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2.1. Materials

2.1.1. Lutein

Lutein isolated from marigold flowers (*Tagetes erecta* L) (80%) was supplied by Omni Active Health Technologies Pvt. Ltd (Mumbai, India) as a 5% solution in sunflower oil. Purity of lutein is 95%.

Figure 2.1. HPLC analysis of lutein

2.1.2. Cell lines used

L929 (mouse lung fibroblast) cell line was obtained from National Centre for Cell Sciences, Pune. Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines were initially procured from Adayar Cancer Institute, Chennai and propagated as transplantable tumors in the peritoneal cavity of BALB/c mice.

2.1.3. Animals

BALB/c mice, Swiss Albino mice and Wistar rats were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. Animals were maintained in ventilated polypropylene cages and fed with normal chow (Sai Durga Feeds and Foods, Bangalore) and water ad libitum. The animal experiments were conducted after getting prior permission from Institutional Animal Ethics Committee (IAEC) and as per the instructions prescribed by the Committee for the
Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

2.1.4. Chemicals

Dulbecco’s Modified Eagle Medium (DMEM) : Hi-Media, Mumbai

Modified Eagle Medium (MEM) without phenol red : 

Rosewell Park Memorial Institute medium (RPMI 1645) : 

Caesin : 

Trypsin : 

Fluid thioglycolate : 

L-Glutamine : 

Thiobarbituric acid (TBA) : 

Dextran : 

Ethidium Bromide : Sigma-Aldrich, St.Louis, USA

Propidium iodide : 

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) : 

2,2-Azobiz-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) : 

2,2-Diphenyl -1-picryl hydrazil(DPPH) : 

Carrageenan : 

1,1,3,3-tetramethoxypropane (malondialdehyde) : 

N’Nitrosodiethylamine (NDEA) : 

Dichloro fluorescein diacetate (DCFH-DA) : 

Bromophenol blue : 

Diethylpyrocarbonate (DEPC) : 

Glycyl glycine : 

Hydroxyproline : 

Silymarin : 

3-Methyl cholantheme (3-MC) : 


7,12-dimethyl benz[a]anthracene (DMBA)
7-ethoxy resorufin (ER)
7-pentoxy resorufin (PR)
7-methoxy resorufin (MR)
Glutathione reduced (GSH) : Sisco Research Ltd (SRL), Mumbai.
Glutathione oxidized (GSSG)
Deoxy ribose
Nicotinamide adenine dinucleotide
Phosphate reduced (NADPH)
Acetyl acetone
Tris Buffer
Tris-HCl
Folin’s reagent
Sodium Azide
Carboxymethyl cellulose
Agarose
Riboflavin
Colchicine
Nitrobluetetrazolium (NBT)
5-5’dithiobis (2-nitrobenzoic acid)(DTNB)
Bovine Serum Albumin (BSA) : E-Merck, Germany
Hydrogen peroxide
p-dimethyl amino benzaldehyde (p-DAB)
Sodium dodecyl sulfate
Formaldehyde
Dimethyl sulfoxide (DMSO)
Triton X-100
Isopropanol
Trisodium citrate
Trypan blue
Geimsa powder
May-Grunwald powder
Eosin
2.1.5. Diagnostic Reagent kit

HaemoChek : Agappe Diagnostics, Thane
Alkaline Phosphatase kit : Span diagnostics, Surat, India.
Serum Glutamate PyruvateTransaminase kit : ”
Serum Glutamate oxaloacetateTransaminase kit : ”
γ-glutamyl transpeptidase (GGT) : ”
Total bilirubin kit : ”
Urea kit : ”
Creatinine kit : ”
pH paper : E-Merck, Germany

2.1.6. Instruments

Inverted microscope : Leica, GermanRadicle, Ambala
Upright research microscope : Meiji, Japan; Labex,Labovision
Horizontal Laminar flow hood : Cleanair, Chennai
Deep freezer, -70ºC, -20ºC : Remi
High speed cooling centrifuge : ”
Spectrophotometer : Elico Ltd, Hyderabad
pH meter : ”
Electrophoresis unit : Biotech,Yercaud;Genei, Bangalore
Gel documentation system : Vilber Lourmat, France
CO₂ Incubator : NAPCO, Canada
Fluorescent microscope : Olympus BX41.

2.1.7. Reagents and stains

(a) Phosphate buffered saline (PBS)
NaCl - 8.00g
KC1 - 0.20g
KH₂PO₄ - 0.20g
Na₂HPO₄. 2H₂O - 1.44g
Dissolved the contents in distilled water, made up to 1000mL. pH was adjusted to 7.2 with 1N NaOH/ HCl. Sterilized by autoclaving at 15 lbs for 15 min.

**(b) Trypsin solution**
Trypsin - 200mg
Dextrose - 20mg
Trypsin was dissolved in 100mL ice-cold PBS-EDTA (20mg% EDTA in PBS) and sterilized by filtration through 0.2μm membrane filter. Stored at -20°C.

**(c) Trypan blue**
Trypan blue stain - 100mg
Normal saline (0.9%NaCl) - 100mL
Trypan blue stain was dissolved in saline followed by filtration using Whatmann No.1 filter paper.

**(d) May-Grunwald stain**
May-Grunwald powder - 250mg
Methanol - 100mL
The stain was dissolved in methanol by stirring and filtered through Whatmann No.1 filter paper and stored at 4°C.

**(e) Leishmann’s stain**
Leishmann’s stain – 150mg
Methanol - 100mL
Leishmann’s stain was dissolved in methanol, filtered and used.

**(f) Turk’s fluid**
Acetic acid - 1.5mL
10% crystal violet - 1.0 mL
Distilled water - 98 mL
Stirred overnight, filtered and used.

**(g) Giemsa stain**
Giemsa powder - 800mg
Glycerol - 50mL
Methanol - 50mL
Giemsa powder was dissolved in glycerol at 60°C with shaking. The mixture was cooled to room temperature and methanol was added. Mixed well for 5 min and allowed to stand overnight. The solution was filtered through Whatmann No.1 filter paper and stored at 4°C.

(h) **Harris haematoxylin**
Haematoxylin - 5g
Ethyl alcohol - 50mL
Potassium alum - 50mg
Potassium iodide - 50mg
Distilled water - 950mL
Haematoxylin was dissolved in ethyl alcohol by gentle heating. The alum was dissolved in distilled water by heating with frequent stirring and kept at 4°C overnight. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium iodide was added and filtered.

(i) **Eosin solution**
Eosin - 500mg
Ethyl alcohol - 100mL
Eosin was dissolved in 5mL of ethyl alcohol and made up to 100 mL with ethyl alcohol.

(j) **Griess Reagent**
Reagent 1 - 0.1% N (1-Naphthyl ethylene diaminodihydrochloride)
Reagent 2 - 1% sulfanilamide in 2% orthophosphoric acid.
Mixed reagent 1 and 2 in 1:1 proportion before use.

(k) **Sorensen’s buffer (pH 6.8)**
Na$_2$HPO$_4$ - 0.42g
KH$_2$PO$_4$ - 0.40g
Distilled water - 100mL
2.2. Methods

2.2.1. Maintenance of cells in the animals

Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were propagated in the peritoneal cavity of BALB/c mice and maintained by transplanting the cells in every two weeks. For this tumor cells were aspirated from the peritoneal cavity, washed with PBS and $1.5 \times 10^6$ cells were injected intraperitoneally to induce ascites tumor.

2.2.2. Determination of cell viability

2.2.2.1. Trypan blue exclusion method

Cell viability was determined by trypan blue dye exclusion method as described by Gupta and Bhattacharya, 1978.

**Principle:** Trypan blue is not permeable in live cells due to the presence of intact plasma membrane. When the cells are dead they will take up the dye and appear as blue color. The method is an index of the dead cells in a cell population.

**Procedure:** 0.1 mL of cell suspension was mixed with 0.1mL of 1% trypan blue, kept for 2-3 minutes and loaded on a hemocytometer. The number of stained and unstained cells was counted separately.

\[
\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Number of viable cells}} \times 100
\]

2.2.2.2. MTT assay

**Principle:** 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) is converted by the microsomal enzymes into a formazan and the amount of formazan formed is an index of live cells present in the cell population (Cole, 1986; Campling et al., 1991).

**Procedure:** $5 \times 10^3$ cells were plated in 96-well flat bottom titer plate in appropriate volume of the medium supplemented with FCS and antibiotics. The drugs were added 24 hr after the plating of the cells and incubation continued. 4 hr before($44^{th}$ hr) the completion of incubation, $20\mu$L of MTT ($5\text{mg/mL}$) was added to each well and the incubation was continued. The plates were centrifuged and supernatant removed. DMSO ($100\mu$L/well) was added to solubilize the formazan crystals and incubated for 15 minutes at room temperature. The optical density was taken in an ELISA reader at 570nm with reference at 630nm.
% dead cells = \[ 100 - \left( \frac{O.D\ of\ drug\ treated\ well}{O.D\ of\ the\ control\ well} \right) \times 100 \]

### 2.2.3. Determination of in vitro antioxidant activity

#### 2.2.3.1. Determination of superoxide radical scavenging activity

Superoxide radical scavenging was determined by the nitroblue tetrazolium (NBT) reduction method of McCord and Fridovich (1969). The reaction mixture contained EDTA (6µM) containing NaCN (3µg), riboflavin (2µM), NBT (50µM), various concentrations of lutein and phosphate buffer (67mM, pH 7.8) in a final volume of 3mL. The tubes were uniformly illuminated with an incandescent visible light (Philips, 40w) for 15 min and the optical density was measured at 530nm before and after illumination. The percentage inhibition of superoxide generation was evaluated by comparing the values of the control and experimental tubes.

#### 2.2.3.2. Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the compounds for hydroxyl radicals generated from the Fe³⁺/ascorbate. EDTA/H₂O₂ system (Kunchandy & Rao, 1990). The hydroxyl radical attacks deoxyribose, which eventually results in TBA reacting substance (TBARS) formation (Ohkawa et al., 1979). The reaction mixture contained deoxyribose (2.8mM) FeCl₃ (0.1mM), EDTA (0.1mM), H₂O₂ (1mM), ascorbic acid (0.1mM), KH₂PO₄-KOH buffer (20-mM, pH 7.4) and various concentrations of lutein in a final volume of 1mL. The reaction mixture was measured as TBARS and percent inhibition was calculated.

#### 2.2.3.3. Determination of inhibition of lipid peroxidation

Reaction mixture (0.5mL) containing rat liver homogenate (0.1mL, 25% w/v) in Tris-HCl buffer (40mM, pH 7.0), KCl (30mM), ferrous ammonium sulphate (0.16 mM), ascorbic acid (0.06 mM) and different concentrations of lutein was incubated for 1h at 37°C. The lipid peroxide formed was measured by TBARS formation (Ohkawa et al., 1979). For this incubation mixture 0.4mL was treated with sodium dodecylsulphate (8.1%, 0.2mL), TBA (0.8%, 1.5mL) and acetic acid (20%, 1.5mL, pH3.5). The total volume was then made up to 4mL by adding distilled water and kept in a water bath at 100°C for 1h. After cooling, 1mL of distilled water and 5mL of a mixture of n-butanol
and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with those of control not treated with lutein.

2.2.3.4. Determination of DPPH radical scavenging assay

In this method a commercially available and stable free radical (DPPH\(^{+}\), 2, 2-diphenyl-1-picrylhydrazyl) which is soluble in methanol, was used (Aquino et al, 2001). DPPH in its radical form has an absorption peak at 515 nm, which disappears on reduction by an antioxidant compound. Lutein was added to 1.5 mL of freshly prepared DPPH solution (0.25 g/L in methanol). Absorbance was measured at 515 nm; 20 min. after the reaction was started. The percentage inhibition of DPPH radical was calculated by comparing with that of control.

2.2.3.5. Determination of ABTS radical scavenging assay

In this method, the radical scavenging activity of lutein was determined using ferryl myoglobin/ABTS protocol (Alzoreky & Nakahara, 2001). The stock solutions of 500 µM ABTS diammonium salt, 400 µM myoglobin (Mb III), 740 µM potassium ferricyanide and 450 µM H\(_2\)O\(_2\) were prepared in phosphate buffered saline (PBS) (pH 7.4). Methmyoglobin was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (2 mL) contained ABTS (150 µM), Mb III (2.25 µM), varying concentrations of lutein and PBS. The reaction was initiated by adding 75 mM H\(_2\)O\(_2\) and lag time in seconds was recorded before absorbance of ABTS\(^{+}\) at 734 nm began to increase. The percentage inhibition was calculated by comparing with control.

2.2.3.6. Determination of nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent (Green et al, 1982). 10 mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of lutein and sodium nitroprusside (1 mM) in PBS in a final volume of 3 mL were incubated at 25°C for 150 min. After incubation, 0.5 mL of the solution was removed and diluted with 0.5 mL of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% naphthyl ethylene diaminedihydrochloride). The absorbance of the chromophore formed during the
diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diaminedihydrochloride was read at 546nm. The percentage inhibition of nitric oxidewas calculated by comparing with the control.

2.2.3.7 Ferric reducing antioxidant power (FRAP)

The ferric reducing ability was measured at low pH(Benzie & Strain, 1996). The FRAP reagent contained 2.5mL 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) solution, 2.5 mL ferric chloride solution (20 mM) and 25 mL acetate buffer. Freshly prepared FRAP reagent (900 μL) was mixed with various concentrations of sample (20-100 μg) and incubated at 37°C for 15 min. Absorbance at 595 nm was read against distilled water. Values are expressed as millimols of ferrous chloride formed.

2.2.4. Haematological parameters

2.2.4.1. Determination of haemoglobin

**Principle:** The haemoglobin is converted to cyanmethaemoglobin in the presence of potassium ferricyanide and potassium cyanide. The absorbance of cyanmethaemoglobinis proportional to the haemoglobin concentration (Drabkin & Austin, 1932).

**Procedure:** The reagents used were procured from Agappe diagnostic kit. 20μL of heparinised fresh blood was mixed with 5mL of Drabkin’s reagent and incubated for 5min at room temperature and followed by the measurement of absorbance at 545nm against reagent blank. The concentration of Hb was calculated by using the following formula.

\[
\text{Hb (gm/dL) = } \frac{\text{O.D of sample} \times N \times 0.251}{\text{O.D of standard}}
\]

Where N= concentration of standard= 60mg/dL.

2.2.4.2. Determination of total white blood cell (WBC) count

**Principle:** The whole blood was diluted using a diluent (Turk’s fluid) where the acetic acid present in the diluent lyses the red cells. All the nucleated cells become intact and are stained by crystal violet.

**Procedure:** Blood (20μL) was mixed with 380μL of Turk’s fluid and kept at room temperature for 2-3 minutes. The cells were mixed gently and loaded on to the
Neubauer chamber, allowed to settle at the bottom of the chamber and the four large corner squares of chamber was counted under a microscope using 10 x objectives. Total leucocyte counts/mm$^3$ = No. of cells counted x dilution factor x depth factor 

Area counted

Were,

Dilution factor = 1/20
Depth = 1/10mm
Area counted = 4sq. mm

Therefore, Total leucocyte counts/mm$^3$ = $N \times 20 \times 10 = N \times 50$

$\frac{4}{4}$

2.2.4.3. Determination of differential count of leucocytes

Procedure: A thin film of blood was made by spreading a drop of blood evenly across a clean glass slide using a glass spreader and air dried. Few drops of Leishmann’s stain was poured over the smear and kept for 3 min. The stain was diluted with distilled water and kept for 7 min, washed with tap water and allowed to air dry. Various types of cells were scored according to the morphology of the nucleus under oil immersion with 100 x objective and a total of 100 cells were counted.

2.2.5. Biochemical parameters

2.2.5.1. Preparation of tissue homogenate

The tissues (liver and kidney) were homogenized in appropriate buffer and 10% or 25% homogenate was prepared. The homogenate was then centrifuged at 12000 rpm for 20 min. at 4°C and the clear supernatant was taken for the assay. It was stored at -70°C till analysis.

2.2.5.2. Estimation of $\gamma$-Glutamyltranferase ($\gamma$-GT) activity in the blood and in the tissue homogenate.

The method of Tate and Meister (1974) was used to assay $\gamma$-GT.

Principle: $\gamma$-glutamyl tranferase catalyses the transfer of the $\gamma$-glutamyl moiety of a $\gamma$-glutamyl donor to the acceptor.

$\gamma$-glutamyl-p-nitroanilide + acceptor $\rightarrow$ $\gamma$-glutamyl acceptor + p-nitroaniline

The formation of p-nitroaniline was determined from the increase in absorbance at 410nm.
**Procedure:** The assay solution (1mL) contained 0.05M Tris-HCl buffer (pH 8), 75mM NaCl, 2.5mM L-γ-glutamyl-p-nitroanilide, 20mM glycyl glycine (pH 8) and enzyme (0.2 to 1µg). The rate of release of p-nitroaniline was read at 410nm.

2.2.5.3. Determination of superoxide dismutase (SOD) activity in the blood and in the tissue homogenate

SOD activity was determined according to the method of McCord and Fridovich (1969).

**Principle:** The photo-illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to $O_2^-$, which is allowed to react with a detector molecule NBT. Upon reaction NBT is reduced to a formazan blue. The SOD in the sample inhibits the formazan production.

**Procedure:** The heparinised blood was centrifuged at 2500rpm to remove the plasma. To the packed RBCs, normal saline was added and again centrifuged and supernatant was removed. The remaining packed RBCs were used for the experiment. The haemoglobin concentration was determined as described in the section 2.2.9.1.1. 100µL of the packed RBCs was haemolysed by 900µL of cold water. The haemolysate was then treated with 250µL of CHCl$_3$ and 500µL of ethanol with vigorous mixing to remove the haemoglobin. The mixture was then centrifuged at 15000 rpm for 60 min. at 4°C. 100µL of the clear supernatant was used for the SOD assay. 100µL of supernatant was mixed with 200µL of 0.1 M EDTA (containing 0.0015% NaCN), 100µL of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.95 mL. After adding 0.05 mL of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. The tubes were then uniformly illuminated with an incandescent lamp for 15 min. and absorbance was taken again at 560nm. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). In the case of tissue homogenate also the volume used was 100µL and methodology followed was same as given above. The volume of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/g Hb in the case of blood and SOD activity was expressed as U/mg protein for tissue.
2.2.5.4. Determination of catalase activity in the blood and in the tissue homogenate

Catalase activity in the blood was determined by the method of Aebi (1974) and tissue catalase was determined according to the method of Beer and Sizer (1952).

**Principle:** The catalase activity was assayed by measuring the decomposition of H$_2$O$_2$. The H$_2$O$_2$ has absorption maxima at 240 nm and absorption decreases with the decomposition of H$_2$O$_2$. The difference in extinction per unit time is a measure of the catalase activity.

**Procedure:** The lysate of packed RBCs was prepared in ice cold water which containing approximately 5g Hb/dL. A 1:500 dilution of this concentrated haemolysate with sodium-potassium phosphate buffer (0.05 M, pH 7) was prepared immediately before the assay. Reference cuvette contained 1 mL of buffer and 2 mL of haemolysate and test cuvette contained 2 mL diluted haemolysate. The reaction was started by addition of 1 mL of H$_2$O$_2$ (30 mM in the buffer, fresh everytime) to the test cuvette, mixed well and the decrease in extinction was measured at 240 nm for 1 minute with an interval of 15 sec. Catalase activity was calculated using the formula and expressed as k/g Hb,

where k is a rate constant of 1st order reaction.

Catalase (k/g Hb) = \[
\frac{2.303 \times (\log E_1 - \log E_2) \times \text{dil. Factor}}{15 \times \text{g Hb/mL of blood}}
\]

E$_1$ is E$_{240}$ at t=0 and E$_2$ is E$_{240}$ at t=15 sec.

Catalase in the tissue: 0.1 mL of the tissue homogenate (approximately 0.1 mg protein) was mixed with 1.9 mL of phosphate buffer. After adding 1 mL of H$_2$O$_2$ solution in buffer, decrease in extinction was measured at 240 nm, at 1 min interval for 3 min. A sample control was placed in the reference cuvette containing 0.1 mL of tissue homogenate and 2.9 mL of the buffer. Activity of catalase was calculated using the molar extinction coefficient of 43.6. Specific activity at 25°C was defined in terms of mmoles of H$_2$O$_2$ consumed/min/mg of protein sample.

\[
\text{mmoles of H}_2\text{O}_2 \text{ decomposed/min/}
\]

mg protein or (U/mg protein) = \[
\frac{\Delta A/\text{min} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}
\]
2.2.5.5. Determination of reduced glutathione (GSH) content in the blood and in the tissue homogenate

Reduced glutathione in blood and tissue was determined according to the method of Moron et al. (1979).

**Principle:** Reduced glutathione forms a yellow colored complex with DTNB with an absorbance at 412 nm.

**Procedure:** Haemolysate of heparinised blood was prepared in distilled water. Haemolysate (500 μL of the tissue homogenate) was mixed with 125 μL of 25 % TCA and cooled on ice for 5 min followed by further dilution of the mixture with 600μL of 5% TCA and these were then subjected to centrifugation at 3000 g for 5 min to settle down the precipitate. 150 μL of the supernatant was mixed with 350μL of sodium phosphate buffer (0.2M, pH 8.0) and 1.0 mL of DTNB (0.6mM in 0.2M, pH 8.0 phosphate buffer). The yellow color obtained was measured at 412 nm against a blank which contained 5%TCA in place of the supernatant. A standard graph was prepared using different concentrations (10-50 nmoles) of GSH. The GSH content of the sample was calculated from the standard graph and expressed as nmol/mL of blood and for tissue it was expressed as nmol/mg protein.

2.2.5.6. Determination of glutathione peroxidase (GPx) activity the tissue homogenate

Glutathione peroxidase activity was determined according to the method of Hafemann et al. (1974).

**Principle:** The GPx enzyme degrades the H₂O₂ in presence of GSH by the following reaction

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

The remaining GSH was measured by its reaction with DTNB.

**Procedure:** 100μL of tissue homogenate was treated with100μL of 5mM GSH, 100μL of 1.2 mM H₂O₂, 100μL of 25 mM NaN₃ and phosphate buffer (1M, pH 7.0) in a total volume of 2.5 mL at 37°C for 6 min. The reaction was stopped by adding 2.0 mL of 1.65% m-H₃PO₄ and the reaction mixture was centrifuged at 3000 rpm for 10 min to settle down the precipitate. Supernatant (2.0 mL) was mixed with 2.0 mL 0.4 M Na₂HPO₄ and 1mL of1mM DTNB (in buffer). The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min. at 37°C against distilled water.
water. A sample without the haemolysate was processed in the same way and was kept as the blank. The activity is expressed as U/mg protein for tissue homogenate.

\[
\text{GPx activity} = \frac{\text{O. D of blank} - \text{O. D of sample} \times 1000}{0.001 \times \text{g Hb or mg protein} \times 10}
\]

One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the nonenzymatic reaction.

2.2.5.7. Determination of glutathione reductase (GR) activity in serum

Glutathione reductase activity was determined according to the method of Racker (1955)

**Principle:** The activity of GR was determined by the amount of NADPH consumed in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH).

The reaction is:

\[
\text{GSSG + NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ 
\]

**Procedure:** The reaction mixture contains 100μL EDTA (10mM), 100μL GSSG (10mM), 50μL of serum and buffer (1M, pH 7.0) in a total volume of 900μL. The reaction mixture was incubated for 5 min at 37°C, followed by the addition of 100μL NADPH (2mM) and decrease in optical density was measured at 340 nm for 5 min with an interval of one min. The activity of GR was calculated using the molar extinction coefficient of 6.22nm⁻¹cm⁻¹ and expressed as nanomoles of NADPH consumed/min/mg protein.

2.2.5.8. Determination of glutathione-S-transferase (GST) activity in liver tissue homogenate

GST activity was determined according to the method of Habig et al. (1974)

**Principle:** Determination of activity of GST is based on the rate of increase in conjugate formation between reduced glutathione and 1-chloro-2, 4-dinitrobenzene (CDNB). The conjugate has maximum absorbance at 340 nm.

**Procedure:** Three ml reaction mixture consists of 0.1M sodium phosphate buffer (pH 6.5), 1mM CDNB (100μl) in ethanol and 1mM GSH (100μl). Reaction started by the addition of diluted 10% cytosolic sample. The initial reading was taken at 340 nm with a reference cuvette containing the complete assay mixture without cytosolic sample and continued for 5 minutes with 1 minute interval. The activity of GST was calculated from the following formula and it is expressed as μmoles or nanomoles of CDNB-GSH.
conjugate formed/min/mg protein. The activity of GST was used as the extinction coefficient 9.6 mµ⁻¹cm⁻¹ between CDNB-GSH conjugate and CDNB.

### 2.2.5.9. Determination of lipid peroxidation in tissue homogenate

The level of lipid peroxidation in tissue was measured as malondialdehyde (MDA) according to the method of Ohkawa et al. (1979).

**Principle:** The malondialdehyde (MDA) is formed mainly from the peroxidation of PUFAs. MDA is a TBA reacting substance (TBARS) and the product formed between the reaction of MDA and TBA is extracted into an organic layer and estimated at 532 nm.

**Procedure:** The reaction mixture (4 mL) containing 100µL of the tissue homogenate, 1.5mL of 0.8 % TBA, 1.5 mL of acetic acid (20%, pH 3.5) and distilled water were kept for 1hr in a boiling water bath at 100°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 5 mL of butanol mix thoroughly and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol. The concentrations of MDA was calculated from a standard graph made by using different concentrations (1-10nmol) of 1,1,3,3-tetramethoxypropane (MDA) and is expressed as nmol of MDA formed/mL of serum and for tissue it was expressed as nmol of MDA formed/mg protein.

### 2.2.5.10. Determination of the total protein in the tissue homogenate

Protein content in the tissue was determined according to the method of Lowry et al. (1951).

**Principle:** The tyrosine and tryptophan residues of proteins cause reduction of the phosphormolybdate and phosphortungstate components of Folin-Ciocalteau reagent in an alkaline medium to give a bluish purple colour with absorbance at 660nm.

**Procedure:** 10µL of the homogenate was mixed with 990µL of distilled water, 5 mL of alkaline CuSO₄ (0.5 % CuSO₄ in 1% sodium potassium tartrate and 2%Na₂CO₃ in 0.1 N NaOH mixed in the ratio 1:50) was kept for 10 min at room temperature. 0.5 mL of 1 N Folin-Ciocalteau reagent was added and absorbance was measured after 30 min at 660 nm against the reagent blank. Protein content was calculated from the standard graph plotted using different concentrations (1-500µg/mL) of bovine serum albumin (BSA).
2.2.6. Micronucleus test

Both femurs from experimental mice were removed and bones were freed from muscles. The proximal ends of the femurs were carefully shortened with scissors until a small opening to the marrow canal became visible. Approximately 5mL PBS with 1000L serum (FCS) were aspirated into a disposable syringe and the needle was inserted a few millimeters into the bone marrow canal. Bone marrow was flushed into a centrifuge tube and mixed gently. Tubes were centrifuged at 1000 rpm for 10 min. The cell button was collected and smears were made. The air dried smears were fixed in methanol for 5 to 10 min and then stained using May-Grunwald Giemsa (undiluted) for 3min, followed by diluted May Grunwald in distilled water (1:1) for 2min and finally with diluted Giemsa (1:6 distilled water) for 10min. The slides were rinsed in distilled water, air dried and mounted in DPX. The slides were screened for 2000 polychromatic erythrocytes and corresponding normochromatic erythrocytes and also for the presence of micronuclei (Schmid, 1975; Thressiamma et al., 1998).

2.2.7. Chromosomal aberration

Animals were killed by cervical dislocation and bone marrow was used for analyzing chromosome abnormalities (Savage, 1983; Preston et al., 1987). For this proximal ends of the femurs were shortened with scissors to visualize the bone marrow canal. 0.5 mL of phosphate buffered saline (PBS) was aspirated into a disposable syringe and the needle was inserted into the bone marrow canal. The bone marrow was flushed out into a centrifuge tube containing PBS and mixed thoroughly. Tubes were centrifuged at 1000 rpm for 8 min. The cell button was suspended in a hypotonic solution of 0.075M KCl, (KCl prewarmed at 37°C), and was incubated for 20 min. at 37°C. Tubes were then centrifuged and cell buttons were resuspended in 5mL ice-cold, freshly prepared methanol:acetic acid fixative (3:1). The chilled fixative was added dropby drop keeping the centrifuge tube on the cyclomixer and then the total volume was made upto 5mL. Tubes were allowed to stand for at least 15min at room temperature to allow the cells to fix. Tubes were centrifuged for 1000rpm for 8min and the treatment with methanol: acetic acid was repeated twice. Cleaned slides were refrigerated (4°C) in distilled water for 2-3h prior to harvesting. With a Pasteur pipette about 4 drops of the cell suspension were dropped onto the slide surface. The slide was held at an angle, such a way that the nuclei burst and the chromosomes were released, while dropping was done. Four slides were prepared for each animal. Slides were aged.
for 2 to 3 days at room temperature before staining (Natarajan & Obe, 1982; George, 1997). The slides were placed in 5% buffered Giemsa (5mL of Giemsa stock in 95mL of Sorenson’s buffer) for 7min and rinsed in 2 changes of distilled water. The slides were air dried and mounted in DPX. The slides were observed under oil-immersion and were screened for metaphase spreads. A minimum of 100 metaphase spreads were scored for aberrations. When breaks involved both the chromatids, it was termed ‘chromosome type’ aberration while ‘chromatid type’ aberrations involved only one chromatid. If the deleted portion had no apparent relation to a specific chromosome, it was called fragment. Three types of aberration were generally scored in metaphase: gaps, breaks and exchanges (Natarajan & Obe, 1982; Savage, 1983).

2.2.8. Alkaline single cell gel electrophoresis (comet assay)

**Principle:** Comet assay is a technique which allows measuring DNA damage like strand breaks, base damage, alkali labile sites etc. at a single cell level. During alkaline single cell gel electrophoresis the RNA gets degraded due to high alkaline condition at the same time the double strand breaks get converted into single strands. Hence all the DNA breaks are measured in a cumulative single strand form.

**Procedure:** Comet assay was performed essentially using method given by Singh (Singh, 2000), with minor modification (Maurya *et al*., 2005). Fully frosted slides were covered with 200μl normal melting agarose (NMA) 1% in PBS, immediately coverslipped and kept at 4°C for 10 minutes to allow the agarose to solidify. The removal of the cover glass from agar layer was followed by the addition of a second layer of 200μl of 0.5% low-melting agarose (LMA) at 40°C containing approximately 1x 10⁵ cells. Cover glasses were placed immediately and the slides were placed at 4°C. After solidification of LMA, the cover slips were removed and slides were immersed in pre-chilled lysing solution containing 2.5mM NaCl, 100mM Na₂-EDTA, 10mM Tris-HCl (pH 10), 1%Sarcosyl and freshly added 1% Triton X 100 for one hour at 4°C. After lysis, slides were drained properly and placed in a horizontal electrophoresis apparatus filled with freshly prepared alkaline buffer containing 300mM NaOH, 1mM EDTA, 0.2% DMSO (pH 13). Slides were equilibrated in the same buffer for 20 min. and the electrophoresis was carried out for 20 min. at 40V. After electrophoresis, the slides were washed gently with 0.4mM Tris-HCl buffer, pH7.4, to remove alkali. 50μl of propidium iodide (20μg/mL) was layered on the slide and the comets were visualized using fluorescent microscope, excitation filter of 546-590 nm at 400X magnification.
The images were captured with a high performance camera. The integral frame grabber used in this system is a PC based card which accepts color composite video output of the camera. Per animal a minimum of fifty randomly selected cells were taken into account. The comet images were analysed using the software CASP which gives percent DNA in tail, tail length and tail moment directly. The parameter tail moment was calculated as the product of the tail length and percent DNA in tail (Konca et al, 2003).

2.2.9. Histopathological analysis

For histopathological analysis the portion of the tissue was fixed in 10% formalin. Specimens were dehydrated by passing through ascending grades of alcohol, cleared in xylene, impregnated and embedded in paraffin. These sections were cut (3-5µm) and were stained using haematoxylin (0.5%) and eosin (1%) and mounted in DPX. Histochemical sections were evaluated by light microscopy.

2.3. Statistical analysis

All data were expressed as mean ± S.D. The statistical analysis was done by one way ANOVA (Tukey) using Graphpad InStat version 3.00 for Windows 98, GraphPad Software, San Diego, California, USA. p-value considered as significant are indicated by “*” “**” and “***” for p<0.05, p<0.01 and p<0.001 respectively.