CHAPTER II

MATERIALS AND METHODS

2.1 Plant material

2.1.1 Plant collection and preparation

*Elephantopus scaber* Linn was collected from Thodupuzha, Kerala, during the month of June identified and a voucher specimen (SBS/BRL-03) was kept in School of Biosciences, Mahatma Gandhi University, Kottayam. Fresh *Elephantopus scaber* L. with commercial maturity (the plant height approximately 50 cm with corolla 8~10 mm long purple flower) were obtained, In order to ensure the sample used was from the same source throughout the experiment, the fresh sample was collected in sufficient quantities (~5 kg) at a time.

Fresh plants were collected and stored using a standard procedure. The plants were thoroughly washed using running tap water followed by rinsing with distilled water. The plants were then cleaned, chopped, shade dried and powdered.

2.1.2 Soxhlet extraction

The soxhlet extraction procedure was carried out using various solvents of increasing polarity, *i.e.* Petroleum ether, Chloroform, ethanol and methanol. About 400ml of the solvent was poured into the round bottom extraction flask and placed on the heating mantle top. About 50g of the dried plant powder was placed on the thimble of the soxhlet apparatus and placed above the round bottom flask. The condenser was placed above the thimble and the parts were fixed vertically. The soxhlet extraction was carried out for
72 hours. The extracts were collected and the solvent evaporated under vacuum in a rotary evaporator. The extraction was repeated with a new set of dried powder until the required quantity was achieved. The extracts were kept under refrigeration and used for \textit{in vitro} and \textit{in vivo} studies.

\section*{2.2 Experimental animals}

Male albino rats of Wistar strain weighing between 120 to 150g were used for the experimental purpose. The animals were housed in polypropylene cages and given standard pellet diet (M/S Hindustan and Lever Ltd Bombay). Animal studies were conducted according to the Institute animal ethics committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Reg No. B2442009/1

\section*{2.3 Chemicals and reagent kits}

Chemicals purchased from Sisco Research Laboratories (SRL), Mumbai, India.

Reduced glutathione (GSH)
Oxidized glutathione (GSSG)
n-hexane
5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB)
1-chloro-2, 4-dinitrobenzene (CDNB)
Trichloroacetic acid (TCA)
Ethylene diamine tetra acetate (EDTA)
Tris-HCl
NADPH
Iodine
Sodium azide
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Nitroblue tetrazolium (NBT)
Bovine serum albumin (BSA)
Folin - Ciocalteau reagent

**Chemicals obtained from Merck, Mumbai, India.**

Sodium hydroxide
Di-sodium hydrogen phosphate
Sodium di-hydrogen phosphate
Trisodium citrate
Sodium chloride
Potassium iodide
Potassium permanganate
Sodium carbonate
Citric acid
Tween-80
Dimethylsulphoxide (DMSO)
Glycerol
Petroleum ether
Chloroform
Ethyl acetate
Methanol
Hydrochloric acid
Sulphuric acid
Glacial acetic acid
Paraffin wax
Xylene and DPX mountant
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Chemicals procured from Central Drug House (CDH), New Delhi, India.
Thiobarbituric acid (TBA) and Ferric chloride

Chemicals purchased from Nice chemicals, Kochi.
Formaldehyde solution
Hematoxylin and Eosin
Hydrogen peroxide (H$_2$O$_2$)

Reagent kits procured from Agappe Diagnostics Ltd. Kerala, India.
The diagnostic Kits for
Glucose
Urea
Creatinine
Cholesterol
Triglycerides

2.4 Instruments

Rotary evaporator : Superfit DB3135H, India.
Spectrophotometer : Hitachi U-2800, Japan.
Weighing balance : Sartorius, Germany.
Cooling centrifuge : Remi, India.
Inverted Microscope : Motic AE 21, Germany.
Deep freezer (-20°C) : Celfrost, India.
pH meter : pH 700, Eutech Instruments, Singapore.
Microscope : Magnus, India.
Semi autoanalyzer : RMS BCA201, India
Rotary microtome : Biotech master precision ultra thin rotary microtome.
Liquid Chromatography (LC): PHENOMENEX RP18
Liquid Chromatography (LC): WATERS e2695
Mass Spectrum (MS): WATERS 3100

2.5 Experimental design

2.5.1 Preliminary Phytochemical analysis

A screening study was conducted to identify the different types of phytochemicals present in the plant extract by different qualitative tests.

2.5.2 In vitro antioxidant study

Antioxidant activity of the petroleum ether, chloroform, ethanol and methanol extracts were conducted in vitro by performing different free radical scavenging and lipid peroxidation assays.

2.5.3 Preliminary pharmacological screening and toxicological analysis

2.5.3.1 Induction of hepatotoxicity by CCl₄

Rats were divided into five groups with six animals in each group.

Group I - Animals served as pairfed control and received subcutaneous administration of liquid paraffin (LP) twice a week at the dose of 3ml/kg body weight.

Group II - Animals constituted the toxic control group which received administration of LP+CCl₄ in the ratio 1:2 (V/V) subcutaneously (s.c) twice a week, on every first and fourth days of the week at the dose of 3ml/kg body weight (bw).

Group III - Animals were Curcumin treated rats at a dose of 75mg/kg body weight orally along with subcutaneous administration of LP+CCl₄ as in group II.
Group IV - Animals were same as in GroupII but received methanolic extract of *E. scaber* at a dose of 75 mg/ kg body weight orally.

Group V - Animals were same as in GroupII but received methanolic extract of *E. scaber* at a dose of 150 mg/ kg body weight orally.

Liver damage was induced in rats by administering CCl$_4$, subcutaneously (s.c) at lower abdomen. A suspension of CCl$_4$ in LP in the ratio 1:2 (V/V) at the dose of 3ml/ kg body weight(bw), was administered twice a week, on every first and fourth days of all weeks.

2.5.3.2 **Induction of hepatotoxicity by paracetamol**

Animals were divided into 5 groups of 6 animals each and treated as follows.

Group I - Animals served as pairfed control and received distilled water.

Group II - Animals served as toxic control and received distilled water + Paracetamol (PCM) in distilled water (3g/kg body weight)

Group III - Animals received methanol extract of *E.scaber*75mg/kg bw + PCM (3g/kg bw).

Group IV - Animals received methanol extract of *E.scaber*150mg/kg bw+ PCM (3g/kg bw).

Group V - Animals received Silymarin 50mg/kg bw + PCM (3g/kg bw).

Liver damage was induced in rats by administering paracetamol as a suspension in distilled water, orally as a single dose of 3gm/kg bw.
2.5.3.3 Induction of inflammation

(i) Carrageenan-induced hind paw edema model for the determination of anti-inflammatory activity.

Rats were divided into four groups.

Group I – received normal saline.

Group II- received diclofenac sodium 10 mg/kg bw.

Group III -received *E.scaber* 100 mg/kg bw.

Group IV- received *E.scaber* 200 mg/kg bw.

Animals were pre-treated with drugs orally one hour before the experiment, each rat was injected with freshly prepared 0.05ml of suspension of carrageenan (0.5 mg/25 µl) in physiological saline (154 nM NaCl) into subplantar tissue of the right hind paw. The control group received 25 µl saline solutions.

(ii) Formalin-induced hind paw edema model for determination of anti-inflammatory activity.

The same procedure as mentioned above in carrageenan-induced paw edema assay was followed except that acute inflammation was produced by administration of 20 µl formalin in physiological saline (154 nM NaCl) into the subplantar area of right hind paw of rat.

2.5.4 Induction of hepatic fibrosis

Thirty rats were divided into five groups with 6 rats each

Group I - Animals served as vehicle control and received oral administration of LP twice a week at the dose of 3ml/kg body weight.
Group II - Animals constituted the toxic control group which received administration of LP+CCl₄ (1:1) twice a week orally at the dose of 3ml/kg body weight.

Group III- Animals were same as in GroupII but received methanolic extract of *E. scaber* at a dose of 100mg/ kg body weight orally.

Group IV- Animals were same as in GroupII but received methanolic extract of *E. scaber* at a dose of 200mg/ kg body weight orally.

Group V- Animals were same as in Group II but received Silymarin at a dose of 50mg/ kg body weight orally.

Hepatic fibrosis was induced in rats by administering CCl₄, orally. A suspension of CCl₄ in LP in the ratio 1:1 (V/V) at the dose of 3ml/kg bw, was administered twice a week, on every first and fourth days of all weeks.

The experiment was carried out in preventive and curative models of hepatic fibrosis. In preventive model, oral treatment with *E.scaber* methanolic extract and silymarin was started one week before the onset of CCl₄ administration and continued upto 10 weeks. In curative model, hepatotoxicity was induced by CCl₄ administration for a period of 10 weeks. After that, oral treatment with *E. scaber* and silymarin were started and continued for a period of two weeks.

### 2.6 Collection of serum and tissue samples

The animals were kept starved overnight before they were sacrificed. They were sacrificed by decapitation and blood was collected by cutting the jugular vein. The blood was allowed to clot and centrifuged at 3000 rpm for 20 minutes and serum was separated and left standing on ice until required. The liver tissue was dissected out, blotted off blood washed in saline and
weighed instantaneously. This was kept in frozen containers. Serum and tissue samples were used for different biochemical estimations.

2.7 Biochemical tests

Sera from different groups of rats were subjected to biochemical estimations of different parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT), total protein (TP), cholesterol, sugar, creatinine, triglycerides, urea and albumin. For assessing the antioxidant efficacy of the drugs, the liver tissue homogenate was subjected to biochemical tests for thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were evaluated.

2.8 Histopathology

Dissected livers were cut into small pieces and fixed in 10% buffered formalin for histopathological analysis. The liver (5-6 mm thick pieces) fixed in buffered formalin for 12 hours was processed for paraffin embedding using the micro technique procedure (Galigher et al., 1971). The tissue blocks were cut into 5 µm thick sections (Rotary microtome, Yorco, New Delhi). The paraffin embedded liver were stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic A.E 21, Germany). The microphotographs were taken using Moticam 1000 digital camera at original magnification of 100X.
2.9 Immunohistochemistry

(Wills and Asha, 2006)

Liver samples were collected, washed with phosphate-buffered saline (PBS) and fixed overnight in 10% buffered formalin. Serial sections (5 µm) were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin wax. For immunohistochemistry, mouse anti-rat Collagen III monoclonal antibody was used. Tissue sections were deparaffinised in three changes of xylene at 60°C for 10 min each and hydrated through a graded series of alcohol. For antigen retrieval, evaluation slides were incubated in citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven. The sections were then allowed to cool to room temperature and then rinsed with IX tris buffered saline and treated with 0.3% H₂O₂ in water for 10 min to block endogenous peroxidase activity. Non specific binding was blocked with 3% BSA in room temperature for one hour. The sections were then immunostained with Collagen III antibody diluted 1:200 with 3% BSA in PBS and incubated overnight at 4°C. Sections were then washed thrice in PBS and incubated with anti-mouse horseradish peroxidase for 45 minutes. After triplicate washing with PBS, sections were incubated for 30 minutes with streptavidin-HRP complex. Sections were then washed with PBS and incubated for 5 – 10 minutes in a solution of 0.02% diaminobenzidine containing 0.01% H₂O₂. Counterstaining was performed with hematoxylin. Images were taken at original magnification of 100× (Motic AE 21, Germany and Moticam 1000 Camera).
2.10 Statistical analysis

The data obtained were analyzed for finding the variation between treated and control using one-way ANOVA followed by tukeys post hoc analysis. The level of significance was set as p<0.05.

2.11 Percentage of hepatoprotection

The percentage of protection for different tests was calculated using the formula

\[
\% \text{ Protection} = \frac{\text{Toxic control} - \text{Treated group}}{\text{Toxic control} - \text{Pairfed control}} \times 100
\]

2.12 Detailed procedures for biochemical analysis

2.12.1 Preliminary Phytochemical analysis

Qualitative phytochemical analysis was conducted on the various dried extracts of *Elephantopus scaber* Linn as follows to identify the various compounds in the extract using standard conventional protocols (Kokate et al., 2009; Evans and Trease, 2002; Khandelwal, 1995).

2.12.1.1 Detection of Alkaloids

(i) Wagner’s (iodine – potassium reagent) test

To 1 ml of the extract 2 ml of Wagner’s reagent (iodine in potassium iodide) was added. Presence of alkaloids was confirmed by the formation of reddish brown colored precipitate.
(ii) *Meyer’s (potassium – mercuric iodide reagent) test*

1ml of Mayer’s reagent (potassium mercuric iodide solution) was added to 1 ml of the extract and the formation of cream colored precipitate confirmed the presence of alkaloids.

(iii) *Dragendroff’s test*

To 1 ml of the extract, 1 ml of Dragendroff’s reagent (potassium bismuth iodide solution) was added. Formation of an orange-red precipitate indicated the presence of alkaloids.

2.12.1.2 *Detection of flavonoids*

Little quantity of extract was treated with amyl alcohol, sodium acetate and ferric chloride. A yellow color solution formed, disappeared on addition of an acid indicated the presence of flavonoids.

2.12.1.3 *Detection of Saponins*

(i) To small quantity of extract 20 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of foam indicated the presence of saponins.

(ii) A suspension of RBC in normal saline was treated with 25mg of extract. Hemolysis indicated the presence of saponins.

2.12.1.4 *Detection of Phenols*

(i) *Ferric chloride test*

To 1 ml of the extract, ferric chloride solution was added. Formation of a dark blue color confirmed the presence of phenolic compounds and tannins.
(ii) **Lead Acetate test**

1 ml of the test solution was mixed with basic lead acetate solution, formation of white precipitate indicated the presence of tannins and phenolic compounds.

### 2.12.1.5 Detection of Tannins

Little quantity of the extract was treated with potassium ferric cyanide and ammonia solution. Formation of a deep red color indicated the presence of tannins.

### 2.12.1.6 Detection of Carbohydrates

(i) **Fehling’s Test**

To 2ml of aqueous solution of the plant material, 1 ml of a mixture of equal parts of Fehling’s solution “A” and “B” were added. The contents were boiled for few minutes. Formation of red or brick red precipitate indicated the presence of carbohydrates.

(ii) **Benedict’s Test**

To 0.5 ml of plant extract, 5 ml of Benedict’s reagent was added and boiled for 5 minutes. Formation of bluish green, yellow or red colour depending on the amount of reducing sugar present in the solution, shows the presence of carbohydrates.

(iii) **Molisch’s Test**

In a test tube containing 2 ml of plant extract, 2 drops of freshly prepared 20% alcoholic solution of α-Naphthol was added and mixed. To this solution, 2 ml of concentrated sulphuric acid was added through the sides of the tube so as to form a layer below the mixture. Formation
of the red violet ring at the junction of the solution indicated the presence of carbohydrates.

2.12.1.7 Detection of Gums and Mucilage

10ml of the extract is slowly transferred to 25ml of absolute alcohol with constant stirring in a beaker. The precipitate was dried in air and examined for its swelling properties and for the presence of carbohydrates.

2.12.1.8 Detection of Fixed Oils

A small quantity of extract was pressed between two filter papers. Oil stains on the paper indicated the presence of fixed oil.

2.12.1.9 Detection of Proteins

(i) Biuret Test

To 2ml of the plant extract added an equal volume of 10% sodium hydroxide followed by few drops of 0.5% copper sulphate solution. Appearance of violet to pink colour indicated the presence of protein.

(ii) Xanthoproteic Test

To 5 ml of the extract, 1 ml of concentrated nitric acid was added and boiled. Then 40% of sodium hydroxide was added. Orange color indicated the presence of aromatic amino acids.

(iii) Ninhydrin test

Added two drops of freshly prepared 0.2% ninhydrin reagent (0.1% solution in n-butanol) to the small quantity of extract solution and heated for 2 minutes. Development of blue color confirmed the presence of proteins, peptides or amino acids.
2.12.1.10 Detection of Thiols

To about 0.5 ml of plant extract, enough ammonium sulphate was added to saturate the solution, 2-4 drops of 5% Sodium nitroprusside was then added, followed by one or more drops of Con. HNO₃.

Formation of Magenta colour showed the presence of thiols.

2.12.1.11 Detection of Phytosterols

(i) Salkowski Test

To 2ml of the extract, 2ml chloroform and 2ml conc. sulphuric acid were added and shaken well. Formation of red fluorescence in the chloroform layer and greenish yellow fluorescence in the acid layer indicated the presence of phytosterols.

(ii) Liebermann- Burchard Reaction

Mixed 2ml of the extract with an equal amount of chloroform. Then 2ml of acetic anhydride and 2 drops of concentrated sulphuric acid were added through the sides of the test tube. Initially red, then blue and finally green colour indicated the presence of phytosterols.

2.12.1.12 Detection of Coumarins

100mg extract was dissolved in methanol and alcoholic potassium hydroxide was added. Appearance of yellow color which disappeared on addition of conc. HCl indicated the presence of coumarins.

2.12.1.13 Detection of Terpenoids

5ml of the extract was mixed in 2ml chloroform. 3ml conc. sulphuric acid was added to form a layer. Reddish brown precipitate at the interface formed indicated the presence of terpenoids.
2.12.2 \textit{In vitro} antioxidant assays

2.12.2.1 Superoxide radical scavenging assay

Assay is based on the ability of the extract to inhibit or scavenge the superoxide radical generated from the photo reduction of riboflavin according to the method of Mc Cord and Fridovich (1969). The reaction mixture contained, EDTA (6mM), 3µg NaCN; riboflavin (2µM); NBT (50µM); KH$_2$PO$_4$-Na$_2$HPO$_4$ buffer (67mM, pH 7.8) and various concentrations of the different extracts in a final volume of 3 ml. The tubes were illuminated under the incandescent lamp for 15 minutes. The optical density (OD) at 560nm was measured before and after illumination against distilled water. The inhibition of the superoxide radical generation was determined by comparing the absorbance values of the control with that of experimental groups. All tests were done in triplicate.

Percentage of inhibition of SOD generation = \( \frac{(C-T)}{C} \times 100 \)

2.12.2.2. Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe$^{2+}$- ascorbate system (Bishayee and Balasubramanian, 1979) in the rat liver homogenate in the presence and absence of extracts to form TBARS. TBARS is measured according to the method of Ohkawa et al., (1979). The reaction mixture contained 0.1ml of rat liver homogenate (25%, v/v) in Tris-HCl buffer (20mM, pH7); KCl (30mM); FeSO$_4$(NH$_4$)$_2$SO$_4$.6H$_2$O (0.16mM); ascorbate (0.06mM) and various concentrations of the extracts of \textit{Elephantopus scaber Linn} in a final volume of 0.5ml. The reaction mixture was incubated for 1 hour at 37°C. After the incubation period, 0.4ml of the reaction mixture was treated with 0.2ml SDS (8.1%); 1.5 ml thiobarbituric acid (0.8%); and 1.5ml acetic acid (20%, pH
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3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95-100°C for 1h. After cooling 1.0ml distilled water and 5.0 ml of n-butanol were added and centrifuged at 4000rpm for 10 minute. The organic layer was removed and its absorbance at 532nm was measured against n-butanol-pyridine mixture. Inhibition of lipid peroxidation was determined by comparing the optical density of treatments with that of control. All tests were done in triplicate.

Percentage inhibition of lipid peroxidation = \( \frac{(C-T)}{C} \times 100 \)

2.12.2.3 Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined by studying the competition between deoxyribose and the extracts of *Elephantopus scaber Linn* for the hydroxyl radicals generated from Fe³⁺ ascorbate-EDTA-H₂O₂ system (Fenton’s reaction). The hydroxyl radical attack deoxyribose, which eventually results in a TBARS. The TBARS thus formed was measured (Ohkawa et al., 1979). The reaction mixture contained deoxyribose (2.8mM); FeCl₃ (0.1mM); K₂HPO₄-KOH buffer (20mM, pH7.4); EDTA (0.1mM); H₂O₂ (1.0mM); ascorbic acid (0.1mM) and various concentrations of the extracts of *Elephantopus scaber Linn* in a final volumes of 1ml. The reaction mixture was incubated at 30°C for 60 min. The TBARS formed was estimated by thiobarbituric acid method of Ohkawa et al., (1979). The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated. All tests were done in triplicate.

Percentage of hydroxyl radical scavenging activity = \( \frac{(C-T)}{C} \times 100 \)
2.12.2.4 Assay of nitric oxide scavenging activity

The nitric oxide scavenging activity was measured according to the method of Sreejayan and Rao 1997, immediately before the experiment, 10mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of *Elephantopus scaber* Linn extracts and sodium nitroprusside (1mM) in a final volume of 3 ml were incubated at 25°C for 150 min. After incubation, 2.5 ml of reaction solution was removed and mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore was read immediately at 546nm against reagent blank. The nitric oxide scavenging activity was determined by comparing the absorbance of control with that of treated. All tests were done in triplicate.

Production of nitrite from solution of 1mM sodium nitroprusside solution incubated in the presence and absence of *Elephantopus scaber* Linn extracts at various time intervals (50, 100 and 150 min) were also studied and referred to the absorbance of standard solutions of sodium nitrite (0.1µM - 5µM) treated similarly with Griess reagent.

\[
\text{Percentage of nitric oxide radical scavenging activity} = \left( \frac{C-T}{C} \right) \times 100
\]

2.12.3 Estimation of Liver markers

2.12.3.1 Estimation of the activity of Aspartate transaminase (AST) (EC 2.6.1.1) and Alanine transaminase (ALT) (EC 2.6.1.2)

Activities of AST and ALT in serum were determined by the method of Mohum and Cook, 1957.
Reagents

- Phosphate buffer (100mM) with $\alpha$-oxoglutaric acid (2mM)
- Substrates  (a) Aspartatetransaminase. 100mM L-aspartic acid was added to the above buffer.
  
  (b) Alanine transaminase. Prepared as in (a) but using 200mM DL-alanine.
- 2, 4 Dinitro phenyl hydrazine (1mM) in 1N HCl
- Sodium hydroxide (400mM)
- Pyruvate standard (2mM). Working standard- diluted 1 in 20.

Procedure

1ml of substrate was pipetted into two tubes and placed in a water bath at 37°C for a few minutes to reach its temperature. To one (test), 0.2ml of serum was added and shaked gently to mix. Exactly 1h later in the case of AST and after 30 min for ALT, with the test tubes still in the bath, 1.0 ml dinitro phenyl hydrazine was added to both, and 0.2 ml serum to the other (control). Allowed to stand for 20 minutes at room temperature. 10 ml 0.4N NaOH was added to all tubes mixed well and absorbance was read at 520nm after 5 minutes in a colorimeter. For standard, 1 ml working standard was taken and made up to 1.2 ml with water and proceeded as above. For blank, 1.2 ml water was taken and proceeded as above.
2.12.3.2: *Estimation of the activity of Alkaline Phosphatase (ALP) (EC 3.1.3.1).*

(Kind and King, 1954)

Reagents

- Disodium phenyl phosphate (0.01M)
- Sodium carbonate-sodium bicarbonate buffer (0.1M).
- Buffered substrate for use prepared by mixing equal volumes of above two solutions pH=10.
- Standard phenol solution- Stock Solution : 100mg phenol/100ml solution
- Working Standard: Stock standard was diluted 1 in 10.
- Sodium hydroxide 0.5M
- Sodium bicarbonate 0.5M.
- 4- amino anti-pyrine 0.6% in water.
- Potassium ferricyanide 2.4g/100ml in water.

Procedure

2ml of buffered substrate was measured into each of two test tubes and placed in a water bath at 37°C for a few minutes. Then to the test 0.1ml of serum was added and incubated for exactly 15 minutes. It was removed from the bath and added 0.8ml of 0.5 N NaOH and 1.2ml of 0.5 M sodium bicarbonate to both tubes and then 0.1ml of sera was added to the second tube (blank). To both tubes, 1 ml of amino anti-pyrine reagent and 1ml of potassium ferricyanide were added. For standard, 1.1ml of buffer and 1ml of phenol standard containing 0.01ml of buffer and 1ml of water were taken.
Then to both tubes NaOH, bicarbonate, amino anti-pyrene and ferricyanide were added as above. Read at 520nm.

2.12.3.3: Estimation of the activity of $\gamma$-glutamyl transpeptidase (GGT) (EC2.3.2.2).

(Naftalin et al., 1969)

Reagents

- Buffer: Prepared by mixing Tris (120mM/l), MgCl$_2$ (90mM/l) and Glycyl-glycine (pH=7.8).
- Substrate: 1.28g L-$\gamma$-glutamyl 4- nitroanilide in 0.15mM/1N HCl and made to 100ml with acid.

Procedure

100$\mu$l of serum or liver extract and 1ml of buffer were warmed to 37$^\circ$C. The reaction was initiated by adding 0.1ml of substrate and mixing well instantaneously. The reaction was monitored continuously at 405nm in 1cm cuvette so as to obtain the change in absorbance per minute.

2.12.3.4 Estimation of Total Protein (TP) by Lowry’s Method

Protein was measured by the method given by Lowry et al., 1951 using bovine serum albumin as standard.

Principle

Aromatic amino acids like tyrosine and tryptophan reduce the phosphomolybdate of Folin’s reagent (Folin Ciocalteau reagent) to give a blue color. The intensity of the purple blue color formed depends upon the amount of the amino acids present and is proportional to the amount of protein that was measured in spectrophotometer at 650 nm.
Reagents

1) 0.4M Tris HCl buffer pH 7.0
2) 10% w/v Trichloroacetic acid (TCA).
3) 0.1 N NaOH (7gm of NaOH in 1750ml distilled water)
4) Alkaline Copper Reagent:
   a) 25gm Na₂CO₃ in 1250ml 0.1 N NaOH.
   b) 125 mg CuSO₄.5H₂O in 25ml distilled water.
   c) 0.25gm sodium potassium tartarate (1%) in 25ml distilled water.
5) Folin Ciocalteau reagent (1:1 dilution).
6) Stock Standard BSA (100mg BSA in 100ml 0.1 N NaOH).
7) Working standard BSA (5ml of stock standard was made upto 50ml with 0.1 N NaOH).

Procedure

To 0.2ml of serum/tissue extract 1.8ml of 0.1 N NaOH and 5ml of alkaline copper reagent were added and kept for 15 minutes. Then 0.5ml of diluted Folin - phenol reagent was added, mixed, kept for 30 minutes and read at 675nm. To the blank, 0.2ml of water and to the standard, 0.2ml of working standard were added instead of serum/tissue extract and treated as above.
2.12.3.5 Estimation of Albumin (Doumas and Peters, 1997)

Principle

Albumin binds with Bromocresol green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the conc. of albumin present, when measured photometrically between 580-630 nm with maximum absorbance at 625 nm.

Reagents

1. Albumin reagent
   a. Bromocresol green 0.08 m mol/L
   b. Succinate buffer (pH 4.2±0.1 at 25°C) 50 m mol/L
   c. Sodium azide 1 gm/L
2. Albumin standard 3.6 g/dl

Procedure

Pippete into tubes labeled test and standard 1ml each of albumin reagent add 10 µl of test sample and standard mix well and take the reading at 625nm after one minute at 37°C.

\[
\text{Albumin} = \frac{\text{Absorbance of test} \times \text{conc. of standard (g/dl)}}{\text{Absorbance of standard}}
\]

2.12.3.6 Estimation of Lactate Dehydrogenase (LDH)(EC 1.1.1.27) (Wroblewski and LaDue, 1955).

Reagents

- Glycine Reagent: 7.505g glycine and 5.85g NaCl were dissolved in about 900 ml of distilled water and made upto 1 litre.
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- Buffered substrate: 125ml of glycine buffer and 75ml of 0.1N NaOH were mixed, and then added into it 4g of lithium lactate / sodium lactate. Mixed well and pH was adjusted to 10.
- NAD\(^+\) Solution: 10 mg of NAD\(^+\) was dissolved in 2.14 g /dl of Nicotinamide solution.

Procedure

10ml of buffered substrate, 0.2ml of NAD\(^+\) solution and 0.2ml of serum were pipetted into a cuvette. The mixture was mixed thoroughly and then readings were taken at 340nm after 45 seconds and then at an interval of 1, 2, 3 minutes. The mean absorbance change per minute was determined

2.12.4 Estimation of Antioxidant Parameters

2.12.4.1 Estimation of Reduced Glutathione (GSH) By Ellmann’s Method.

GSH was estimated by Ellmann’s method of Moron et al., 1979.

Principle

Reduced glutathione (GSH) was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB) that gives a yellow colored complex with absorption maximum at 412nm.

Reagents

- Phosphate Buffer 0.2M (pH-8.0).
- 25% w/v Trichloroacetic acid (TCA).
- 5% w/v Trichloroacetic acid (TCA).
- Dithio-bis-2-nitrobenzoic acid (DTNB) 0.6mM.
- Standard Glutathione (GSH) – 5mg reduced glutathione was diluted to 50ml with distilled water.
Procedure

To 500µl of the homogenate, 125µl of 25% (w/v) TCA was added to precipitate proteins. The tubes were cooled on ice for 5 minutes and the mixture was further diluted with 600µl of 5% TCA and centrifuged for 10 minutes. 300µl of the resulting supernatant was taken for GSH estimation. The volume of the aliquot was made up to 1ml with 0.2 M phosphate buffer, pH 8.0 and 2ml freshly prepared DTNB (0.6 mM) was added to the tubes. The intensity of yellow color formed was measured at 412nm. Values are expressed in mmol/mg tissue.

2.12.4.2 Estimation of Catalase (EC 1.11.1.6)

The catalase activity was determined by the method of (Machly and Chance, 1954). Catalase assay was measured by the disappearance of peroxide.

Principle

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

The uv absorption of H2O2 is measured between 230 and 250nm. On decomposition, H2O2 by catalase, the absorption decreases with time. The enzyme activity can be calculated from this decrease.

Reagents

- Phosphate Buffer 0.1M pH 7.0.
- H2O2 - 30mM in phosphate buffer (340µl H2O2 made up to 100mL with phosphate buffer).
Procedure

Tissue was homogenized in 0.025M Tris HCl buffer of pH 6.5 and centrifuged at 1500 rpm for 10 minutes. The supernatant was used for the assay. To about 25 µl tissue extract 1 ml of phosphate buffer of 0.1M, pH 7.0 and 250 µl of H$_2$O$_2$ was added. Change in optical density (at zero time, after 30 sec and after 60 sec) was measured at 240nm. Specific activity is expressed as µmol of H$_2$O$_2$ consumed/ min/ mg protein.

2.12.4.3 Estimation of Thiobarbituric Acid Reactive Substances

TBARS was estimated by Thiobarbituric acid assay method of Nichanus and Samuelson, 1968.

Principle

In this method, malondialdehyde and other TBARS were measured by their reactivity with Thiobarbituric acid in the acidic condition to generate a pink colored complex which was read at 535 nm.

Reagents

Tris-HCl Buffer 0.25M, pH – 7.5.

- 15% w/v Trichloroacetic acid.
- 0.25 N hydrochloric acid.
- 0.375% w/v Trichloroacetic acid in 0.25N Hydrochloric acid.
- TCA – TBA – HCl reagent – 125mL TBA in HCl is mixed with 125ml 15% TCA.
- Stock Standard -0.16mL of 3M 1,1’,3,3’ tetramethoxypropane made upto 100mL.1mL of stock standard diluted to 100mL was used as working standard.
**Materials and Methods**

**Procedure**

The tissue homogenate was prepared in 0.1µl of Tris HCl buffer (pH-7.5). 1.0ml of the tissue homogenate was combined with 2.0 ml of TCA – TBA - HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. The flocculent precipitate was removed by centrifugation at 2000 rpm for 10 minutes. The absorbance of the sample was read at 535 nm against a blank without tissue.

2.12.4.4 Estimation of Conjugated Dienes (CD) (Beuje and Aust, 1978).

**Reagents**

- Chloroform
- Methanol
- Cyclohexane

**Procedure**

To 1ml of tissue homogenate 5ml of chloroform methanol reagent (2:1 v/v) was added, mixed thoroughly and centrifuged for 5 minutes. 3ml of lower layer was then evaporated to dryness. To this 1.5ml of cyclohexane was added and the absorbance was read at 233 nm against a cyclohexane blank.

2.12.4.5 Assay of Super Oxide Dismutase (SOD) (EC 1.15.1.1) (Kakkar et al., 1984).

**Reagents**

- Sodium pyrophosphate buffer (0.052 M, pH 8.3).
- Phenazine methosulphate (PMS) 186µM
• Nitroblue tetrazolium (NBT); 300µM.
• NADH 780µM
• Glacial acetic acid
• Sucrose
• n- butanol
• Ammonium sulphate (90%)
• Tris-HCl buffer (0.0025M; pH 7.4).

Procedure

Tissues were homogenized in 0.25M sucrose and differentially centrifuged before estimating the activity of SOD, an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and dialyzing against 0.0025M Tris HCl buffer (pH 7.4). Supernatant was used as the enzyme source.

The assay mixture contained 1.2ml of sodium pyrophosphate buffer 0.1ml of PMS, 0.3ml of NBT, 0.2ml of NADH, approx. diluted enzyme preparation and water in a total volume of 3ml. Reaction was initiated by the addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 1ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml of n-butanol. The mixture was allowed to stand for 10 minutes. The intensity of the chromogen in the butanol was measured at 560nm against butanol. A system devoid of enzyme served as control.

1 unit of enzyme is defined as the enzyme concentration required to inhibit the optical density at 560nm of the chromogen by 50% in 1 minute.
under the assay condition and expressed as specific activity in units per mg protein.

2.12.5 Estimation of Hydroxyproline in liver tissue

(Jamall et al., 1981).

Homogenized 200mg of liver tissue and the homogenate was mixed with 6N HCl and then hydrolysed at 110°C for 16 h. After cooling 100 µl of each sample was made up to 2ml with acetate–citrate buffer. Added 1ml of chloramine-T reagent (0.56% buffered) and kept for 20 min at room temperature. Then added 1ml of freshly prepared Ehrlich’s reagent and incubated at 60°C for 15 min and cooled. Read the absorbance at 560 nm against a reagent blank which contained the complete system without the tissue sample.