Chapter 5

Materials and methods
LIST OF CHEMICALS:

<table>
<thead>
<tr>
<th>Name of the chemicals</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 2-di(4-tert-octylphenyl)-1-pierylhydrazyl (DPPH), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), Phenyl methyl sulphonyl fluoride (PMSF), 2’-7’-dichlorofluorescin diacetate (DCFDA), Bovine serum albumin (BSA), GSH, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), 5, 5’-dithio-bis (2-nitro benzoic acid) (DTNB), 2, 4, 6-tripyridyl-s-triazine (TPTZ), Quercetin, Epicatechin and Kaempferol.</td>
<td>Sigma (St Louis, MO, USA)</td>
</tr>
<tr>
<td>Nuclear factor-kappa B (NF-κB) antibody.</td>
<td>Imgenex, San Diego, CA, USA</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid disodium salt (EDTA)</td>
<td>E Merck Co. (Darmstadt, Germany).</td>
</tr>
</tbody>
</table>

All other chemicals used were of highest purity grade available.

COMPOSITION OF BUFFERES:

<table>
<thead>
<tr>
<th>Serial no</th>
<th>Name of the buffers</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphate Buffer Saline (PBS)</td>
<td>137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄ (pH 7.4).</td>
</tr>
<tr>
<td>2</td>
<td>Electrophoresis buffer (Comet assay)</td>
<td>300 mM NaOH, 1mM EDTA, (pH 13.5)</td>
</tr>
<tr>
<td>3</td>
<td>Tissue homogenization buffer</td>
<td>50 mM Tris-Hcl (pH 8), 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% glycerol, 1μg/mL each of leupeptin and aprotinin in distilled water</td>
</tr>
</tbody>
</table>
### Materials and methods

#### 4. Nuclear extraction buffer
20 mM HEPES, 10 mM KCl, 1mM EDTA, 20% glycerol, 10 mM NaCl, 1μg/ml each of leupeptin and aprotinin, 1mM PMSF in distilled water.

#### 5. 1X Tris glycine buffer
25 mM Tris, 250 mM glycine, 0.1% SDS

#### 6. Tris Buffer Saline (TBS)
25 mM Tris, 0.15 M NaCl (pH 7.5)

#### 7. Washing Buffer
TBS containing 0.05% tween 20

#### 8. 50X TAE
2 M Tris-Acetate, 0.05 M EDTA, pH 8.3

### LIST OF REAGENTS:

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Name of the reagents</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nash Reagent</td>
<td>75 g of ammonium acetate, 3ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water.</td>
</tr>
<tr>
<td>2.</td>
<td>Fe-EDTA reagent</td>
<td>0.6 mM Fe-ammonium sulfate and 1.2 mM EDTA (Mixed in 1:1 volume).</td>
</tr>
<tr>
<td>3.</td>
<td>Lowry reagent</td>
<td>2% Na₂CO₃ in 0.1 N NaOH, 1% Sodium potassium tartrate in H₂O, 0.5% CuSO₄,5 H₂O in water prepared immediately before use by mixing the following stock solutions in the proportion 98:1:1 (by volume).</td>
</tr>
<tr>
<td>4.</td>
<td>Griess reagent</td>
<td>0.2% naphthylene diamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric acid.</td>
</tr>
</tbody>
</table>
5. FRAP reagent

| 300 mM acetate buffer, 10 mM TPTZ solution, 20 mM FeCl₃·6H₂O solution in a ratio of 10:1:1 |

6. NBT-BCIP reagent

| NBT (7.5 mg in 250 µl water) and BCIP (3.75 mg in 250 µl water) in NBT/BCIP buffer (1.21 g Tris, 1.02 g MgCl₂ and 0.584 g NaCl and water upto 100 ml) |

**MORINGA LEAF EXTRACT PREPARATION:**

The leaves of *Moringa oleifera* were collected from a specific tree in the month of September and certified by the Botanical Survey of India, Howrah, India (Voucher no. CNH/I-I/10)/2009/Tech.II/352).

![Herbarium sheet of Moringa oleifera](image-url)

Leaves were air-dried, powdered and extracted with three different solvents i.e. aqueous, aqueous-ethanol (50% v/v) and ethanol. The extracts obtained were then filtered. The filtrates were vacuum evaporated to remove solvent completely and powdered form was obtained. Finally, aqueous (AQ-LE), aqueous-ethanolic (AQ-ET) and ethanolic (ET) solution of these powders were prepared at 50 mg/ml of concentration. This aqueous-ethanolic ((AQ-ET)) *Moringa oleifera* leaf extract (50 mg/ml) has been referred to as MoLE.

**DPPH SCAVENGING ACTIVITY:**

**Principle:** The scavenging reaction between (DPPH·) and an antioxidant (H-A) can be written as:
Antioxidants react with DPPH\(^{\cdot}\), which is a stable free radical and is reduced to DPPH-H and as a consequence the absorbance decreased from the DPPH\(^{\cdot}\) radical to the DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

**Procedure:** The free radical scavenging activity of the extract was measured by DPPH using the method described by Oktay *et al.* \(^{66}\) About 1ml 0.2 mM of ethanolic DPPH solution was added to 3 ml leaf extract and mixed well. After 30 minutes, absorbance was measured at 517 nm. Radical scavenging activity was expressed as percentage of inhibition and radical scavenging activity was calculated using the formula:

\[
\text{% of Radical Scavenging Activity} = \left( \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \right) \times 100
\]

**\(\text{H}_2\text{O}_2\) SCAVENGING ACTIVITY:**

This activity was determined according to a previously described method with minor changes \(^{67}\). An aliquot of \(\text{H}_2\text{O}_2\) (2 mM) and various concentrations (5-200 g/ml) of samples were mixed (1:0.6 v/v) and incubated for 10 min at room temperature. After incubation, the absorbance of hydrogen peroxide at 230 nm was determined against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction.

The percentage scavenging activity of hydrogen peroxide was calculated as follows:

\[
\text{% of scavenging activity} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of standard}}{\text{Absorbance of control}} \right) \times 100
\]

**HYDROXYL RADICAL (OH\(^{\cdot}\)) SCAVENGING ACTIVITY:**

**Principle:** Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid-iron EDTA. The hydroxyl radicals were formed by the oxidation; react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. It is shown that the actual colour-forming agent in the Nash method is an isolable iminodione formed by reaction of the two components.
**Materials and methods**

**Procedure:** The hydroxyl radical scavenging activity was determined according to the method described by Murthy et al. Various concentrations (2.5, 5, 10, 20, 30, 40 µg/ml) of MoLE in ethyl alcohol were taken in different test tubes. 1 ml of iron-EDTA solution (0.1% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA (0.018%) and 1 ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-90°C for 15 min. The reaction was terminated by the addition of 1 ml of ice cold TCA (17.5% w/v). 3 ml of Nash reagent was added to all of the tubes and left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm against reagent blank.

The percentage scavenging activity of hydrogen peroxide was calculated as follows:

\[
\% \text{ Hydroxyl Radical Scavenging activity} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100
\]

**Fe\(^{2+}\) CHELATION ACTIVITY:**

The Fe\(^{2+}\) ion chelating activity was evaluated by a standard method with minor changes. The reaction was carried out in 4-(2-hydroxyethyl)-1 piperazineethane sulfonic acid (HEPES) buffer (20 mM, pH 7.2). Briefly, increasing concentrations of test samples were added to 12.5 mM ferrous sulfate (FeSO\(_4\)) solution and the reaction was initiated by the addition of ferrozine (75 mM). The mixture was shaken vigorously and incubated for 20 min at room temperature. The absorbance was measured at 562 nm. Ethylene diamine tetra acetic acid (EDTA) was used as a positive control.

**REDUCING ACTIVITY:**

**Principle:** Substances, which have reduction potential, react with potassium ferricyanide (Fe\(^{3+}\)) to form potassium ferrocyanide (Fe\(^{2+}\)), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

**Procedure:** Different concentrations (in µg) of the extracts in 1ml of distilled water were mixed with 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. Then 10% TCA was added to the mixtures which were then centrifuged. The upper layer of the solution was mixed with distilled water and 0.1% FeCl\(_3\). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixtures indicated increased reducing power. Ascorbic acid was used as positive control.
DETERMINATION OF ASCORBIC ACID:
Principle: Ascorbic acid is oxidized by the blue coloured dye, 2, 6-dichlorophenolindophenol to dehydroascorbic acid; at the same time, the dye is reduced to a colourless compound so that the end point of the reaction can be easily determined.
Procedure: Ascorbic acid was quantitatively determined according to the 2, 6-dichlorophenolindophenol dye method. 10 g powdered sample of leaves was extracted by grinding with a small amount of sand and 6% metaphosphoric acid (v/v). The extract was made up accurately to a suitable volume (15 ml), mixed and centrifuged at 3000 g for 15 min at room temperature (24°C). Five milliliters of the supernatant was titrated against standard 2, 6-dichlorophenolindophenol dye, of which 1 ml=0.2 mg of ascorbic acid; the dye was previously standardized by titration against a 0.02% standard solution of ascorbic acid in 6% metaphosphoric acid. Results are presented as the amount of ascorbic acid present in 100 g of the dry leaves.

ESTIMATION OF TOTAL POLYPHENOL CONTENT:
Principle: The Folin-Ciocalteu assay of polyphenols relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined at 765 nm. The colour development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valency.
Procedure: Total polyphenol content of the extracts were quantified according to the method of Taga et al. with modifications. The test samples were mixed with 2% Na₂CO₃ and allowed to stand at room temperature for 2 minutes. After the incubation 50% Folin-Ciocalteu’s phenol reagent was added and the reaction tube was allowed to stand for another 30 minute at room temperature prior to read the absorbance at 720 nm. Gallic acid was used as the standard for a calibration curve. Polyphenol content of the extracts was expressed in terms of gallic acid equivalent.

TOTAL ANTIOXIDANT CAPACITY ASSAY:
Principle: Cu²⁺ is converted to Cu⁺ by both small molecules (ascorbate, uric acid, GSH, Vitamin E) and protein. The protein mask prevents Cu²⁺ reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu⁺ is chelated with a colourimetric probe giving a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity.
**Materials and methods**

**Procedure:** 0, 4, 8, 12, 16, 20 µl of Trolox Standard was added to individual wells in ELISA plate. Sample was diluted 1:1 with protein mask. Volume was adjusted 100 µl with distilled water. 100 µl Cu²⁺ working solution was added to all standard and sample wells. The plate was covered and the reaction mixture was incubated at room temperature for one and half hour. Reading was taken at 570 nm using a plate reader.

Calculation: \( \frac{S_a}{S_v} = \text{nmmol}/\mu \text{l or mM trolox equivalent} \)

Where \( S_a \) is the sample amount (in nmoles) read from the standard curve and \( S_v \) is the undiluted sample volume added to the wells.

**REVERSE PHASE HIGH PERFORMANCE LIQUID CROMATOGRAPHY (RP- HPLC) ANALYSIS:**

**Principle:** Compounds bind to RP-HPLC columns in high aqueous mobile phase and eluted from RP-HPLC columns with high organic mobile phase. In RP-HPLC compounds are separated based on their hydrophobic character.

**Procedure:** Chromatographic separation of flavonoids was done by a Thermo Hypersil (Runcorn, UK) ODS (4.6×125 mm, 3µm) column with a C-18 guard column (Bandapak C18, 4.6×10 mm, 10µm). Both columns were placed in a column oven set at 35°C. The HPLC system consisted of a Merck Hitachi (Hitachi, Tokyo, Japan) Lachrom Pump L-7100, Lachrom auto sampler L-7200, diode array detector (DAD) L-7450 and interface D-7000. Wavelengths used for identification of catechins and identification and quantification of other flavonoids and phenolic acids, with a diode array detector, were 280 nm for catechin, epicatechin and 340 nm for ferulic acid, ellagic acid and myricetin. Gradient elution was employed with a mobile phase consisting of 50 mM H₃PO₄, pH 2.5 (solution A) and acetonitrile (solution B) as follows: isocratic elution 95% A/ 5% B, 0-5 min; linear gradient from 95% A/ 5% B to 50% A/ 50% B, 5-55 min; isocratic elution 50% A/ 50% B, 5-65 min; linear gradient from 50% A/ 50% B to 95% A/ 5% B, 65-67 min; post time 6 min before next injection. The flow rate of the mobile phase was 0.7 ml/min and the injection volumes were 10 µl of the standards and sample extracts. The samples were prepared and analyzed in triplicate.

**ANIMAL CARE AND HANDLING:**

Swiss albino male mice (*Mus musculus*), 6-8 weeks old with an average body weight of 24 ± 2 g were obtained from Bengal chemical and Pharmaceuticals Ltd. (Kolkata, India). Animals were housed in groups of five in each polypropylene cage. Mice were maintained according to the guidelines set by Institutional Animal Ethical Committee (IAEC, India), maintained
under the controlled conditions of temperature (23±2°C), humidity (50±5%) and a 12-h light-dark cycle. Animals were given standard mice feed (procured from Hindustan Lever Ltd., Mumbai) and water ad libitum.

**IRRADIATION:**

Mice were irradiated with $^{60}$Co source at Saha Institute of Nuclear Physics, Kolkata, India. Unanaesthetized animals were restrained in well-ventilated Perspex boxes and exposed whole body to gamma radiation (5 Gy), at a dose-rate of 1 Gy/min and a source-to-surface distance of 77.5 cm.

**EXPERIMENTAL DESIGN:**

**Design 1:** Mice selected from an inbred colony were divided into four groups of 8 animals each. These groups are

Control: Mice given double distilled water through oral gavages once in a day for 15 consecutive days.

IR: Mice were given distilled water for 15 days and then exposed to 5 Gy of gamma radiation.

LE: Mice were treated with 300 mg/kg body weight of MoLE through oral gavages for 15 consecutive days. ($^{7, 74}$)

LE+IR: MoLE was given 300 mg/kg body weight of mouse orally for 15 days and exposed to 5 Gy dose of gamma radiation.

**Design 2:** Mice were selected from an inbred colony and divided into 6 groups with 8 animals in each group. These groups were-

Control: The control group received only distilled water.

IR: Mice were given distilled water for three consecutive days before exposing them to a single dose of 5 Gy $^{60}$Co gamma irradiation.

QN: Mice were treated with quercetin (100 mg/kg body weight) through oral gavages for 3 consecutive days ($^{75, 76, 77, 78}$).
EN: Mice were treated with epicatechin (100 mg/kg body weight) through oral gavages for 3 consecutive days \(^{(79)}\).

QN+IR: Quercetin (100 mg/kg body weight) was given for 3 consecutive days and 1 hour after the last dose, animals were exposed to single dose of 5 Gy gamma irradiation.

EN+IR: Epicatechin (100 mg/kg body weight) was given for 3 consecutive days and 1 hour after the last dose, animals were exposed to single dose of 5 Gy gamma irradiation.

After 24 hours of the irradiation, blood, liver and testis were collected and mice were sacrificed following the regulations set forth by IAEC.

**PREPARATION OF PACKED CELL VOLUME:**

Blood (100 µl) were collected from mice by the retro-orbital sinus vein puncture into vial containing 40U/ml sodium heparin. RBC was separated by centrifugation (Superspin R-V/FM, Mumbai, India) (180 g, 20 minute, 22°C), washed 3 times with a saline buffer and used for different studies \(^{(80)}\).

**MEASUREMENT OF ROS GENERATED WITHIN RBC BY FLOW CYTOMETRY:**

Intracellular accumulation of ROS level was measured after incubation of RBC with a membrane permeable fluorescent probe, DCFDA. It passively diffuses into the cell and it is converted to 2',7'-Dichlorodihydrofluorescin (DCFH) after deacetylation in presence of intracellular esterases. DCFH after subsequent oxidation reaction with ROS produces fluorescence. The oxidation of DCFDA is conveniently monitored to determine the level of intracellular oxidative stress \(^{(81)}\). 10 l of washed erythrocytes (5×10^6 erythrocytes) were incubated with PBS with saturating concentration of DCFDA (1 g/ml) in the dark for 30 minutes at room temperature. The DCFDA fluorescence signal was detected using 480 nm excitation and 530 nm emission light on a FACS caliber instrument (BD Bioscience, Mountain View, CA, USA). For each sample, auto fluorescence signal of unstained erythrocytes were measured and used to adjust the fluorescence intensity of DCFDA stained erythrocytes. Data were analyzed using Flow JO software (version7.6.5) attached with the flow cytometer. Analysis of FL-1(FITC channel) fluorescence was performed with gating on

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the total unstained RBC population to identify the live RBC population (46.6%). Same gate was used for all the samples to measure the FL-1 fluorescence intensity.

**DETERMINATION OF MEMBRANE LPO:**

RBC was centrifuged and resuspended in phosphate buffer saline (PBS). The RBC membrane was prepared by the method of Dodge et al. (82). Concentration of thiobarbituric acid reactive substance (TBARS) was measured by spectrophotometric (BIO-RAD Smartspec™ plus, spectrophotometer, USA) method based on the reaction of lipid peroxides with thiobarbituric acid (83). Absorbance of these conversions was done at 532 nm and the calculations were done using the molar extinction coefficient of 1.56×10⁵ M⁻¹ cm⁻¹. The level of LPO was expressed as thiobarbituric acid reactive substances (TBARS) content in nmoles / mg of membrane protein. Protein was estimated according to the Lowry et al. (84).

**PREPARATION OF RBC LYSATE AND ESTIMATION OF INTRACELLULAR GSH LEVEL:**

RBC was separated from whole blood in the same way as mentioned above. Then 10ml distilled water was added to the RBC (10:1) and incubated for 2 hours at 4ºC. A mixture of chloroform- ethanol (3:5, v/v) and 0.3 ml of water added to the lysate. Hemoglobin was removed from the cell lysate after centrifugation at 3000 g for 10 minutes at 4ºC [Dani et al. 2005]. The reduced glutathione level was estimated in the lysates according to Moron et al. (86).

**DETERMINATION OF MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (MCHC):**

The MCHC is the hemoglobin concentration in a given volume of RBC (87). The hemoglobin concentration was estimated using Drabkin’s reagent and cyanmethemoglobin as standard.

**NORMAL RED BLOOD CELL HEMOLYSIS AT ISOTONIC SALINE:**

Normal red blood cell hemolysis was evaluated by measuring the hemoglobin released from red blood cells relative to the total cellular hemoglobin content. 10 μl fresh blood was added to 5 ml normal saline and then incubated for 30 minutes. After centrifugation the supernatant
was aspirated and absorbance was taken at 540 nm. The percentage of hemolysis was taken against complete hemolysis. It can be expressed as:

$$\% H = \frac{A_{\text{sample}}}{A_{100\%\text{lysis}}} \times 100$$

Where $A_{\text{sample}}$ and $A_{100\%\text{lysis}}$ are the absorbance of hemoglobin released from red blood cells in normal saline and after complete hemolysis after incubation in distilled water (88).

**OSMOTIC FRAGILITY TEST:**

Osmotic fragility of RBC was determined by the modified method of (89). Fresh blood was added to the different hypotonic saline (pH 7.4) ranging from 0 to 0.9% (0, 0.1%, 0.2%, 0.3%, 0.5%, 0.6% and 0.9%) in the ratio of 1:100 respectively. Then the mixtures were gently shaken, incubated at room temperature for 30 min and centrifuged to precipitate the nonhemolized RBC. The osmotic lysis of RBC was determined by the release of hemoglobin into extracellular fluid. The absorbance of the supernatant was measured at 540 nm using a spectrophotometer. The percentage of hemolysis against each NaCl concentration relative to the blank salt concentration was calculated (89, 90). The osmotic fragility curves obtained from experimental data were fitted with Boltzmann equation to evaluate the average osmotic fragility ($H_{50}$) (NaCl concentration at which 50% hemolysis occurred). Other parameters can be evaluated from differentiation of the Boltzmann fitted curves of the experimental data, which represents the Gaussian curve [rate of hemolysis (dH/dC) versus NaCl concentration]. These parameters are position of the peak, height of the peak, area of the curve (rate of hemolysis) and width at half of maximum height of peak. The position of the peak on the X axis represents average osmotic fragility ($H_{50}$). The maximum height of the peak denotes the maximum rate of hemolysis (dH/dC$_{max}$). The area and the width of the curve signify the rate of hemolysis and dispersion of hemolysis respectively.

**DATA FITTING:**

The osmotic fragility curves obtained from experimental data were fitted by Origin pro8 software (76). Followings are the two applied functions:

Boltzmann function:
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\[ y = \frac{A_1 - A_2}{1 + e^{-\frac{(x-x_0)}{dx}}} + A_2 \]

Where \( A_1 \) and \( A_2 \) are the initial and final value of hemolysis (at 95% and 5% respectively), \( x_0 \) is the centre and \( dx \) is a constant.

Gaussian function:

\[ y = y_0 + \frac{A}{W} \left[ e^{-\frac{(x-x_c)^2}{2w^2}} \right] \]

Where \( y_0 \) is the offset, \( A \), \( x_c \) and \( W \) are the area, centre and width at half of maximum height respectively.

**MORPHOLOGY (SIZE AND ROUGHNESS OF THE MEMBRANE) AND SHAPE CHANGE STUDY BY AFM:**

**Sample preparation for Atomic Force Microscopic study (AFM)**

Blood was diluted 5 fold with isotonic buffer containing 5 mM glucose and thin film was prepared on glass cover slip \(^{(91)}\).

**AFM image acquiring and analysis**

AFM study was performed with VEECO Multimode system having Nanoscope IIIa controller (Veeco, Santa Barbara, CA, USA). All the images were taken using taping mode (TM) in air (at room temperature, 75% humidity) with Resolution Taping Mode Etched Silicon Probe (RTESP) tip silicon probes at resonant frequency 302.87 kHz. 200 µm J scanner was used. The cover slip carrying the blood sample was mounted on XY stage of scanner. The images obtained were analyzed by Nanoscope Software version 5.12 in offline mode.

AFM image analysis was employed to measure the change in membrane roughness of RBC quantitatively \(^{(91)}\). In the present study we also observed the roughness of RBC membrane of different group by AFM image analysis. The roughness value of the surface can be described in terms of \( R_{\text{rms}} \) (root-mean-square of the height distribution) value in roughness analysis window and the vertical height of the RBC was analyzed in the Section analysis window of the offline image analysis Nanoscope software (version 5.12).
The roughness of the membrane can be described in terms of the measure of the root-mean-square of the height distribution. It can be expressed as

\[ R_{\text{rms}} = \sqrt{\frac{\sum_{i=1}^{n} (Z_i - Z_m)^2}{n-1}} \]

Where \( n \) is the total number of data points, \( Z_i \) is the height of the \( i \)th point and \( Z_m \) is the mean height. The roughness value is dependent on the area taken into consideration for measurement (91). To avoid this problem we have considered a fixed 500 \( \times \) 500 nm\(^2\) square box at 30 different areas on the membrane surface for each RBC image.

**ISOLATION OF LYMPHOCYTE AND BONE MARROW:**

Whole blood (30 \( \mu l \)) was mixed with Dutch modified RPMI 1640 medium (1 ml) supplemented with 10\% (v/v) FCS, under layered with Histopaque 1077 (100 \( \mu l \)) and centrifuged at 200Xg for 3 min at 4\^\circ\ C. 100 \( \mu l \) of buffy coat were washed in PBS, pH 7.4. This buffy coat contains lymphocytes.

Femurs from the mice were dissected out and cleaned. The heads were cut off, and bone marrows flushed out and diluted with mice serum, using a syringe.

**SINGLE-CELL GEL ELECTROPHORESIS (ALKALINE COMET ASSAY):**

**Principle:** Comet assay used for the quantification and analyzing DNA damage in individual cells. The head of comet composed of intact DNA while the tail consists of damaged or broken piece of DNA. Individual cells are embedded in a thin agarose gel on microscope slide. All cellular proteins removed from the cells by lysis. The DNA is allowed to unwind under alkaline/neutral conditions. Following the unwinding the DNA undergoes electrophoresis, allowing broken DNA away from the nucleus. The gel is stained using ethidium bromide. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage and that corresponds to the length of the tail. Comet assay detects single, double strand break, alkali labile sites, oxidative base damage and DNA cross linking with DNA or protein.

**Procedure:** Comet assay using lymphocyte cells was performed under alkaline condition following the method of Singh *et al.* (92) with minor modifications. Lymphocytes were incubated with MoLE (100 \( \mu l \)) for 30 min at 37\^\circ\ C. The cells were subsequently washed with...
PBS, pH 7.4, centrifuged at 4°C and incubated in 1 ml PBS, pH 7.4, containing H₂O₂ (200 µM) for 5 min on ice. Next, slides were covered with 400 ml of 0.75% normal melting point agarose in PBS was pre-warmed to 50°C. A cover glass was placed over the agarose solution, and the agarose was allowed to solidify. The cover glass was then removed, and 85 µl of cell-agarose suspension was placed over the first agarose layer and allowed to solidify under a clean cover glass. After removing the cover glass 100 µl of 0.5% low melting point agarose was added and allowed to solidify in a chilled condition. After the cover glass was removed, the slides were gently immersed in a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100, pH adjusted to 10 with NaOH) and kept at 4°C in the dark for 1 hour. The slides were placed on the horizontal gel electrophoresis unit filled with fresh, cold electrophoresis buffer for 20 min. Electrophoresis was conducted for the next 20 min at 18V (1.0 V/cm, 250 mA). The slides were then drained, placed on a tray and flooded slowly with three changes of neutralization buffer (0.4M Tris-HCl, pH 7.5), each for 5 min. The slides were stained with ethidium bromide (10 mg/ml), covered with a cover glass, and analyzed within 1 hour at 400X magnification using a fluorescent microscope (Motic BA 400, Germany) with green filter. The photograph was taken through the attached digital camera.

**DNA FRAGMENTATION ASSAY:**

**Principle:** This method is based on the notion that extensively fragmented double-stranded DNA can be separated from chromosomal DNA upon centrifugal sedimentation. The protocol includes the lysis of cells and the release of nuclear DNA, a centrifugation step with the generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA, hydrolysis and colourimetric quantitation upon staining with diphenylamine (DPA), which binds to deoxyribose.

**Procedure:** DNA Fragmentation assay was done according to Burton. The heads were cut off and bone marrows flushed out and diluted with mice serum, using a syringe. After centrifugation bone marrow cells were obtained. A total of 2×10⁶ bone marrow cells were transferred to a micro centrifuge tube. The cells were lysed with 0.5 ml ice-cold lysis buffer (10 mM Tris-HCl, pH-7.5, containing 1 mM EDTA and 0.2% Triton X-100). Fragmented DNA was separated from intact chromatin by centrifugation for 10 min at 13000× g, 4°C (preparation B). The supernatant was carefully transferred to a tube (preparation A). 0.5 ml lysing buffer was added to pellet containing preparation B. 0.5 ml of 25% trichloroacetic acid (TCA) was added to the A and B preparations and vortexed vigorously. The tubes were
placed at 4°C and precipitate was left over night. The precipitates were centrifuged for 10 min at 13000× g. The supernatants were aspirated and discarded. After complete removal of supernatant, 80 µl of 5% TCA was added to each pellet, the DNA was hydrolyzed by heating for 20 min at 83°C in a water bath. 160 µl of diphenylamine solution was added to the test tubes and to a blank containing 80 µl 5% TCA. All tubes were vortexed and then left overnight at room temperature. In order to read the optical density (OD), the collected supernatants were transferred to 96 well plate and optical densities were read at 620 nm by ELISA reader. The percentage of fragmented DNA was calculated according to the following formula:

\[
\% \text{ fragmented DNA} = \left( \frac{\text{OD}_{620 \text{ nm tube A}}}{\text{OD}_{620 \text{ nm tube A}} + \text{OD}_{620 \text{ nm tube B}}} \right) \times 100
\]

**HEMATOLOGICAL STUDY:**

**RBC Count**

Blood is diluted 1:200 with the RBC diluting fluid and cells are counted under high power (40X objective) by using a haemocytometer. The cell count is expressed as cells/mm³ of whole blood.

**WBC Count**

WBC diluting fluid contains Gentian violet and Glacial acetic acid. Glacial acetic acid destroys the RBC by hemolysis. Gentian violet stains the nucleus of WBC. For WBC counting 980 µl of WBC diluting fluid and 20 µl of blood were mixed and cell count was done using a haemocytometer.

**BIOCHEMICAL ESTIMATIONS USING TISSUE HOMOGENATES:**

**Estimation of protein:**

**Principle:** The peptide bonds of proteins react with copper under alkaline condition to produce Cu+, which reacts with Folin reagent, result in phosphomolybdotungstate which is reduced to hetero- polymolybdenum blue by the copper catalyzed oxidation of aromatic amino acids. The reaction result in a strong blue colour.

**Procedure:** The protein content was determined from tissue extract by Lowry’s method. Tissue protein amount was determined by using Lowry reagent. About 1 ml of Lowry reagent and NaCl was mixed with 4 µl tissue extract. After 10 minute of incubation, 100 µl Folin reagent was added. After 30 minute incubation absorbance was taken.

**Determination of lipid peroxidation (lpo):**

**Principle:** Thiobarbituric acid reactive substance (TBARS) such as Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as an index for determining
the extent of the peroxidation reaction. TBARS reacts with TBA to give a red species absorbing at 535 nm.

**Procedure:** TBARS in the tissue homogenate was estimated by using standard protocol \(^{83}\). Briefly, the homogenate was incubated with 15% TCA, 0.375% TBA and 5 N HCL at 95°C for 15 min, the mixture was cooled, centrifuged and the absorbance of the supernatant measured at 535 nm against appropriate blank. The amount of lipid peroxidation was determined by using \(\varepsilon = 1.56 \times 10^5 /\text{M/cm} \).

**Determination of tissue nitrite concentration:**

**Principle:** NO exists primarily as NO\(_2\) and NO\(_3\) in biological systems. Nitrite reductase is utilized for the enzymatic reduction of nitrate to nitrite. In acidic solution nitrite is converted to nitrous acid which diazotizes sulfanilamide. This sulfanilamide-diazonium salt is then reacted with naphthyl ethylenediamine dihydrochloride (NED) to produce a chromophore which is measured at 540 nm.

**Procedure:** Tissue nitrite concentration was determined by using Griess reagent. 990 µl of Griess reagent was mixed with 10 µl of the tissue extract. After 5 minute of incubation at 37°C in the dark the reading was taken at 540 nm using spectrophotometer and the value was calculated from standard curve using 1 mM NaNO\(_2\) solution \(^{94}\).

**Determination of superoxide dismutase (sod) activity:**

**Principle:** SOD activity was estimated by measuring percentage inhibition of auto-oxidation of pyrogallol by the action of SOD.

**Procedure:** Superoxide dismutase (SOD) activity was determined by utilizing the involvement of superoxide anion radical in the autoxidation of pyrogallol \(^{95}\). 3 ml Tris buffer (50 mM) mixed with 50 µl samples. The mixture incubated at 25°C for 10 minutes. 100 µl pyrogallol was added to it and the reading was taken at 420 nm using spectrophotometer.

**Determination of catalase activity:**

**Principle:** Catalase activity measured by monitoring the decrease in absorbance resulting from the elimination of H\(_2\)O\(_2\) by the action of catalase.

**Procedure:** The standard reaction mixture contained 50 mM Potassium phosphate buffer (pH 7.0), 30 mM Hydrogen peroxide and 3µl of liver homogenate for a total volume of 1.0 ml. The reaction was run at 20°C and only the initial linear catalytic reaction for a period of one minute was considered as the catalase activity. The enzyme activity was determined from its ability to decompose 1 µmol of H\(_2\)O\(_2\) per minute and taken as 1 U activity using the \(\varepsilon\) for H\(_2\)O\(_2\) at 240 nm, i.e., 43.6 M\(^{-1}\)cm\(^{-1}\) \(^{96}\).
**Materials and methods**

**Determination of reduced glutathione (GSH):**

**Principle:** The sulphydryl group of GSH reacts with DTNB and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB provides accurate estimation of GSH in the sample.

**Procedure:** Glutathione (reduced) was determined according to the method described by Moron *et al.* Tissue homogenates were treated with 0.1 ml of 25% trichloroacetic acid (TCA) and the resulting precipitate was pelleted by centrifugation at 3900× g for 10 min. Free endogenous sulfydryl was assayed in a total 3 ml volume by adding 2 ml of 0.5 mM 5, 5′-dithio-bis (2-nitro benzoic acid) (DTNB) prepared in 0.2 M phosphate buffer (pH 8) to 1 ml of the supernatant. The GSH reacts with DTNB and forms a yellow-coloured complex with DTNB. The absorbance was read at 412 nm.

**Determination of ferric reducing antioxidant power (FRAP):**

**Principle:** In FRAP assay reductants in the sample reduce the Fe (III)/Tripyridyltriazine complex to a blue ferrous form which causes increase in the absorbance at 593 nm.

**Procedure:** FRAP level was determined according to the methods described by Benzie & Stain. Briefly, 1 ml FRAP reagent and 10 µl sample (Tissue homogenate) were mixed and kept in water bath at 37°C for 4 minutes. The optical density was measured at 593 nm. Concentration was calculated against a FeSO₄ standard curve. FRAP unit is equal with 100 mol/dm³ Fe²⁺.

**HISTOLOGY:**

**Principle:** Hematoxylin and Eosin are the principal stains used for the demonstration of nucleus and the cytoplasmic inclusions. Alum acts as a mordant and Hematoxylin containing alum stains the nucleus light blue which turns red in the presence of acid. The cell differentiation is achieved by treating the tissue with acid solution. The counter staining is performed using eosin solution which imparts pink colour to cytoplasm.

**Procedure:**

1. Fixation: Tissues were fixed using 10% formal saline.
2. Dehydration: The tissues were dehydrated using 80% for 2 hours, 90% for 1 hour and 100% alcohols for 3 hours.
3. Cleaning: The dehydrated sections were kept in Xylene for 2 hours for cleaning.
4. For infiltration and impregnation, sections were kept in Paraffin for 2 hours at the temperature of 56°C.
5. Embedding: Leuckhard box (consisting of two L shaped pieces of heavy metallic material brass) was arranged on a glass plate. The specimen was placed at the bottom of the cavity. Paraffin wax (with high melting point, 56°C to 58°C) was melted and filtered through course filter paper. The filtered paraffin was then poured into the cavity of the box containing the specimen. The box (mold) was then placed in a container of cold water or kept in a refrigerator; until the wax hardened. Now the hardened block was ready for the section cutting on the microtome.

5. Section cutting of Paraffin wax embedded tissue: paraffin block was trimmed and attached to the microtome. Next the sections were cut into 5 µm. The sections were fixed on the slides by albumin solution.

6. Deparaffinization of the section: Slides were flamed on a burner and placed in xylene for 45 minutes. Xylene treatment is repeated with agitation.

7. Hydration: Sections were hydrated by soaking in graded alcohol baths. The alcohol solution used were-100%, 90%, 80% and 70%.

8. Sections were placed for 30 seconds in each of these alcohol solutions, washed in tap water and rinsed in distilled water. Sections were drained well before staining.

9. Staining:  
   i) Sections were stained with Hematoxylin solution and kept for 3 minutes in water bath and then washed in running tap water.
   ii) Slides were quickly dipped in and out of 0.5% (v/v) hydrochloric acid (the differentiation is checked using a microscope. The nuclei should appear dark purple and rest of the tissue should appear pale.
   iii) The slides were quickly rinsed in tap water for 60 seconds.
   iv) It was washed in tap water and rinsed in 95% alcohol.
   v) The slides were agitated in eosin for 20 seconds. The staining solution was then drained off.

10. Dehydration: The slides were placed in 70% alcohol for 30 seconds. They were placed in 95% alcohol for 60 seconds. Then they were placed in absolute alcohol (2 changes, 60 seconds each).

11. Clearing: The slides were placed twice in xylene for 60 seconds each.

12. Mounting: The excess xylene was drained and mounted using Canada balsam with a cover slip.
Materials and methods

13. The stained slide for each group was observed using light microscope (Olympus, Tokyo, Japan) at 200X magnification. Cell nuclei appeared Blue colour and Cytoplasm was varying shades of pink.

**IMMUNOHISTOCHEMICAL ANALYSIS:**

Immunohistochemistry was performed according to the modified protocol of Descargues et al.98, 99 with some modifications.

1. Liver tissues were fixed in 10% neutral-buffered formalin. Fixed tissues were paraffin-embedded.
2. Embedded livers were sectioned into 4 µm. Sections were washed with xylene to remove paraffin.
3. Sections washed with PBS (1X). 100%, 90%, 80%, 70%, 60%, 50% alcohols were added to the sections and kept for 4 minutes each. Sections were washed with PBS (1X) again.
4. 100 µl Triton X-100 (0.5%) was added to the sections and kept for half an hour, for permealization.
5. Sections were kept at 90°C in Sodium citrate buffer (10 mM, pH 6) for unmasking of antigen.
6. The processed liver sections were incubated with NF-κB primary antibody for overnight at 4°C.
7. A specific signal was detected by using DAPI and FITC tagged antibody.

**WESTERN BLOT ANALYSIS (NF-κB):**

**Preparation of tissue homogenate:** Liver was weighed and sonicated using tissue homogenization buffer (50 mM Tris-Hcl, 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% glycerol, 1 g/mL each of leupeptin and aprotinin, distilled water) using a tissue homogenizer (Sono Plus, Germany). Homogenate was centrifuged at 12,000× g (30 min at 4°C) and pellet was collected.

**Extraction of nuclear proteins:** Nuclear extraction buffer (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 20% glycerol, 10 mM NaCl, 1 g/ ml each of leupeptin and aprotinin, 1mM PMSF, distilled water) was added to the pellet. It was vortexed and centrifuged to collect the supernatant containing nuclear proteins. The supernatant was separated and stored at −70°C for analysis of nuclear protein.

**Protein estimation:** Total nuclear protein content was determined by Lowry’s method 84.

**SDS-PAGE analysis of proteins:** The tissue homogenate was subjected to 10% Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to resolve the cellular protein
for western blot analysis. Equal amounts of protein (50 µg) was loaded into SDS-PAGE and run with 1X Tris glycine buffer at 20 mA current with voltage 130 V.

**Immunoblotting:** After completion of the separation by gel electrophoresis, proteins were subjected to electro transferred (100V, 1 hour) to a nitrocellulose membrane using a mini-trans blot assembly (Bio-Rad, USA). The nitrocellulose membrane was blocked in a blocking solution (3%, w/v, BSA in TBS) for 2 h at room temperature. Nuclear translocation of NF-κB (p65) was analyzed by probing with respective mouse monoclonal primary antibodies (1:1000 dilution) against the former (Imgenex, San Diego, USA). Following three washes of 15 min each in washing buffer (TBS, 0.2% Tween 20); membranes were incubated in TBS containing 1:10,000 dilution of goat anti-mouse IgG alkaline phosphate conjugated secondary antibodies. The membranes were again washed (three times each for 15 minutes) with washing buffer and then treated with NBT-BCIP reagent for 20 minutes. The protein bands obtained were further subjected to densitometric analysis using Gel Documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

**MEASUREMENT OF CYTOKINE LEVELS:**
The levels of murine serum TNF-α and IL-6 were measured using a sandwich ELISA Kit purchased from Endogen Inc. (Rockford, IL, USA). In brief, wells of high-binding Elisa plates (Millipore, Bedford, MA, USA) were coated with 100 µl of the capture antibody for mouse TNF-α and IL-6 (1 µg/ml). After overnight incubation at 25°C, plates were washed and blocked for 2 hours with 1% bovine serum albumin in phosphate-buffered saline. After wells were washed four times, 100 µl of serum or various concentrations of standard (recombinant mouse TNF-α and IL-6 proteins) was incubated at 48°C overnight. The wells were washed before addition of 100 µl of biotinylated antibody to wells. After 2 hours at 25°C, plates were washed, and the immune complex was determined using the streptavidin horseradish peroxidase-tetramethylbenzidine detection system (Endogen). The reactions were terminated by addition of 100 µl of 2N H₂SO₄, and absorbance was determined using a microtiter plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA) at 450 nm. The concentration of TNF-α and IL-6 in the unknown samples was calculated by comparison of the absorbance of the unknown samples to the standard curve.

**DETERMINATION OF ASPARTATE AMINOTRANSFERASE (AST), ALANINE AMINOTRANSFERASE (ALT), ALKALINE PHOSPHATASE (ALP) AND ACID PHOSPHATASE LEVELS:**
**Materials and methods**

**Principle:** α-Ketoglutarate and L-Aspartate produce Oxaloacetate in presence of Aspartate aminotransferase. Oxaloacetate is coupled with 2, 4-Dinitrophenyl hydrazine to produce corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colourimetrically.

Alanine aminotransferase catalyzes the transamination of L-Alanine and α-Ketoglutarate to form Pyruvate. Pyruvate is coupled with 2, 4-DNPH to form a corresponding hydrazone, a brown coloured complex in alkaline medium and this can be measured.

Alkaline Phosphatase from serum converts Phenyl phosphate to inorganic Phosphate and Phenol at pH 10.0. Phenol so formed reacts with 4-Aminoantipyrine in presence of oxidizing agent Potassium ferricyanide and forms an Orange-Red coloured complex, which can be measured. The colour intensity is proportional to the enzyme activity.

Acid Phosphatase Assay Kit utilizes para-nitrophenyl phosphate (pNPP) as a chromogenic substrate for the enzyme. In the first step, AP dephosphorylates pNPP. In the second step, the phenolic OH-group is deprotonated under alkaline conditions resulting in p-nitrophenolate that yields an intense yellow colour which can be measured at 405-414 nm.

**Procedure:** AST, ALT and ALP were estimated using enzymatic kit of Span diagnostic Ltd. (Surat, India) according to the manufacturer’s protocol. ACP was estimated using enzymatic kit of Cayman Chemicals Co. according to the manufacturer’s protocol.

**STATISTICAL ANALYSIS:**

The values are given as Mean±standard error of the mean (SEM). Analysis of variance (ANOVA) with Tukey’s post hoc test was done for statistical evaluation of the data and for the determination of level of significance in various groups of animals. P<0.05 was considered as the level of significance.