 ml distilled water.

Buffered substrate for use:

Prepared by mixing equal volumes of the above two Reagents.

<table>
<thead>
<tr>
<th>Sodium hydroxide</th>
<th>0.5 N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>0.5M</td>
</tr>
<tr>
<td>4-amino antipyrene(sigma)</td>
<td>0.6%</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

Procedure

Measured 2 ml of buffered substrate into each of the two test tubes and was incubated at 37°C for few minutes in a water bath. Then 0.1 ml of serum was added to one of the test tubes (test) and further incubated for 15 min. At the end of incubation, removed the tubes from the bath and added 0.8 ml of Sodium hydroxide, 1.2 ml of sodium hydroxide and 1.2 ml of sodium bicarbonate to both tubes. Then 0.1 ml of serum was added to the second tube (Blank). To both the tubes, added 1 ml of amino antipyrene reagent and 1 ml potassium ferricyanide solution. For standard 1.1 ml of the buffer and 1 ml of phenol solution containing 0.01 mg of phenol, and for the standard blank 1.1 ml buffer and 1 ml distilled water were taken, instead of buffered substrate and serum. All other procedures were same as in the case of test samples. The absorbance was read at 520 nm. Values are expressed in King Amstrong units/100 ml serum.

- Acid Phosphatase

Reagents

Citric acid-sodium citrate buffer : 0.01 M (pH 4.9)

All other reagents were same as for alkaline phosphatase.
Procedure

Procedure was same in the case of alkaline phosphatase except that the citric acid-sodium citrate buffer was used for the preparation of buffered substrate and incubation time is 1hr. For developing the colour with aminoantipyrine, 1 ml sodium hydroxide and 1 ml sodium bicarbonate were added. Values are expressed in King Armstrong units/100 ml serum.

3.11.6 Haemoglobin

Estimation of Haemoglobin (Cyanmethaemoglobin method).

Reagents

Drabkin's Solution : Ferricyanide-Cyanide Reagent.
Cyanmethaemoglobin standard : 60 mg/100 ml.

Procedure

Added 0.02 ml of blood to 4 or 5 ml of the Drabkin's Reagent. Allowed to stand at least 4 min and read against reagent blank at 540 nm. Read the standard in the same way. Haemoglobin is expressed as grams of haemoglobin/100 ml blood.

3.11.7 Estimation of proteins

Protein content of the serum and tissues were assayed by the method of Lowry et al. 119

Reagents

Sodium hydroxide solution : 0.1 N
Sodium Carbonate solution : 2% in 0.1 N Sodium Hydroxide
Copper Sulphate solution : 0.5%
Sodium potassium tartrate solution: 1%.

Alkaline copper reagent: A mixture of 50 ml sodium carbonate solution and 0.5 ml of each of copper sulphate solution and sodium potassium tartrate solution.

Folin’s phenol reagent : 1:1 dilution with distilled water.
Standard protein solution : 100 mg % in 0.1 NaOH.

Procedure

Pipetted out 0.2 ml of serum or extract to the test tube and added 1.8 ml of sodium hydroxide solution and 5 ml of alkaline copper reagent. Shaken well and kept the mixture for 10 min. After 10 min, 0.5 ml of Folin’s phenol reagent was added and mixed well. The mixture was kept for another 30 minutes. The optical density was measured at 675nm, in a spectrophotometer. The system devoid of sample was used as the blank.

All were estimated using UV spectrophotometer (UV-1601-shimadzu).

3.11.8 Statistical analyses

All statistical analysis was done using a computer software created by Russel E. Freed (Crop & Soil Sciences Department, Michigan State University) named MSTATC-C (Version 1.2).