HEPATOPROTECTIVE STUDIES

Worldwide several people are suffering from hepatic damage induced by alcohol and drug abuse. The liver plays a major role in the digestion, metabolism and storage of nutrients. Today an increasing impact of liver disease and liver injury is being recognized. Especially liver injury due to pharmacological treatment plays a significant role was noticed by Gerbes et al. (2006) and reported that during recent years new insights have been brought into the pathomechanisms of liver injury. In certain cases this provides the basis for novel therapeutic strategies.

Carbon tetrachloride (CCl₄) is a toxic substance that is used to induce liver damage in rats, and according to Ohta et al. (1998). CCl₄ by itself does not have cytotoxic effects on the liver but its metabolic products are responsible for the toxicity. CCl₄ can damage a number of tissues particularly the liver and kidney of many species (Drill, 1952). Administration of CCl₄ can cause cirrhosis (Cameron and Karunaratne, 1936) and ultimately lead to hepatic carcinoma (Reuber and Glover, 1970).

It has been reported that CCl₄ intoxication results in the peroxidation of lipids and lipid membranes of rats. Ohta et al. (1997) observed an increase in lipid peroxidation (LPO) as a result of CCl₄ treatment. Therefore CCl₄, a hepatotoxin for evaluating hepatoprotective agents, is commonly used to induce liver damage by producing free radical intermediates.
Studies on hepatotoxicity induced by CCl₄ indicated that hepatic damage can probably be prevented by some herbal extracts. Herbs are also known to play a vital role in the management of various hepatic disorders (Venkateswaran et al., 1998).

Hepatoprotective studies by Mitra et al. (1998) showed that plants have active ingredients that are capable of free radical scavenging in living systems. The dependence of humankind on plants is as ancient as evolutionary history. Plants play a significant role in maintaining human health and improving the quality of human life. Many synthetic antioxidants, such as Butylated hydroxyanisole, butylated hydroxytoluene, t-butyldihydroquinone and propyl gallate, are used to retard lipid peroxidation (Wanita and Lorenz, 1996). However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compound (Park et al., 2001).

It was therefore decided to investigate the in vivo, hepatoprotective efficacy of the ethanolic extracts of M. azedarach and P. longum and the combined biherbal formulation made up of equal concentrations of M. azedarach and P. longum the traditional Indian medicinal plant, on CCl₄-induced hepatotoxicity and to elucidate the mechanism underlying these protective effects in rats.

It was also found important to determine the acute toxicity value (LD₅₀) of the Biherbal extract in mice and to determine histologically the chronic toxicity effects of Biherbal extract on the internal organs of rats.
Animals

Adult albino male rats of Wister strain (150-175g) and mice (27-35g) obtained from animal house in Madras Medical College, Chennai, India were used in the pharmacological and toxicological studies. The animals were maintained in well-ventilated room temperature with natural 12±1 hr day-night cycle in the propylene cages. They were fed with balanced rodent pellet diet from Poultry Research Station, Nandanam, Chennai, India and tap water ad libitum was provided throughout the experimental period. The animals were sheltered for one week prior to the experiment for getting acclimatized to laboratory temperature. The protocol has got the ethical committee clearance from IAEC (Institute Animal Ethical Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Chemicals

Ammonium thiocyanate, Ferric chloride, Ferrous sulphate (FeSO₄), Potassium ferricyanide [K₃ Fe(CN)₆], Potassium thiocyanate, Sodium carbonate, Sodium dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA), Thiobarbituric acid (TAB), Sodium nitroprusside, Sulphanilamide, Phosphoric acid, Naphtyl ethylene diamine dihydrochloride, Reduced Glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), Glutathione oxidised (GSSG), Epinephrine, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) were obtained from Sd Fine Chemicals Ltd., India. Bovine Serum Albumin (BSA), Folin-Ciocalteu reagent, 5,5-
Acute toxicity

Biherbal extract (100mg/ml), will be used as a stock solution in the entire study. A stock solution will be prepared by dissolving 100mg in 10 ml of 2% Tween 80 as a vehicle (Adjuvant). Either sex of albino mice, weighing 27 – 35 g will be used in both acute toxicity experiments.

1. Acute toxicity test was performed according to the World Health Organization (WHO) guideline (WHO, 2000) and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001).

2. An initial test will be carried out to determine the approximate lethal and non-lethal doses of the Biherbal extract according to Turner, 1965.

3. Eight groups of eight mice each will be used in the experiments. The Biherbal extract, in doses of 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 5.0g/kg body weight respectively will be administered orally, using intragastric tubes, to the animals as a single dose. The control group will be given an equal volume of 2% Tween 80. All the animals will be observed at the first, second, fourth and sixth hours and thereafter once daily over 14 days for (Rhodes, 1999), clinical signs of toxicity such as respiratory pattern, colour of body surfaces, frequency and nature of movement, marked involuntary contraction.
or seizures of contraction of voluntary muscle, and loss of reflex etc, and the number of dead mice will be recorded and used in the calculation of the acute toxicity value (LD$_{50}$). The mice will also be observed for other signs of toxicity, such as, excitation, tremors, twitches, motor coordination, righting reflex and respiratory changes. Twenty-four hours after the oral dosing the animals were lightly anaesthetized with ether and blood was withdrawn from the orbital plexus. They were then killed by cervical dislocation and vital organs were dissected out. Organ to body weight ratio, various haematological and biochemical variables were studied. Tissues of vital organs viz., lung, liver, kidney, spleen, heart and testis or ovaries were fixed in 10% buffered formalin for microscopic examination. Standard procedures were used for the analysis of haematological, biochemical and histological parameters.

**Chronic toxicity study**

Male and female Wistar rats were kept in polypropylene cages with maximum of four animals per cage at constant conditions. Temperature was set to 23 ± 2º C, relative humidity to 60 [+ or -] 10%. The animals were kept under artificial illumination with a photoperiod of 12 h. Water was available *ad libitum*. Each cage was labelled with the allocation of the animals to the respective trial group, and the diet to be used. Whenever more than one animal was kept in a cage, the animals obtained colour markings for differentiation. The cages were tended at regular intervals. The care and treatment of the rats were in accordance with the institutional ethical committee guidelines. All the animal experimentation was carried out with
the prior approval from CPCSEA. Basic diet was dry pellet feed (Sai Meera Foods, Bangalore).

Thirty six weanling rats (18 female, 18 male) with an average initial body weight of 100 (female rats) and 110g (male rats) were allocated to three groups. Each group consisted of six male and female randomly allocated animals. The first group served as control and obtained standard diet. The second group obtained diet with Biherbal extract 25mg/kg, the third group obtained diet with Biherbal extract 50mg/kg. After 3 months, four male and four female animals of each group were sacrificed for toxicity testing. The remaining two male and female animals were set to standard diet and observed for additional 4 weeks in order to detect deviations in spontaneous behaviour or growth parameters prior to sacrificing and submission to the same toxicological examination.

Twenty-four hours before the animals were sacrificed, blood samples were taken for the examination of haematological and biochemical parameters. Laboratory parameters such as blood glucose, total blood protein, ALT, AST and total cholesterol, red and white blood cell count and differential leucocyte count were carried out according to standard procedures. The animals were killed by cervical decapitation under ether anaesthesia. The major organs (liver, lungs, kidneys, heart, testis and ovaries) were removed and macroscopically examined for physiological abnormalities. The organs were then submitted to histological examination.
HEPATOPROTECTIVE EFFICACY STUDIES

Experimental protocol

The rats were divided into seven groups of six animals each and were given dose schedule as given below:

Group 1 : Animals were given a single administration of 0.5 ml vehicle 2% v/v aqueous Tween-80 p.o daily for 14 days. This group served as normal control.

Group 2 : Animals were given a single dose of 2ml/kg, p.o CCl4 in 2% v/v aqueous Tween-80 daily for 7 days.

Group 3 : Animals were pre-treated with 50 mg/kg, p.o of BHE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl4 on days 7 to 14.

Group 4 : Animals were pre-treated with 50 mg/kg, p.o of MAE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl4 on days 7 to 14.

Group 5 : Animals were pre-treated with 50 mg/kg, p.o of PLE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl4 on days 7 to 14.

Group 6 : Animals received 50 mg/kg, p.o Silymarin in 2% v/v aqueous Tween-80 daily for 14 days and administered with CCl4 on days 7 to 14. This group served as positive control.
Group 7: Animals received only 50 mg/kg, p.o of BHE in 2% v/v aqueous Tween-80 daily for 14 days.

On the 15th day after the experimental period the body weight of the animals were recorded. The animals were sacrificed after 12 hrs fasting under mild pentobarbitone anaesthesia. Blood was collected and the serum was separated by centrifuging at 3,000 rpm for 10 min. The serum was used for analysing various biochemical parameters. A portion of blood was used for certain biochemical estimation. Liver was excised from the animals, washed in ice-cold saline, and dried gently on the filter paper. The weight of the liver was taken. A 10% liver homogenate was prepared in Tris HCl buffer [0.1M pH 7.4]. The homogenate was centrifuged and the supernatant was used for the assay of various parameters.

**Biochemical analysis - Blood and Serum**

**Estimation of Glucose**

The blood glucose was estimated by Orthotoluidine method by Hyvarimen and Nikkila (1962).

**Reagents**

1. 10% TCA

2. Orthotoluidine reagent: 12.5gms of thiourea and 12.0 gms of boric acid were dissolved in 50ml of distilled water by heating. 75ml of Orthotoluidine (redistilled) and 375 ml of glacial acetic acid were mixed separately. These two solutions were mixed
and the total volume was made up to 500ml with distilled water. The reagent was left overnight in the refrigerator and filtered.

3. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

**Procedure**

To 0.1ml whole blood 1.0 ml of 10% TCA was added. The above mixture was centrifuged to precipitate the proteins. To 1ml of the above supernatant 4.0 ml of orthotoluidine reagent was added. Aliquots of standard containing different concentrations of glucose were made up to 1.0ml with water and 4.0 ml of orthotoluidine reagent was also added. Mixed well and kept in boiling water bath for 8mts. Cooled and read at 610nm against the blank which contained 1ml of water and 4.0 ml of orthotoluidine reagent. The values were expressed as mg/dl blood.

**Estimation of Urea**

Blood urea was determined by the method of Bousquet et al. (1971).

**Reagents**

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. DAM-TSC (Diacetyl monoxime –Thiosemicarbazide) reagent: 36mM Diacetyl monoxime and 61.7mM thiosemicarbazide were dissolved in100 ml of 2% glacial acetic acid.
4. Acid ferric reagent: 3.6ml sulphuric acid, 0.12mg of ferric chloride and 38.6ml Orthophosphoric acid were mixed and made up to 100ml with distilled water.

5. Standard Urea: 10mg of urea was dissolved in 100ml of distilled water.

**Procedure**

In a test tube 0.1ml of blood was taken. To that 3.3ml of water, 0.3ml of 10% sodium tungstate and 0.3ml of 2/3N sulphuric acid were added to precipitate the proteins. The mixture was centrifuged and to 1ml of the supernatant fluid 1ml of water, 0.4ml of DAM reagent and 1.6ml of acid ferric reagent were added and placed in a boiling water bath for 30 minutes. Aliquots of the standard urea and blank containing 2.0ml of water were also treated in the similar manner. After cooling the colour developed was read at 520nm using spectrophotometer.

The values were expressed as mg/dl.

**Estimation of Bilirubin**

Serum bilirubin was estimated by the method of Malloy and Evelyn (1937).

**Reagents**

1. Absolute methanol
2. 1.5% HCl
3. Diazo reagent
   Solution A: Dissolve 1gm of sulphanilic acid in 15ml conc.
   HCl and made up to 1 litre with distilled water.
   Solution B: 0.5gm of sodium nitrite dissolved in 100ml distilled
   water.
   Solution C: Mixed 10ml of solution A and 0.3ml of solution B.

4. Stock Standard: 10 mg of bilirubin was dissolved in 100ml of
   chloroform.

5. Working standard: Stock solution was diluted 1 in 5 times.

Procedure

In a test tube 0.2 ml of serum was taken, and 1.8ml of distilled water
was added. A blank was set up by adding 0.2ml of 1.5% hydrochloric
acid. To all the test tubes 1.0 ml of diazo reagent was added. Finally to
all the test tubes 2.5 ml of methanol, was added and allowed to stand at
room temperature for 30 mts in dark and read at 540nm. For the
standard curve, pipetted out various concentrations of bilirubin in to a
series of test tubes, made up the volume in all the tubes with 2.5ml of
methanol and added 1ml of diazo reagent was added and read
colorimetrically.

Estimation of Total Protein

Estimation of total protein was carried out by the method of Gornall
(1949).
Reagents

1. Biuret reagent. Weighed 1.50 gm of cupric sulphate and 6.0 gm of sodium potassium tartrate transferred to a dry 1 litre volumetric flask, and dissolved it in about 500 ml of water with constant swirling. Added 300 ml of 10 per cent sodium hydroxide. Finally the volume was made up to 1 litre with water, mixed, and stored in a cool dry place.

2. 22.6% sodium sulphate.

3. Std Protein solution: 400mgs of BSA was dissolved in 100 ml of distilled water.

Procedure

In to the test tube marked as “B” pipetted 2.0 ml of sodium sulphate solution. In to the “t” test tube, 0.5 ml. of serum and 9.5 ml of sodium sulphate were taken. Stoppered the tube and mixed thoroughly by inversion. From that mixture 2 ml was transferred to another test tube. Standard protein solution was also treated in the same way. Now into each of the three test tubes pipetted 8.0 ml of biuret reagent and mix thoroughly. Allowed these to stand for 30 minutes at room temperature. Using a photoelectric colorimeter, or spectrophotometer, the colour intensity was measured at 540 nm.

The amount of serum protein was expressed as g / dl.
Estimation of Albumin

The albumin present in the serum was estimated by the method of Reinhold (1953).

Reagents

1. Biuret reagent – Prepared as mentioned earlier
2. Ether
3. Sulphate – Sulphite Reagent: About 20.8g of sodium sulphate and 7.0g of sodium sulphite were dissolved in about 90ml water, with constant stirring. Then 0.02ml of conc. H₂SO₄ was added and made up to 100ml with distilled water.

Procedure

To 0.4ml of serum, 5.6ml of sodium sulphate-sulphite reagent and 3ml ether was added, the test tubes were stoppered and shaken well. Care should be taken not to shake more vigorously, otherwise the albumin may denature. The tubes were capped and centrifuged for 5mts. The pipette was inserted into the clear solution carefully below the globulin layer, and 3ml of solution was taken, 5ml of biuret reagent was added and incubated for 30 min at room temperature. The violet color was read at 540nm.

The amount of serum albumin was expressed as g / dl.
**Estimation of Globulin**

Albumin was subtracted from the total protein to obtain the amount of globulin. The amount of serum globulin was expressed as g/dl.

**Estimation of Cholesterol**

Estimation of serum cholesterol was carried out by the method of Wybenga *et al.* (1970).

**Reagents**

1. Cholesterol reagent. Dissolved 520 mg of Ferric per chlorate in 600 ml ethyl acetate, contained in a 1-liter Erlenmeyer flask. Placed the flask in an ice bath and cooled the contents to 4°C. Added gradually 400 ml of cold concentrated sulphuric acid in small portions, mixed after each portion is added, and not allowed the temperature to exceed 45°C.

2. Cholesterol standard: 200 mgs of cholesterol per 100 ml of glacial acetic acid.

**Procedure**

Added 50 µl of cholesterol standard and 50µl of serum was taken to the vials marked “Standard” and “Test,” respectively, and added 5 ml of cholesterol reagent mixed the contents of each vial thoroughly for at least 10 seconds. Cooled and the absorbance is read in the spectrophotometer at
560 nm against the blank, which contained 50 µl of glacial acetic acid and 5 ml of cholesterol reagent.

Cholesterol concentration was expressed as mg/dl of serum.

**Estimation of Triglycerides (TGL)**

The triglycerides were estimated by the method of based on the method of Rice and Vanhandle (1970).

**Reagents**

1. Chloroform : methanol mixture 2:1 (v/v)
2. Saturated sodium chloride
3. Activated silicic acid: Silicic acid was washed with 4N HCl and then distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
4. 0.4% potassium hydroxide in ethanol.
5. Sodium meta periodate 0.1 M: 2.149 g of sodium meta periodate was dissolved in 100 ml of water.
6. Sodium meta arsenate 0.5M: 6.496 g sodium meta arsenate was dissolved in 100 ml of water.
7. Chromotropic acid: 1.14 g of chromotropic acid was dissolved in 100 ml of distilled water and stored as a stock solution in a brown bottle. Before use this solution was mixed with 45 ml of sulphuric acid – water mixture in the ratio of 2:1.

8. 0.2N sulphuric acid

9. Tripalmitin standard: 100 mg of Tripalmitin was dissolved in 100 ml of chloroform in a standard flask. The stock solution was diluted to 1 in 10 times with chloroform.

**Procedure**

About 0.2 ml of serum was mixed with 9.8 ml of chloroform:methanol mixture and left for 30 min. It was centrifuged and 4 ml of the lipid extract was added to the tubes containing 8 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for an hour and centrifuged. The supernatant containing saline – methanol phase was discarded. The washed chloroform phase was filtered in to a dry tube. 200 mg of silicic acid was added to chloroform phase shaken vigorously and allowed to stand for 30 min. It was centrifuged. 0.5 ml of supernatant was taken, to which 0.5 ml of potassium hydroxide solution was added and the mixture was saponified in a water bath at 60°C for 20 min, to the above mixture, 0.5 ml of 0.2 N sulphuric acid was added and kept in a boiling water bath for 10 min. After cooling the tubes 0.1 ml of sodium meta periodate was added and allowed to stand for 10 min. The excess sodium meta periodate was reduced
by the addition of 0.1ml sodium meta arsenate, finally 0.5 ml of Chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 min. The colour developed was estimated at 540nm.

Triglycerides concentration was expressed as mg/ dl of serum.

**Estimation of Phospholipids (PL)**

The phospholipids content in serum was determined by the method of Zilversmith and Davis (1950).

**Reagents**

1. Ethanol- ether 3:1(V/V mixture)
2. 3% Ammonium molybdate
3. 3% Ascorbic acid
4. 70% Perchloric acid
5. Standard Phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100ml of water to give a concentration of 80 µg/ml.
6. Working Standard solution: A concentration of 8 µg/ml was prepared by diluting the stock solution in the ratio of 1: 10 with distilled water.
**Procedure**

To 1.0 ml of serum, 3.0 ml of the ethanol- ether mixture was added and mixed well. The protein precipitate was separated by centrifugation and the supernatant solvent was transferred to another tube. 0.1 ml of the lipid extract was dissolved in 1 ml of perchloric acid and digested on a sand bath till the solution becomes colourless. After cooling the solution was made up to 5.0 ml with double distilled water. In the standard 1 ml of working standard solution was taken. Blank contained 1 ml of water. To all the tubes 0.5 ml of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 mts. The blue color developed was read at 710 nm using a spectrophotometer. The values were expressed as mg/dl of serum after multiplication by factor 25.

**Serum Lipoproteins**

**Fractional precipitation of lipoproteins**

Lipoproteins were fractionated by a dual precipitation technique of Wilson and Spiger (1973).

**High-density lipoprotein (HDL)**

**Reagents**

1. Heparin – manganese chloride reagent: 3.167 g of manganese chloride was added to 1 ml solution of heparin containing 20,000 units. This mixture was made up to 8 ml with distilled water.
Procedure

About 2ml serum was added to 0.18ml heparin- manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged at 2000 rpm and maintained at 10°C for 30 minutes. The supernatant contained the HDL fraction. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

HDL- Cholesterol concentration was expressed as mg/dl of serum.

Low-density lipoprotein (LDL)

Reagents

1. Sodium- dodecyl sulphate(SDS): 10% solution of SDS was prepared in 0.15 M sodium chloride and the pH was adjusted to 9 with sodium hydroxide.

Procedure

To 2ml of serum was added to 0.15ml of sodium-dodecyl sulphate. The contents were mixed well and incubated at 37°C for 2 hrs. The contents were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 mts. The VLDL aggregated as pelleted. The supernatant contained the HDL and LDL fractions. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

LDL-Cholesterol concentration was expressed as mg/dl of serum.
Very low-density lipoprotein (VLDL)

The pelleted portion contains the VLDL cholesterol. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

VLDL- cholesterol was expressed as mg/ dl.

Assay of serum Aspartate amino transferase (AST) (Glutamate oxaloacetate transaminase, E.C.2.6.1.1)

Assay of serum aspartate amino transferase was carried out by the method of Reitman and Frankel (1957).

Reagents

1. Phosphate buffer - 0.1M, pH 7.4.
2. Substrate: 2.66 gms of DL aspartate and 38 mg of α-keto glutartate were dissolved in 20.5ml of 0.1N NaOH with gentle heating. This was made up to 100 ml with buffer.
3. 2,4-Dinitrophenyl hydrazine reagent (DNPH): 1.0mM DNPH in 2.0 N HCl.
4. 0.4 N NaOH
5. Standard Pyruvate: 11 mg of sodium pyruvate was dissolved in 100ml of phosphate buffer. This contained 1 µmole pyruvate/ml.
**Procedure**

In different tubes 1.0 ml of the buffered substrate was added. To one tube 0.1ml of serum was added and incubated at 37°C for 1 hr. Then 1ml of DNPH reagent was added to arrest the reaction. To the ‘Blank ‘tube 0.1ml of serum was added only after the addition of DNPH reagent. The tubes were kept aside for 15 minutes, and then 0.4 N NaOH was added and read at 520 using the spectrophotometer. Aliquots of standard were also treated in the same manner.

Enzyme activity was expressed as U / Litre.

**Assay of serum Alanine amino transferase (ALT) (Glutamate pyruvate transaminase, E.C.2.6.1.2)**

The reagents and method used were same as those used for the assay of aspartate amino transferase except for the substrate solution and incubation time was reduced for 30 minutes.

**Reagents**

1. Substrate: 1.78 gms of DL alanine, 38 mg of α-ketoglutarate were dissolved in phosphate buffer, and 0.5ml of 0.1 N NaOH was added and the volume was made up to 100ml with buffer.

Enzyme activity was expressed as U / Liter.
Estimation of Alkaline Phosphatase (ALP) (ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.1)

The serum alkaline phosphatase was assayed by the method of Kind and King (1954).

Reagents

1. Carbonate – bicarbonate buffer 0.1M, pH 10.0: 6.36gm of sodium carbonate and 3.36gm of sodium bicarbonate were dissolved in 1000 ml of distilled water.

2. Substrate 0.01M: 254mg of disodium phenyl phosphate was dissolved in 100 ml of water.

3. 0.5 N Sodium bicarbonate

4. 0.5N NaOH

5. 4-Amino-antipyrine (0.6%): 0.6 g was dissolved in water and made to 100 ml.

6. Potassium Ferricyanide (2.4%): 2.4 g was dissolved in water and made to 100 ml.

7. Stock Phenol Standard (1 mg/ ml): Pure crystalline phenol, 1 g was dissolved in and made to 1 liter with 0.1 N HCl.

8. Working Phenol Standards (0.01 mg/ml) 1 ml, stock standard was diluted to 100 ml with 0.1 N HCl.
Procedure

For the enzyme estimation four test tubes were taken and they were marked as “B” (Blank), “T” (Test), “C” (Control) and “S” (Standard). In the “T” test tube, 1 ml buffer were added to 1 ml of substrate and warmed at 37°C for three min. Then 0.1 ml of serum were added and mixed. The solution was incubated at 37°C for 15 min. To the incubated solution 0.8 ml of Sodium hydroxide and 1.2 ml of sodium bicarbonate were added. Then 1 ml 0.6% 4-Amino-antipyrine was added and mixed. Finally 1 ml Potassium Ferricyanide was added and mixed. The Control was treated same as test, except that serum was added after the NaOH addition. Aliquots of standard was also treated in the similar manner. Blank was set up same as standard, but water is substituted for phenol. The colour developed was measured at 620 nm using the colorimeter.

The enzyme activity was expressed as IU/litre.

Estimation of Acid Phosphatase (ACP) (ortho-phosphoric monoester hydrolase, E.C.3.1.3.2)

The serum acid phosphatase was assayed by the method of Kind and King (1954).

Reagents

1. Citrate Buffer 0.1M, pH 4.8.
2. Substrate 0.01M: 254mg of disodium phenyl phosphate was dissolved in 100 ml of water.

3. 0.5 N Sodium bicarbonate

4. 0.5N NaOH

5. 4-Amino-antipyrine (0.6%): 0.6 g was dissolved in water and made to 100 ml.

6. Potassium Ferricyanide (2.4%): 2.4 g was dissolved in water and made to 100 ml.

7. Stock Phenol Standard (1 mg/ml): Pure crystalline phenol, 1 g was dissolved in and made to 1 liter with 0.1 N HCl

8. Working Phenol Standards (0.01 mg/ml): 1 ml, stock standard, diluted to 100 ml with 0.1 N HCl.

Procedure

Four test tubes were taken and they were marked as “B” (Blank), “T” (test), “C” (Control) and “S” (Standard). In the “T” test tube, 1 ml of buffer was added to 1 ml of substrate warmed at 37°C for three min. Then 0.1 ml of serum was added and mixed. The solution was incubated at 37°C for one hour. To the incubated solution 1.0 ml of Sodium hydroxide and 1.0 ml of sodium bicarbonate, 1 ml 0.6% 4-Amino-antipyrine was added and mixed. Finally 1 ml Potassium Ferricyanide was added and mixed. The Control was treated same as test, except that serum was added after the NaOH. Aliquots of standard were made up of 1.1 ml in alkaline buffer and treated similarly.
Blank was set up same as standard, but water was substituted for phenol. The colour developed was measured at 620 nm using the colorimeter.

The enzyme activity was expressed as K.A units.

**Estimation of Lactate dehydrogenase (LDH) (L-lactate:NAD oxidoreductase, E.C.1.1.1.27)**

The Lactate dehydrogenase present in the serum was assayed by the method of King (1965).

**Reagents**

1. Glycine buffer 0.1M, pH 8.5: 750.5mg of glycine and 585mg of NaCl were dissolved in 100ml of distilled water.
2. Buffered Substrate: 125ml of glycine buffer, 75ml of 0.1N NaOH and 4.0g of lithium lactate were added, mixed well and kept in cold room.
3. Nicotinamide adenine dinucleotide (NAD\(^+\)): 10mg of NAD\(^+\) was dissolved in 2.0ml of water.
4. 2, 4- Dinitrophenyl hydrazine (DNPH) 0.02%: 20mg of DNPH was dissolved in 100ml of 1N HCl.
5. 0.4N NaOH
6. Standard: 11mg of sodium pyruvate was dissolved in 100ml of buffer. This contained 1 µ mole of pyruvate/ ml.
Procedure

Pipetted out 1.0ml of the buffered substrate and 0.1ml of serum into two tubes and 0.2ml of distilled water was added to the blank, to the test, 0.2ml of NAD⁺ solution was added and shaken well. The tubes were incubated at 37°C for 15mts. Exactly after that time the reaction was stopped by adding 1.0ml of DNPH solution. NAD⁺ was added to the control tubes. It was left at 37°C for another 15mts. About 5.0ml of 0.4N NaOH was added and the color developed was read at 420nm within 5mts. A set of standards were also treated in a similar manner.

The enzyme activity was expressed as U/L.

Estimation of Acetyl cholineesterase (ACE) (Acetylcholine acetylhydrolase, E.C.3.1.1.7)

Determination of Acetylcholine esterase was carried out by the method of Biggs et al. (1958).

Reagents

1. Stock buffer; Dissolved 12.37 gms of Sodium barbitone, 1.361 gms of Potassium dihydrogen phosphate and 175.35 gms of sodium chloride in water and made to a litre

2. Stock–buffer indicator solution: Dissolve 100mg of bromothymol blue in 2 ml of 2 N NaOH and washed in to the 1 litre flask with 150 ml of stock buffer and diluted to 950 ml
with water. Adjust the pH to 8.0 by adding 16 ml of HCl and then diluted to 1 litre with water.

3. Working–buffer indicator solution: Diluted 476.2 ml of the Stock–buffer indicator solution to 1 litre with water.

4. Standard acetyl choline bromide solution: (15 %) 15 gms acetyl choline bromide dissolved in 100 ml of water.

5. Acetic acid, 0.15 N: Diluted 1 in 10 for use.

**Procedure**

Measured 4.2 ml of Working–buffer indicator solution in to a suitable tube and added 0.1 ml of serum and 0.2 ml of substrate. Mixed thoroughly and read the absorbance at 620 nm. Incubated at 37°C for 30 minutes and read again. Units of enzyme activity were expressed as micromoles of acetic acid liberated from acetylcholine by 1 ml of serum in 30 minutes. To obtain the calibration curve a series of dilutions of acetic acid to water was prepared which corresponds to 0–150 units of enzyme activity in steps of 10 units. A series of these tubes containing 2 ml of stock–buffer indicator solution, 1.4 ml of water, 0.1 ml of normal serum and 1 ml of above series of diluted acetic acid was added and treated as the same way as test. A standard curve was plotted from which units of activity was calculated.

Enzyme activity expressed as U/l.
Estimation of Gamma glutamyl transferase (\(\gamma\) GT) (5 Glutamyl peptide amino acid 5 glutamyl transferase, E.C. 2.3.2.2)

The assay of \(\gamma\)-Glutamyl transferase was carried out by the method of Szasz (1969).

**Reagents**

1. **Substrate:** 30.4 mg of L-\(\gamma\) glutamyl-p-nitro anilide was added to 10 ml of water, heated to dissolve at 50- 60\(^\circ\) C.
2. **Tris-HCl buffer:** 0.1M, pH 8.5.
3. **Glycyl-glycine:** 13.2 mg was dissolved in 10 ml of water.
4. **Standard:** 13.8 mg of p-nitro anilide was dissolved in 100ml of distilled water.
5. **10 \% acetic acid**

**Procedure**

Into three test tubes marked as “B”, “C” and “T” 1.0 ml of buffer and 2.2 ml of glycyl glycine was added. 0.5ml of substrate solution was added in the “B” and “T” test tubes. About 0.2ml of enzyme was added to “C” and “T” test tubes. Incubated at 37\(^\circ\)C for 30 minutes and 1.0 ml of 10\% acetic acid was added to all the test tubes. 0.5ml of substrate solution was added in to the “C” tube and the absorbance was measured at 410 nm.

Enzyme activity was expressed as U/Liter.
Estimation of 5'-Nucleotidase (5'-NT) (5'-Ribonucleotide phosphohydrolase, E.C. 1.1.1.42)

The 5'-Nucleotidase was assayed by the method of Luly et al. (1972), with slight modification.

Reagents

1. Tris HCl buffer: 184 mM pH 7.5
2. Magnesium sulphate : 50mM
3. Potassium chloride: 650mM
4. EDTA :1mM
5. 2.5% Ammonium molybdate
6. 5’ Adenosine monophosphate (5’AMP) 40mM
7. ANSA - 500mg of Amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium meta bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.
8. Stock Standard Phosphorous: 35.1 mg potassium dihydrogen phosphate in 100ml of water.
9. Working Standard Phosphorous: Dilute the stock standard 1 in 10 times which contain 8mcg/1ml.
**Procedure**

The assay medium contained 1.0ml Tris HCl buffer, 0.1ml of Magnesium sulphate, 0.1ml potassium chloride, 0.1ml of EDTA and 0.1ml of 5’AMP. The reaction was initiated by addition of 0.2ml of enzyme preparation and incubated at 37°C for 15mts. The reaction was terminated by the addition of 2.0 ml of 10% TCA. After centrifugation the phosphorus in the supernatant was estimated as discussed earlier in the phospholipids.

Enzyme activity was expressed in U/ litre.

**Assay of Isocitrate Dehydrogenase (ICD) (Isocitrate NADP⁺ oxidoreductase, E.C. 1.1.1.42)**

Assay of Iso citrate dehydrogenase was carried out by the method of Belljoice and Baron (1960).

**Reagents**

1. 0.15 M Sodium chloride
2. Tris-HCl buffer 0.1 M in 0.15 M Sodium chloride pH 7.5.
3. Buffered substrate: Dissolved 1.845 g of trisodium isocitrate in 100 ml of Tris buffer.
4. NADP - 10 mg /ml in 0.15 M Sodium chloride
5. Reduced NADP - 10 mg /ml in 0.15 M Sodium chloride
6. Manganous chloride - 0.03 M in 0.15 M Sodium chloride
7. Standard $\alpha$-oxoglutaric acid - Stock containing 70 mg/100ml water. Diluted this 1 in 10 for use.

8. EDTA-Dissolved 5.6 gms in 100ml of water by adjusting the pH 8.0 using NaOH pellets.

9. 2,4-Dinitrophenyl hydrazine - Dissolved 19.8 mg 2,4-Dinitrophenyl hydrazine in 1 N HCl and made upto 100ml with water.

10. 0.4 N NaOH.

**Procedure**

The assay medium contained 0.5ml $\alpha$-oxoglutaric acid, 0.1ml of MnCl$_2$ solution and 0.1ml of NADP to all the three test tubes marked as “B”, “C” and “T”. To the control tube 0.033ml of NADPH$_2$ was added. About 0.3ml of 0.15 M NaCl$_2$ was added to all the tubes. The reaction was initiated by addition of 0.2ml of enzyme preparation and incubated at 37°C for 1hr. At the end of incubation period added 1.0 ml of EDTA to the blank and standard test tubes and 1ml of DNPH to all the test tubes immediately. Allowed to stand for 20 minutes, and then added 10 ml of 0.4n sodium hydroxide. Stood for 15 minutes and read against the reagent blank at 390nm.

Enzyme activity was expressed as IU / L.
Biochemical analysis - Liver tissues

Estimation of Total lipid

Total lipids were extracted from the liver tissue according to the method of Folch et al. (1957).

Procedure

The tissues were washed with saline and dried with a filter paper. A weighed amount of tissue (500mg) was homogenized with 7.0 ml of chloroform: methanol (1:2) mixture in a potter Elvehjem homogeniser and filtered through a whatman No 1 filter paper into a conical flask. The residue after filtration was scrapped and homogenized with 10ml chloroform-methanol mixture 2:1 v/v and the resulting filtrate was evaporated to dryness. The weight of the flask with and without the dried lipid was recorded and the differences in weight gave the total lipid content of the tissues.

The total lipids were expressed as mg/ gm of wet tissue.

“Folch” wash

Reagents

1. 0.1N potassium chloride

2. Folch’s reagent: 0.1N HCl: Methanol: Chloroform (10:10:1)

The lipid extract from above procedure was redissolved in 2ml of Folch’s reagent and 1.0ml of 0.1N potassium chloride was added and the
contents were shaken well. The upper aqueous phase containing ganglioside and other water soluble compounds were separated. The lower phase containing neutral and phospholipids in chloroform was again washed 3 times with 2.0ml of Folch’s reagent and the upper aqueous phase was aspirated. The lower chloroform phase was made up to known volume. Aliquots were taken for analysis of cholesterol, triglycerides and phospholipids.

**Estimation of Cholesterol**

The total cholesterol present in the tissue homogenate after Folch’s wash was estimated by the method of Wybenga *et al.* (1970), as mentioned earlier.

The total cholesterol was expressed as mg/ gm of wet tissue.

**Estimation of Triglycerides (TGL)**

The triglycerides present in the tissue homogenate after Folch’s wash was estimated by the method of Rice and Vanhandle (1970) as mentioned earlier.

The triglycerides was expressed as mg/ gm of wet tissue.

**Estimation of Phospholipids**

The tissue phospholipids were estimated by the method of Rouser *et al.* (1970).
Reagents

1. 3% Ammonium molybdate
2. 3% Ascorbic acid
3. 70% Perchloric acid
4. Standard Phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100ml of water to give a concentration of 80 µg/ml
5. Working Standard solution: A concentration of 8 µg/ml was prepared by diluting the stock solution from 1 to 10ml-distilled water.

Procedure

0.1ml of the lipid extract was dissolved in 1ml of perchloric acid and digested on a sand bath till the solution becomes colorless. After cooling the solution was made up to 5.0ml with double distilled water. In the standard 1ml of working standard solution was taken. Blank contained 1ml of water. To all the tubes 0.5ml of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 mts. The blue color developed was read at 710 using a photochem colorimeter. Expressed as mg/gm of wet tissue after multiplication by factor 25.
Estimation of Glycogen

The liver tissue glycogen was extracted and estimated by the method of Morales et al. (1973).

Reagents

1. 30% potassium hydroxide solution
2. Absolute alcohol
3. Anthrone reagent: 0.2% anthrone in con. Sulphuric acid was prepared just before use.
4. 1.0M Ammonium acetate solution
5. Saturated ammonium chloride
6. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

Procedure

The alkali extract of the tissue was prepared by digesting 50mg of fresh tissue with 3.0 ml of 30% potassium hydroxide solution in boiling water bath for 15 min. The tubes were cooled and mixed with 5.0ml of absolute alcohol and a drop of 1.0M ammonium acetate to precipitate glycogen and left in the freezer overnight for complete precipitation. Glycogen was collected by centrifugation at 2000g for 20 mts. The precipitate was dissolved in water with the aid of heating in a boiling water bath for 5 mts. Aliquots of glycogen solution were taken up after suitable dilution and 4.0ml of anthrone
reagent was added heated in the boiling water bath cooled the tubes in an ice bath. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20mts. After cooling the absorbance was read at 640nm in spectrophotometer against a water blank treated in a similar manner. Standard glucose solutions were also treated similarly.

The glycogen content was calculated from the amount of glucose present in the sample and was expressed as mg/g fresh tissue.

**Estimation of Protein**

The liver tissue protein was estimated by the method Lowry *et al.* (1951) as mentioned earlier in Chapter I.

The protein contents of the tissues were expressed as mg/g of fresh tissue.

**Estimation of Aspartate Aminotransferase (AST) Glutamate oxaloacetate transaminase, E.C.2.6.1.1)**

The Aspartate Aminotransferase in the tissue homogenate was estimated by the method of Reitman and Frankel (1957) as mentioned earlier.

The enzyme activity is expressed as µ moles of pyruvate liberated/min/mg protein.
Estimation of Alanine Aminotransferase (ALT) Glutamate pyruvate transaminase, E.C.2.6.1.2)

The alanine aminotransferase in the tissue homogenate was estimated was estimated by the method of Reitman and Frankel (1957) as mentioned earlier.

The enzyme activity is expressed as µ moles pyruvate liberated/min/mg protein.

Estimation of Alkaline Phosphatase (ALP) ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.1)

The Alkaline Phosphatase present in the tissue homogenate was estimated by the method of Kind and King (1954) as mentioned earlier.

The results were expressed as µ- moles of phenol liberated /min/ mg protein.

Estimation of Acid Phosphatase (ACP) (ortho-phosphoric monoester hydrolase, E.C.3.1.3.2)

The Acid Phosphatase present in the tissue homogenate was estimated by the method of Kind and King (1954) as mentioned earlier.

The results were expressed as µ- moles of phenol liberated /min/ mg protein.
Estimation of Lactate Dehydrogenase (L-lactate: NAD oxido-reductase E.C.1.1.1.27)

The liver tissue Lactate dehydrogenase was estimated by the method of King (1965) as mentioned earlier.

The enzyme activity is expressed as µ moles of pyruvate liberated /min/ mg protein.

Estimation of Gamma Glutamyl Transferase(5 Glutamyl peptide aminoacid 5 glutamyl transferase, E.C. 2.3.2.2)

The Gamma Glutamyl transferase in the liver tissue was assayed by the method of Szaz (1969) as mentioned earlier.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 micro mole of p- nitroanilide/minute under incubation conditions.

Estimation of 5'-Nucleotidase (5'-Ribonucleotide phosphohydrolase, E.C. 1.1.1.42)

The 5'-Nucleotidase present in the tissue homogenate was estimated by the method of Luly et al. (1972) as mentioned earlier.

The results were expressed as µ moles of phosphorous liberated/minute mg of protein.
Estimation of Xanthine Oxidase (xanthine:NAD+ oxidoreductase, E.C. 1.17.32)

Estimation of Xanthine oxidase was carried out by the method of Fried and Fried (1957).

Reagents

1. Phosphate buffer- 0.1 M, pH 8.2
2. EDTA - 10 mM
3. Gelatin -1 %
4. Phenazine metho sulphate (PMS): The solution of PMS in the phosphate buffer at the concentration of 0.2 mg / ml was prepared at the time of the assay and was protected from light
5. Nitroblue tetrazolium salt (NBT): NBT solution containing 5 mg/ ml was prepared in phosphate buffer just before use and was protected from light
6. 1mM Xanthine solution: prepared in phosphate buffer just before use.

Procedure

To 0.6ml of buffer , 0.4ml of EDTA , 0.4 ml of gelatin , 0.1ml of PMS and 0.3ml of NBT were added in a test tube . To this 0.2ml of enzyme was added and incubated at room temperature for 5 minutes. Then 0.5ml of
buffer and 0.5ml of substrate were added and the increase in the optical density was measured in 532 nm at 2 minutes interval for 10 minutes.

The activity of enzyme was expressed as Unit / mg of protein. One unit corresponds to the amount of the enzyme required to bring about change in optical density 0.01/ min.

**Estimation of Lipid peroxidation**

**Basal**

Lipid peroxidation in the liver homogenate was assayed by the method of Ohkawa and Yagi (1979).

**Reagents**

1. 8.1% Sodium dodecyl sulphate (SDS)
2. 0.8% Thiobarbituric acid (TBA)
3. 20% Acetic acid
4. 15:1 v/v n- Butanol: Pyridine mixture

**Procedure**

The reaction mixture contained 0.2 ml of liver homogenate, 1.5 ml of TBA, 0.2ml SDS, 1.5ml of acetic acid and 0.8ml of distilled water. The above solution was kept in the boiling water bath at 90°C for 1 hr and cooled in tap water. After cooling 1ml of distilled water and 5ml of mixture of n-butanol: pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation at
4000 rpm for 10 min, the organic layer was taken and its absorbance was read at 532 nm.

The lipid peroxide concentration was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

**Hydrogen Peroxide induced Lipid peroxidation**

Hydrogen Peroxide induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

**Reagents**

1. 0.15M Tris–HCl buffer, pH 7.4
2. 10mM KH₂PO₄
3. 10% Trichloroacetic acid (TCA)
4. 10mM Hydrogen peroxide
5. 1% Thiobarbituric acid (TBA)

**Procedure**

The peroxidation system consists of 1.4 ml of Tris buffer, 0.2ml of H₂O₂, 0.2ml of KH₂PO₄ and 0.2ml of homogenate. The tubes were incubated at 37°C with constant shaking for 20 minutes. The reaction was stopped by the addition of 1.0 ml of 10 % TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.
The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

**Ascorbate induced Lipid peroxidation**

Ascorbate induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

**Reagents**

1. 0.15M Tris-HCl Buffer, pH 7.4
2. 10 mM KH$_2$PO$_4$
3. 10% Trichloroacetic acid (TCA)
4. 1mM Ascorbic acid
5. 1% Thiobarbituric acid (TBA)

**Procedure**

The peroxidation system consisted of 1.4 ml of Tris buffer, 0.2ml of ascorbate 0.2ml of KH$_2$ PO$_4$ and 0.2ml of homogenate. The tubes were incubated at 37 °C with constant shaking for 20 minutes. The reaction was arrested by the addition of 1.0 ml of 10 % TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.

The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.
**FeSO₄ induced Lipid peroxidation**

FeSO₄ induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

**Reagents**

1. 0.15M Tris-HCl Buffer, pH 7.4
2. 10 mM KH₂PO₄
3. 10% Trichloroacetic acid (TCA)
4. 10mM ferrous sulphate
5. 1% Thiobarbituric acid (TBA)

**Procedure**

The peroxidation system consists of 1.4 ml of Tris buffer, 0.2ml of FeSO₄ 0.2ml of KH₂PO₄ and 0.2ml of homogenate. The tubes were incubated at 37°C with constant shaking for 20 minutes. The reaction was arrested by the addition of 1.0 ml of 10% TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.

The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.
Lipid peroxidation products

Estimation of Conjugated dienes (CD)

Dienes conjugates were estimated by the method of Klein and Klein (1983).

Extraction of Lipids

Lipids were extracted from the liver homogenate with chloroform/methanol (2:1) according to the method described by Folch et al. (1957) with the modification.

Procedure

The lipid in chloroform was isolated and dried. Aliquots of lipid extracts were evaporated to dryness. The lipid residue was suspended in methanol and the absorbance at 213 nm and 233 nm were measured against the blank. The extent of peroxidation is determined by the measurement of conjugated diene content which is arrived by computing the ratio of absorbance at wavelengths 233 nm and 213 nm.

Units expressed as $\Delta^{233}$/mg of protein.

Estimation of Lipid hydro peroxide (LOOH)

Lipid hydro peroxide (LOOH) assay was performed essentially as described by Nourooz-Zadeh et al. (1996) with minor modifications.
Reagents

1. Triphenylphosphine (10mMol/L)

2. 90% Methanol

3. FOX2-reagent: FOX2-reagent was prepared by dissolving 38 mg Xylenol Orange 440 mg of Butylated hydroxy toluene in 450 ml HPLC-grade methanol with stirring. 49 mg Ammonium ferrous sulphate dissolved in 50 ml of 250-mmol/l of sulphuric acid was added to the methanol solution.

Procedure

Aliquots of sample were incubated for 30 minutes, at 20-25°C in 10 µL of triphenylphosphine to remove hydroperoxides and to generate a blank, and 90 µL of tissue samples were incubated in 10 µl methanol to generate a test sample. Both the blank and the test sample were mixed with 900 µL FOX2 reagent and incubated for 30 minutes, at 20-25°C. After centrifugation at 12000 rpm for 10 min, the absorbance of the supernatants was monitored at 560 nm The hydroperoxide concentration of each sample was calculated from the difference between the absorbance of the blank and test samples.

The LOOH content of the sample was expressed as µ moles of LOOH liberated / min / mg of Protein.
Assay of Nitric oxide end products (NO)

Nitric oxide end products like Nitrite and Nitrate determination was carried out by the method of Miranda et al. (2001).

Reagents

1. 10% TCA
2. Griess reagent: 1% sulfanilamide in 1N HCl, 15% N-1-naphtylethylenediamine dichloride
3. 50 mM Potassium phosphate buffer
4. Vanadium (III) chloride (8 mg/ml)

Procedure

Approximately 4.0 ml of tissue homogenate was treated with 2.5 ml of 10% TCA solution, and centrifuged at 3000 rpm for 30 min. After centrifugation at room temperature, 100µl of supernatant was applied to a clean tube, 100 µL vanadium (III) chloride was added to each tube (for reduction of nitrate to nitrite) and this was followed by addition of 450µl of Griess reagent. After mixing well, all tubes were left in a dark place for 30 minutes at room temperature. At the end of the reaction time, the absorbance was measured on a spectrophotometer at a wavelength of 550nm. A blank was prepared in the same way but 150µl potassium phosphate buffer (50mM) was used instead of supernatant. Aliquots of sodium nitrite were also treated in a similarly for standard calibration.

Nitric oxides of the sample were expressed as nmol/mg of protein.
Determination of Enzymic antioxidant systems

Assay of Catalase (CAT) (H$_2$O$_2$ Oxidoreductase, E.C. 1.11.16)

The antioxidant enzyme Catalase was assayed by the method of Sinha (1972).

Reagents

1. 0.01M Phosphate buffer, pH 7.00
2. Dichromate acetic acid reagent: This reagent was prepared by mixing 5% solution of potassium dichromate with glacial acetic acid in the ratio of 1:3.
3. 0.2M Hydrogen peroxide

Procedure

To 0.1ml of the homogenate was added to 1.0ml of phosphate buffer. To this 0.5ml of hydrogen peroxide was added. The reaction was stopped at “0”, “30” and “60” seconds by the addition of 2.0 ml of the dichromate acetic acid reagent. The tubes were boiled for 10 minutes, cooled and read at 620nm. For standards different amounts of hydrogen peroxide, ranging from 20-100 μmoles were taken and processed as above.

Enzyme activity were expressed as n moles of H$_2$O$_2$ decomposed/min/mg protein.
**Estimation of Superoxide dismutase (SOD) (Superoxidase dismutase; Copper-zinc superoxide dismutase, E.C. No. 1.15.1.1)**

The Super oxide dismutase was estimated according to the method of Misra and Fridovich (1972).

**Reagents**

1. 0.1 M Carbonate – bicarbonate buffer, pH 10.2 containing 5.7 mg EDTA / 100ml
2. 3mM Epinephrine
3. Absolute ethanol
4. Chloroform

**Procedure**

In the test tube labelled as “Test” 0.5ml of liver homogenate was taken and to this 0.25 ml of cold ethanol and 0.15 ml of chloroform was added and kept for 15 minutes in a shaker and centrifuged. To 0.5ml of the supernatant 2.0 ml, of the buffer was added. The reaction was initiated by the addition of 0.4ml of epinephrine and change in the optical density per min was measured at 480 nm. 100% auto oxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.

The enzyme unit was defined as the enzyme required to give 50 % inhibition of epinephrine auto oxidation.
Assay of Glutathione-S-Transferase (GST) (RX: Glutathione R-transferase, E.C.2.5.1.18)

The enzyme Glutathione-S-Transferase was estimated by Habig et al. (1974).

Reagents

1. 0.5 M Phosphate buffer pH 6.5
2. 25 mM 1-Chloro 2,4 dinitrobenzene (CDNB)
3. 30 mM Reduced Glutathione

Procedure

To 1.0 ml of phosphate buffer, 0.1ml of 1-Chloro 2,4 dinitrobenzene, 1.7 ml of water and 0.1ml of liver homogenate were added. After 5 minutes of incubation at 37°C, 0.1 ml of reduced glutathione was added and the change in the optical density was measured for 3 minutes for 30 secs interval. Complete assay mixture without enzyme was used as control. Optical density was measured at 340nm.

Enzyme activity was expressed as n moles of CDNB conjugate formed/ min/ mg of protein.

Assay of Glutathione Reductase (GR) (Glutathione: NADP+ oxidoreductase, E.C.1.8.1.7.)

The enzyme Glutathione reductase activity was carried out by the method of Dobler (1981).
Reagents

1. 0.3 M Phosphate buffer, pH 6.8
2. 25 mM EDTA
3. 12.5mM Glutathione oxidised (GSSG)
4. 3mM Nicotinamide adenine dinucleotide phosphate (NADPH)

Procedure

The reaction mixture containing 1.5 ml of buffer, 0.5ml of EDTA, and 0.2 ml of GSSG was incubated at 37°C for 10 minutes. To the incubated mixture 0.1ml of the homogenate and 0.1 ml of NADPH solution was added. The change in the optical density was monitored at 340 nm at 37°C for 3 minutes at 30 secs interval.

The enzyme activity was expressed as n moles of GSSG utilized / min/ mg protein of liver homogenate.

Assay of Glutathione Peroxidase (GPx) (Glutathione: hydrogen-peroxide oxidoreductase, E.C.1.11.1.9.)

Glutathione Peroxidase was estimated by the method of Necheles et al. (1968).

Reagents

1. 0.4 M Phosphate buffer, pH 7.0
2. 10.mM Sodium azide
3. 4.0 mM Reduced glutathione
4. 2.5 mM Hydrogen peroxide
5. 10% TCA
6. 0.3 M Phosphate solution
7. 0.4 mM EDTA
8. 1.0 mM 5,5′-Dithio-bis-2 Nitro benzoic acid

Procedure

The incubation mixture consisted of 0.2ml of liver homogenate, 0.4 ml of buffer, 0.1ml of sodium azide, 0.2ml of reduced glutathione, and 0.2ml of EDTA, mixed well. To this mixture 0.1ml of hydrogen peroxide was added and incubated at “0”, “1.5” and “3.0” minutes. Then 1.0 ml of TCA was added to arrest the reaction. It was centrifuged and to 1.0 ml of the supernatant 4.0ml of phosphate solution and 0.5ml of DTNB were added and the residual glutathione was measured at 412 nm. Non enzymic oxidation of glutathione was measured in the blank containing all the reagents with buffer substituted for the enzyme source.

The activity of glutathione peroxidase was expressed as n moles of GSH oxidised / min/ mg protein liver homogenate.

Determination of non Enzymic antioxidant systems

Estimation of liver ascorbic acid

Estimation of liver ascorbic acid was carried out according to the method of Omaye et al. (1971).
Reagents

1. 10% TCA
2. Dinitrophenyl- Thiourea- copper sulphate reagent: 3 gms of 2,4-Dinitrophenyl hydrazine, 0.4gms of thiourea and 0.05 gms of copper sulphate were dissolved in 100ml of 9N H$_2$SO$_4$
3. 3. 65% H$_2$SO$_4$

Procedure

To 0.5ml of the homogenate, 0.5 ml of water , 1ml of TCA, are added, mixed and centrifuged. To 1ml of the supernatant, 0.2ml of DTC reagent was added and incubated at 37° C for 3hrs. Then 1.5 ml of ice cold H$_2$SO$_4$, was added, mixed well and the solution was allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520 nm. Standards of ascorbic acid were treated similarly.

The level of ascorbic acid was expressed as mg/g wet tissue.

Estimation of Vitamin E

Estimation of Vitamin E was carried out according to the method of Desai (1984).

Reagents

1. Ethanol
2. Petroleum ether
3. Batho phenanthroline reagent: 0.2% 4.6 diphenyl 1,10-phenanthroline in ethanol.
4. 0.001 M ferric chloride in ethanol
5. 0.001M O-Phosphoric acid in ethanol.

Procedure

To 1ml of the tissue homogenate, 1ml of ethanol was added and thoroughly mixed. Then 3ml of petroleum ether was added, shaken rapidly and centrifuged. 2ml of the supernatant was taken and evaporated to dryness. To this 0.2 ml of diphenyl 1,10-phenanthroline was added. The assay mixture was protected from light and 0.2ml of ferric chloride was added followed by 0.2ml of O- phosphoric acid. Total volume was made upto 3ml with ethanol. The colour developed was read at 530 nm. Standards were treated similarly.

The level of Vitamin E was expressed as mg/g wet tissue.

Estimation of Vitamin A

Estimation of Vitamin A was carried out by the method of Kaser and Stekol (1943).

Reagents

1. Absolute ethanol
2. Light petroleum ether
3. Chloroform
4. Acetic anhydride
5. Carr-price reagent: This contains 25% solution of antimony trichloride in chloroform. Kept it in the room temperature in a tightly stoppered brown bottle.

**Procedure**

To 3ml of homogenate in a stoppered flask added 3ml of ethanol, slowly drop by drop with shaking, to precipitate the protein. Added 6ml of light petroleum and shake vigorously for ten minutes. Poured the emulsion into a centrifuge tube, cork and spin at low speed for about one minute. The petroleum layer gets separated. To 4ml of the petroleum layer taken in the colorimeter tube evaporate off the solvent by placing in a water bath at 40-60°C. Dissolve the residue in 0.5ml of chloroform and added a drop of acetic anhydride to remove the water present. With colorimeter adjusted with the chloroform blank added quickly 3ml of Carr-price reagent and read the colour developed at 620 nm.

The level of serum Vitamin A was expressed as mg/g of wet tissue.

**Determination of Ceruloplasmin activity**

Ceruloplasmin activity was determined according to the method of Raven (1961).

**Reagents**

1. p-Phenylene diamine hydrochloride: 0.5% for purification of Phenylene diamine hydrochloride was dissolved in minimum volume of hot distilled water, decolourised with charcoal,
filtered hot and allowed to crystallize. The crystals were stored over calcium chloride.

2. 0.04M Acetate buffer: pH 5.3
3. 0.5% Sodium azide

**Procedure**

0.1ml of fresh serum was taken into 15ml test tube. 1.0 ml of 0.5% sodium azide was added to the control. Then 8.0 ml of acetate buffer was added to each tube, followed by 1.0ml of the p-phenylene diamine hydrochloride. The solution was mixed and placed in the water bath at 37°C for one hour. After incubation, the tubes were removed and added 1.0 ml of sodium azide to each of the tubes. The contents were mixed and cooled at 4-10°C for 30 minutes. The colour intensity was measured at 530 nm against reagent blank.

The level of serum Ceruloplasmin was expressed as mg / dl.

**Determination of serum Uric acid**

Determination of serum uric acid was carried out according to the method of Caraway (1963).

**Reagents**

1. Colouring reagent: 50 gms of sodium tungstate was dissolved in 400 ml of distilled water, to that 40 ml of phosphoric acid was added and refluxed for 2hrs. A drop of bromine was added, cooled and diluted to 500ml with water.
2. 20% sodium carbonate

3. Standard Uric acid: 100mgms of uric acid was dissolved in 150 ml of water containing 60 mg of lithium carbonate by heating at 60 °C, the solution was cooled at room temperature and added 2ml of formaldehyde diluted to about 500ml.

4. Working standard: 1.0 ml of the stock standard and 2.0 ml of 300 mg/1ml BSA were diluted to 10 ml with water. The working standard was prepared fresh. Albumin was added to account for the positive error induced by co precipitation of uric acid and proteins.

**Procedure**

To 0.6ml of serum 5.4 ml of diluted tungstic acid was added and centrifuged. Into three test tubes 3ml of each supernatant, standard, and water were taken and labelled as “T”, “S” and “B”. 0.6ml of sodium carbonate and 0.6ml of phosho tungstic acid reagent were added, mixed and placed in a water bath at 25°C for 10 minutes. The blue colour developed was read at 700 nm.

The level of serum Uric acid was expressed as mg / dl.

**Assay of liver Glutathione**

Assay of liver Glutathione was carried out by the method of Ellman (1959).
Reagents

1. 0.3M Disodium hydrogen phosphate
2. 5,5'-Dithiobis-2-nitrobenzoic acid reagent (DTNB): 40 mg of DTNB was dissolved in 100 ml of 1% sodium citrate.

Procedure

To the test tube marked as “T” 1.0 ml of the homogenate was taken and 1 ml of water is added. To that 1 ml of 10% TCA was added. The blank test tube contained 1 ml of TCA. Both the test tubes were centrifuged. To 1 ml of the supernatant 4.0 ml of phosphate reagent and 0.5 ml of DTNB solution was added and the color developed was read at 412 nm.

Liver glutathione was expressed as micromole GSH/mg of protein.

Assay of Total thiols

Assay of total thiols was carried out by the modified method of Sedlak and Lindsay (1968).

Reagents

1. 0.2M Tris-HCl buffer in EDTA, pH 8.2
2. 0.01M 5,5'-dithiobis-2-nitrobenzoic acid, reagent (DTNB)
3. Methanol
4. 0.02 M EDTA
Procedure

100 mg of tissue was homogenized in 4ml of 0.02M EDTA. To 1ml of the homogenate 1.5 ml of 0.2 M Tris buffer and 0.1 of 0.01 M DTNB (5,5′-dithiobis-2-nitrobenzoic acid) were added the mixture was brought to 6.5 ml with methanol. The test tubes were capped and left to stand for 20 min, then centrifuged at 3000 rpm/min at room temperature for 10 min. The absorbance of the clear supernatant was read at 412 nm.

Liver total thiols were expressed as µmoles of GSH mg/g of protein.

Estimation of Iron in liver tissue

Estimation of Iron in the liver tissue was carried out by the method of Ramsay (1957).

Reagents

1. Stock ferric chloride solution: 145 mg of ferric chloride in 100ml of 0.5N HCl
2. Working ferric chloride solution: Dilute 1 in 100 of the stock ferric chloride solution
3. Sodium sulphite: 2.25 gms of anhydrous sodium sulphite in 100 ml of water.
4. 0.2% 2-2 Dipyridyl in 3% glacial acetic acid
5. Stock standard 100µgm/ml: Dissolve 0.48 gms of ferrous sulphate in water and 1ml of conc H₂SO₄ and made up to 1 litre in water.

6. Working standard 5µgm/ml: 5 ml of the stock diluted to 100ml with distilled water.

7. Chloroform

**Procedure**

About 1.0 ml of the tissue homogenate was taken and 0.5ml of bipyridyl solution and 0.5ml of sodium sulphite were added blank contained 1.0 ml of distilled water. Mixed well and kept in boiling water bath for 5 minutes cooled and then added chloroform. Mixed well and centrifuge and read supernatant at 520 nm in spectrophotometer against blank. Aliquots of standard were also treated in the similar manner.

Iron in the liver tissue was expressed as mg/ g wet tissue.

**Determination of membrane bound enzymes**

Liver membrane was prepared by the method of Song et al. (1969).

Frozen rat liver samples of about 2 g were homogenized in 2 volumes each of ice-cold 1 mM sodium carbonate, pH 7.50, using 20 strokes of a loose-fitting homogeniser. The homogenates were diluted and filtered through 3 layers of surgical gauze. After centrifugation at 1500 g for 10 min, the pellets were resuspended in buffer and 5.5 volumes of 70.7% sucrose were
added, mixed, and then distributed into centrifuge tubes. 8 ml of 48.2% and 4 ml of 42.5% sucrose were layered over this suspension. After centrifuging for 60 min at 12,000 rpm, the material which accumulated around the interface of 42.5% and 48.2% sucrose was collected, diluted, and washed. The final pellet was resuspended in 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8), and stored at –80°C.

**Estimation of Total Adenosine triphosphatase (ATP Phosphohydrolase E.C. No. 3.6.1.4)**

Total adenosine triphosphatase activity was estimated by the method of Evan (1969).

**Reagents**

1. 0.1M Tris-HCl buffer pH 7.0
2. 0.1M Magnesium chloride
3. 0.1M Potassium chloride
4. 0.1M Sodium chloride
5. 0.1M calcium chloride ATP
6. 0.01M ATP
7. 10% TCA
8. Ammonium molybdate - 2.5 gm of ammonium molybdate was dissolved in 100ml of 5 N Sulphuric acid
9. ANSA - 500mg of Amino naphth Sulphonic acid was dissolved in 195ml of 15% sodium meta bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.
**Procedure**

The incubation mixture contained 1ml of Tris-HCl buffer, 0.2ml each of magnesium chloride, calcium chloride, potassium chloride, sodium chloride, ATP and homogenate. The mixture was incubated at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as μ moles of phosphorous liberated/ min/ mg of protein.

**Estimation of Na⁺/ K⁺ ATPase (Adenosine tri phosphatase EC 3.6.1.37)**

Estimation of Na⁺, K⁺ ATPase was estimated by the method of Bonting (1970).

**Reagents**

1. 0.09M Tris-HCl buffer - pH 7.5
2. Sodium chloride - 0.60M
3. EDTA - 0.001M
4. ATP - 0.04M
5. 10% TCA
6. Ammonium molybdate - 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid.
7. ANSA - 500mg of amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

**Procedure**

The incubation mixture contained 1ml of Tris-HCl buffer, 0.2ml each of potassium chloride, sodium chloride, EDTA, ATP and homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as µ moles of phosphorous liberated/ min/ mg of protein.

**Estimation of Ca⁺-ATPase (ATP phosphohydrolase EC 3.1.3.1)**

The activity of Ca⁺ - ATPase was assayed according to the method of Hjerten and Pan (1983).

**Reagents**

1. Tris-HCl buffer – 125mM, pH 8.0
2. Calcium chloride - 50mM
3. ATP - 10mM
4. 10% TCA
5. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid

6. ANSA - 500mg of amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

**Procedure**

The incubation mixture contained 0.1ml of each of Tris- HCl buffer, calcium chloride, ATP and enzyme preparation. After incubation at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as µ moles of phosphorous liberated/ min/ mg of protein.

**4.2.9.4 Estimation of Mg²⁺-ATPase (ATP phosphohydrolase EC 3.6.1.3)**

The activity of Mg²⁺-ATPase was assayed by the method of Ohnishi *et al.* (1982).

**Reagents**

1. Tris-HCl buffer – 375mM, pH 7.6
2. Magnesium chloride- 25mM
3. ATP - 10mM
4. 10% TCA

5. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid

6. ANSA - 500mg of amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

**Procedure**

The incubation mixture contained 0.1ml each of Tris-HCl buffer, Magnesium chloride, ATP and enzyme preparation. After incubation at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated

The enzyme activity is expressed as µ moles of phosphorous liberated/ min/ mg of protein.

**Estimation of Glycolytic enzymes**

**Estimation of Hexokinase (ATP: D-Hexose-6-Phosphotransferase E.C.2.7.1.1)**

Hexokinase was analysed by the method of Branstrup (1957).
Reagents

1. 0.01M Tris-HCl buffer, pH 8.0
2. Substrate 0.005M Glucose
3. ATP – 0.072M
4. 0.05M Magnesium chloride
5. 0.01M Potassium Chloride
6. 0.5M sodium fluoride
7. 0.0125M Potassium hydrogen phosphate
8. 10% TCA
9. Ortho toluidine reagent - 940 ml of acetic acid was dissolved in 60 ml of Ortho toluidine, to this added 3g of thiourea
10. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

Procedure

The incubation mixture was taken in two tubes marked as “0” minute and “30” minute which contained 2.5ml buffer, 1ml substrate, 0.5ml ATP, 0.1ml magnesium chloride, 0.1ml sodium fluoride, 0.4ml potassium hydrogen phosphate and 0.4ml of potassium chloride were pre incubated at 37°C for 5 minutes. 0.5ml of enzyme was added in both. To the” 0” minute tube added 1ml of the 10 % TCA immediately to stop the reaction. To the “30” minute tube 10 % TCA was added after 30 minutes. The two tubes were incubated at 37°C for 30 minutes. Both the tubes were centrifuged. Taken 1ml of the two supernatant separately and 1ml of water as blank, 4ml of ortho toluidine
reagent was added in all three tubes kept in boiling water bath for 8 minutes, cooled and read at 620nm. Standard containing varying concentrations of glucose and reagents were also similarly treated.

The enzyme activity is expressed as nmoles of Glucose utilized /min /mg of protein.

**Estimation of Phospho gluco isomerase (n-glucose-g-phosphate ketol-isomerase, EC 5.3.1.9)**

The enzyme was assayed by the method of Horrocks *et al.* (1963). The assay is based on the estimation of fructose using fructose thiourea reagent.

**Reagents**

1. 0.1M Borate buffer pH 7.8.
2. Buffered substrate- 3mg disodium glucose-6-phosphate was dissolved in 1ml buffer. This was prepared fresh before use.
3. 30% HCl
4. Resorcinol – thiourea reagent: 100mg of resorcinol and 250mg of thiourea were dissolved in 100ml of glacial acetic acid. This was stored in brown bottle.
5. Colour reagent: 30% HCl, resorcinol- thiourea reagent, and H₂O were mixed in the proportion 7:1:1. The solution was used on the same day as prepared.
6. Standard solution of fructose: 54 mg of fructose was dissolved in 100 ml of 0.25% benzoic acid.

**Procedure**

In to each of the tubes labelled “test” and “blank”, 1ml of buffered substrate was added. A suitable amount of the enzyme extract was added in to the test and the tubes were incubated at 37°C for 30 minutes. After the period of incubation enzyme was added to the blank tube, and 9ml of colouring reagent was immediately added to all tubes. The tubes were heated in the boiling water bath maintained at 70°C for 15 minutes. Standards containing varying concentrations of fructose and a reagent blank were similarly treated. The tubes were cooled in running water and the colour was read at 410 nm.

The enzyme activity is expressed as n moles of fructose utilized /min /mg of protein.

**Estimation of Aldolase (Ketose-1-phosphate aldehyde lyase E.C.4.1.2.7)**

Aldolase activity was estimated by the method of King (1965a).

**Reagents**

1. 0.1M Tris-HCl buffer: pH 8.6
2. Fructose 1,6 diphosphate: 8.33 mg dissolved in 5ml of 0.05M in buffer, prepared just before use.
3. Hydrazine sulphate ; 0.56 N, pH 8.6
4. 0.1% DNPH in 2N HCl
5. 0.75N Sodium hydroxide
6. 10% TCA
7. Standard DL glyceraldehyde: 123 mg was dissolved in 1.0 litre of water. This was left at room temperature for 2-4 days to permit polymerization.

**Procedure**

To 2.0 ml of fructose-1,6 diphosphate, 0.25 ml of hydrazine sulphate and 1.0 ml of the buffer was added to 1ml of enzyme extract and incubated at 37°C for 15 minutes and the reaction was stopped by addition of 1.0 ml of 10% TCA. 0.1ml of enzyme was added to the blank tubes and were centrifuged. 1.0 ml of the supernatant was transferred to the tubes containing 1.0 ml of the 0.75 N sodium hydroxide and left in the room temperature for 10 minutes. 1ml of the colour reagent was added in the test and the blank tube and incubated at 37°C for 1 hr. The colour developed by the addition of 0.75 N sodium hydroxide was read at 540 nm in UV spectrophotometer.

The enzyme activity was expressed as n moles of glyceraldehyde formed /min/ mg protein

**Determination of Glyconeogenic enzymes**

**Estimation of Glucose–6-phosphatase (D-glucose-6-phosphate: NADP+ 1-oxidoreductase, E.C. 3.1.3.9)**

Estimation of Glucose-6-phosphatase was carried out by the method of King (1965a).
Reagents

1. Citrate buffer - 0.1M, pH 6.5
2. Glucose–6 Phosphate : 0.01M
3. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
4. ANSA - 500mg of amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.
5. 10 % TCA.

Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.5ml of substrate and 0.2ml of the enzyme. Incubation was carried out at 37° C for 60 minutes. The reaction was arrested by the addition of 1.0 ml of TCA and centrifuged. The phosphorous content of the supernatant was estimated.

Enzyme activity was expressed as n moles of phosphorous liberated / mg of protein / min.

Estimation of Fructose 1,6-diphosphate (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, E.C. No. I. I. I .37)

Estimation of Fructose 1,6-diphosphate was carried out by the method of Gancedo and Gancedo (1971).
Reagents

1. Tris-HCl Buffer : 1 M, pH 7.0
2. Fructose 1, 6 diphosphate 0.05 M
3. Magnesium chloride: 0.1 M
4. Potassium chloride: 0.1 M
5. EDTA : 0.001 M
6. TCA 10 %
7. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
8. ANSA - 500mg of amino napthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The assay medium in the final volume of 2.0 ml contained 1.2 ml of buffer, 0.1ml of substrate solution, 0.25 ml of magnesium chloride, 0.1ml of potassium chloride, 0.25 ml of EDTA and 1ml of enzyme source. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of TCA. The suspension was centrifuged and phosphorus content was estimated.

The enzyme activity was expressed as n moles of phosphorous liberated / mg of protein / min.
Protein bound carbohydrate complexes

Isolation of Glycoprotein from liver tissues

About 500mg of the liver tissue was homogenised in 7.0 ml of methanol, filtered and scarped from the filter paper. To that material 10ml of the chloroform was added and it was filtered. To the residue 4.0 ml of the chloroform was added and once again it was filtered. To the residue 7.0 ml of Chloroform: Methanol (2:1) mixture was added, homogenised and filtered. The defatted tissue was taken for the estimation of glycoprotein.

Estimation of Hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. Orcinol – Sulphuric acid reagent:
   Solution A: 60ml of concentrated sulphuric acid was mixed with 40ml of distilled water.
   Solution B: 1.6g of orcinol (recrystallized from benzene) was dissolved in 100ml of distilled water. 7.4ml of solution A was mixed with 1ml of solution B just before use.

2. Standard: 50mg of each of galactose and mannose were dissolved in 100ml of distilled water. This solution is diluted to a proportion of 1:10 to give concentration of hexose 100micrograms/ ml.
Procedure

For hydrolysis 25 mg of the defatted tissue was mixed with 2ml of 3N HCl and hydrolysed at 100°C for 4 hrs. The hydrolysate was neutralized with sodium hydroxide. From the hydrolysed sample 0.5ml of the neutralized solution was made up to 1.0 ml with distilled water and 8.5ml of ice-cold orcinol reagent was added very slowly. The mixture was heated at 80°C for 15mts, cooled and left in the dark for 25mts for color development. Standard solutions containing 25-100µgms were treated in the similarly. Then the absorbance was read at 540nm.

The hexose content is expressed as mg/100mg of defatted tissue.

Estimation of Hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagents

1. Acetyl acetone reagent:
   Solution A - Trisodium phosphate 0.1M: 4.1g of trisodium phosphate was dissolved in 25ml of distilled water.
   Solution B - Potassium tetraborate 0.5N: 305.5 mg of potassium tetraborate was dissolved in 2ml of distilled water.
   3.5ml of acetyl acetone was added to the mixture of solution A and solution B in the ratio of 98:2 (v/v).
2. Ehrlich’s reagent: 320mg of p-dimethyl amino benzaldehyde was dissolved in 21ml of isopropanol and 3ml of concentrated HCl was added to it.

3. Standard: 100 mgs galactosamine was prepared in 100ml of water. This solution was diluted to a proportion of 1:10 to give concentration of hexosamine 100micrograms/ ml in distilled water.

**Procedure**

For hydrolysis 25 mg of the defatted tissue was mixed with 2ml of 3N HCl and hydrolysed at 100°C for 4 hrs. The hydrolysate was neutralized with sodium hydroxide.

0.5ml of the neutralized sample was made up to 1ml with distilled water.0.6ml of acetyl acetone reagent was added to all the tubes and heated in a boiling water bath for 30mts. After cooling, 2ml of Ehrlich’s reagent was added and the contents were shaken well. The pink color developed was measured at 540nm against the reagent blank. Standard solution containing 10-40 µg of galactosamine were also treated in the similar manner.

The content of hexosamine in tissues is expressed as mg/100ml of defatted tissue.

**Estimation of Sialic acid**

Sialic acid was determined by the method of Warren (1959).
Reagents

1. Periodic acid 0.25 M: 14g of sodium periodate was dissolved in 100ml of 0.1N sulphuric acid.
2. Sodium meta arsenite 4%: 4gm of sodium meta arsenite was dissolved in 100ml of 0.5N hydrochloric acid.
3. Thiobarbituric acid: 144mg of thiobarbituric acid was dissolved in 20ml of distilled water. The pH of the solution was adjusted to 9 with 6 N Sodium hydroxide. This reagent was prepared just before use.
4. Acidified butanol: 5ml of concentrated hydrochloric acid was added to 95ml of n- butanol.
5. Standard: 10mg of N-acetyl neuraminic acid was dissolved in 100ml of distilled water.

Procedure

About 25 mg of defatted tissue was mixed with 0.5ml of 0.1N Sulphuric acid and hydrolysed at 80°C for 1hr. 0.5ml of the neutralized samples were taken along with standards (in the range of 10-50µg). Blank contained 0.5 ml of 0.1N sulphuric acid, 0.25ml of periodate was added to all tubes and incubated at 37°C. After 30mts, 0.25ml of arsenite solution was added to inhibit the reaction. Contents were mixed and 2ml of thiobarbituric acid was added and the tubes were heated in a boiling water bath for 6mts. After cooling, pink color developed was extracted into 5ml of acidified butanol phase, and was measured at 540nm against a reagent blank.
The sialic acid content is expressed in tissues as mg/100mg of defatted tissue.

**Histopathological Investigations**

The rats were sacrificed liver, kidney, intestine, and heart were dissected out and cleaned well with cold physiological saline to remove blood and adhering tissues. The samples were then fixed in 10% formalin-saline and embedded in paraffin. Serial sections were cut at 5mcm and stained with haemotoxylin and eosin. The sections were examined under light microscope and photographs were taken.

**Statistical Analysis**

Results will be expressed as mean ± standard error of mean (S.E.M.). Statistical significance is determined by one-way analysis of variance (ANOVA). The data obtained from toxicity studies will be analyzed using Dunnet’s ‘t’ test P values less than 0.05 will be considered significant.
RESULTS

In the acute toxicity studies death was recorded during the treatment period in treated groups given 500mg/kg of Biherbal extract orally. The animals showed changes in general behavior and other physiological activities like giddiness, sniffing, aggressiveness, tachypnoea, and convulsion finally at the dose level of 500mg/kg (Table I). There is a significant difference in the organs like lung, liver (**p<0.01) and heamatological parameters like Hb (*P<0.05) and W.B.C. (**P<0.01) were observed (Tables II and III). Similarly there was a remarkable alteration in biochemical parameters like Glucose, Sodium, AST and ALP (**p<0.01) were observed (Table IV). Pathological examinations of the tissues on a gross and macroscopic basis indicated that there were no detectable abnormalities. From the above toxicity studies the ED$_{50}$ dose of the BHE was calculated and it was fixed as 50 mg/kg body weight.

In chronic toxicity studies were no significant differences in food consumption and variation in the body weight was negligible. In the 3 month experiment, no mortality was observed in any of the treatment group. There was no relevant difference in body weight development, hematological (Table VI) or biochemical parameters (Table VII) and organ weights (Table V) for all groups.

For the male rats of the Biherbal extract treated group, values of AST, ALT and cholesterol were slightly elevated as compared to control but not statistically significant (Table VII). They were, however, still in the normal
range. No macroscopical abnormalities were detected in the examined organs. Histologically, there were only minor pathological findings of inflammatory or degenerative origin (Figure I to X). Inflammatory processes, especially in the respiratory system, showed the same frequency and extent, in the BHE treated groups as in control groups. A harmful effect of the Biherbal extract to the organs could be ruled out. In particular, no histological changes were found for all groups in hearts, testes or ovaries. Slight inflammatory infiltrates or signs of bronchopneumonia with diffuse foci were found in the lungs of single animals of both, control and treatment groups.

Similarly, slight inflammatory infiltrates in the connective tissue or the glomeruli of the kidneys were observed in single animals, as well as mild turbid swellings in the liver lobules. In all cases, the degree of inflammatory or degenerative processes was only minor, and the phenomena occurred in all treatment groups with the same frequency. It can therefore be assumed that the observations were unrelated to the ingestion of Biherbal extract. The withdrawal of the Biherbal extract ingestion after 3 months did not induce any detectable change in behavioural patterns, nor were there any differences to controls for the hematological, biochemical, anatomical or histological parameters.

Table VIII shows the effect of BHE on body weight, liver weight, blood glucose, urea and serum bilirubin levels in various experimental rats. In the present investigation a significant (p < 0.01) reduction in the liver weight was shown in-group III BHE pre-treated animals when compared to that of group II rats intoxicated with CCl₄. Decreased level of blood glucose in CCl₄
induced rats were found to be normalized on BHE supplementation. Urea (p<0.01) and bilirubin (p<0.001) levels were found to be significantly increased in group II rats, and on BHE pretreatment these parameters were found to be normalized.

Figure XI shows the effect of the plant extract on Serum total proteins, albumin and globulin levels. The serum levels of total proteins, albumin and globulin were significantly decreased in CCl₄ intoxicated rats (p<0.001) when compared to control animals. The total protein levels were increased in BHE pretreated, CCl₄ intoxicated group III animals showing the regeneration of hepatic cells. This increase in the protein level are more pronounced in BHE treated group III animals, when compared with the group IV and V, which received its individual preparations MAE and PLE.

Table IX depicts the change in serum levels of the liver marker enzymes like AST, ALT, ALP, ACP, ICD and LDH. A significant increase in the serum transaminases AST and ALT levels was seen in the group II CCl₄ intoxicated animals. These enzymes were brought back to near normal levels in BHE pretreated group III animals (p<0.001). Similarly the elevated ALP, ACP and LDH enzyme levels in group II CCl₄ intoxicated animals were also significantly decreased in the group III BHE pretreated and CCl₄ intoxicated animals (p<0.01, p<0.001). BHE treatment reverted the increased levels of these enzymes to near normalcy (p<0.001) which was comparable to that of silymarin a standard drug used in the present study. Comparison of group I control rats with that of group VII which received only BHE showed no
significant variation in the marker enzyme levels indicating no adverse side effects due to the administration of Tween-80 and BHE alone.

Figure XII shows the protective role of the BHE on the serum enzyme levels of ACE, γ GT and 5’-Nucleotidase. The enzymes like γ-GT (p<0.001) and 5’-Nucleotidase (p<0.05) were significantly increased in group II CCl₄ intoxicated animals. These increased levels are brought back to near normal levels in BHE treated group III animals. The probable mechanism by which the BHE extract exerts its protective action against CCl₄ induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration. Serum acetyl choline esterase levels in the present study decreased in CCl₄ treated group II animals. BHE treatment reverted the decreased levels to near normalcy (p<0.001,) which was comparable to that of standard silymarin drug. The BHE was effective in correcting these marker enzyme levels when compared with its individual preparations like MAE and PLE extracts. Comparison of group I and VII shows no significant variation in these enzyme levels indicates no appreciable adverse side effects due to the administration of Tween–80 and BHE only.

Table X shows the changes in liver tissue levels of ALT, AST, LDH, ALP and ACP in different groups of experimental animals. All the marker enzyme levels were significantly (p<0.001) increased in CCl₄ intoxicated rats. Being cytoplasmic in location the marker enzymes AST, ALT, ALP and LDH are increased when hepatocytes get damaged as in the case of CCl₄ damage. The stabilization of AST, ALT, LDH, and ALP levels by BHE is a clear indication of the improvement of the functional status of the liver cells.
The changes in liver tissue levels $\gamma$GT, 5’Nucleotidase and Xanthine oxidase in different groups of experimental animals were shown in Table XI. All these enzymatic parameters were significantly ($p<0.001$) increased in the group II CCl$_4$ intoxicated animals. In this study, the elevated levels of XO, $\gamma$GT, 5’Nucleotidase activity in the CCl$_4$ administered group was shown to be effectively counteracted by the administration of BHE.

The BHE was effective in correcting these marker enzyme levels when compared with those of its individual preparations like MAE and PLE extracts. Comparison of group I and VII showed no significant variation in these enzyme levels indicated no appreciable adverse side effects due to the administration of Tween –80 and BHE only.

The serum lipid profile of the control and the experimental animals were depicted in Figure XIII. The concentration of triglycerides and cholesterol ($p<0.001$) were significantly decreased and phospholipids were increased in CCl$_4$ intoxicated Group II animals. This showed that a block in the secretion of hepatic triglycerides and cholesterol into the plasma, a major mechanism underlying the fatty liver induced by CCl$_4$ and other toxins in the rats. The concentration of phospholipids was significantly ($p<0.001$) increased in CCl$_4$ intoxicated group II animals. The decreased levels of cholesterol, triglycerides and increased level of phospholipids were brought back to near normal by the treatment of BHE. This observed restoration of the CCl$_4$evoked changes in the serum lipid profile shows the protective nature of BHE.
Table XII shows the serum lipoprotein profile of the control and the experimental animals. Increased levels of LDL and VLDL cholesterol (p<0.01) and decreased level of HDL in CCl₄ intoxicated rats were normalized on BHE treatment. The recovery towards normalization of serum lipoproteins caused by BHE is almost similar to that caused by silymarin, in the present study which was used as a positive control.

The BHE was effective in correcting these serum lipid and lipoprotein profile when compared with its individual preparations like MAE and PLE extracts. Comparison of group I and VII shows no significant variation in these parameters indicates no appreciable adverse side effects due to the administration of Tween-80 and BHE only.

Table XIII depicts the concentration of total lipids, cholesterol, phospholipids and triglycerides in liver tissue. Significant enhancement in the concentrations of total lipids, cholesterol and triglycerides were observed in the tissues of group II rats, which received CCl₄ alone. Remarkable increase in the concentration of phospholipids was also noticed in the liver of group II rats. The altered biochemical parameters in the liver tissue were significantly brought towards normalization by co-administration of BHE. The combinational preparation of BHE was found to be more effective, when compared with its individual preparation that was given to the group IV and group V animals respectively.

Figure XIV shows the values of liver glycogen and protein in different experimental rats. The animals that received CCl₄ alone showed
decrease in the glycogen level (p<0.001). It has been observed that there was a significant hypoglycemia with a drop in hepatic glycogen content. In the present study a significant (p<0.001) decrease in the liver protein was observed in CCl₄ intoxicated group II rats. These protein and glycogen levels were returned to near normal in the BHE treated groups, which shows the protective nature of the drug against the CCl₄ damage.

Comparison of group I and VII shows no significant variation in these parameters indicates no appreciable adverse side effects due to the administration of Tween-80 and BHE only.

The levels of lipid peroxidation LPO products malonaldehyde conjugated dienes, nitric oxide and hydro peroxides were significantly increased (p<0.01) in the liver tissues of CCl₄ intoxicated group II rats (Table XIV). Pretreatment with BHE significantly reversed the increased levels in group III animals. Increased production of Reactive Oxygen Species [ROS] due to oxidative stress plays an important role in liver diseases. CCl₄ has been reported to induce lipid peroxidation and alter the antioxidant defence system through formation of free radicals, which in turn causes damage, and degeneration of hepatic tissues. The significant (p<0.01) increase in the level of LPO products in-group II animals could be due to the damage caused by CCl₄ which were decreased in BHE treated group III animals showing the protective nature of the drug by scavenging the free radicals.

Figure XV shows the changes in the \textit{in vitro} lipid peroxidation in liver cells in the presence of inducers like ascorbate, FeSO₄ and H₂O₂ in
different groups of experimental animals. The hepatic tissue of untreated CCl₄ intoxicated rats showed a 2-fold rise in basal lipid peroxidation levels as well as a 1.3-fold, 1.8-fold and 1.5 fold increase in LPO in the presence of inducers such as ferrous sulphate, ascorbate and H₂O₂ respectively.

Table XV shows levels of various liver antioxidant enzymes in normal, CCl₄ intoxicated, and BHE pre-treated groups. The enzymes like SOD, CAT, GPx, GST, and GR activities were significantly decreased (p<0.01) in CCl₄ intoxicated group II rats when compared to those of normal control rats. But the oral pretreatment with BHE increased the activities of SOD, CAT, GPx, GST and GR.

Table XVI and Figure XVI shows the levels of non-enzymic parameters in normal, CCl₄ intoxicated and plant drug treated groups of different experimental animals. The decreased levels of vitamin E, Vitamin A, total thiols, glutathione, uric acid, and cereloplasmin were observed in CCl₄ treated group II rats. The group III CCl₄ intoxicated and BHE pretreated animals showed near normal levels of vitamin E Vitamin A, thiols, and cereloplasmin activities (p<0.01) when compared to group II animals. Thus the free radical scavenging property of BHE could have maintained the near normal levels of non-enzymic antioxidants in-group III animals Decreased activities of non-enzymic antioxidants in CCl₄ treated group II rats may increase their susceptibility to oxidative injury. Elevated levels of these non-enzymic antioxidants in BHE treated group III animals offer protection against the oxidative injury caused by the free radicals.
Table XVII shows the activities of membrane bound ATPases in the liver of various experimental animals. The levels of various membrane bound enzymes were significantly decreased in the liver tissues of CCl₄ intoxicated group II rats. The decreased level of Na⁺/K⁺-ATPase, Mg²⁺-ATPase and Ca²⁺-ATPase in CCl₄ intoxicated rats may be due to the alteration of membrane lipid composition due to the release of free radicals. The rats which received BHE, MAE, and PLE restored the enzyme levels to near normal levels, which could be due to the ability of plant extracts to protect the membranes from oxidative damage through inhibition of lipid peroxidation.

Table XVIII shows the effect of BHE on the glycolytic enzymes in the various experimental group animals. The activities of glycolytic enzymes, in liver were found to be significantly decreased in CCl₄ intoxicated rats. The BHE treatment restored these enzymes.

Table XIX shows the effect of BHE in the gluconeogenic enzymes in the various experimental group animals. The activities of gluconeogenic enzymes, glucose-6-phosphatase and fructose-1, 6-diphosphatase in liver were found significantly decreased in CCl₄ intoxicated rats. The BHE restored the normal levels of these enzymes, indicating that the plant drug has got protective action on the mitochondria from damage enabling them to secrete the enzymes and keeping the blood glucose in normal levels.

Figure XVII shows the average values of liver Glycoprotein in different experimental rats. Glycoprotein are linear polymers of amino acids with branching chain of carbohydrates that may include hexose, hexoseamine
and sialic acid. There was a significant decrease in the levels of Glycoprotein (\( p < 0.001 \)) was observed in the group II CCl\(_4\) treated animals due to the increased load of the toxic metabolites. The increase in glycoprotein content of BHE treated group II animals suggested the cytoprotective nature of the formulation.

The Figures XVIII to XXI depicts the histopathological changes in the liver, kidney, heart and intestinal tissues. CCl\(_4\) selectively damages the liver which is shown in Figure XIX(b) by the formation of centrilobular necrosis. The BHE treated liver cells showed near normal architecture. The histopathological changes were negligible as far as kidney heart and intestinal tissues are concerned.
DISCUSSION

Liver regulates various important metabolic functions. Hepatic damage is associated with distortion of these metabolic functions (Wolf, 1999). Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are effective and sometimes can have serious side effects. This is one of the reasons for many people in the world over including those in developed countries turning to complimentary and alternative medicine (CAM). Many traditional remedies employ herbal drugs for the treatment of liver ailments.

Liver detoxifies and excretes destructive agents in many toxic cases. The toxins are converted into the intermediate reactive radicals, prior to their hepatotoxic effects. CCl₄-induced hepatotoxicity in rats represents an adequate experimental model of cirrhosis in man and it is used for the screening of hepatoprotective drugs (Al-Shabanah et al., 2000). It is well established that CCl₄ induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl₄ is bio-transformed by the cytochrome P450 system in the endoplasmic reticulum to produce trichloromethyl free radical (•CCl₃). Trichloromethyl free radical then combined with cellular lipids and proteins in the presence of oxygen to form a trichloromethyl peroxyl radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethyl peroxyl free radical leads to elicit lipid peroxidation, the destruction of Ca²⁺ homeostasis, and finally,
results in cell death. According Parola et al., 1992 a number of investigators have utilized this chemical to produce liver cirrhosis in experimental animals.

The age old traditional values attached with the medicinal plants have gained tremendous importance in the present century according to Stein (2004); Kala (2004). India and China are two of the largest countries in Asia, which have the richest arrays of registered and relatively well-known medicinal plants as reported by Raven (1998). The use of natural remedies for the treatment of liver diseases has a long history, starting with the ayurvedhic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver diseases by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization of herbal products and randomized placebo controlled clinical trials to support clinical efficacy. In spite of the availability of more than 300 preparations for the treatment of jaundice and chronic liver diseases in Indian systems of medicine using more than 87 Indian medicinal plants, only few terrestrial plants have been scientifically elucidated while adhering to the internationally acceptable scientific protocols according to Robert Borris, 1996.

With the above scenario, the Biherbal extract (BHE) made up equal quantities of leaves of *Melia azedarach* and seeds of *Piper longum* were subjected to various pharmacological and biochemical studies to evaluate the hepatoprotective nature of the formulation.
The hepatoprotective nature of any formulation was evaluated after fixing the LD$_{50}$ value by acute toxicity studies. In addition to acute toxicity studies chronic toxicity effects of the BHE was evaluated according to OECD (2001) guidelines. In acute toxicity about 50% animal death was recorded during the treatment period in treated groups given in 500 mg/kg of BHE orally. Hence it can be concluded that BHE is practically toxic or lethal after an acute exposure at the dose range of 500 mg/kg. This test limit for acute oral toxicity is generally considered to be 5.0 g/kg body weight. The LD$_{50}$ value of BHE was 500 mg/kg body weight, one tenth of the LD$_{50}$ value was considered as ED$_{50}$ value for any pharmacological studies. So for hepatoprotective efficacy studies 50 mg/kg body weight BHE was used which was considered as its ED$_{50}$ value.

Significant difference in the organ weight of lung and liver was observed in the test animals which received the lethal dose drugs. The histological parameters such as Hb, WBC and biochemical parameters like glucose, Na, AST and ALP also showed significant difference. The similar results were shown by Caisey and King (1980).

There were no significant differences in food consumption and variation in the body weight was negligible. In the 3 month experiment, no mortality was observed in any of the treatment group. There was no relevant difference in body weight development, hematological or biochemical parameters as mentioned by Carol, 1995 and organ weights for all groups.
For the male rats of the Biherbal extract treated group, values of AST, ALT and cholesterol were slightly elevated as compared to control but not statistically significant (Barry, 1995).

The 3 month daily oral application of Biherbal extract to rats yielded no signs of toxicity. In addition, no behavioural or physiological changes were observed on discontinuation of Biherbal extract feeding after 3 months treatment. In rats, the extract tested was proven non-toxic under the experimental conditions. The dosage range and study duration applied in this study are relevant for long-term human use, and represent dosage schemes by far exceeding the normal human application. The results of this study do not allow the conclusion of any toxicity of Biherbal extract, particularly to the liver.

In the present investigation a significant reduction in the liver weight was shown in-group III BHE pretreated animals when compared to that of group II CCl$_4$ intoxicated animals. This is due to the accumulation of lipids the triglycerides in the CCl$_4$ rats as stated by Saxena and Garg (1979) which was reduced in the BHE, treated rats leading to the decreased weight of the liver. The decrease in glucose level was significant in CCl$_4$ treated groups. According to Ohyashiki et al., 1995 this might be due to the glucose 6-phosphatase deficiency in the CCl$_4$ administered rats. Urea and bilirubin levels were found to be significantly high in CCl$_4$ intoxicated rats. Rao (1973) reported a defective excretion of bile by the liver that is reflected in their increased levels in serum due to hepatotoxin in liver injury. The same effect was observed in our study also. Hyperbilirubinaemia is a very sensitive test to
substantiate the functional integrity of the liver and severity of necrosis of hepatocytes as reported by Singh et al. (1998).

These biochemical parameters were decreased in BHE pretreated group III animals, when compared with the CCl₄ intoxicated group II animals. The preliminary phytochemical screening of the BHE showed the presence of many phytochemicals including phenolic compounds and flavonoids which can be stated for the protection of hepatocytes from damage. Several plant extracts and different classes of phytochemicals have been shown to have protective effect on liver ailments (Vani et al., 1997). The combinational preparation (BHE) was found to be effective, when compared with the drugs given individually to the group IV and group V.

Reduced levels of serum total proteins, albumin and globulin confirmed the diseased liver state, in CCl₄ intoxicated group II animals. In hepatotoxicity, as reported by Dubey et al. (1994); Bickerton et al. (1996) a decrease in total protein is observed due to the liver inflammation leading to a depression of hepatic protein synthesis similar to our results. According to Clawson (1989), this is due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl₄ administration. Administration of BHE at a dose of 50 mg/kg body weight prevented this change and shows the regeneration of hepatic cells. This might be due to the promotion of the assembly of ribosome on endoplasmic reticulum to facilitate uninterrupted protein biosynthesis as stated by Kheir-Eldin (1992).
An increase of globulin was observed in the BHE administered rats when compared to CCl₄ intoxicated group II animals showed the plant does not reduce the power of the subjects to fight infection. According to Christina et al. (2006) *Piper longum* which is one of the ingredient of BHE contains flavonoids which might have scavenged the free radical offering hepatoprotection. This increase in the protein levels is more pronounced in BHE treated group III animals, when compared with the group IV and V, which received the single drugs. Comparison between group I and VII shows no significant variation indicates no appreciable adverse side effects due to the administration of tween-80 and BHE alone. Group comparison between group III and group VI shows no significant variation in these parameters indicating that the BHE has got the same effect as that of silymarin which was considered as the positive control.

CCl₄-induced hepatotoxicity in rats represents an adequate experimental model of cirrhosis in man and it is used for the screening of hepatoprotective drugs as reported by Plaa and Hewitt (1989). The plasma levels of the liver marker enzymes like AST ALT, ALP, ACP, \( \gamma \)GT 5′-NT, ICD and LDH were significantly increased in CCl₄ intoxicated group II animals According to Recknagel et al. (1991) the increased serum levels of AST and ALT have been attributed to the damaged structural integrity of the liver, because these enzymes are cytoplasmic in location and are released into circulation after cellular damage.

Tanaka and Iizuka (1968) reported that acid phosphatases are frequently employed as the marker enzymes to assess the lysosomal changes.
The rise in serum levels of γGT 5′-NT and ICD has been attributed to the damaged structural integrity of the liver according to Chenoweth and Hake (1962). In agreement with the above investigators, our present study elicited a significant increase in the activities of liver marker enzymes in CC14 treated rats indicating considerable hepatocellular injury.

Pretreatment with the BHE extract attenuated these increased enzyme activities produced by CC14 and a subsequent recovery towards normalization of these enzymes. This strongly suggests the possibility of BHE extract being able to condition the hepatocytes so as to cause an accelerated regeneration of parenchymal cells, thus protecting against membrane fragility decreasing the leakage of marker enzymes into the circulation. The antihepatotoxic activity of BHE against CCl4 might be due to the inhibitory effects on microsomal enzymes or on lipid peroxidation, stimulatory effects on hepatic regeneration and free radical scavenging effects. This is supported by the view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes by Thabrewet et al. (1987).

Tamaoki et al. (2002) reported that the P. longum contains various constituents, such as piperine, piperidine, pipernonaline and piperlonguminine which could account for this hepatic regeneration and free radical scavenging effects of the BHE through their presence of phenolic structures. The BHE was effective in correcting these marker enzyme levels when compared with its individual preparations like MAE and PLE. In support of the present study.
Khopde et al. (2001) stated that compounds in their natural formulations are more active than their isolated form. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations.

Serum acetyl Choline esterase levels in the present study decreased abruptly in CCl₄ treated group II animals. Zakut et al. (1988) reported that Pseudocholinesterase is low also in some instances of liver disease, including decompensated cirrhosis, hepatitis, metastatic carcinoma, chemical toxicity, and in malnutrition. BHE treatment reverted the decreased levels to near normalcy.

Lipid peroxidation is accepted to be one of the principal causes of CCl₄-induced liver injury. Therefore, according to Campo et al. (2001) the antioxidant activity and/or the inhibition of free radicals generation are important in terms of protecting the liver from CCl₄-induced damage. Arora et al. (2003) in their study stated that antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet. It has been assumed that nutritional intervention to increase intake of phyto-antioxidants may reduce threat of free radicals.

The liver protective role of the BHE has also been reflected in the normal levels of marker enzymes present in the liver tissues that were severely altered in the CCl₄-intoxicated animal liver. Liver regulates various important metabolic functions and contains host of enzymes. In his study
Wells, 1988 reported that in tissues, AST and ALT are found in higher concentrations in cytoplasm and AST in particular also exists in mitochondria. Most of the hepatotoxic chemicals including CC1₄ damage liver mainly by inducing lipid peroxidation directly or indirectly. Chang et al., 1994 in their study mentioned that lipid peroxidation was known to cause destabilization and disintegration of the cell membrane, leading to liver injury and arteriosclerosis in liver. So the marker enzymes ALT, AST γGT, 5′Nucleotidase, xanthine oxidase and LDH present in the liver tissues were increased, when hepatocytes get damaged as in the case of CCl₄ damage. The stabilization of these marker enzyme levels by BHE is a clear indication of the improvement of the functional status of the liver cells.

It is well documented by Krauskopf et al. (2005) that xanthine oxidase is an important prerequisite factor in the process of O₂ generation in CCl₄ toxicity. Our phytochemical experimental results demonstrated that BHE exercises free radical scavenging activity upon the superoxide radical generated using the NBT-PMS system. It was also observed that the concentration of xanthine oxidase was high in CCl₄ intoxicated group II animals showing the increased production of super oxide radicals which was found to be minimized in BHE pretreatment through the decreased production of xanthine oxidase.

According to Kugelman et al. (1994) cellular γGT has a central role in glutathione homeostasis by initiating the breakdown of extracellular glutathione (GSH)—the critical antioxidant defense for the cell. So increase in the γGT enzyme level indicates the increased breakdown of glutathione in
the CCl₄ intoxicated rats and due to this increased deprivation of glutathione which cannot cope up with the synthesis, the antioxidant defense mechanism was altered leading to hepatotoxicity. Neutralizing reactive oxygen species by nonenzymatic mechanisms may be one of the main mechanisms of action of BHE against chemical-induced cytotoxicity. The quantitative analysis of these extracts showed the presence of considerable amount of phenolic compounds and flavonoids, which may be effective in protecting the rats from CCl₄ injury. Many plants exhibit efficient antioxidant properties owing to their phenolic constituents. (Larson, 1988). Moreover, the hepatoprotective activity of BHE was much stronger than that of the reference drug silymarin, administered at the same concentrations.

The serum and liver tissue lipid profile of the control and the experimental animals were discussed below. The serum triglycerides and cholesterol were significantly decreased in CCl₄ intoxicated group II animals. At the same time significant enhancement in the concentrations of total lipids, cholesterol and triglycerides were observed in the liver tissues of the same. Littleton and John (1979) reported a similar work where there exists a parallel decrease in the concentration of plasma lipids and lipoproteins along with the increase of these in liver tissues. This is because of the block in the secretion of hepatic triglycerides in to the plasma. According to Recknagel and Lombardi (1961), the accumulation of triglycerides in liver of CCl₄-treated rats is not due to the interference with the formation triglycerides by the liver, but due to the inhibition or destruction of triglycerides secreting mechanism. Moreover the hepatic triglycerides is not released as such, but it is combined
with lipoprotein. Due to the interference with the synthesis of protein moiety by CCl4, the synthesis of lipoprotein also, gets affected leading to the decreased levels of cholesterol in the serum. These same effects were observed in our results also. Both the serum as well as liver tissue concentration of phospholipids were significantly increased in CCl4 intoxicated group II animals.

Weissberger et al. (1940) reported that hepatotoxic treatment produces an increase in the level of phospholipids in serum, which may be due to the decrease in mitochondrial fat oxidation and according to Vercesi et al. (1997), the peroxidation of membrane phospholipids induced by reactive oxygen species and/or free radicals leads to alterations in the membrane structure and functions. These degenerative changes can affect dynamic properties of the membranes such as fluidity and permeability, and consequently the activity of various membrane-associated enzymes. In our study the serum and liver lipid profile levels were brought back to near normal levels by BHE administration suggesting that its hepatoprotective activity might be due to its effect against the loss of functional integrity of the cell. Pennington and Styles (1975) reported that the main class of small molecular metabolites produced by the M. azedarach consists of modified triterpenoids known as limonoids, of which azadiractin is the most well known owing to its insecticidal properties. These phytochemicals might be accounted for the hepatoprotective activity of the BHE. In the present study the recovery towards normalization of these lipid profile caused by BHE is almost similar to that caused by silymarin, a known hepatoprotective drug.
which was studied by Morazzoni and Bombardelli (1995) and reported similar results.

Decrease in liver total protein and glycogen was observed in the rats treated with CCl₄. This might be associated with the decrease in the number of hepatocytes, which in turn might result in the decreased hepatic capacity to synthesize protein and glycogen. Apart from this ROS generated as a result of •CCl₃ radical will affect the amino acids histidine, methionine, tyrosine, thereby interfering the synthesis of protein. The protein and glycogen levels were returned to near normal levels in the BHE treated groups which shows the protective nature of the drug against the CCl₄ damage. The compounds such as melaartenin and 12-hydroxyamoorastatin have already been isolated by Carpinella et al. (2002) in the M. Azedarach and the compounds piperlonguminine, piperine, apigenin, dimethyl ether, and β sitosterol were isolated by Parmar et al. (1997) in Piper longum. The chemical structure of these compounds showed the presence of many OH and phenol-containing groups could be accounted for the liver protective role of BHE.

According to Brattin et al. (1985) requires bio transformation by hepatic microsomal cytochrome P450 to produce the hepatotoxic metabolite, trichloromethyl free radicals (•CCl₃ and/or CC1₃OO⁻) Trichloromethyl free radicals can react with sulphhydryl groups, such as glutathione (GSH) and protein thiols. According to Williams and Burt, 1990 this covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events, which eventually leads to membrane lipid peroxidation and finally cell necrosis. Although several isoforms of P450 may metabolize
CC14, according to Zangar *et al.*, 2000 attention has been focused largely on the P450 2E1 isoform, which is ethanol inducible. Alternations in the activity of P450 2E1 affect the susceptibility to hepatic injury from CC14 as stated by Jeong (1999). The reactive intermediates formed during the metabolism of therapeutic agents, toxicants and carcinogens by this enzyme are frequently capable of binding covalently to tissue macromolecules, which may in turn cause tissue damage according to Eaton *et al.* (1995).

Hermes-Lima (2004) reported that pro-oxidant reactions are ordinary outcomes of the environmental, physiological and metabolic networks in which most contemporary organisms survive. These reactions are the result of a delicate balance between generation of reactive species of oxygen and nitrogen (ROS and RNS, respectively), and the presence and activity of a variety of antioxidant systems. Variation from this equilibrium may have two main consequences: 1) Under regulated conditions, cells may display a redox signaling which requires activation of specific redox-sensitive receptors and transcriptional factors and 2) during an imbalance between oxidants and antioxidants, a pathophysiological condition termed oxidative stress can be produced. Rhee *et al.* (2003) stated that pro-oxidant reactions could be quantified by measuring the oxidation of membrane phospholipids by the process known as lipid peroxidation (LPO). According to De Zwart *et al.* (1999) LPO is a chain reaction which can occur by enzymatic or non-enzymatic reactions, in most cases catalyzed by transition metals, where active oxidants cause the breakdown of polyunsaturated fatty acids in membrane phospholipids.
There are several assays for the determination of LPO, but two of the more frequent techniques to measure this process are to quantify the presence of Conjugated dienes (CD,\textsubscript{}) and to determine thiobarbituric acid-reactive substances (TBARS). Conjugated dienes are formed by the rearrangement of double bonds of the PUFAs during the peroxidative process, and they are considered an estimation of \textit{in vivo} LPO, whereas the TBARS assay relies on the adducts formed between thiobarbituric acid and the carbonyl end products of lipid peroxidation progression, mainly malonaldehyde.

The \textit{in vivo} oxidants such as CD, hydroperoxide, iron and nitric oxide metabolites were also found to be increased in CCl\textsubscript{4} treated rats. The increase in the LPO substances in the liver of CCl\textsubscript{4} intoxicated rats indicated the enhanced lipid peroxidation leading to tissue injury and failure of defence mechanisms to prevent the formation of excess free radicals. Geller (1993) have suggested that increased NO production and plasma nitrite/nitrate levels are also found during chronic hepatic inflammation, implicating a role for NO in the hepatic response to inflammatory stimuli. It has been demonstrated by Beckman (1990), that nitric oxide (NO) reacts with O\textsubscript{2} in pathological states to produce peroxynitrite, a potent oxidizing agent. Peroxynitrite can initiate intracellular LPO formation and NP-SH oxidation, resulting in the production of an extreme cellular membrane damage. In the present study, BHE extract was effective in reducing the production of these LPO products.

Several previous studies have demonstrated that CC1\textsubscript{4}-induced hepatotoxicity can be modulated by substances that influence the activity of P450 2E1. According to Day \textit{et al.}, 1993; Kim \textit{et al.}, 1997 compounds or
drugs that induce P450 2E1 potentiate the hepatic toxicity of CC14 and compounds that inhibit P450 2E1 protect against CC14-induced toxicity. From the above studies it can be concluded that the BHE might have played an important role in the inhibition of P450 2E1 enzyme system and generation of free radicals.

Similar studies were conducted by Emmanual et al. (2001) to evaluate the hepatoprotective activity of plants showed that the presence of antioxidant such as phenols and flavonoids were responsible for reducing the LPO in vivo by blocking radical formation and transition metals reactions. The presence of several amides Guineensine Pipernonaline Pellitorine Piperine Piperanine Piperlonguminine were reported in Piper longum and Sheela Ghoshal et al. (1996) reported the anti-amoebic action of the plant Piper longum Linn, is due to the action of these compounds this could also be accounted for its free radical scavenging properties. According to these studies, the P. longum is the one of the constituent present in the formulation of BHE might account for the LPO scavenging property.

According to Abuja and Albertini, 2001 in vitro lipid peroxidation in a liver homogenate can proceed in a nonenzymatic way. The process is induced by the presence ascorbate, Fe$^{2+}$/Fe$^{3+}$ and H$_2$O$_2$. Based on the studies by Chan (1993) reported that ascorbic acid is a critical antioxidant that acts as a free radical scavenger and may regenerate other antioxidants, including vitamin E. However, the reducing capacity of ascorbic acid can potentially lead to redox cycling of transition metals, which in turn can generate hydroxyl radicals in the presence of hydrogen peroxide. It has been reported that the
addition of ascorbic acid to iron greatly increases oxidative damage in vitro. Markers of lipid peroxidation, protein modification and DNA damage are all enhanced by ascorbic acid. In addition, ascorbic acid has been suggested to directly produce genotoxic lipid hydroperoxides in the absence of metals.

In order to clarify the mode of action of BHE, the *in vitro* LPO experiments were carried out using the LPO inducers like ascorbic acid, FeSO₄ and H₂O₂. The hepatic tissue of untreated CCl₄ intoxicated rats showed a 2-fold rise in basal lipid peroxidation levels as well as a 1.3-fold, 1.8-fold and 1.5 fold increase in LPO in the presence of inducers such as ferrous sulphate and ascorbate and hydrogen peroxide, respectively. According to the results obtained, BHE inhibited *in vitro* LPO in the presence of inducers of LPOs in liver homogenate, which is shown by the decreased levels of MDA, produced. It is obvious in this study that BHE played a significant role in the elimination of iron, H₂O₂ and hydroxyl radicals generated in the present *in vitro* LPO experiments. Michele Ambrosio and Antonio Guerriero (2002) reported that the leaves of *M. azedarach* contain limonoids such as teracrylmelazolide A, melazolide A, teracrylmelazolide, pyroangolensolide, fraxinellone and its derivatives, 30-hydroxyfraxinellone, 9a- and 9b-hydroxyfraxinellone could account for the inhibition of LPO.

The levels of various antioxidant enzymes such as SOD, CAT, GPx, GST, and GR activities were significantly decreased in CCl₄ intoxicated group II rats when compared to normal control group I rats. But the oral pretreatment of BHE to such CCl₄ treated rats increased the activities of SOD, CAT, GPx, GST and GR. This indicates the antilipid peroxidative nature of
the system against CCl\textsubscript{4} treatment is enhanced by BHE. The enzyme superoxide dismutase and glutathione constitute the first line of defence against free radical induced damage. In states of oxidative stress, GSH is converted into GSSG and depleted leading to lipid peroxidation. GSSG is reduced to GSH by GR, which is NADPH dependent. It plays a role in maintaining adequate amounts of GSH. Accordingly, the reduction of GR results in decrease of GSH (Reckengel \textit{et al.}, 1991). Reduction in liver GSH was observed in CCl\textsubscript{4} treated group II rats due to the increased utilization or lower expression of GSH which leads to the damage of the liver cells. Sinclair (1991) suggested that the unavailability of GSH decreases the activities of GR, GPx and GST.

Masukawa and Iwata (1986) stated that GST is a soluble protein which is located in cytosol, which plays an important role in the detoxification and excretion of xenobiotics. GST catalyzes the conjugation of the thiol functional groups of glutathione to electrophilic xenobiotics and results in increasing solubility. The xenobiotic–GSH conjugate is then either eliminated or converted to mercapturic acid. Since GST increases solubility of hydrophobic substances, it plays an important role in the excretion of xenobiotics. Compounds which increase the activity of GST, which metabolizes toxic compounds to non-toxic compounds, means they have an increasing protective activity on the liver. This effect was shown by our plant extracts. In CCl\textsubscript{4} intoxicated rats, the activity of GST decreased drastically compared to that of normal group. Reconstitution of the levels of GSH, and
GST activity in the rats treated with BHE proves the protective and antioxidant efficiency of the drug.

GPx is a selenium-containing enzyme. It is believed to reduce hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent to form water and corresponding alcohols, respectively. According to Ho et al., 1997 cellular hydroperoxides can otherwise serve as substrates for the metal mediated Fenton reaction to generate the highly reactive hydroxyl radical. According to Harman, 1991 increase in CAT and GPX activities are essential if a beneficial effect from increase in SOD activity is to be expected. The enzymes like SOD and CAT plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues. SOD removes superoxide by converting it to H₂O₂, which can be rapidly converted to water by CAT as stated by Halliwell et al. (1986).

Bhattacharya (1997) stated that potential antioxidant therapy should therefore include either natural free radical scavenging antioxidant enzymes or agent, which are capable of augmenting the activity of these enzymes. A restoration of these CAT, SOD activity and glutathione level by the BHE may account for its protective effects. The components present in the leaves of *Melia azedarach* and seeds of *Piper longum* in BHE contain active principles like piperine, piperidine and azadirachtin, meliacin which are known for hepato protective and antioxidant activity. As reported by Khajuria et al. (1998); Surh (1999) piperine and piperidine which is present in the BHE could reduce the tert-butyl hydroperoxide and CC14 induced lipid
peroxidation both *in vitro* and *in vivo* resulting in significant hepatoprotection in rats.

Several experimental studies investigated the role of antioxidative vitamins, minerals, drugs and plant-derived compounds in the prevention and therapy of liver fibrosis. Decreased activities of non-enzymic antioxidants like vitamin C, vitamin E Vitamin A, thiols, uric acid, and cereloplasmin levels in CCl₄ treated group II rats may increase their susceptibility to oxidative injury. Elevated levels of these non-enzymic antioxidants in BHE treated group III animals offer protection against the oxidative injury caused by the free radicals. According to Parola *et al.* (1993) Vitamin E inhibited m-RNA expression of tumour growth factor-b and α₂ procollagen in CCl₄-induced animals and prevent fibrosis. Seifert *et al.* (1995) also observed beta-carotene treatment also decreased liver hydroxyproline level during CCl₄ administration and reduced liver fibrosis by inhibition of lipid peroxidation in animal studies.

The mechanism of uric acid action as an antioxidant may relate to the formation of a urate-free radical after oxidant exposure. This urate-free radical is being scavenged by ascorbate. Thus it is suggested that urate and ascorbate interact as plasma antioxidants Recently it has been shown by Abul *et al.* (2002) that uric acid can be used as a scavenger of peroxynitrate (ONOO⁻), a toxic product of the free radicals nitric oxide and superoxide (ONOO⁻) has been implicated in the pathogenesis of central nervous system. According to Denkob, 1979 cereloplasmin is produced in the liver in response to tissue injury and released into the circulation. Frei *et al.* (1988) stated in his study
that ceruloplasmin is considered a preventive plasma antioxidant because it sequesters transition metals, thereby preventing them from participating in free radical reactions. Plasma total sulfhydryl groups have also been suggested to contribute significantly to the antioxidant capacity of plasma. According to Carlo et al. (1999) Plant polyphenolic compounds, such as flavonoids, are described as scavengers of reactive oxygen species (ROS), via inhibition of oxido-reductases. The presence of a number of potent pharmaceutical limonoids, flavonoids, and triterpenoids have been isolated from *Melia azedarach* may responsible for the prevention of hepatotoxic activity by BHE.

The hepatic necrosis is associated with damage to sub cellular organelles including mitochondria was reported by Dixon (1984). The mitochondrial oxidative process plays a central role in the cellular energy metabolism by providing about 95% of cellular energy needs in the form of ATP was reported by Erecinska and Wilson (1982). The Na⁺, K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase are the membrane bound enzymes responsible for the transport of these ions respectively across the cell membrane at the expense of ATP. The deleterious effect of a xenobiotic on mitochondrial energy metabolism can, hence, have serious consequences for the vitality of the cell. The levels of membrane bound enzymes were significantly decreased in the liver tissues of CCl₄ intoxicated group II rats. Marin et al. (1992) reported that during lipid peroxidation process the activity of different membrane-bound enzymes are changed which ultimately leads to changes in membrane permeability leading to the destruction of cells or whole cell systems. According to Frank and Massaro (1980) and Tinrmestein and Nelson
the decreased level of these enzymes in CCl₄ rats may be due to alteration in membrane lipid composition and/or content, lipid peroxidation, disturbance in calcium homeostasis and oxidation and alkylation of thiol groups of glutathione and proteins.

The rats which received BHE, MAE and PLE retained the levels of TBA reactive substances and the activities of these enzymes were also restored to the near normal levels which could be due to the ability of plant extracts to protect the membranes from oxidative damage through inhibition of lipid peroxidation. Therefore the possible hepatoprotective effect of BHE on the chemical-induced liver injuries may be due to: (1) inhibiting Cytochrome P-450 activity, (2) preventing the process of lipid peroxidation, (3) stabilizing the hepatocellular membrane and (4) enhancing protein and glycoprotein biosynthesis. Silymarin is a known hepatoprotective compound, which was considered as a positive control in our present study exhibited the same effect, was studied by Ramellini and Meldolesi (1976).

Liver is the candidate organ involved in glucose homeostasis. It is the main site for glycolysis, a process where glucose is degraded and gluconeogenesis, where glucose is synthesized from lactate, amino acids and glycerol. According to Bhavapriya and Govidasamy (2000) these are the two important complementary events that balance the glucose load in our body. The activity of gluconeogenic enzymes, glucose-6- phosphatase and fructose-1, 6-diphosphatase were found significantly decreased in CCl₄ intoxicated rats. The BHE treatment restored the normal levels of these enzymes which has got
protective action on mitochondria enabling them to secrete the enzymes for keeping the blood glucose normal.

The activity of Hexokinase and phosphofructokinase enzyme are ATP dependent are reported to be under regulation by citrate. In CCl₄ damage ATP depletion was also considered as an important factor, the enzymes which are ATP dependent were also decreased. Aldolase, another key enzyme in the glycolytic pathway, decreases in liver diseases and this may be due to cell impairment and necrosis according to Koster et al. (1995). BHE treatment restored these enzyme levels to near normal showing the regenerating ability of the plant extracts.

Glycoprotein are linear polymers of aminoacids with branching chain of carbohydrates that may include hexose, hexoseamine and sialic acid. The various suggestion offered by Kishore et al. (1983) that elevated glycoprotein may be due to the tissue necrosis, rapidly metabolizing tumur cell destruction from normal connective tissue and non specific stimulus in the process similar to that observed in many infectious diseases. The main change observed during the necrotic stage of CCl₄ poisoning was a highly significant reduction in the sialyl transferase activity followed by a considerable decrease in the sialic acid content. The similar effect was observed in our study also. According to Molnar et al. (1964) the microsomal portion only is responsible for the glycoprotein synthesis and the increased load of the toxic metabolites in the system might affect the microsomes which may further responsible for the decreased glycoprotein components in the CCl₄ treated rats. The increase
in glycoprotein content of BHE treated group III animals suggested the cytoprotective nature of the formulation.

Histopathological examination of the livers provided supportive evidence for this study. Liver of rats administered with CCl₄ showed centrilobular necrosis with mononuclear infiltration in the portal area, fatty deposition and loss of cell boundaries. In animals treated with the BHE, there was much lesser hepatocellular necrosis, mononuclear infiltration and loss of cell architecture in comparison to livers from control animals.

The histopathological observations which show a faster regeneration of the hepatic cells in rats treated with BHE seems to suggest the possibility of BHE being able to condition the hepatic cells towards accelerated regeneration. Similar histopathological observations observed with silymarin seem to suggest that the ability to cause accelerated regeneration may be a feature common to certain medicinal plants to protect against liver dysfunction.

The mechanism by which BHE exerts its protective action against CCl₄ induced alterations in the liver is not clear. However, the fact the BHE when given prior to CCl₄ administration, can produce more rapid recovery of the liver when compared with those exposed to CCl₄ only, indicates that the protective action may be due to an antioxidant property as in the case of other agents known to oppose the hepatotoxic effects of CCl₄.
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