Chapter Two

Materials and Methods

2.1 Plant materials

2.1.a Coscinium fenestratum

Stem portion of Coscinium fenestratum was collected from Mannarghat area of Palghat district, Kerala during April-May. The plant was identified and authenticated by experts in the Post Graduate and Research Department of Botany, St. Thomas College, Pala, Kottayam. The collected material was chopped, air-dried at 35-40°C for a week and pulverized in electric grinder. The powder obtained was successively extracted in petroleum ether (60-80°C), benzene, chloroform, methanol and distilled water, by using Soxhlet extractor. The extracts were then made to powder with the help of rotary evaporator under reduced pressure. C. fenestratum stem yielded 0.8, 1.2, 2.8, 2.6 and 3.1 % w/w powdered extract with petroleum ether, benzene, chloroform, methanol and distilled water respectively. These powdered extracts were stored in refrigerator for further use.

2.1.b Curculigo orchioides

Rhizomes of C. orchioides were collected from Thattekkad, Ernakulam district of Kerala during June-July. The procedure for preparing various extracts of C. orchioides was the same as that for C. fenestratum. Rhizomes of C. orchioides yielded 0.6, 0.9, 2.2, 2.6 and 2.4 % w/w powdered extract with petroleum ether, benzene, chloroform, methanol and distilled water respectively.

2.2 Experimental Animals

Male albino rats of Sprague-Dawley strain weighing 100 - 120 g were used for the experiment. They were purchased from Small Animals’ Breeding Centre of Kerala Agriculture University, Mannuthy, Trichur. The animals were housed in
polypropylene cages kept in the animal house of School of Biosciences, Mahatma Gandhi University, Kottayam. They were maintained in controlled temperature (27 ± 2°C) and light cycle (12 h light and 12 h dark). The animals were fed with Amrut Laboratory animal feed manufactured and supplied by Nav Maharashtra Chakan Oil Mills Ltd, Pune. [Feed composition: crude protein: 21.98%, crude oil: 4.05%, crude fibres: 3.6%, ash: 7.8% and sand silica: 1.52%]. Food and water were provided *ad libitum*. They were given a week's time to get acclimatized to the laboratory conditions. Initial body weight of each animal was recorded.

2.3 Chemicals

- α-oxoglutaric acid
- 1-chloro-2,4-dinitrobenzene
- 2-dinitrophenyl hydrazine
- 4-amino antipyrine
- Acetic acid
- Acetone
- Alloxan*
- Ammonium molybdate
- Ammonium sulphate
- Aniline
- Benzene
- Bovine serum albumin
- Carbon tetrachloride

- Chloroform
- Cholesterol
- Chromotropic acid
- Citric acid
- Copper sulphate
- Cyclohexane
- Diethyl ether
- Dipotassium hydrogen phosphate
- Disodium phenyl phosphate
- DL-alanine
- Eosin
- Ethanol
- Ethylene diamine tetrachloride
- Ferric chloride
- Florisil
- Folin phenol reagent
- Formaldehyde
- Glucose-6-phosphate
- Glutathione (oxidised)*
- Glutathione (reduced)*
- Glutathione reductase*
- Glycine
- Glycyl glycine
- Haematoxylin
- Hydrochloric acid
- Hydrogen peroxide
- L-γ-glutamyl-4-nitroanilide*
- L-aspartic acid*
- Liquid paraffin
- Lithium acetate
- Magnesium chloride
- Methanol
- Metol (p-methyl aminophenyl sulphate)
- n-butanol
- Nicotinamide adenine dinucleotide (reduced)*
- Nicotinamide adenine dinucleotide phosphate (reduced)*
- Nitroblue tetrazolium
- Olive oil
- Ortho phosphoric acid
- Perchloric acid
- Petroleum ether
- Phenazine methosulphate
- Phenol
- Potassium dihydrogen phosphate
- Potassium monohydrogen phosphate
- Potassium ferricyanide
- Potassium hydroxide
- Sodium acetate
- Sodium arsenite
- Sodium bicarbonate
- Sodium chloride
- Sodium hydroxide
- Sodium metaperiodate
- Sodium potassium tartarate
- Sodium pyrophosphate
- Sodium pyruvate
- Sucrose
- Sulphuric acid
- t-Butyl hydroperoxide*
- Thiobarbituric acid
- Trichloro acetic acid
- Tris (Hydroxy methyl amino methane)
- Tris - HCl
- Vanillin*

* Chemicals obtained from Sigma Chemical Company, St Louis, MO, USA. All other chemicals were of analytical grade.

2.4 Instruments used

1. Soxhlet extraction apparatus - Boroglass

2. Rotary evaporator - Kika Werke RV 05 ST

3. Research centrifuge - R 24, Remi Equipments of India

4. Shimadzu UV - visible spectrophotometer UV 1601

5. Water bath - Kika Labortechnik HB 4 basic

6. Bruker Avance DPX 300 FT NMR spectrometer

7. Nicolet Magna 560 FT IR spectrometer

8. Shimadzu UV-3101 PC NIR scanning spectrophotometer
9. Shimadzu GCMS 5050 A spectrophotometer

10. Trinocular Research Microscope with 35 mm SLR camera; model Olympus 779A


12. Biotech master precision ultra thin rotary microtome

2.5 Experimental Design

2.5.1 Induction of Hepatopathy

Liver damage was induced in rats by administering CCl₄ sub-cutaneously (sc), at lower abdomen. A suspension of CCl₄ in liquid paraffin (LP) in the ratio 1:2 (v/v) at the dose of 1 ml/kg body weight (bw) of CCl₄ was administered twice a week, on every first and fourth days of all weeks.

2.5.2 Preliminary evaluation of anti-hepatotoxic effect of extracts of C. fenestratum

Rats were divided into 7 groups of 6 animals each as follows:

Group-I animals served as paired control and received sc administration of LP only at the dose of 3 ml/kg bw, twice a week.

Group-II animals constituted the hepatotoxic rats, and received sc administration of LP + CCl₄ twice a week as described earlier.

Group-III animals were the drug-administered ones which received sc administration of LP + CCl₄ as in Group-II. Besides, they received orally by intubation powdered petroleum ether extract of C. fenestratum stem (PECF), in a suspension of 1 ml water, at the dose of 75 mg/kg bw, daily for 90 days.

Group-IV animals were the same as in Group-III, but received benzene extract of C. fenestratum (BECF) at the same dose.
Group-V rats were same as that of Group-III, but received powdered chloroform extract of *C. fenestratum* (CECF) at similar dose.

Group-VI animals were also same as that of Group-III, but received powdered methanol extract of *C. fenestratum* (MECF) at the same dose.

Group-VII rats too were same as in Group-III, but received powdered aqueous extract of *C. fenestratum* (AECF) at similar dose.

**2.5.3 Preliminary evaluation of anti-hepatotoxic effect of extracts of *C. orchiodes***

The experimental design was same as in the case of 2.5.2. The only difference was that, instead of *C. fenestratum* stem extracts, rhizomes of *C. orchiodes* were used for the experiment.

**2.5.4 Hepatoprotective effect of *C. fenestratum* - a dose-dependent study with methanol extract**

The experimental design was the same as that of 2.5.2, with the difference that Group-III, IV and V were co-administered daily with methanol extracts of *C. fenestratum* stem at doses 40, 60 and 80 mg/kg bw respectively. These extracts were administered orally by intubation in a suspension of 1 ml of distilled water.

**2.5.5 Hepatoprotective effect of *C. orchiodes* - a dose-dependent study with methanol extract.**

The experimental protocol was the same as that of 2.5.4, except that rats of Group-III, IV and V were given daily, po, methanol extracts of *C. orchiodes* rhizomes at doses 40, 70, 100 mg/kg bw respectively.

**2.5.6 Hepatoprotective efficacy of *C. fenestratum* - a detailed study**

In this study, the animals were divided into 3 groups of 8 rats each as follows:
Groups-I and II were as in 2.5.1. But, Group-III rats received daily, po, methanol extract of *C. fenestratum* stem, at the dose of 60 mg / kg bw, daily in addition to CCl₄. The extracts were administered in a suspension of 1ml of distilled water. The body weight of animals was recorded weekly. Besides, the daily food consumption of each group of animals was also noted. For that, a measured amount of fresh food was replenished daily at 10:30 am, and their food intake of previous day was measured. From the daily food consumption, mean weekly food consumption was calculated.

### 2.5.7 Hepatoprotective efficacy of *C. orchioiides* - a detailed study

The experimental design was the same as in 2.5.6, except that methanol extract of *C. orchioiides* rhizomes at the dose of 70 mg / kg bw was used for this experiment instead of *C. fenestratum* extract.

### 2.5.8 Antioxidant effect of *C. fenestratum*

Experimental protocol was the same as in 2.5.6. But daily food consumption and weekly body weight were not noted, as food was given *ad libitum* in this case.

### 2.5.9 Antioxidant effect of *C. orchioiides*

The experiment was designed as in 2.5.8, with the only difference that methanol extract of *C. orchioiides* rhizomes was used for the experiment.

### 2.6 Collection of serum and tissue samples

The animals were kept starved on the 89th day. On the next day, after recording their body weight, the animals were sacrificed by decapitation and blood was collected by cutting the jugular vein. The blood was allowed to clot, and then centrifuged at 3000 rpm for 20 min. Sera samples were collected and kept standing on ice until required.
The liver and kidney in each case were dissected out, blotted off blood, rinsed in phosphate buffered saline (pH 7.4) and rushed to ice-cold containers. These liver, kidney and sera samples were used for the various 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) and gamma glutamyl transpeptidase (GGT). The liver and kidney homogenates/extracts as well as sera were subjected to estimations of total proteins, total lipids, triglycerides, phospholipids and cholesterol. For assessing the antioxidant efficacy of the drugs, the collected sera as well as liver and kidney homogenates were subjected to biochemical tests for thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione -S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPX). Activities of glucose-6-phosphate dehydrogenase (G-6-PD) in liver and lactate dehydrogenase (LDH) in serum were also evaluated.

2.8 **Histopathology**

A portion of the liver in 2.5.6 and 2.5.7 was fixed in 10% formalin (formalin diluted to 10% with saline), and proceeded for histopathology. The fixed liver tissues were embedded in paraffin wax and blocks were prepared. Serial sections of 5μ thickness were made from the blocks, and then stained with Haematoxylin and Eosin to evaluate the details of hepatic architecture in each group microscopically.
2.9 Statistical Analysis

The results were presented as mean ± SEM (standard error of the mean). Student's 't' test was used to analyse statistical significance. P values less than 0.01 were considered significant.

2.10 Percentage of hepatoprotection

The percentage of hepatoprotection offered by the different extracts was calculated by using the following formula:

\[ H = \left(1 - \frac{T - V}{C - V}\right) \times 100 \]

where

\[ T = \text{mean value of group treated with herbal preparation + CCl}_4 \]
\[ C = \text{mean value of group treated with CCl}_4 \]
\[ V = \text{mean value for pairfed control animals.} \]

2.11 Procedure for biochemical tests

ESTIMATION OF THE ACTIVITY OF ASPARTATE AMINOTRANSFERASE AND ALANINE AMINOTRANSFERASE (AST & ALT)\(^{226}\)

Reagents

- Phosphate buffer: 100 mM with α oxoglutaric acid (2 mM)
- Substrates (a): AST:100 mM L-aspartic acid was added to the above buffer.
  (b): ALT: Prepared as in (a) but using 200 mM in 1 N HCl
- 2,4 dinitro phenylhydrazine (1 mM) in 1 N HCl
- Sodium hydroxide (400 mM)
Procedure

1 ml of substrate was pipetted into two tubes, and placed in a water bath at 37°C for a few min to reach this temperature. To one (the test), 0.2 ml of serum was added and shaken gently to mix. Exactly one hour later in the case of AST, and after 30 min for A1P, with the test tubes still in the bath, 1.0 ml dinitrophenyl hydrazine was added to both, and 0.2 ml sera to the other (control). This was allowed to stand for 20 min at room temperature. 10 ml of 0.4 N sodium hydroxide was added to all tubes, mixed well and was read at 520nm after 5 min in a colorimeter. For standard, 1 ml of working standard was taken and made up to 1.2 ml with water and proceeded as above. For standard blank, 1.2 ml of water was taken and processed as above.

ESTIMATION OF THE ACTIVITY OF ALKALINE PHOSPHATASE (ALP)

Reagents

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

**Procedure**

2 ml of buffered substrate was measured into each of two test tubes and placed in a water bath at 37°C for a few min. Then to one (the test) 0.1 ml of serum was added and incubated for exactly 15 min. It was removed from the bath and added 0.8 ml of 0.5 N sodium hydroxide and 1.2 ml of 0.5 M sodium bicarbonate to both tubes, and then 0.1 ml of sera was added to the second tube (the blank). To both tubes, 1.0 ml of amino-antipyrine reagent and 1.0 ml of potassium ferricyanide were added. For standard, 1.1 ml of buffer and 1.0 ml of phenol standard containing 0.01 ml of buffer and 1.0 ml of water were taken. Then to both, sodium hydroxide, bicarbonate, amino-antipyrine and ferricyanide were added as above. Read at 520 nm.

**ESTIMATION OF THE ACTIVITY OF GAMMA GLUTAMYL TRANSPEPTIDASE (GGT)**

**Reagents**

- Buffer: Prepared by mixing tris (120 mM/L), magnesium chloride (90 mM/L), and glycyl glycine: pH 7.8.
- Substrate: 1.28 g L-γ-glutamyl-4-nitroanilide in 0.15 mM/1 N HCl and made to 100 ml with the acid.

**Procedure**

100 µl of serum/liver extract and 1 ml of buffer were warmed to 37°C. The reaction was initiated by adding 0.1 ml of substrate, and mixing well instantaneously. The reaction was monitored continuously at 405 nm in 1 cm cuvette so as to obtain the change in absorbance per min.
ESTIMATION OF PROTEIN

**Reagents**

- 0.5% copper sulphate
- 0.1 N sodium hydroxide
- 2% sodium carbonate in 0.1 N sodium hydroxide
- 1% sodium potassium tartarate
- Alkaline copper reagent: prepared by mixing 0.5 ml of copper sulphate, 0.5 ml of sodium potassium tartarate and 50 ml of sodium carbonate.
- Folin-phenol reagent: diluted 1:1 with distilled water.
- Standard protein solution (100 mg %) bovine serum albumin in 0.1 N sodium hydroxide.
- Working standard (100 μg / ml): prepared by diluting stock standard 1 in 10 using 0.1 N sodium hydroxide.

**Procedure**

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

**EXTRACTION OF TISSUE FOR LIPID ESTIMATION**

Lipids were extracted from tissues by the following method:

**Reagents**

- Alcohol: Ether mixture (3:1)
Chloroform: Methanol mixture (1:1)

Procedure

About 1g of the tissue was ground in a mortar using pestle with a little alcohol-ether mixture. The extract was then transferred to a test tube and thorough washing of pestle was made. The washing of 10ml was pooled.

The test tube was placed at 60°C in a water bath for 2h. The contents were initially agitated using a glass rod. The supernatant was then transferred to a 25ml standard flask. The process was repeated three times with alcohol-ether mixture. Then a little chloroform-methanol mixture was added, mixed and kept at 60°C for 1h. It was centrifuged and the supernatant was transferred to a standard flask. The process was repeated once more with chloroform-methanol mixture. The extract was then made up to 25ml with either of the two solvents.

ESTIMATION OF TOTAL LIPIDS

Reagents

- Concentrated sulphuric acid
- Vanillin (0.6%)
- Phospho-vanillin reagent: 200 ml of 0.6% vanillin in 800 ml of concentrated ortho-phosphoric acid.
- Standard: Olive oil
  - Stock: 1 g % in ethanol
  - Working standard: 400 mg % in ethanol

Procedure

Three test tubes were labelled as test, blank and standard. 0.1 ml of serum / lipid extract of tissues, 0.1 ml of distilled water and 0.1 ml of working standard
were taken respectively in three test tubes. 2.0 ml of conc. sulphuric acid was added to each tube and they were heated in boiling water bath for 10 min. It was cooled and then 0.1 ml of the digested mixture was pipetted from each tube and transferred them into another three tubes. 0.1 ml of phospho-vanillin reagent was added to all tubes and incubated at 37°C for 15 min. The optical densities were measured at 540 nm in a colorimeter.

**ESTIMATION OF CHOLESTEROL**

**Reagents**

- Ferric chloride (0.5% solution of FeCl₃.6H₂O in acetic acid)
- Sulphuric acid (AR)
- Standard:
  - Stock: Cholesterol (100 mg in 100 ml acetic acid)
  - Working standard: The stock standard was diluted 1 to 25 with ferric chloride-acetic acid reagent.
- Acetone-ethanol reagent (1:1)

**Procedure**

0.1 ml of serum was added to 10 ml of ferric chloride-acetic acid reagent in a stoppered centrifuge tube. It was mixed and allowed to stand for 10-15 min for the proteins to flocculate. 5 ml of clear supernatant fluid was transferred to a stoppered centrifuge tube after centrifugation. For standard, 0.1 ml of physiological saline was mixed with 10 ml of the cholesterol standard and 5 ml of ferric chloride-acetic acid reagent. 3.0 ml of sulphuric acid was added to all the three tubes, stoppered and mixed. It was then allowed to stand for 20-30 min. The unknown and the standard were read against the blank at 560 nm in a spectrophotometer.
ESTIMATION OF PHOSPHOLIPIDS

Reagents

- Trichloro acetic acid (TCA) : 5 % (w/v) in water.
- Digestion mixture : 50 ml of distilled water, 25 ml of conc. sulphuric acid and 25 ml of 70 % perchloric acid.
- Sodium acetate : 50 % of the trihydrate (w/v) in water.
- Ammonium molybdate : 2.5 % in water
- Metol (p-methyl aminophenyl sulphate) : 1 g in 100 ml of 3 % sodium hydrogen sulphate
- Standard :
  
  Stock solution : 1 mg phosphorus per ml (4.394 g anhydrous potassium dihydrogen sulphate in a litre of the solution containing 2 ml of conc. sulphuric acid.)
  
  Working standard : 1 ml = 4 µg of phosphorus. The stock standard was diluted 1 to 250.

Procedure

0.2 ml of serum / lipid extract of tissue was pipetted into a test tube and 5 ml of 5 % trichloro acetic acid was added, drop by drop while shaking. It was centrifuged to give a tightly packed precipitate. The supernatant was decanted off and the tube was kept inverted on a filter paper. 1.0 ml of the digestion mixture was added and heated gently until the liquid becomes colourless or almost so. It was allowed to cool and then 1.0 ml of distilled water was added and boiled for 15 seconds to convert pyrophosphate to orthophosphate. 1.0 ml of 50 % sodium acetate was added and
made to 10 ml with water. Then 1.0 ml of ammonium molybdate and 1 ml of metol were added. The mixture was mixed well, kept for 15 min and read with a red filter or at 700 nm against a reagent blank prepared by mixing 0.25 ml of conc. sulphuric acid, 1 ml of 50% sodium acetate, 1 ml of molybdate, 1 ml of metol and 8.75 ml of distilled water. As standard, 5.0 ml of working standard (containing 20 μg phosphorus) was taken and 0.25 ml of conc sulphuric acid, 1 ml of acetate, 1 ml of molybdate, 1 ml of metol and 3.75 ml of distilled water were added and read against the reagent blank.

**ESTIMATION OF TRIGLYCERIDES**

**Reagents**

- Chloroform
- Florisil
- Ethanollic potassium hydroxide (0.4%)
- Sulphuric acid (0.2 N)
- Sodium metaperiodate (0.05 M)
- Sodium arsenite (0.5 M)
- Chromotropic acid: 2 g chromotropic acid was dissolved in 200 ml of distilled water. Then 600 ml of conc. sulphuric acid was added slowly to 300 ml of distilled water which was already chilled in ice. This chilled and diluted acid was added to chromotropic acid solution.
- Standard: Glycerol (9 mg/ml)

**Procedure**

2 g of florisil was taken in a glass stoppered tube and 3 ml of chloroform was added. An aliquot of the extract/serum was layered on the top of the florisil,
mixed and chloroform was then added to this to a total volume of 10 ml. It was then stoppered, shaken intermittently for about 10 min and then filtered. After filtration, 1 ml of the filtrate was pipetted into each of the three tubes. 1.0 ml of working standard of glycerol (9 mg/ml) was pipetted into each of the three tubes. The solvent was evaporated at 60-70°C, and then 0.5 ml of ethanolic potassium hydroxide was added to two out of three tubes (saponified samples), and 0.5 ml of ethanol was added to the third tube (unsaponified sample). The tubes were then closed and kept at 60-70°C for 15 min. Then 0.5 ml of 0.2 N sulphuric acid was added to each tube and the tubes were then placed in a gently boiling water bath for 15 min to remove alcohol. They were then cooled to room temperature. 0.1 ml of sodium metaperiodate was added to each tube and kept for 10 min. 0.1 ml of sodium arsenite was then added and mixed. An yellow colour of iodine appeared and vanished within a few min. 5.0 ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 min. They were then cooled and the absorbance was read at 570 nm.

**ESTIMATION OF THE ACTIVITY OF LACTATE DEHYDROGENASE (LDH)**

**Reagents**

- Glycine reagent: 7.505 g of glycine and 5.85 g of sodium chloride were dissolved in about 900 ml of distilled water and made up to a litre.

- Buffered substrate: 125 ml of glycine buffer and 75 ml of 0.1 N sodium hydroxide were mixed, and then added into it 4 g 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 per dl of nicotinamide solution.

**Procedure**

10 ml of buttered substrate, 0.2 ml of NAD$^+$ solution and 0.2 ml of serum were pipetted into the cuvette. The mixture was mixed thoroughly and then readings were taken at 340 nm after 45 seconds, and then at an interval of 1, 2, 3 min. The mean absorbance change per min was determined.

**ESTIMATION OF THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD)**

**Reagents**

- Glucose-6 phosphate: 0.02 M
- NADP$: 1.5 \times 10^{-3}$ M
- Magnesium chloride: 0.1 M
- Glycyl glycine buffer: 0.04 M adjusted to pH 7.5

**Procedure**

Chilled liver tissue was homogenised with three volumes of 0.04 M glycyl glycine buffer. Homogenate was centrifuged at 2000 g at 0°C for 10 min. Supernatant was used as the enzyme source.

To 1.0 ml of the substrate in a quartz cell, added 0.1 ml of NADP$, 0.25$ ml of buffer and 0.2 ml of magnesium chloride. To this mixture was added 0.05 ml of the enzyme source and the absorbance was read immediately at 340 nm and at one min intervals thereafter.
ESTIMATION OF THIobarbitUric ACID REACTIVE SUBSTANCES

( TBARS )

Reagents

- Trichloro acetic acid - thiobarbituric acid - hydrochloric acid (TCA-TBA-HCl) reagent: 15 % ( w/v ) trichloroacetic acid and 0.375 % ( w/v ) thiobarbituric acid in 0.2 N hydrochloric acid.

Procedure

1 ml of tissue homogenate was combined with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance of the sample was read at 535 nm against a blank that contained no tissue homogenate. The extinction co-efficient of malondialdehyde is 1.56 x 105 m⁻¹.

ESTIMATION OF CONJUGATED DIENES ( CD )

Reagents

- Chloroform
- Methanol
- Cyclohexane

Procedure

To 1.0 ml of tissue homogenate, 5.0 ml of chloroform-methanol reagent ( 2 : 1, v/v ) was added, mixed thoroughly and centrifuged for 5 min. 3.0 ml of lower layer was then evaporated to dryness. To this, 1.5 ml of cyclohexane was added and the absorbance was read at 233 nm against a cyclohexane blank.
ESTIMATION OF REDUCED GLUTATHIONE (GSH) 

Reagents

- Alloxan (0.1 M)
- Phosphate buffer (0.5 M; pH 7.5)
- Sodium hydroxide (0.5 N)

Procedure

Tissue was homogenised in 0.5 M phosphate buffer (pH 7.5). The reaction mixture contained 50 μl of tissue extract, 50 μl of 0.1 M alloxan, 50 μl of 0.5 M phosphate buffer and 50 μl of 0.5 N sodium hydroxide.

The mixture was incubated at 25°C for 6 min. Reaction was stopped by addition of 50 μl of sodium hydroxide. The absorbance was noted at 305 nm in a quartz cuvette of 1 cm light path. Glutathione level is expressed as mg of glutathione per 100 g tissue.

ASSAY OF CATALASE (CAT)

Reagents

- Phosphate buffer (0.1 M; pH 7.0)
- Hydrogen peroxide (H₂O₂)

Procedure

Tissue was homogenised in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 1500 rpm for 10 min. The supernatant was used for the assay. The reaction mixture contained 2 ml of buffer, 20 (50) μl of tissue extract and 0.5 ml of H₂O₂. Change in optical density (at zero time, after 30 seconds and after 60 seconds) was measured at 240 nm.
ASSAY OF SUPEROXIDE DISMUTASE (SOD) 240

Reagents

- Sodium pyrophosphate buffer (0.025 M; pH 8.3)
- Phenazine methosulphate (PMS): 186 μM
- Nitroblue tetrazolium (NBT): 300 μM
- NADH: 780 μM
- Glacial acetic acid
- Sucrose
- n-butanol
- Ammonium sulphate: 90 %
- Tris-HCl buffer: 0.0025 M; pH 7.4

Procedure

Tissues were homogenised in 0.25 M 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 dialysing against 0.0025 M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of NADH, approximately diluted enzyme preparation and water in a total volume of 3.0 ml. Reaction was initiated by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to
stand for 10 min. The intensity of the chromogen in the butanol was measured at 560 nm against butanol. A system devoid of enzyme served as control.

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

ASSAY OF GLUTATHIONE-S-TRANSFERASE (GST)

Reagents

- K$_2$HPO$_4$ / KH$_2$PO$_4$ (phosphate buffer) : 0.5 M, pH 6.5
- 1-chloro-2,4- dinitrobenzene (CDNB) in 95 % ethanol : 25 nM
- Reduced glutathione (GSH) : 20 mM

The reaction mixture contained 200 µl of phosphate buffer, 20 µl of CDNB and 680 µl of distilled water. The mixture was incubated at 37°C for 10 min. Then 50 µl of GSH was added and mixed well. Afterwards, 50 µl of tissue extract was added and the change in absorbance was read at 340 nm. To the blank, 50 µl of water was added instead of tissue extract.

ASSAY OF GLUTATHIONE PEROXIDASE (GPX)

Reagents

- Buffer : Prepared by mixing tris-HCl (1 M) and EDTA (5 mM), pH : 8.0
- Glutathione (reduced) : 10 µl / ml
- Glutathione reductase : 10 µl / ml
- NADPH : 2 M
- T-butyldihydroperoxide : 7 mM
Procedure

The reaction mixture contained 100 µl of buffer, 20 µl of glutathione, 100 µl of glutathione reductase, 100 µl of NADPH, 10 µl of tissue extract and 660 µl of water. To the blank, 10 µl of water was added instead of tissue extract. The mixture was incubated at 37°C for 10 min. Then 10 µl of tert-buty1 hydroperoxide (7 mM) was added and the change in absorbance per minute was measured at 340 nm.

ASSAY OF GLUTATHIONE REDUCTASE (GR) 243

Reagents

- Tris-HCl : 1 M
- Ethylene diamine tetraacetic acid (EDTA) : 5 mM, pH 8.
- Glutathione oxidase (GSSG) : 20.421 mg/ml
- Reduced nicotinamide adenine dinucleotide phosphate (NADPH) : 2 mM

Procedure

50 µl of Tris-HCl-EDTA, 10 µl of serum/tissue homogenate and 790 µl of distilled water were mixed together, and incubated at 37°C for 10 min. To this mixture was added 100 µl of GSSG, and incubated for a further period of 10 min. Afterwards 50 µl of NADPH was added and absorbance was read at 340 nm, at 1 min intervals.