3. MATERIALS AND METHODS

3.1. CHEMICALS AND REAGENTS

Ethanol, Egg yolk lecithin, 2-thiobarbituric acid (TBA) and diphenyl carbazide were purchased from Hi Media Laboratories Private Limited, Mumbai Ltd, India. 1-amino, 2-naphthol, 4-sulphonic acid (ANSA), ammonium molybdate, sodium metaperiodate, acetyl acetone, cytochrome C, 2,6 -dichlorophenol indophenol, dicoumarol, trichloroacetic acid (TCA), oxidized glutathione (GSSG) and 1-chloro-2, 4-dinitrobenzene (CDNB), hematoxylin and eosin stain, morin hydrate, IL-6, NF-κB, CD14 and GAPDH primers were obtained from Sigma Chemical Company, MO, St. Louis, USA. TNF-α, TGF-β, β-actin antibodies were purchased from Santa-cruz Biotechnology, USA. COX-2 antibody was purchased from Cell Signaling Technology, USA and iNOS antibody was purchased from Sigma-Aldrich Co. St. Louis, Missouri, USA. Diphenyl carbazide, 2,2’ dipyridyl and 1,1,3,3’ tetra methoxy propane were purchased from Ranbaxy Private Ltd, Mumbai, India. Triolein was purchased from from Sisco Research Laboratory Private Ltd., Mumbai, India. Cholesterol, reduced glutathione chromotrophic acid, digitonin, dextran sulphate, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS) and glycerol were obtained from S.D. Fine Chemicals Private Ltd., Mumbai, India.

3.2. ANIMALS

Adult male albino Wistar rats (150-170 g) were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, (RMMC&H), Annamalai University. The rats were housed in plastic cages under controlled conditions of 12-h light-dark cycle, 50% humidity and temperature of 28 °C. They were all fed standard pellet diet (Lipton I ever Private Limited Mumbai, India) and water *ad libitum*. Animal handling and experimental procedures were approved by
the Institutional Animal Ethics Committee, Annamalai University and animals were cared for in accordance with the Indian National Law on animal care and use.

3.3. PROCESSING OF BLOOD AND TISSUE SAMPLES

3.3.1. Isolation of serum

Blood collected in dry tubes were allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min.

3.3.2. Preparation of plasma and hemolysate

Blood was collected in heparinized tubes and the plasma was separated by centrifugation at 2000×g for 10 min. After the separation of plasma, the packed cells (RBCs) were washed thrice with cold physiological saline. Erythrocyte membrane was prepared by lysing a known volume of erythrocytes with hypotonic phosphate buffer, pH 7.4 and centrifuged at 3000×g for 10 min at 2 °C, and the hemolysate was separated.

3.3.3. Preparation of tissue homogenate

Immediately after sacrifice, the liver was quickly excised, rinsed with saline, blotted dry on filter paper and weighed. Subsequently 10% (w/v) tissue homogenates with appropriate buffer using a tissue homogenizer was prepared and the supernatants were used for the various biochemical estimations.

3.3.4. Preparation of cytosol and microsomal fractions

Cytosolic and microsomal fractions were prepared from tissues by homogenizing in 0.25 M sucrose and centrifuged at 9000×g for 20 min. The supernatant fluid was collected, 0.2 mL (v/v) of 0.1 M CaCl₂ in sucrose was added to each, and the samples were kept on ice for 30 min, centrifuged at 27,000×g for 20 min, which yielded clear cytosolic fractions that were promptly assayed for phase II enzymes and ADH. Microsomal pellets were washed twice by suspending in 7 mL of 10 mM Tris HCl (pH 7.4) in 0.25 M sucrose, centrifuged at 9000×g for 20 min, which yielded microsomal fractions, that were promptly assayed for phase I enzymes.
3.3.5. Lymphocytes preparation

Lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by layering heparinised blood onto equal volume of histopaque gradient (Ticol histopaque-1077) and centrifuged at 1000 rpm for 10 min. Lymphocytes were aspirated from the gradient-plasma interfaces and washed twice in phosphate buffered saline (PBS). The final cell pellets were resuspended in PBS and used for the estimation of DNA damage.

3.4. EXPERIMENTAL DESIGN

3.4.1. Phase – I

3.4.1.1. In vitro assay

In vitro assay : 1,1-diphenyl-2-picrylhydrazyl (DPPH\(^*\)), 2, 2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), superoxide and hydroxyl radical scavenging assay.

3.4.1.2. In vivo assay (Preliminary study)

The experiments were performed in two phases. In phase I the animals were divided into seven groups of 6 rats each. All animals were fed with standard pellet diet. Rats in groups 1 and 2 received isocaloric glucose from a 40% glucose solution. Animals in groups 3-7 received 20% ethanol (equivalent to 6 g/kg/body weight) as an aqueous solution by intragastric intubation for 60 days. Group 2 animals received aqueous solution of morin (120 mg/kg BW). In addition to ethanol group 4, 5, 6 and 7 animals received aqueous solution of morin (15, 30, 60 and 120 mg/kg BW) for the last 30 days of the experiment. The experimental protocol is represented as below.

Group 1 : Control rats, received isocaloric glucose from a 40% stock glucose solution twice in a day for a period of 60 days.

Group 2 : Control rats, received glucose from a 40% stock glucose solution twice daily, which was isocaloric to ethanol and morin (120 mg/kg BW) from the 30\(^{th}\) day along with glucose in the morning.

Group 3 : Rats received ethanol (6 g/kg BW) from 30% stock solution twice in a day daily for a period of 60 days.

Group 4 : Rats received ethanol (6 g/kg BW) twice in a day and morin (15 mg/kg BW) from the 30\(^{th}\) day along with ethanol in the morning.

Group 5-7 : Rats were treated as for group 4 but with morin equal to 30, 60 and 120 mg/ kg BW respectively.
3.4.2. Experimental protocol for Phase II and III

In phase II and III the animals were divided into four groups of 6 rats each. All the animals were fed with standard pellet diet. Rats in groups 1 and 2 received isocaloric glucose from a 40% glucose solution. Animals in groups 3 and 4 received 20% ethanol (equivalent to 6 g/kg BW) as an aqueous solution by intragastric intubation for 60 days. In addition to isocaloric glucose and ethanol group 2 and 4 animals received aqueous solution of morin (60 mg/kg BW) for the last 30 days of the experiment. The experimental protocol is represented as below:

Group 1 : Control rats, received isocaloric glucose from a 40% stock glucose solution twice in a day for a period of 60 days.

Group 2 : Control rats, received glucose from a 40% stock glucose solution twice daily, which was isocaloric to ethanol and morin (60 mg/kg BW) from the 30th day along with glucose in the morning.

Group 3 : Rats received ethanol (6 g/kg BW) from 30% stock solution twice in a day for a period of 60 days.

Group 4 : Rats received ethanol (6 g/kg BW) twice in a day and morin (60 mg/kg BW) from the 30th day along with ethanol in the morning.

3.5. LIST OF PARAMETERS ANALYZED

3.5.1. Phase I

Phase I : Dose fixation study

Serum : Markers of liver function
Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT) alkaline phosphatase (ALP) and bilirubin.

Lipid profile
Total cholesterol (TC), free fatty acids (FFA), triacylglycerols (TG) and phospholipids (PL).

Lipoprotein metabolism
High density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL).

Markers of kidney function
Urea, uric acid and creatinine.

Plasma and lysate : Lipid peroxidative markers: Thiobarbituric acid reactive substance (TBARS), lipid hydroperoxides (LOOH).
Enzymic antioxidants: Superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx).

Histopathology : Liver
Those rats administered with morin (60 mg/kg BW) showed more significant results with regard to hepatonephritic markers, oxidative markers and antioxidants in plasma, liver mitochondria and erythrocytes as well as histological alterations, when compared to rats treated with low dose. On the other hand the higher concentration might have resulted in the production of more byproducts that would have interfered with the activity. Therefore, 60 mg/kg of morin was chosen for the further study.

### 3.5.2. Phase II and III

#### SERUM
- Total protein
- Albumin
- Globulin
- Albumin/Globulin (A/G) ratio

#### PLASMA
- Lipoprotein lipase (LPL)
- Leceithin cholesterol acyl transferase (LCAT)

#### LIVER MICROSCOPES AND CYTOSOL
- Microsomal cytochrome P450
- Microsomal cytochrome b5 reductase
- Microsomal NADH cytochrome P450 reductase
- Microsomal cytochrome P4502E1
- Cytosolic DT-diaphorase
- Cytosolic glutathione S-transferase (GST)

#### LYMPHOCYTES
- DNA damage (comet assay)

#### LIVER
- HMG CoA reductase
- Alcohol dehydrogenase (ADH)
- Aldehyde dehydrogenase (ALDH)
- Iron
- Nitrite
- PCR Analysis - NF-κB, CD14 and IL6
- Western blot analysis - TNF-α, TGF-β
- COX-2 and iNOS
- Apoptotic Markers - Bcl-2 and Bax expression by immunohistochemistry
- Lipid peroxidation - FTIR
- Markers of fibrosis - collagen by Masson’s Trichrome Staining
3.6. **IN VITRO ASSAY**

3.6.1. *In vitro* antioxidants assay

3.6.1.1. **DPPH**\(^*\) scavenging activity of morin

The ability of morin in scavenging the stable free radical, DPPH\(^*\) was determined *in vitro* by the method of Mensor et al., (2001). To a methanolic solution of DPPH\(^*\), an equal volume of morin (50-250 \(\mu\)M) dissolved in 0.5% DMSO was added and made up to 1.0 mL with methanolic DPPH\(^*\). An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Systronics UV-visible spectrophotometer.

\[
\% \text{ Scavenging of DPPH}^* = \frac{\text{Control-OD} - \text{Test-OD}}{\text{Control-OD}} \times 100
\]

3.6.1.2. **ABTS**\(^*\) scavenging activity of morin (Total antioxidant activity)

Total antioxidant potential of morin was determined by scavenging ABTS\(^*\), as described by Miller, (1996). The reaction mixture contained ABTS\(^*\), morin (50-250 \(\mu\)M) and phosphate buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm in a Systronics UV visible Spectrophotometer.

\[
\% \text{ Scavenging of ABTS}^* = \frac{\text{Control-OD} - \text{Test-OD}}{\text{Control-OD}} \times 100
\]

3.6.2. *In vitro* free radical scavenging assay

3.6.2.1. **Superoxide anion scavenging effect of morin**

Superoxide anion (O\(^2-\)) scavenging activity of morin *in vitro* was determined by the method of Nishikimi et al., (1972) with slight modification. One mL of nitroblue tetrazolium (100 \(\mu\)M of nitroblue tetrazolium in 100 mM phosphate buffer, pH 7.4), 1 mL of reduced nicotinamide adenine dinucleotide solution (14.68 \(\mu\)M of reduced nicotinamide adenine dinucleotide in 100 mM phosphate buffer, pH 7.4) and varying volumes of morin (50-250 \(\mu\)M) were mixed well. The reaction was started by the addition of 100 \(\mu\)M of phenazine methosulphate (60 \(\mu\)M/100 mM of phosphate buffer, pH 7.4). The reaction mixture was incubated at 30 °C for 15 minutes. The absorbance was measured at
560 nm in a spectrophotometer with a reagent blank containing double distilled water instead of morin. Decreased absorbance of the reaction mixture indicates increased O$_2^{•−}$ scavenging.

\[
\% \text{ Scavenging of O}_2^{•−} = \frac{\text{Control-OD} - \text{Test-OD}}{\text{Control-OD}} \times 100
\]

3.6.2.2. Hydroxyl radical scavenging effect of morin

The Hydroxyl radical (OH$^{•−}$) scavenging activity of morin in vitro was determined by the method of Halliwell et al., (1987). The incubation mixture in a total volume of 1 mL contained 0.1 mL of 100 mM potassium dihydrogen phosphate-dipotassium hydrogen phosphate buffer, varying volumes of morin (50-250 µM), 0.2 mL of 500 mM ferric chloride, 0.1 mL of 1 mM ascorbic acid, 0.1 mL of 1 mM ethylene diamine tetra acetic acid, 0.1 mL of 10 mM hydrogen peroxide and 0.2 mL of 2-deoxyribose. The contents were mixed thoroughly and incubated at room temperature for 60 min. Then, 1 mL of 1% TBA (1 g in 100 mL of 0.05 N sodium hydroxide) and 1 mL of 28% trichloro acetic acid were added. All the tubes were kept in a boiling water bath for 30 min. The absorbance was read in a Spectrophotometer at 532 nm with a reagent blank containing double distilled water instead of morin. Decreased absorbance of the reaction mixture indicates increased OH$^{•−}$ scavenging activity.

\[
\% \text{ Scavenging of OH}^{•−} = \frac{\text{Control-OD} - \text{Test-OD}}{\text{Control-OD}} \times 100
\]

3.7. ASSESSMENT OF THE LIVER FUNCTIONS

3.7.1. Estimation of serum bilirubin

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

Serum was diluted with water, and methanol was added in an amount insufficient to precipitate the proteins, yet sufficient to permit all the bilirubin to react with diazo reagent.
Reagents

1. Absolute methanol

2. Hydrochloric acid: 1.5%

3. Diazo-reagent: Prepared freshly before use by adding 0.3 mL of solution B to 10.0 mL solution A.

   Solution A: 1.0 g of sulphanilic acid was dissolved in 15 mL of conc. HCl and made up to one litre with water.

   Solution B: 0.5 g of sodium nitrite was dissolved in water and made up to 100 mL.

4. Bilirubin standard: 10.0 mg of bilirubin was dissolved in 100 mL chloroform.

Procedure

Two test tubes were taken and 0.2 mL serum and 1.8 mL of distilled water were added to each. 0.5 mL of diazo-reagent was added to the test and 0.5 mL of 1.5% HCl to the blank. Finally, to each tube 2.5 mL methanol was added and left aside for 30 min and the colour developed was read at 540 nm.

Serum bilirubin levels are expressed as mg/dL of serum.

3.7.2. Assay of serum aspartate transaminase (AST, EC 2.6.1.1) and alanine transaminase (ALT, EC 2.6.1.2)

Activities of AST and ALT were assayed by the method of Reitman and Frankel (1957).

Principle

AST catalyses the transfer of an amino group from dL-aspartate (dL-ASP) to α-ketogluutarate (α-KG) to yield oxaloacetate and L-glutamate. ALT catalyses the transfer of an amino group from dL alanine (dL-Ala) to α-ketogluutarate to yield pyruvate and L-glutamate.
The liberated oxaloacetate reacts with aniline-citrate to form pyruvate. Pyruvate reacts with 2,4-dinitrophenyl hydrazine (DNPH) to form 2,4-dinitrophenyl hydrazone, which was read at 540 nm.

\[
\begin{align*}
\text{L-ASP + } & \overset{\text{AST}}{\alpha-\text{KG}} \rightarrow \text{oxaloacetate + L-glutamate} \\
\text{L-Ala + } & \overset{\text{ALT}}{\alpha-\text{KG}} \rightarrow \text{pyruvate + L-glutamate}
\end{align*}
\]

**Reagents**

1. Phosphate buffer - 0.1 M, pH 7.5

2. Substrate
   
   a) For AST: 0.30 g of dL-aspartic acid and 50mg of \(\alpha\)-keto glutaric acid were dissolved in 20 mL of phosphate buffer. The pH was adjusted to 7.5 with 1 N sodium hydroxide and made up to 100 mL with phosphate buffer.

   b) For ALT: 5.0 g of dL-alanine and 20 mg of \(\alpha\)-ketoglutaric acid were dissolved in 20 mL of phosphate buffer. The pH was adjusted to 7.5 with 1 N sodium hydroxide and made up to 100 mL with phosphate buffer.

3. DNPH: 200 mg of DNPH was dissolved in hot 1 N hydrochloric acid.

4. Aniline-citrate reagent: 50 g of citric acid were dissolved in 50 mL of distilled water and to this added an equal volume of redistilled aniline.

5. Sodium hydroxide - 0.4 N

6. Standard pyruvate solution: 12.5 mg of sodium pyruvate was dissolved in 100 mL of phosphate buffer.

**Procedure**

One mL of substrate was incubated for few min at 37 °C. Then 0.2 mL of serum was added and incubated for one hour in case of AST and 30 min for ALT, then added 2 drops of aniline-citrate reagent to both the test and control. To the
control, serum was added after incubation. The reaction was arrested using 1.0 mL of DNPH solution and the tubes were kept at room temperature for 20 min. 1.0 mL of 0.4 N sodium hydroxide was added to all the tubes. Sets of standards were also treated in a similar manner. The colour developed was read at 540 nm.

Activities of aspartate and alanine transaminase are expressed as IU/L.

3.7.3. Assay of $\gamma$-glutamyl transferase (GGT, EC 2.3.2.2)

The enzyme activity was assayed according to the method of Rosalki and Ran (1972).

Principle

GGT hydrolyses peptide bonds, in which a terminal glutamic acid residue is linked by its $\gamma$-carboxyl group to an amino group. The enzyme has low specificity for non-glutamyl moiety, so that synthetic substrates such as $\gamma$-glutamyl-1-nitroanilide are acted upon by GGT which catalyse, the simultaneous transfer of glutamyl residue to an amino acid or peptide acceptor. Glycyl glycine is chosen for this purpose, to form a yellow product p-nitroanilide.

Reagents:

1. Tris-HCl buffer: 0.1 M, pH-8.2

2. Substrate: 30.3 mg of L- $\gamma$-glutamyl-p-nitroanilide/10 mL. The substrate was sparingly soluble and was dissolved by warming to 50-60 °C. The substrate solution was used within 2 h of its preparation.

3. 13.2 mg of glycyl glycine was dissolved in 10 mL of distilled. This was used as a second substrate.

4. Acetic acid 10%

5. Standard p-nitroaniline: 13.8 mg of p-nitroaniline/100 mL of distilled water.
Procedure

0.5 mL of serum was added to the incubation mixture containing 0.5 mL \(\gamma\)-glutamyl-p-nitroanilide, 2.0 mL glycyl glycine and 1 mL buffer. After incubation for 30 min at 37 °C the reaction was arrested by the addition of 1 mL of 10% acetic acid. The amount of p-nitroaniline liberated in the supernatant was measured as the difference in OD at 410 nm, between samples, with and without substrate. The substrate incubated in the absence of serum under the same conditions was used as the reference blank.

The enzyme activity is expressed as IU/L.

3.7.4. Assay of serum alkaline phosphatase (ALP, EC 3.1.3.1)

Alkaline phosphatase was assayed by the method of Kind and King’s (1954) using disodium phenylphosphate as the substrate. Disodium phenylphosphate is hydrolysed by alkaline phosphatase with the liberation of phenol, which reacts under alkaline conditions with Folin - phenol reagent to form a blue colour, which was estimated colorimetrically at 680 nm.

Reagents

1. Sodium carbonate bicarbonate buffer–0.1 M, (pH 10)

2. Substrate – 0.01 M, phenylphosphate disodium salt solution

3. Folin-phenol reagent: Folin - Ciocalteau reagent: 100 g sodium tungstate, 25 g sodium molybdate, 700 mL water, 50 mL 85% O-phosphoric acid and 100 mL conc. HCl were taken in a 1500 mL round bottomed flask. The mixture was refluxed gently for 10 h. To this, 150 g lithium sulphate, 50 mL water and a few drops of bromine were added. The mixture was boiled for 15 min to remove excess bromine. This was diluted in the ratio 1:2 with double distilled water just before use.

4. Sodium carbonate – 10%

5. Standard phenol solution – 5 \(\mu\)g/mL
Procedure

An incubation mixture containing 150 mL of bicarbonate buffer and 10 mL of substrate in 2.9 mL distilled water was preincubated at 37 °C for 10 min. 0.2 mL of serum was added to this and incubated at 37 °C for 15 min. The reaction was arrested by the addition of 1.0 mL of Folin phenol reagent. The suspension was centrifuged and 2.0 mL of 10% sodium carbonate was added to the supernatant. The solution was incubated at 37 °C for 10 min. Standard phenol solutions (2.5 μg -10.0 μg) were also treated with dilute Folin - phenol reagent and sodium carbonate. The blue colour developed was read at 680 nm.

The enzyme activity is expressed as U/L.

3.7.5. Estimation of total protein and albumin

Total proteins and albumin in the serum were estimated by Biuret method (Reinhold, 1953).

Proteins form a purple coloured complex with cupric ions in alkaline solution. The reaction takes its name from the simple compound biuret which reacts in the same way. The intensity of the purple colour is proportional to the amount of protein present in the sample.

Reagents

1. Stock biuret reagent: 45 g of sodium potassium tartrate was dissolved in 400 mL of 0.2 N sodium hydroxide and 15 g of copper sulphate was added and stirred. 5.0 g potassium iodide was then added, dissolved and made up to 1.0 L with 0.2 N sodium hydroxide.

2. Dilute biuret reagent: 200 mL of stock biuret reagent was added to 800 mL of 0.2 N sodium hydroxide containing 4.0 g potassium iodide.

3. Standard egg albumin: 500 mg/100 mL distilled water (small quantity of alkali was added to dissolve albumin).

4. Sodium sulphite solution: 28%.
**Procedure**

0.5 mL of sample was taken in a test tube and 9.5 mL of sodium sulphite solution was added and mixed. After mixing, 3.0 mL of the mixture was transferred into a tube for total protein estimation to which 5.0 mL of biuret reagent was added.

To the rest of the mixture, 3.0 mL of ether was added, stoppered, shaken well for 20 sec and then centrifuged for 5 min. 3.0 mL of the clear supernatant was taken for the estimation of albumin and treated with 5 mL of biuret reagent. Simultaneously, 2.0 mL of standard egg albumin was mixed with 1.0 mL of water and treated with 5.0 mL of biuret reagent. The purple colour developed was read at 540 nm after 15 min using reagent blank.

Values were expressed as g/dL.

**Estimation of globulin**

Serum globulin concentration was calculated using the following formula after the estimation of total protein and albumin.

\[
\text{Globulin} = \text{Total protein} - \text{Albumin}
\]

**3.8. ESTIMATION OF NEPHRITIC MARKERS**

**3.8.1. Estimation of urea**

Serum urea was estimated by using the diagnostic kit based on the method of Fawcett and Scott (1960).

**Principle**

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, the ammonia so formed reacts with hypochlorite and sodium salicylate in the presence of sodium nitroprusside to form a green coloured chromophore. The intensity of the colour produced is proportional to the concentration of urea in the sample.
Reagents

1. Buffered enzyme: Phosphate buffer, urease, sodium nitroprusside and ethylene diamine tetraacetic acid.

2. Colour developing reagent: Buffer, sodium hypochlorite, sodium salicylate and sodium hydroxide.

3. Urea standard: 40 mg/dL

Procedure

To 1.0 mL of buffered enzyme, 10 μL of sample was added, mixed well and kept at 37 °C for 5 min. 10 μL of standard and 10 μL of distilled water (blank) were also processed simultaneously. To all the tubes, 1.0 mL of colour developing reagent was added and mixed well. Exactly after 5 min of incubation at 37 °C, 1.0 mL of distilled water was added and the colour developed was read at 600 nm.

The values are expressed as mg/dL.

3.8.2. Estimation of uric acid

Serum uric acid was estimated by using the diagnostic kit based on the enzymic method described by Caraway, (1955).

Principle

Uric acid in the sample is oxidized by uricase to allantoin. In this reaction 1 mole of hydrogen peroxide is formed for every mole of uric acid oxidized. Hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxybenzene sulphonic acid and 4-aminoantipyrine to give quinoneimine dye. Intensity of the colour of this dye is proportional to the concentration of uric acid in the sample.

Reagents

1. Enzyme reagent: 4-Aminoantipyrine (4mM), 3,5-dichloro-2-hydroxybenzenesulphonate (2.0 mM), microbial uricase (150 U/L), horseradish peroxidase (10,000 U/L).

2. Standard uric acid: 5.0 mg/100 mL
Procedure

To 1 mL of the enzyme reagent, 25 μL of sample was added and mixed by inversion. 25 μL of standard and 25 μL of distilled water (blank) were also processed simultaneously. The tubes were incubated at 37 °C for 5 min and the colour developed was read at 510 nm.

The values are expressed as mg/dL.

3.8.3. Estimation of creatinine

Serum creatinine was estimated using the diagnostic kit based on the method of Tietz (1987) using Jaffe’s (1886) colour reaction.

Principle

The assay of creatinine was based on the reaction of creatinine with alkaline picrate as described by Jaffe. Most of the contaminants reacting with the Jaffe reagent produce a colour at a lower rate than does creatinine. The initial rate of colour formation is proportional to the concentration of creatinine in the sample.

Reagents

1. Saturated picric acid
2. Sodium hydroxide: 0.75 N
3. Creatinine standard: 2.0 mg/dL

Procedure

0.1 mL of serum was added to a reagent mixture containing 0.5 mL of picric acid solution and 0.5 mL of sodium hydroxide. The tubes were mixed well and incubated for 20 sec. The colour developed was measured spectrophotometrically at 510 nm after at 20 sec (A1) and after 45 sec (A2). Change in the absorbance (A2-A1) was measured for test and standard, which was used to determine the creatinine concentration in the test sample.

The values are expressed as mg/dL.
3.9. ESTIMATION OF LIPIDS

3.9.1. Estimation of total cholesterol

The total cholesterol was estimated by the method of Siedel et al., (1983).

**Principle**

Sample is treated with ferric chloride-acetic acid reagent to precipitate the protein. The protein free filtrate containing cholesterol ferric chloride is treated with conc. H₂SO₄. The reaction involves the 3-OH group of the cholesterol molecule, it is first dehydrated to form cholesterol 3-5 diene and then oxidized by sulphuric acid to link two molecules together as bis-cholesterol 3-5 diene. This material is sulphonated by sulphuric acid to form a red coloured disulphonic acid in the presence of ferric ion as catalyst (Salkowski’s reaction). The colour developed was read at 560 nm using suitable standard and a reagent blank.

**Reagents**

1. Acetic acid
2. Ferric chloride – 0.05% in acetic acid (w/v)
3. Conc. H₂SO₄
4. Stock standard: 100 mg of purified cholesterol in 100 mL of purified acetic acid.
5. Working standard: one volume of stock standard was diluted to 25 volumes with the ferric chloride-acetic acid reagent. The solution was kept in a cool dark place.

**Procedure**

0.5 mL extract was evaporated to dryness and 5.0 mL ferric chloride-acetic acid reagent was added, mixed well and 3.0 mL H₂SO₄ was added. The absorbance was read after 20 min at 560 nm against a reagent blank.

Values are expressed as **mg/g tissue** for tissue and **mg/dL** for plasma.
3.9.2. Estimation of free fatty acids (FFAs)

Non-esterified FFAs were estimated by the method of Falholt et al. (1973).

**Principle**

In the presence of phosphate buffer, the extract was shaken with a high density copper reagent (pH 8.1). The free fatty acids in the copper solution were determined colorimetrically with diphenyl carbazide.

**Reagents**

1. Extraction solvent: Chloroform: heptane : methanol (5:5:1)
2. Phosphate buffer: 33 mM, pH 6.4
3. Stock copper solution: 500 mM
4. Triethanolamine – 1 M
5. Sodium hydroxide – 1 M
6. Copper reagent: 10 mL of copper solution was mixed with 10 mL of triethanolamine and 6 mL of sodium hydroxide and diluted to 100 mL, then 33g of sodium chloride was added and the pH was adjusted to 8.1.
7. Diphenyl carbazide solution: 1.5 M in ethanol
8. Standard palmitic acid: 2 mM.

**Procedure**

0.1 mL of lipid extract/serum was evaporated to dryness and 1.0 mL of phosphate buffer, 6.0 mL of extraction solvent and 2.5 mL of copper reagent were added. All the tubes were shaken vigorously. 200 mg of activated sialic acid was added and left aside for 30 min. The tubes were centrifuged and 3.0 mL of the copper layer was transferred to another tube containing 0.5 mL of diphenyl carbazide and mixed carefully. The absorbance was read at 550 nm immediately.

Values are expressed as **mg/g tissue** for tissue and **mg/dL** for plasma.
3.9.3. Estimation of triglycerides (TGs)

Triglycerides were estimated by the method of Foster and Dunn, (1973).

**Principle**

Triglycerides are extracted by isopropanol, which upon saponification with potassium hydroxide yields glycerol and soap. The glycerol liberated is treated with metaperiodate, which releases formaldehyde, formic acid and iodide. The formaldehyde released reacts with acetyl acetone and ammonia forming yellow coloured compound, the intensity of which is measured at 405 nm.

**Reagents**

1. Isopropanol
2. Activated aluminium oxide (Neutral)
3. Saponification reagent: 5.0 g of potassium hydroxide was dissolved in 60 mL of distilled water and 40 mL of isopropanol was added to it.
4. Sodium metaperiodate reagent: 77 g of anhydrous ammonium acetate was dissolved in about 700 mL of distilled water; 60 mL glacial acetic acid was added to it followed by 650 mg of sodium metaperiodate. The mixture was diluted to 1.0 litre with distilled water.
5. Acetyl acetone reagent: 0.75 mL of acetyl acetone was dissolved in 60 mL of distilled water and 40 mL of isopropanol was added to it.
6. Standard triolein solution: 1 g of triolein was dissolved in 100 mL of isopropanol. 1.0 mL of stock standard was diluted to 100 mL to prepare a working standard of concentration 100 μg triolein/mL.

**Procedure**

To an aliquot of serum/lipid extract, evaporated to dryness. 0.1 mL of methanol was added followed by 4.0 mL of isopropanol. 0.4 g of alumina was added to all the tubes and shaken well for 15 min. Centrifuged and then 2.0 mL of the supernatant fluid was transferred to labelled tubes. The tubes were placed in a water bath at 65°C for 15 min for saponification after adding 0.6 mL of the saponification reagent followed by 1 mL of metaperiodate reagent and 0.5 mL of acetylacetone
reagent. After mixing, the tubes were kept in a water bath again at 65 °C for one hour, the contents were cooled and the absorbance was read at 405 nm. A series of standards of concentrations 8–4 μg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405 nm.

Values are expressed as mg/g tissue for tissue and mg/dL for plasma

3.9.4. Estimation of phospholipid (PL)

Phospholipids content was estimated by the method of Zilversmit and Davis (1950).

**Principle**

Principle of PL estimation involves the conversion of organic phosphorus to inorganic phosphorus, which reacts with ammonium molybdate to form phosphomolybdic acid. This on treatment with 1-amino,2- naphthol 4-sulphonic acid (ANSA) forms a stable blue colour, which is read at 680 nm.

**Reagents**

1. Conc. H$_2$SO$_4$ : 5N
2. Conc. HNO$_3$.
3. Ammonium molybdate solution: 2.5 g of ammonium molybdate was dissolved in 100 mL of 3N H$_2$SO$_4$
4. 1-amino, 2-naphthol, 4-sulphonic acid (ANSA): 500 mg of ANSA was dissolved in 195 mL of 15% sodium bisulphite solution and 5 mL of 20% sodium sulphite was added for complete solubilisation. The solution was filtered and stored in a brown bottle.
5. Standard phosphorus solution: 35.1 mg of potassium dihydrogen phosphate was dissolved in water. To this 1.0 mL of 10N H$_2$SO$_4$ was added and made upto 100 mL with distilled water.10 mL of this solution was diluted to100 mL, to prepare a working standard containing 8μg phosphorus/mL.
Procedure

An aliquot of the lipid extract was evaporated to dryness. 1.0 mL of 5 N H₂SO₄ was added and digested till light brown. Then 2 to 3 drops of concentrated HNO₃ was added and the digestion was continued till it became colourless. After cooling, 1 mL of water was added and heated in a boiling water bath for about 5 min. Then, 1.0 mL of ammonium molybdate and 0.1 mL of ANSA were added. The volume was then made upto 10.0 mL with distilled water and the absorbance was measured at 680 nm within 10 min. Standards in the concentration range of 2-8 mg were treated in the similar manner. The values obtained were multiplied with a factor 25 to convert inorganic phosphorus to its phospholipid equivalents.

Values are expressed as mg/g tissue for tissues or mg/dL for plasma

3.9.5. Estimation of HDL

HDL-cholesterol was estimated using the diagnostic kit based on the enzymatic method described by Izzo et al. (1981).

The VLDL and LDL fractions of plasma samples were precipitated using phosphotungstic acid and then HDL in the supernatant was separated by centrifugation and its cholesterol content measured.

Reagents

1. Precipitating reagent
2. Enzyme reagent
3. HDL-cholesterol standard: 50 mg%

Procedure

0.1 mL of plasma was mixed with 0.1 mL of precipitating reagent, allowed to stand at room temperature for 5 min and centrifuged at 2000-3000 rpm for 10 min. In the clear 50 µL of supernatant, cholesterol was estimated as described earlier.

The values were expressed as mg/dL of plasma.
3.9.6. Estimation of VLDL- and LDL-cholesterol (Friedewald et al., 1981)

These were calculated using the formula

\[
\text{VLDL cholesterol} = \frac{\text{TG}}{5}
\]

\[
\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL-C} + \text{VLDL-C})
\]

The values were expressed as mg/dL of plasma.

3.10. DETERMINATION OF LIPID METABOLIC ENZYMES

3.10.1. Assay of lipoprotein lipase (LPL, EC 3.1.1.34)

Lipoprotein lipase was assayed in tissues by the method of Korn, (1962).

**Principle**

LPL catalyses the breakdown of glycerol trioleate into glycerol and free fatty acids. Albumin was added to the incubation tube to bind the unesterified fatty acids which otherwise will inhibit the reaction. Ammonium ions were also added to provide the necessary activating cation. The amount of glycerol liberated was determined colorimetrically.

**Reagents**

*a. For incubation*

1. Ammonium sulphate: 0.5 M
2. 2% BSA adjusted to pH 8.5 with NaOH
3. Enzyme source: plasma homogenate prepared in 0.2 M Tris-HCl buffer, pH 8.5.
4. Substrate: 13.2 mg glycerol trioleate and 18 mg BSA were homogenized in 2.0 mL of 0.2 M Tris-HCl buffer of pH 8.5.
b. **For glycerol determination**

1. 0.05 M sodium periodate: 11.4 g of periodic acid was dissolved in 900 mL of water, adjusted to pH 8.5 with 1 N NaOH and made up to 1 L with H₂O.

2. 0.5 M sodium arsenate: 2.25 g NaOH and 50 g arsenic oxide were dissolved in 1 L distilled water.

3. Chromotropic acid (1,8 dihydroxy naphthalene disulphonic acid): 1.0 g of chromotropic acid was dissolved in 100 mL water and added to 400 mL H₂SO₄ (2:1 conc. H₂SO₄ with water). The reagent was cooled throughout the mixing procedure and prepared fresh every day.

**Procedure**

*a. Preparation of sample*

A known amount of the sample (plasma or tissue) was homogenized using 20mL of acetone. This was filtered and the residue in the filter paper was scrapped off, weighed and suspended in 1.0 mL of 0.025 M ammonia for 30 min at 0 °C for complete extraction of enzymes. This extract was used as the enzyme source.

*b. Incubation*

The incubation mixture consisted of 0.4 mL albumin, 0.1 mL ammonium sulphate, 0.1 mL substrate, 0.1 mL enzyme and sufficient water to make up a final volume of 1.0 mL. The reagents were kept cold while mixing and the tubes were incubated at 37 °C for 1 h, removing aliquots of 0.2 mL at 15 or 30 min intervals. The first sample was removed before placing the tubes in the water bath. The samples were transferred directly into a 10 mL conical tip centrifuge tube containing 0.1 mL of 1 N H₂SO₄.

*c. Glycerol determination*

0.1 mL periodate was added to the centrifuge tube, mixed well and allowed to stand at room temperature for 5 min. Then 0.1 mL sodium arsenate reagent was
added, mixed well and again allowed to stand at room temperature for 10 min. This was followed by the addition of 9.0 mL chromotropic acid, mixed by inversion (covering the top of the tube by paraffin) and placed in a boiling water bath for 30 min, cooled and the volume adjusted to 10 mL with water. The optical density was read at 570 nm. The assay was standardized against a glycerol solution of known molarity.

The activity of lipoprotein lipase is expressed as \textit{\textmu}moles of glycerol liberated/h/mL plasma.

3.10.2. Assay of lecithin cholesterol acyl transferase (LCAT, EC 2.3.1.43)

The enzyme was assayed in plasma by the method of Hitz et al., (1983)

\textit{Reagents}

1. Dextran sulphate:0.2%

2. Isopropanol

3. Acetone

4. Digitonin-5.0 mg/mL in 50% ethanol.

5. Preparation of substrate: A pool of human plasma was heated at 56 °C for 30 min to inactivate LCAT. Plasma was then incubated at 4 °C for 15 min with 0.2% dextran sulphate, which eliminated 2/3\textsuperscript{rd} of lipoproteins (1.0L, VI.1L)

This was sedimented by centrifugation at 1750×g for 15 min. The supernatant containing HDL was used as the substrate.

\textit{Procedure:}

1.0 mL plasma was mixed with 0.5 mL of 0.2% dextran sulphate. The mixture was incubated at 4 °C for 15 min and centrifuged at 1750×g for 15 min. The supernatant was separated and used as the enzyme source and its activity was assayed as given below.
0.6 mL substrate was mixed with 0.6 mL of enzyme. 0.2 mL of this mixture was mixed with 1.0 mL isopropanol, and the remaining mixture was incubated at 27 °C for 90 min. The precipitate was removed by centrifugation and the supernatant was used for the estimation of free cholesterol. The free cholesterol content estimated represent the amount of free cholesterol present in the test sample at zero time.

After 90 min, 0.2 mL of the incubation mixture was mixed with 1.0 mL of isopropanol and the remaining mixture was incubated at 27 °C for a further period of 90 min. At the end of 180 min, 0.2 mL of the incubation mixture was treated with 1.0 mL of isopropanol to arrest the reaction. The precipitated protein in all the tubes were separated by centrifugation and the free cholesterol content in the supernatant was estimated as described previously.

Control tubes containing only the substrate were treated similarly to check for complete inactivation of plasma during substrate preparation. LCAT activity was expressed as a function of disappearance of free cholesterol during the incubation period.

LCAT activity is expressed as \( \mu \text{moles of cholesterol esterified/h/mL plasma} \).

### 3.10.3. Assay of 3-hydroxy 3-methyl glutaryl coenzyme A reductase (HMG-CoA reductase) (EC.1.1.34)

The ratio between HMG-CoA and mevalonate in tissues was taken as an index of the activity of HMG-CoA reductase as described by Rao and Ramkrishnan (1975).

**Reagents**

1. Saline-arsenate: 1 g of sodium arsenate/ L in physiological saline.
2. Dilute perchloric acid: 50 mL of perchloric acid was diluted upto 1 litre with water.
3. Hydroxylamine hydrochloride reagent (For mevalonate): Equal volumes of hydroxylamine hydrochloride reagent and water were mixed freshly before use.
4. Hydroxylamine hydrochloride reagent (For HMG-CoA): Equal volumes of hydroxylamine hydrochloride reagent and 4.5 M NaOH were mixed freshly before use.

5. Ferric chloride reagent: 5.2 g of trichloroacetic acid and 10 g of ferric chloride were dissolved in 50 mL of 6.5 N HCl and diluted to 100 mL with water.

Procedure

Equal volumes of fresh 10% tissue homogenate and dilute perchloric acid were mixed, kept for 5 min and centrifuged at 2000×g for 10 min. 1.0 mL of filtrate was mixed with 0.5 mL of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA) and after 5 min. 1.5 mL of FeCl₃ was added and shaken well. Readings were taken after 10 min at 540 nm against a similarly treated saline-arsenate blank. The ratio of HMG-CoA to mevalonate was calculated.

HMG CoA reductase activity is expressed as a ratio of HMG CoA to mevalonates.

3.11. ESTIMATION OF LIPID PEROXIDATION PRODUCTS

3.11.1. Estimation of thiobarbituric acid reactive substances (TBARS)

The level of TBARS in plasma, RBC lysate and tissue homogenates were estimated by the method of Niehaus and Samuelsson, (1968).

In this method, malondialdehyde and other TBARS were measured by their reactivity with thiobarbituric acid in an acidic condition to generate a pink coloured chromophore which was read at 535 nm.

Reagents

1. Trichloroacetic acid: 15%

2. Hyrdochloric acid: 0.25 N
3. Thiobarbituric acid (TBA): 0.375% in hot distilled water.

4. TCA-TBA-HCl reagent: 5% TCA, 0.25 N HCl and 0.375% TBA were mixed freshly in the ratio of 1:1:1.

5. Standard: 1,1',3,3'-tetramethoxy propane (commercially available).

Procedure

The tissue homogenate was prepared in tris-HCl buffer (0.025 M, pH 7.8). To 1.0 mL of the tissue homogenate or 0.5 mL of plasma, 2.0 mL of TCA-TBA-HCl reagent was added and mixed thoroughly. The mixture was kept in a boiling water bath for 15 min. After cooling the tubes were centrifuged at 1000×g for 10 min and the colour developed in the supernatant was measured at 535 nm against the reagent blank. A series of standard solutions in the concentration of 2.5 to 10 nmol was treated in the similar manner.

Values are expressed as \textit{nmol/mg protein} for plasma, \textit{nmol/mg Hb} for RBC lysate and \textit{mmol/mg tissue} for tissues

3.11.2. Estimation of lipid hydroperoxides (LOOH)

Plasma, lysate and tissue LOOH were quantified by the method of Jiang et al., (1992).

Principle

This method is based on the rapid peroxide-mediated oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) under acidic conditions in the presence of xylene orange. The Fe^{3+}-xylene orange complex was measured spectrophotometrically at 560 nm.

Reagents

1. Fox reagent: 100 µM xylene orange, 4 mM butylated hydroxytoluene, 25 mM H_2SO_4 and 250 µM ammonium ferrous sulphate were added to 90 mL methanol and 10 mL of H_2SO_4 (250 mM).
**Procedure**

0.9 mL of Fox reagent was mixed with 0.1 mL of methanol extracted sample and incubated for 30 min at room temperature. The absorbance was read in a colorimeter at 560 nm. The amount of hydroperoxides produced was calculated by using the molar extinction coefficient of $4.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

The levels of LOOH are expressed as **mmoles/mg protein** for plasma, **mmoles/mg Hb** for RBC lysate and **mmoles/mg of tissue** for tissues.

**3.11.3. Estimation of conjugated dienes (CD)**

Conjugated dienes (CD) in the tissue lipid extracts were estimated by the method of Recknagel and Glende (1984).

**Principle**

Lipid peroxidation is associated with the rearrangement of double bonds in polyunsaturated fatty acids leading to the formation of CD, which absorb light at the wavelength of 233 nm. The oxidation index of the lipid sample at 233 nm and 215 nm (A233/A215), was computed which reflects the diene content and the extent of peroxidation.

**Reagents**

1. Chloroform: methanol mixture (C:M, 2:1,v/v)

2. Cyclohexane

**Procedure**

0.5 mL tissue homogenate was made up to 7.0 mL with chloroform and then 14.0 mL of methanol was added, mixed thoroughly and kept at room temperature for 10 min with occasional shaking. After centrifugation at $260 \times g$ for 10 min, the supernatant was made up to a known volume with chloroform: methanol (C:M) mixture. Then 10 mL of distilled water was added and centrifuged at $260 \times g$ for 10 min to separate the two phases. The lower chloroform phase was
evaporated to dryness. The lipid residue was suspended in 3.0 mL of cyclohexane and the absorbance at 215 nm and 233 nm were measured against cyclohexane blank.

CD content was expressed as a ratio of A233/A215. The concentration of conjugated diene is expressed as \text{nmol/mg protein} for plasma, \text{nmol/mg Hb} for RBC lysate and \text{nmol/mg tissue} for tissues.

3.12. ESTIMATION OF ANTIOXIDANTS

3.12.1. Assay of superoxide dismutase [SOD, EC 1.15.1.1]

SOD was assayed by the method of Kakkar et al. (1984).

\textbf{Principle}

The assay is based on the inhibition of NADII - phenazine methosulphate nitroblue tetrazolium formazan formation. The reaction is initiated by the addition of NADH. After 90 sec incubation, the reaction is stopped by adding glacial acetic acid. The colour developed at the end of the reaction is extracted into n - butanol layer and the absorbance was measured at 520 nm.

\textbf{Reagents}

1. Sodium pyrophosphate buffer, 0.052 M, pH 8.3
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS), 186 \text{µM}
6. Nitroblue tetrazolium (NBT), 300 \text{µM}
7. Reduced nicotinamide adenine dinucleotide (NADH), 780 \text{µM}

\textbf{Procedure}

Tissues were homogenized in sodium pyrophosphate buffer. To 0.5 mL erythrocyte lysate or tissue homogenate, 1.0 mL water followed by 2.5 mL ethanol and 1.5 mL chloroform (chilled reagents) were added, shaken for 90 sec at 4 \text{oC}
and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL PMS, 0.3 mL NBT and appropriately diluted enzyme preparation in a total volume of 3.0 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30 °C for 90 sec, the reaction was stopped by the addition of 1.0 mL glacial acetic acid. The reaction mixture was stirred vigorously, shaken with 4.0 mL n-butanol and was allowed to stand for 10 min, centrifuged and the n-butanol layer was separated. The colour intensity of the chromogen in n-butanol layer was measured in a colorimeter at 520 nm. A system devoid of enzyme served as control. The enzyme concentration required to produce 50% inhibition of chromogen formation in one minute under standard conditions was taken as one unit.

The specific activity of the enzyme is expressed as enzyme required for 50% inhibition of NBT reduction /min/mg Hb for RBC lysate or enzyme required for 50% inhibition of NBT reduction /min/mg protein for plasma and tissues.

3.12.2. Assay of catalase [CAT, EC 1.11.1.6]

The activity of CAT was determined in erythrocyte lysate and tissue homogenate by the method of Sinha, (1972).

**Principle**

Dichromate in acetic acid was reduced to chromic acetate, when heated in the presence of H₂O₂ with the formation of perchromic acid as an unstable intermediate. The CAT preparation is allowed to split H₂O₂ for different periods of time. The reaction is stopped at different time intervals by the addition of dichromate acetic acid mixture in hot conditions. The remaining H₂O₂ forms H₂O₂ - chromic acetate which can be determined colorimetrically at 590 nm.

**Reagents**

1. Phosphate buffer, 0.01 M, pH 7.0

2. Hydrogen peroxide (H₂O₂), 0.2 M

3. 5% Potassium dichromate (w/v)
4. Dichromate-acetic acid reagent: 5% Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this, 1.0 mL was diluted again with 4.0 mL acetic acid.

5. Standard hydrogen peroxide: 0.2 mM

Procedure

Tissue homogenate was prepared in phosphate buffer. To 0.9 mL phosphate buffer, 0.1 mL erythrocyte lysate or tissue homogenate and 0.4 mL H$_2$O$_2$ were added. The reaction was arrested after 15, 30, 45 and 60 sec by adding 2.0 mL dichromate - acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the colour developed was read at 590 nm. Standards in the concentration range of 20-100 µM were processed for the test.

The specific activity of the enzyme is expressed as µmoles of H$_2$O$_2$ utilized /min/mg Hb for RBC lysate or µmoles of H$_2$O$_2$ utilized /min/mg protein for plasma and tissues.

3.12.3. Assay of glutathione peroxidase [GPx, E.C 1.11.1.9]

The activity of GPx was assayed in erythrocyte lysate and tissue homogenate by the method of Rotruck et al., (1973).

Principle

A known amount of enzyme preparation is allowed to react with H$_2$O$_2$ and GSH for a specified time period. The GSH content remaining after the reaction is measured by Ellman’s reaction.

$$2 \text{ GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2 \text{ H}_2\text{O}$$

Reagents

1. Tris-HCl buffer, 0.4 M, pH 7.0
2. Sodium azide solution, 10 mM
3. 10% TCA
4. EDTA, 0.4 mM
5. H₂O₂, 1.0 mM
6. Reduced glutathione (GSH), 2.0 mM

Procedure

To 0.2 mL of Tris-HCl buffer, 0.2 mL EDTA, 0.1 mL of sodium azide and 0.2 mL enzyme preparation (erythrocyte lysate or tissue homogenate) were added and mixed well. To this 0.2 mL GSH followed by 0.1 mL of H₂O₂ were added. The contents were mixed well and incubated at 37 °C for 10 min along with a tube containing all reagents except the homogenate. After 10 min the reaction was arrested by the addition of 0.5 mL of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH calorimetrically at 340 nm.

The activities are expressed as \text{\mu moles of GSH utilized/min/mg Hb} for RBC lysate or \text{\mu moles of GSHII utilized/min/mg protein} for plasma and tissues.

3.12.4. Assay of glutathione S-transferase [GST, EC 2.5.1.18]

The activity of GST was assayed by the method of Habig et al. (1974).

Principle

Activity of GST was measured in erythrocyte lysate and tissue homogenate by following the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as standards.

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

Reagents

1. Phosphate buffer, 0.3 mM, pH 6.5
2. GSH, 30 mM
3. CDNB, 30 mM in 95% ethanol
Procedure

The reaction mixture contained 1.0 mL phosphate buffer, 0.1 mL CDNB, 0.1 mL erythrocyte lysate/tissue homogenate and 0.7 mL distilled water. The reaction mixture was preincubated at 37 °C for 5 min and then the reaction was started by the addition of 0.1 mL GSH. The change in absorbance was read at 340 nm every min for 5 min. Reaction mixture without the enzyme was used as the blank.

Calculation

\[
\text{Activity} = \frac{O.D \times 3 \times 1000}{9.6 \times 5 \times \text{protein in mg}}
\]

Values are expressed as \( \mu \text{moles of CDNB-GSH conjugate formed/min/mg protein} \).

3.12.5. Assay of glutathione reductase [GR, EC. 1. 6. 4. 2]

GR activity was assayed by the method of Carlberg and Mannervik, (1985).

Principle

The enzyme activity was assayed by measuring the GSII formed when the oxidized glutathione (GSSG) is reduced by nicotinamide adenine dinucleotide phosphate (NADPH).

Reagents

1. Phosphate buffer: 0.1 M, pH 7.4
2. Flavine adenine dinucleotide (FAD): 250 mM
3. NADPH: 4 mM
4. Ethylene diamine tetra acetic acid (EDTA): 80 mM

Procedure

2.0 mL phosphate buffer, 0.1 mL enzyme sample (erythrocyte or tissue homogenate), 0.1 mL FAD and 0.5 mL EDTA solution were taken in a test tube. A blank was set up using all the reagents except FAD. The tubes were incubated at 37 °C for 15 min followed by the addition of 0.1 mL of NADPH solution to each
tube. The reaction rate was then continuously monitored in a spectrophotometer at 340 nm for 5 min and the linear absorbance change was measured.

Values are expressed in \( \text{\mu moles of NADPH oxidized / min/mg protein} \).

3.12.6. Estimation of reduced glutathione (GSH)

Reduced glutathione in the plasma and tissues was estimated by the method of Ellman, (1959). This method was based on the formation of 2-nitro-5-thiobenzoic acid (a yellow colour compound) when 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphydryl groups.

Reagents

1. Phosphate buffer : 0.1 M, pH 8.0
2. TCA: 5%
3. Ellman’s reagent: 34 mg of DTNB in 10 mL of 0.1% sodium citrate.
4. Disodium hydrogen phosphate: 0.3 M
5. Standard glutathione solution: 10.0 mg/100 mL.

Procedure

A known weight of tissue was homogenized in phosphate buffer (0.1 M pH 7.0). 0.5 mL of homogenate or plasma was pipetted out and precipitated with 2.0 mL of 5% TCA. 2.0 mL of supernatant was taken after centrifugation and 1.0 mL of Ellman’s reagent and 4.0 mL of 0.3 M disodium hydrogen phosphate were added. The yellow colour developed was read in a Spectronic 20 at 412 nm. A series of standards (20-100 \( \mu g \)) was treated in a similar manner along with a blank containing 1.0 mL of buffer.

The amount of glutathione was expressed as mmole/mg Hb for RBC lysate and mmoles/mg protein of plasma and tissue.
3.12.7. Estimation of ascorbic acid (Vitamin C)

Ascorbic acid was estimated according to the method of Roe and Kuether (1943)

Principle

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. These products are treated with DNPH to form the derivative bis-2,4-dinitrophenylhydrazone. This compound, in strong sulphuric acid, undergoes rearrangement to form a product with an absorption band that can be measured at 520 nm. The reaction is performed in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 10% TCA
2. 65% H₂SO₄
3. DNPH-thiourea-copper sulphate reagent (DTC): This reagent was prepared by dissolving 0.4 g thiourea, 0.05 g copper sulphate and 3.0 g 2,4-dinitrophenylhydrazine (DNPH) in 100 mL of 9 N H₂SO₄.
4. Stock standard: 100 mg L-ascorbic acid was dissolved in 100 mL of 5% TCA.
5. Working standard: 1 in 10 dilution with 5% TCA to obtain a concentration of 0.1 mg/mL.

Procedure

To 0.5 mL of plasma or tissue homogenate, 1.5 mL ice-cold TCA was added, mixed and centrifuged for 10 min at 1800×g. To 0.5 mL of the supernatant, 0.1 mL DTC reagent was added, mixed well and the tubes were incubated at 37 °C for 3 h. 0.75 mL of ice-cold 65% H₂SO₄ was added and the tubes were allowed to stand at room temperature for an additional 30 min. A set of standards containing 10-50 µg
Ascorbic acid was made up to 0.5 mL and were processed in similar manner along with a blank containing 0.5 mL TCA. The colour developed was read at 520 nm.

The amount of ascorbic acid is expressed as mg/dL of plasma and mg/100 g of tissue.

3.12.8. Estimation of α-tocopherol (vitamin-E)

α-Tocopherol in the plasma and tissues was estimated by the method of Baker et al., (1980). The method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured complex with 2, 2′ dipyridyl. Absorbance of the chromophore was measured at 520 nm.

**Reagents**

1. Petroleum ether : 60-80 °C
2. Double distilled ethanol.
3. 2, 2′ dipyridyl solution: 0.2% in double distilled ethanol.
4. Ferric chloride solution: 0.5% in double distilled ethanol.
5. Stock standard: 10 mg of α-tocopherol in 100 mL of distilled ethanol.
6. Working standard: Stock solution was diluted with ethanol to a concentration of 10 μg/mL.

**Procedure**

To 0.5 mL of sample, 1.5 mL of ethanol was added, mixed and centrifuged. The supernatant was evaporated and to the precipitate, 3.0 mL of petroleum ether, 0.2 mL of 2, 2′ dipyridyl solution and 0.2 mL of ferric chloride solution were added. Mixed well and kept in dark for 5 min. An intense red colour was developed. 4.0 mL of n-butanol was added to all the tubes and mixed well.

Standard tocopherol in the range of 10-100 μg was taken and treated similarly along with a blank containing only the reagent. The colour in the n-butanol layer was read at 520 nm.

The values were expressed as mg/dL of plasma and mg/100g for tissue.
3.13. DETERMINATION OF NITRITE

Nitrite concentrations as an index of nitric oxide levels was determined by the method of Moshage et al., (1995) based on Griess reaction.

Principle

Nitric oxide has a short half-life, and thus, the plasma and urine concentrations of the stable end products of nitric oxide, nitrite plus nitrate, were used as an index of nitric oxide production. Nitrites and nitrates (NOx) are the principal oxidation products of NO in an aqueous solution. NOx concentrations in plasma was measured based on Griess reaction.

Reagents

1. 10% (v/v) Sulphosalicylic acid
2. Griess reagent: 1 g/L sulphanilamide, 25 g/L phosphoric acid and 0.1 g/L N-(1-naphthyl)-ethylenediamine
3. Nitrate reductase (1 IU/L)
4. 0.25 M NADPH

Procedure

For the determination of nitrite, plasma was deproteinised with 10% sulphosalicylic acid, the supernatant was aspirated into a microtitre plate well, followed by 100 μL Griess reagent. After 10min of colour development at room temperature, the absorbance was measured on a microplate reader at a wavelength of 540 nm. Plasma nitrate was measured after enzymic reduction by an improved method. Another set of plasma sample was incubated with nitrate reductase and NADPH. After reduction, the samples were deproteinized and the supernatant assayed in same way as that for the measurement of nitrate. The results were calculated using a calibration curve prepared with sodium nitrite in distilled water. Values obtained by this procedure represent the sum of nitrite and nitrate.

The values are expressed as nmoles/mg of protein
3.14. DETERMINATION OF IRON

Iron content was estimated by the method of Ramsay, (1957)

Reagents

1. 2,2'-dipyridyl : 0.1% in 3% acetic acid (v/v)

2. Sodium sulphite (0.1 M): Dissolved 1.26 g of anhydrous sodium sulphite or 2.53 g of Na$_2$SO$_3$·7H$_2$O in water and made up to 100 mL. This reagent was prepared fresh.

3. Chloroform (AR)

4. Standard iron solution: Dissolved 498 mg of ferrous sulphate (FeSO$_4$·7H$_2$O) in water. To this was added 1 mL of concentrated sulphuric acid and the total volume was made up to 1 L. This contained 100 µg iron per mL.

5. Working standard: 3 mL of the stock solution was diluted to 100 mL with water to obtain a solution containing 3.3 mg per mL.

Procedure

Equal volumes of test sample, 0.1 M sodium sulphite and dipyridyl reagent were mixed in glass-stoppered centrifuge tubes. The tubes were heated in a boiling water bath for 5 min. The contents were cooled and 20 mL of chloroform was added to each tube. The tubes were stoppered and shaken vigorously for 30 sec and centrifuged for 5 min at 1000 rpm. The colour intensity was measured at 520 nm in a Shimadzu-UV spectrophotometer. Blank containing ferrous sulphate was also treated similarly.

The results are expressed as µg/g of tissue.
3.15. ESTIMATION OF ALCOHOL METABOLIZING ENZYMES

3.15.1. Assay of alcohol dehydrogenase (ADH, EC 1.1.1.1)

The activity of ADH was estimated by the method of Agarwal and Goedde, (1990).

**Principle**

\[
\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+
\]

**Reagents**

1. Sodium pyrophosphate buffer 50 mM, pH - 8.8
2. 95% (v/v) Ethanol
3. 15 mM Nicotinamide adenine dinucleotide solution oxidized form (NAD\(^+\))
4. 10 mM Sodium phosphate
5. Phosphate buffer 10 mM, pH-7.5

**Procedure**

To 2.9 mL of reaction mixture containing 1.3 mL of buffer, 0.1 mL of ethanol and 1.5 mL of NAD\(^+\), 0.1 mL of enzyme solution was added and mixed by inversion and the increase in absorbance at 340 nm was measured at 1 min intervals for 6 min. (NAD at 340 nm=6.22) using a blank containing buffer and ethanol.

Values are expressed as \(\mu\)moles of NAD utilized/min/mg protein

3.15.2. Assay of aldehyde dehydrogenase (ALDH EC 1.2.1.3)

The activity of ALDH was estimated by the methods of Agarwal and Goedde, (1990).

**Principle**

\[
\text{Acetaldehyde} + \text{NAD}^+ \xrightarrow{\text{ALDH}} \text{Acetic Acid} + \text{NADH} + \text{H}^+
\]
Reagents

1. Tris-HCl buffer 1 M, pII 8.0
2. 20 mM nicotinamide adenine dinucleotide, oxidized form, (NAD+)
3. 100 mM acetaldehyde solution
4. 3 M Potassium chloride solution (KCl)
5. 1 M 2-mercaptoethanol solution (2-ME)

Procedure

To 2.9 mL of reaction mixture containing 2.32 mL deionised water, 0.3 mL buffer, 0.1 mL NAD⁺, 0.1 mL KCl, 0.05 mL acetaldehyde, 0.03 ml mercaptoethanol and 0.1 mL of enzyme solution were added and mixed by inversion and the increase in absorbance at 340 nm was measured at 1 min intervals for 5 min using a blank containing deionised water in place of enzyme. Extinction coefficient for NADH is 6.22 at 340 nm.

Values are expressed as μmoles of NAD utilized/min/mg protein

3.16. DETERMINATION OF XENOBIOTIC METABOLIZING ENZYMES

3.16.1. Assay of microsomal cytochrome P450

Microsomal cytochrome P450 was measured by the method of Omura and Sato, (1964).

Principle

Carbonmonoxide (CO) adducts are formed by the reaction of reduced cytochrome P450 with CO. This property enables the estimation of the cytochrome P450. The absorbance was measured at 450 nm.

Reagents

1. Tris-HCl, 50 mM containing 150 mM KCl and 10 mM MgCl₂, pH 7.4.
2. Sodium dithionate
3. Carbon monoxide (CO) gas; CO can be generated freshly by the action of concentrated H₂SO₄ and formic acid.
**Procedure**

To 0.1 mL of microsomal sample, Tris-HCl, 50 mM containing 150 mM KCl and 10 mM MgCl₂, pH 7.4 was gently bubbled for 1 min. The sample was then divided equally between two cuvettes and allowed to stand for 3-5 min. The baseline was recorded between 400 to 500 nm. Few milligrams of sodium dithionite was added to the contents in the sample cuvette and the resulting spectrum was recorded between 400 to 500 nm. The cytochrome P450 content was determined from the spectrum using the molar extinction coefficient 104 mM⁻¹ cm⁻¹.

Values are expressed as μmoles/mg protein

**3.16.2. Assay of microsomal cytochrome b₅**

Cytochrome b₅ content was measured by the method of Omura and Sato, (1964).

**Principle**

Cytochrome b₅ was calculated by measuring the spectral difference between reduced and oxidized cytochrome b₅.

**Reagents**

1. Phosphate buffer: 0.2 M, pH 7.4

2. NADH, 2% (W/V)

**Procedure**

To a mixture containing 1 mL of buffer, 0.5 mL of tissue homogenate was added and the absorbance was read starting from 400 till 500 nm before and after the addition of 0.025 mL of NADH. The difference in absorption spectrum was used to calculate cytochrome b₅ content using the molar extinction coefficient 185 mM⁻¹ cm⁻¹.

The cytochrome b₅ content is expressed as μmoles/mg protein.
3.16.3. Assay of microsomal NADH-cytochrome b5 reductase (EC 1.6.2.2)

Microsomal NADH-cytochrome b5 reductase was measured by the method of Mihara and Sato, (1972).

**Principle**

The rate of reduction of potassium ferricyanide by NADH was measured at 420 nm.

**Reagents**

1. 0.1 M phosphate buffer, pH 7.5
2. 0.1 mM NADH
3. 1 mM potassium ferricyanide
4. Microsomal sample

**Procedure**

The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 0.1 mM NADH, 1 mM potassium ferricyanide and microsomal sample in a final volume of 1 mL. The reaction was started by the addition of NADH. The enzyme activity was calculated using the extinction coefficient of 1.02 mM⁻¹cm⁻¹.

One unit of enzyme activity is defined as that causing the oxidation of one mole of ferricyanide/min/mg protein.

3.16.4. Assay of microsomal NADPH-cytochrome P450 reductase (EC 1.6.2.4)

Microsomal NADPH-cytochrome P450 reductase was measured by the method of Omura and Takesue, (1970).

**Principle**

The assay measures the rate of oxidation of NADPH at 340 nm.

**Reagents**

1. 0.3 M phosphate buffer, pH 7.5
2. 0.1 mM NADPH
3. 0.2 mM potassium ferricyanide
4. Microsomal sample
**Procedure**

The reaction mixture contained 0.3 M potassium phosphate buffer (pH 7.5), 0.1 mM NADPH, 0.2 mM potassium ferricyanide and microsomal sample in a final volume of 1 mL. The reaction was started by the addition of NADPH. The enzyme activity was calculated using the molar extinction coefficient 6.33 mM$^{1}$cm$^{-1}$.

One unit of enzyme activity is defined as that causing the oxidation of one µmole of NADPH/min/mg protein.

**3.16.5. Assay of cytochrome P4502E1**

Cytochrome P4502E1 (CYP4502E1) activity was measured by the method of Watt et al., (1997).

**Reagents**

1. 40 mM p-nitrophenol
2. 0.1 M Phosphate buffer, pH 7.5
3. 10 mM NADPH
4. 20% TCA
5. 10 mM NaOII

**Procedure**

The assay mixture contained 100 µg microsomal protein, 40 mM p-nitrophenol and 0.1 M phosphate buffer. The reaction was initiated by the addition of 10 mM of NADPH and incubated at 37 °C for 60 min. The reaction was stopped with 20% TCA and centrifuged at 1000 rpm for 5 min. 10 mM NaOII was added to the supernatant and the absorbance was measured at 450 nm.

Values are expressed as µmoles of p-nitrocatechol formed/min/mg protein.

**3.16.6. Assay of DT-diaphorase (EC 1.6.99.2)**

DT-diaphorase activity was measured by the method of Emster et al., (1979).

**Principle**

The reaction was initiated with NADPH as the electron donor and 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor. The reduction of DCPIP was measured spectrophotometrically at 600 nm.
Reagents

1. Tris-HCl buffer, pH 7.4
2. 0.1 mM NADPH
3. 0.04 mM 2,6 DCPIP
4. 0.07% Bovine serum albumin (BSA)
5. 33 μM cytochrome C
6. 10 μM dicoumarol

The reaction mixture contained the following in a final volume of 3 mL: 25 mL Tris-HCl, pH 7.4, 0.02 mL of 0.07% BSA, 0.1 mM of NADPH, 0.01 mL of cytochrome C and an appropriate amount of the cytosolic enzyme sample. The electron acceptor, 2,6-DCPIP (0.04 mM), was added to initiate the reaction. The reduction of DCPIP was measured spectrophotometrically and the absorbance was followed for 5 min at 600nm. A system devoid of enzyme served as the blank and the reagents along with dicoumarol served as a control. The enzyme activity was calculated using the extinction coefficient 21 mM⁻¹cm⁻¹.

Calculation

Activity = \frac{O.D \times 3 \times 1000}{2.1 \times 5 \times \text{protein in mg}}

The values are expressed as μmoles of 2, 6 DCPIP reduced/min/mg protein.

3.17. ASSAY OF MEMBRANE BOUND ATPases

3.17.1. Assay of total ATPase

The activity of total ATPase was assayed in the tissues by the method of Evans, (1969).

Reagents

1. Tris-HCl buffer-0.1 M, pH 7.4
2. Sodium chloride-0.1 M
3. ATP-0.01 M
4. Potassium chloride-0.1%
5. Magnesium chloride-0.1%
6. TCA-10%
Procedure

The incubation mixture in a total volume of 2 mL contained 0.1 mL buffer, 1.5 mL of potassium chloride, 0.1 mL of sodium chloride, 0.1 mL of magnesium chloride, 0.1 mL of ATP and 0.1 mL of tissue sample. The mixture was incubated at 37 °C for 20 min. The reaction was arrested by addition of 1 mL of 10% TCA and then centrifuged. The phosphorous liberated was estimated by the method of Fiske and Subbarow, (1925).

The activity of enzyme in was expressed in **μmole of phosphorous liberated/mg protein/hour**

3.17.2. Assay of Na⁺/K⁺ dependent ATPase

The activity of sodium/potassium dependent adenosine triphosphatase (Na⁺/K⁺ ATPase) in the tissues was assayed according to the procedure of Bonfing, (1970).

Reagents

1. Tris-HCl buffer-184 mM, pH 7.5
2. Magnesium sulphate-50 mM
3. Potassium chloride-50 mM
4. Sodium chloride-600 mM
5. EDTA-1 mM
6. ATP-40 mM
7. TCA-10%
8. Fiske and Subbarow reagent

Procedure

The incubation mixture contained 1.0 mL of buffer, 0.2 mL of magnesium sulphate, 0.2 mL of potassium chloride, 0.2 mL of sodium chloride, 0.2 mL of EDTA, 0.2 mL of ATP and 0.2 mL of tissue homogenate. The contents were incubated at 37°C for 15 minutes. 1.0 mL of ice cold 10% TCA was added at the
end of 15 min to arrest the reaction. The content of inorganic phosphorus liberated was estimated by Fiske and Subbarow, (1925).

The enzyme activity was expressed as $\mu$M of phosphorous liberated/mg protein/h.

### 3.17.3. Assay of Ca$^{2+}$ dependent ATPase

The activity of calcium dependent adenosine triphosphatase (Ca$^{2+}$-ATPase) in the tissues was assayed according to the method of Hjerten and Pan, (1983).

**Reagents**

1. Tris-HCl buffer-125 mM, pH 8.0
2. Calcium chloride-50 mM
3. ATP-10 mM
4. Ammonium molybdate-2.5% in H2SO4
5. ANSA - 0.25%
6. TCA- 10%

**Procedure**

The incubation mixture contained 0.1 ml of buffer, 0.1 ml of calcium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of tissue homogenate. The contents were incubated at 37°C for 15 minutes. The reaction was then arrested by addition of 0.5 ml of ice-cold 10% TCA. The amount of inorganic phosphorus liberated was estimated by the method of Fiske and Subbarow, (1925).

The enzyme activity was expressed as $\mu$M of phosphorous liberated/mg protein/h.

### 3.17.4. Assay of Mg$^{2+}$ dependent ATPase

The activity of magnesium dependent adenosine triphosphatase (Mg$^{2+}$-ATPase) in the tissues was assayed by the method of Ohnishi et al., (1982).
Reagents

1. Tris-HCl buffer-375 mM, pH 7.6
2. Magnesium chloride-25 mM
3. ATP-10 mM
4. TCA-10%
5. Fiske and Subbarow reagent

Procedure

The incubation mixture contained 0.1 ml of buffer, 0.1 ml of magnesium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of tissue homogenate. The contents were incubated at 37 °C for 15 min. The reaction was arrested by the addition of 0.5 ml of ice-cold 10% TCA. The content of inorganic phosphorous liberated was estimated by the method of Fiske and Subbarow, (1925).

The enzyme activity was expressed as $\mu$M of phosphorous liberated/mg protein/h

3.18. ESTIMATION OF HAEMOGLOBIN

Haemoglobin was determined by the method of Drabkin and Austin, (1932)

Principle

The basis of the method was dilution of blood in an alkaline solution containing potassium cyanide and potassium ferricyanide. Haemoglobin was oxidized to methaemoglobin that combines with cyanide to form cyanmethaemoglobin that was measured at 540 nm.

Reagents

1. Drabkin’s reagent

2. Cyanmethaemoglobin standard solution (16 g/dL); This was obtained commercially
Procedure

0.02 mL blood was mixed well with 5.0 mL Drabkin’s reagent and allowed to stand for 10 min. The colour developed was read in a spectrophotometer at 540 nm together with the standard solution of cyanmethaemoglobin, against a reagent blank.

Values are expressed as g/dL

3.19. ESTIMATION OF TOTAL PROTEIN

The protein content of tissue homogenate and RBC membranes were estimated by the method of Lowry et al., (1951).

Principle

Proteins react with Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids in the protein sample.

Reagents

1. 10% TCA

2. NaOH, 0.5 N

3. Alkaline copper reagent
   i. Solution A: 2% sodium carbonate in 0.1 N NaOH.
   ii. Solution B: 0.5% copper sulphate in water.
   iii. Solution C: 1% sodium potassium tartarate in water - 50 mL of solution A was mixed with 0.5 mL of solution B and 1.0 mL of solution C just before use.
4. Folin-Ciocalteau reagent: 100 g sodium tungstate, 25 g sodium molybdate, 700 mL water, 50 mL 85% O-phosphoric acid and 100 mL conc. HCl were taken in a 1500 mL round bottomed flask. The mixture was refluxed gently for 10 h. To this, 150 g lithium sulphate, 50 mL water and a few drops of bromine were added. The mixture was boiled for 15 min to remove excess bromine. This was diluted in the ratio 1:2 with double distilled water just before use.

5. Stock standard: A stock solution was prepared by dissolving 100 mg BSA in 100 mL water in a standard flask. Small quantities of NaOH were added to complete the dissolution of BSA.

6. Working standard: 10 mL of the stock was diluted to 100 mL to obtain a working standard of concentration 100 µg/mL.

Procedure

An aliquot of plasma or tissue homogenate was diluted to 1.0 mL with saline, then 1.0 mL TCA was added. The mixture was centrifuged, supernatant were discarded and the precipitate was dissolved in 1.0 mL NaOH. From this, aliquots were taken for the estimation. 4.5 mL alkaline copper reagent was added and the contents were allowed to stand at 37 ºC for 10 min. Then, 0.5 mL dilute Folin-Ciocalteau reagent was added and mixed. A series of standards of concentration ranging from 20-100 µg and a blank were processed in the same way as that of the test. The blue colour developed was read at 620 nm after 20 min.

Values are expressed as mg/g tissue

3.20. ASSAY OF STRUCTURAL AND FUNCTIONAL CHANGES IN THE LIVER

Assay of structural and functional changes in the liver was performed using Fourier Transform Infrared (FT-IR) Spectrometry. FT-IR spectrometry is based on a simple mathematical technique to resolve a complex wave into its frequency components. FT-IR spectrometry is rapidly becoming a common feature in modern spectroscopy laboratories. With the advent of inexpensive microcomputers, wide
ranges of commercial FT-IR spectrometers with very different specifications are now available.

**Principle**

In frequency domain spectroscopy the radiant power $G(\omega)$ is recorded as a function of frequency ($\omega$). On the other hand, the change in the radiant power $f(t)$ is recorded as a function of time ($t$) in the case of Time domain spectroscopy. The conventional spectroscopy is based on the former technique while Fourier Transform spectroscopy converts the Time domain plot into a Frequency domain spectrum. Data in time domain are converted into frequency domain by Fourier Transform technique. The actual calculation of Fourier Transform of usual system is done by means of high-speed computers.

The absorbance spectra were recorded using the Spectrum RX I FT-IR System (Nicolet Instrument Corporation, Madison, USA). For each spectrum, 8 scans were recorded, at a spectral resolution of 4 cm$^{-1}$. The frequencies for all the sharp bands were accurate to 0.01 cm$^{-1}$. The spectrometer was continuously purged with dry nitrogen. The absorption intensity of the peak was calculated using the baseline method. Each observation was confirmed by taking at least three replicates. The spectra were recorded in the range 4000-400 cm$^{-1}$ (Jagadeesan et al., 2005). Peak normalization was done with respect to 1654 cm$^{-1}$.

**3.21. ASSAY OF CD14, NF-κB and IL-6**

CD14, NF-κB and IL-6 were analyzed by using reverse transcriptase-polymerase chain reaction

**RNA isolation**

Total RNA from tissues were extracted using single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction, according to the modified protocol of Chomczynski and Sacchi (1987).

**Principle**

A single step isolation method (liquid phase separation) was employed to selectively extract total RNA from tissues. The tissues were lysed using 4 M
guanidinium isothiocyanate. The lysed tissues were mixed sequentially with 2 M sodium acetate (pH 4.0), phenol, and finally chloroform/isoamyl alcohol or bromochloropropane. The resulting mixture was centrifuged and the upper aqueous phase contained total RNA. In this single step extraction the total RNA was separated from protein and DNA that remain in the inter phase and in the organic phase. Following isopropanol precipitation, the RNA pellet was redissolved in denaturing solution (containing 4 M guanidine thiocyanate), reprecipitated with isopropanol, and washed with 75% ethanol.

**Table 4. Oligonucleotide primers and thermocycling conditions for reverse transcriptase (RT-PCR)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer and reverse primer</th>
<th>Initial Denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>F: 5'-CCGAGGTGGCAGGGGCAATG-3', R: 5'-R: 5'-AGTGCAAGGCTGCTGTCGGA-3'</td>
<td>95°C, 2 min</td>
<td>95°C, 1 min</td>
<td>60°C, 1 min</td>
<td>60°C, 1 min</td>
<td>60°C, 7 min</td>
<td>32X</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5'-TCAGGCCCAGAAGGAAA-3', R: 5'-TGGCTGAAAGTCTCTTTCG-3'</td>
<td>94°C, 2 min</td>
<td>94°C, 1 min</td>
<td>59°C, 1 min</td>
<td>72°C, 1 min</td>
<td>72°C, 7 min</td>
<td>37X</td>
</tr>
<tr>
<td>CD14</td>
<td>5'-CTCAACCTAGAGCCTTCT-3', R: 5'-CAGGAATGTICAGACGGTCT-3'</td>
<td>93°C, 2 min</td>
<td>93°C, 1 min</td>
<td>57°C, 1 min</td>
<td>70°C, 2 min</td>
<td>70°C, 7 min</td>
<td>32X</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-TCGAGTCTACTGCGCTCT-3', R: 5'-ATGAGGCCCTCCACGAT-3'</td>
<td>94°C, 2 min</td>
<td>95°C, 1 min</td>
<td>60°C, 1 min</td>
<td>72°C, 1 min</td>
<td>72°C, 7 min</td>
<td>32X</td>
</tr>
</tbody>
</table>

**Reagents**

1. DEPC treated water or freshly deionized formamide
2. Denaturing solution: 4 M guanidium isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroyl sarcosine, 0.1 M β-mercaptoethanol, pH 7.0
3. 2 M sodium acetate
4. Water-saturated phenol
5. 25:24:1 (v/v/v) phenol, chloroform and isoamyl alcohol
6. Ethanol (96%-100%)
7. 1% agarose gel
Procedure

Total RNA from collected tissue was extracted using single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction, according to the modified protocol of Chomczynski and Sacchi, (1987). All the reagents and glassware were made free from RNase by suitably treating with 0.1% DEPC followed by autoclaving or by heating to 180 °C for 8 h. All the steps involved in RNA extraction were done at 4 °C. To 1 g of tissue, 10 mL of denaturing solution was added and homogenized using a mortar and pestle. The homogenized mixture was transferred into a fresh RNase free sterile tube. To the mixture, 500 µL of 2 M sodium acetate was added and vortexed thoroughly. To the mixture, 10 mL of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added, vortexed and incubated on ice for 15 min. The mixture was centrifuged at 10,000 rpm 15 min at 4°C. 60% of the supernatant was transferred into a fresh tube and equal amounts of ice cold isopropanol was added and the tubes were incubated at −20°C for 30 min and centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was discarded and washed with 75% ethanol and the pellet was air-dried. Pellet was dissolved in 200 µL of sterilized DEPC treated water. Quantification of total RNA was determined by spectrophotometry at 260 nm and then stored at −70 °C until use. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA using cDNA synthesis kit (Bangalore GENEI, India) according to the manufacturer’s protocol and was used for polymerase chain reaction amplification to estimate the expression of NF-κB, IL-6 and CD14. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Oligonucleotide primers, thermocycling conditions and the resultant PCR products (Gene expressed) are listed in table 4. RT-PCR products were electrophoresed on a 1.5% agarose gel, and the bands were visualized with ethidium bromide and photographs taken using gel documentation system (Wealtec Corp. Model: CCD CAMERA 201, Made in USA). Band intensities scanned by densitometer.

PCR primer design

Gene sequence information was obtained from the Entrez Nucleotide database, and all primer sequences were designed using the online tool Primer 3-BLAST from the GenBank and the primers were obtained from Bangalore GENEI, India. Sense and antisense primers for the gene of interest (Table 4) were designed.
to have a closely matched melting temperature. All primer sets underwent preliminary testing to ensure that they were effective.

3.22. ASSAY OF TNF-α, TGF-β, COX-2 and iNOS

TNF-α, TGF-β, COX-2 and iNOS expression were analysed by Western blotting after resolution by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

SDS PAGE

SDS-PAGE of all proteins was performed as described by Laemmli, (1970).

Reagents

1. Acrylamide stock: 30% acrylamide, 0.8% N,N′-methylene bisacrylamide.
2. Separating buffer: 2.25 M Tris, 0.6% sodium dodecylsulphate (SDS), pH 8.8
3. Sample buffer: 0.063 M Tris, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8.
4. 10% ammonium per sulphate
5. N,N, N′, N′- tetramethylethylenediamine (TEMED).
6. Running gel buffer (5X): 0.25 M Tris, 0.5% SDS, 1.92 M glycine, 30.3 g Tris, 5.0 g SDS and 144.1 g glycine were dissolved in 700 mL of distilled water. pH was adjusted to 8.3 with conc. HCl and diluted to 1 L with water. The working running buffer was prepared by making a 1:5 dilution of the stock 5 x buffers with distilled water.
7. Water-saturated isobutanol
8. β-mercaptoethanol
9. Staining and fixing solution: 2.5 g coomassie brilliant blue R250 was dissolved in 1 L solution containing methanol, acetic acid and distilled water in the ratio 5:1:4.
10. Destaining solution: 100 mL of absolute methanol and 100 mL of glacial acetic acid were mixed with 800 mL of distilled water.
**Gel preparation**

Protein concentration in the supernatant extracted from the liver tissue of each rat was determined by the method of Lowry et al., (1951). To prepare the separating gel and stacking gel the desired acrylamide concentration was mixed with their respective buffer and distilled water in the required proportions. Polymerization was initiated by the addition of TEMED and ammonium per sulphate. After polymerization, the gel was placed in the vertical electrophoresis apparatus containing 1x running buffer. Additional buffer was added to the upper chamber, and the sample loaded wells were also filled with the buffer.

**Western blotting**

Separated proteins on 10% gel were transferred onto polyvinylidene fluoride membrane (PVDF, Millipore) for 1 h at 50V. Then the membrane was blocked with 5% non-fat dried milk for 1 h and 30 min at room temperature. The membrane were incubated with primary antibodies β-actin (Goat polyclonal anti-β-actin (dilution 1:1000; TNF-α, TGF-β, COX-2 and iNOS (1:1000 dilution) in PBS containing 0.2% Tween-20 overnight at 4 °C. After 3–5 washes, the membranes were incubated with secondary antibodies (1:5000 dilutions) [TNF-α, TGF-β, COX-2 and iNOS for 1 h. Then they were stained with diaminobenzidine and the intensity of bands were captured with a Bio-Rad Documentation system (Bio-Rad Lab, Hercules, CA), and quantified using Quantity One software (Bio-Rad Lab, Hercules, CA). β-actin was used as a loading control.

**3.23. ESTIMATION OF DNA DAMAGE**

DNA damage was estimated by alkaline single cell gel electrophoresis (Comet assay) according to the method of Singh et al., (1988). In this method, the cells were first lysed to form nucleoids. During electrophoresis DNA fragments (from damaged DNA) streamed towards anode while the undamaged DNA trapped within the nucleus. When they are stained with ethidium bromide, damaged DNA gave the appearance of a comet tail and undamaged DNA gave spherical appearance.
Reagents

1. Normal melting point agarose: 1%

2. Low melting point agarose: 1%

3. Lysis solution: 7.3 g of sodium chloride, 1.8 g of EDTA and 0.06 g of tris was mixed with 35 mL of distilled water. After mixing, 0.06 g of sodium hydroxide, 1.0 mL of triton X-100 was added and the solution was made up to 50 mL with distilled water.

4. Electrophoresis buffer: 6.0 g of sodium hydroxide and 186 mg of EDTA was dissolved in 500 mL of distilled water. The solution was stored in the refrigerator 1 hr before electrophoresis.

5. Neutralising buffer: 0.4 M tris-HCl, pH 7.4

6. Staining solution: ethidium bromide

Procedure

Prefrosted slides were prepared by pouring 3.0-5.0 mL of 1% normal agarose over clean glass slides. It was allowed to dry at room temperature and placed in hot-air oven at 70-180 °C for 30 min. A freshly prepared 10 μL of whole blood in 1% low melting point agarose (LMPA) (1:3 ratio) was cast on to prefrosted microscopic slides, immediately covered with cover slip and kept for 10 min in a refrigerator to solidify. Then the cover slip was removed and a top layer of 100 μL of LMPA was added and the slides were again cooled for 10 min. The cells were then lysed by immersing the slides in the lysis solution for 1 h at 4 °C. After lysis, slides were placed in a horizontal electrophoresis tank. The unit was filled with electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to the alkaline electrophoresis solution for 20 min to allow DNA unwinding. Electrophoresis was conducted in a cold condition for 20 min at 25 V and 300 mA. After electrophoresis, the slides were placed horizontally and neutralised with tris-Cl buffer. Finally, 50 μL of ethidium bromide was added to each slide and analysed (CASP software) using a fluorescence microscope. To
prevent additional DNA damage, all steps were conducted under dimmed light or in the dark. Twenty five images were randomly selected from each sample and were examined at 200 magnification in a fluorescence microscope connected to a personal computer-based image analysis system, Casp. The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length and tail moment (% tail DNA × tail length) were linearly related to DNA break frequency.

3.24. IDENTIFICATION OF COLLAGEN

Liver sections were stained with Masson's trichrome for collagen by the method of Masson, (1929).

Reagents:

Solution A

2.25 g Potassium dichromate, 2.25 mL concentrated HCl and 25 mL of 95% ethanol were mixed together with distilled water.

Solution B

0.1 mL acid fuchsin was mixed with 100 mL distilled water

Solution C

1 g Phosphomolybdic acid mixed with 100mL distilled water

Solution D

2 g Orange G and 1 g phosphomolybdic acid were mixed with 100 mL distilled water

Solution F

1 mL glacial acetic acid mixed with 100 mL distilled water

Solution F

0.1 g Fast green and 0.2 mL glacial acetic acid were mixed with 100 mL distilled water
Procedure

The unstained samples were immersed in a series of trays consisting of xylene for 5 min, 4 min and absolute ethanol for 3 min, 2 min respectively, after incubation in an oven at 60 °C for 30 min. The unstained samples were placed into solution A for 5 min and rinsed with distilled water. The slides were subsequently placed in solution C for 1-5 min and rinsed with distilled water, then in solution D for 5-10 min and rinsed with distilled water. Then the slides were placed in solution E for 2 min, solution F for 5-10 min, and in solution E for 3 min. Again the slides were dehydrated with ethanol. The slides were cleared with xylene and mounted with a resinous medium.

3.25. ASSAY OF BCL-2 AND BAX

Assay of Bcl-2 and Bax were performed by using immunohistochemistry, as described by Rocha et al., (2009) using super sensitive polymer-HPD detection system kit, from Biogenex, USA. The super sensitive polymer-HPD detection system is a novel detection system using a non-biotin polymeric technology that makes use of two major components: Super Enhancer™ and a Poly-HPD reagent. As the system is not based on the biotin-avidin system, the problems associated with endogenous biotin are completely eliminated.

The detection of antigens in tissues by immunostaining is a two-step process. The first step involves the binding of an antibody to the antigen of interest and the second step involves the detection and visualization of bound antibody by one of a variety of enzyme chromogenic systems. The choice of detection system will dramatically impact the sensitivity, utility and use of the method.

Procedure

Paraffin-embedded tissue was cut to obtain sections of about 5 μm thicknesses. The mounted paraffin-embedded slices are deparaffinized in xylene and rehydrated using an ethanol/H₂O gradient. Heat mediated antigen retrieval step was carried out for 10 min and then the slides were allowed to cool to room temperature for another 20 min. This was followed by peroxidase block treatment (to block endogenous peroxidase enzyme activity) for 10-15 min and then power block
treatment (to block non-specific binding of antibodies to highly charged sites) for another 15 min. The sections were incubated with the concerned diluted primary antibody solution (Bcl-2 and Bax) (for 2 h (1:200) followed by treatment with the super enhancer solution (for 30 min) and super sensitive Poly-HRP solution (for 30 mins). After colour development with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstaining with haematoxylin, the sections were observed under the microscope and photographs were taken.

3.26. HISTOPATHOLOGICAL STUDIES

For histopathological studies, tissues slices were fixed for 48 h in 10% formalin and processed for paraffin embedding following the standard microtechnique. 5μm sections were de-waxed with xylene, three washes each for 5 min. Sections were rehydrated by passing them through alcohol grades viz; 100%, 90%, 70%, 50%, 30%. After giving a last dip in water, sections were incubated in stain for 2 min. Alkaline water (0.3% NH₄OH) treatment was given to sections for 5-10 min till appearance of intense blue colour. Sections were partially dehydrated and incubated with eosin stain for 5 min. Dehydration steps were continued up to 100% alcohol. Slides were dipped once in xylene, air-dried and mounted with neutral distyrene dibutyl phthalate xylene medium (DPX) under a light microscope and examined.

3.27. STATISTICAL ANALYSIS

All quantitative measurements were expressed as means ± SD for control and experimental rats. The data were analyzed using one way analysis of variance (ANOVA) on SPSS/PC (Statistical Package For Social Sciences, Personal Computer) Version 10 and the group means were compared by Duncan’s Multiple Range Test (DMRT). The results were considered statistically significant if the p value is less than 0.05.