Chapter 2

Materials and methods
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2.1. Materials

2.1.1. Turmeric essential oil (TEO)
TEO isolated by steam distillation from the rhizome of *Curcuma longa* L., was supplied by Kancore Ingredients Limited., Angamali, Kerala, India. The colour and appearance of TEO was pale yellow to orange yellow liquid (Sample No. TONE-00321). Essential oil was stored at 4°C away from direct light and heat.

Refractive Index at 20°C : 1.5136
Specific gravity at 25°C : 0.9214

2.1.2. Cell lines
Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were initially procured from Adayar Cancer Institute, Chennai and propagated as transplantable tumors in the peritoneal cavity of Balb/C mice. L929 (Mouse Lung Fibroblast), HeLa (Cervical Cancer Cells), HepG2 (Human Hepatocellular Liver Carcinoma cell lines) and Vero cell lines (Normal African green monkey kidney cells), were obtained from National Centre for Cell Science (NCCS), Pune.

2.1.3. *Salmonella typhimurium* strains
Auxotrophic *Salmonella* strains, TA-98, TA-100, TA-102, TA-1535 were kindly supplied by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chadigarh, India and were used for antimutagenic studies. Frozen cultures of the tester strains (9% DMSO as a cryopreservative agent) were stored at -20°C. The bacterial culture was inoculated on fresh nutrient broth and grown for 12 h at 37°C before each experiment.

2.1.4. *Aspergillus flavus* (MTCC 2799)
*Aspergillus flavus* was supplied by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chadigarh, India, and was used for the studies on aflatoxin production *in vitro*. *Aspergillus flavus* Culture was maintained on Sabouraud’s Dextrose Agar.
2.1.5. Animals

Young adult male and female Wistar rats (6-7 weeks old), male/female Swiss and Balb/C mice (6-8 weeks old) as well as one day old ducklings were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala, India. They were housed in the animal house of Amala Cancer Research Centre, in well ventilated sterile polypropylene cages. They were maintained at a controlled temperature of 22 ± 2°C and relative humidity 60 ± 10% and provided with 12 hours light/dark cycles. Experiments were started after acclimatizing the animals for one week. They were fed with normal pelleted rat chow (Krish Scientists Shopee, Bangalore, India) and water ad libitum. The composition of the feed is maize 36%, rice bran 34%, rice polish 4.97%, cotton seed extract 4%, soybean meal 10%, groundnut extract 4%, dried yeast 2%, salt 5%, mineral mixture 0.025%, vitamin 0.01%, mean energy (Kcal/kg) 3600, pellet size 12mm. Ducklings were fed with starter diet (Corn 56%, Soybean meal 37%, Soybean oil 3%, Dicalcium phosphate 1.1%, Limestone 1.4%, Sodium chloride 0.3%, dl-Methionine 0.2%, Vitamin-mineral 1%)

All animal experiments were conducted after getting prior permission from Institutional Animal Ethics Committee and which followed 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 (Sanction No. 149/1999/CPCSEA).

2.1.6. Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient broth</td>
<td>Hi-media, Mumbai.</td>
</tr>
<tr>
<td>Sabouraud’s Dextrose Agar</td>
<td>&quot;</td>
</tr>
<tr>
<td>Thiobarbituric acid (TBA)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dextran</td>
<td>&quot;</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>&quot;</td>
</tr>
<tr>
<td>Trypsin</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Agar agar : Sisco esearchLaboratories (SRL), Mumbai

L- Histidine : “
Biotin : “
Nicotinamide adenine dinucleotide (NAD) : “
Nicotinamide adenine dinucleotide phosphate (NADP) : “
Nicotinamide adenine dinucleotide phosphate reduced (NADPH) : “
Adenosine triphosphate (ATP) : “
Phenazine methosulphate : “
Glucose-6-phosphate : “
2,6 dichlorophenol indophenols (DCPI) : “
Sodium succinate : “
Oxaloacetate : “
Dimethyl sulphoxide (DMSO) : “
Sodium azide (NaN₃) : “
Nitro blue tetrazolium (NBT) : “
Glutathione (GSH) : “
Glutathione oxidized (GSSG) : “
5-5’Dithiobis 2-niotrobenzoic acid (DTNB) : “
Tris Buffer : “
Tris-HCL : “
Colchicine : “
Riboflavin : “
Agarose : “
Agarose low EEO : “
Agarose low melting : “
Carboxymethyl cellulose : “
Folin’s reagent : “
Deoxy ribose : “
Dimethyl sulfoxide (DMSO) (Case no.67-68-5) : Merck specialties Private Ltd.,
Mumbai, India

Liquid paraffin (CAS No. 8012-95-1) : “
Hydrogen peroxide : ”
Dodium dodecyl sulfate : ”
Formaldehyde : ”
Triton X-100 : ”
Trypan blue : ”
Geimsa powder : ”
May-Grunwald powder : ”
Leishman’s stain : ”
Eosin : ”
Haematoxylin : ”
Bovine Serum Albumin (BSA) : ”
Hydrogen peroxide : ”
Sodium dodecyl sulphate : ”
Ethylene diamine tetra aceticacid (EDTA) : ”
Ferrous ammonium sulphate (FeSO₄) : ”
Tween 20 : ”
Hoechst Stain : ”
N-Methyl-N’-nitro-N-nitrosoguanidine (MNNG) : Sigma Aldrich, St.
Louis, MO, USA

4-Nitro-O-phenylenediamine (NPD) : “
2-Acetamidoflourene (2-AAF) : “
7, 12-Dimethylbenz (a) anthracene (DMBA) : “
3-Methyl cholanthrene (3-MC) : “
Nitrosodiethylamine (NDEA) : “
7-Ethoxy resorufin (ER) : “
7-Pentoxy resorufin (PR) : “
7-Methoxy resorufin (MR) : “
Dextran : “
Carrageenan : “
2,2-Diphenyl-1-picrylhydrazyl (DPPH) : “
2,2-Azobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) : “
Ethidium Bromide : “
Propidium iodide : “
3-(4,5-Dimethyl-2-thiazolyl)-2,5-
Diphenyl-2H-tetrazolium bromide (MTT) : “
Aflatoxin B1 : “
Fructose 1,6-diphosphate : “
Trisodium isocitrate : “
Protenase K : “
RPMI (Roswell Park Memorial Institute) : “
MEM (Modified Eagle Medium) : “
Diclofenac : Torrent Lab Pvt. Ltd, Ahmedabad
Cyclophosphamide : Neon Laboratories Limited.
Aflatoxin mix kit : Supelco Analytical, USA
Ampicillin : Ranbaxy Laboratories Ltd.
Phenobarbitone (Gardenal® 60, Batch No.B03007) : Nicholas-Piramel, India Ltd, Gujarat, India.
Phorbol-12-myristate-13-acetate (PMA) : Gift from Dr. Allen Conney, USA.
Foetal Calf Serum : Biological industries, Kibbutz, Israel.
Goat Serum : From local slaughter house.
Croton oil was prepared from the seeds of *Croton tiglium L.* by light ether petroleum extraction. All other reagents were of analytical grade.

### 2.1.7. Diagnostic Reagent Kits

- **Haemo Chek** : Agape Diagnostics, Thane.
- **Total Bilirubin Kit** : Span Diagnostics, Surat, India.
- **Alanine aminotransferase (ALT)**
- **Aspartate aminotransferase (AST)**
- **Total Bilirubin Kit**
- **Alkaline Phospha tase (ALT)**
- **Urea Kit**
- **Creatinine Kit**
- **Gamma glutatmyl transferase (GGT)** : Merck, Germany
- **pH Paper**
- **Magistik-GP urinalysis strip** : Peerless Biotech Pvt, Ltd, Chennai, India.
- **Medium filtering assembly unit** : Millipore, USA
- **Polycarbonate membrane filter** : Whatman, USA.
- **Tissue culture flask (25cm²)** : Greiner, Germany.
- **96 well flate bottom ELISA plate**
- **Cellular syringe filter**
  - (0.2µm pour size)
- **Aflatoxin DNA adduct Competitive ELISA Kit (AKR-351)** : CELL BIOLABS, INC. USA
- **cDNA direct** kit : Genei, Banglore
- **SYBER® Premix Ex Taq**
  - (Tli RNaseH Plus)RT PCR Kit : TaKaRa
### 2.1.8. Instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Brand/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inverted microscope</td>
<td>Leica, German Radicle, Ambala</td>
</tr>
<tr>
<td>Upright research microscope</td>
<td>Meiji, Japan, Labex, Labovision, India.</td>
</tr>
<tr>
<td>Horizontal Laminar flow hood</td>
<td>Cleannair, Chennai and Rotex, India.</td>
</tr>
<tr>
<td>Deep Freezer, -20</td>
<td>Remi, Chennai, India.</td>
</tr>
<tr>
<td>High speed cooling centrifuge</td>
<td></td>
</tr>
<tr>
<td>Cyclomixer</td>
<td></td>
</tr>
<tr>
<td>Homogenizer</td>
<td></td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>Elico Ltd, Hyderabad, India.</td>
</tr>
<tr>
<td>pH meter</td>
<td></td>
</tr>
<tr>
<td>Electrophoresis unit</td>
<td>Biotech, Yercaud, Genei, Banglore.</td>
</tr>
<tr>
<td>Fluorescent microscope</td>
<td>Olympus BX41.</td>
</tr>
<tr>
<td>Gel documentation system</td>
<td>Vilber Lourmat, France</td>
</tr>
<tr>
<td>Fluorescent spectrophotometer</td>
<td>Nano Drop ND-3300, Thermo Scientific, USA</td>
</tr>
<tr>
<td>Ultracentrifuge</td>
<td>Sorvall WX80, Thermo Scientific, USA</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Tarsons products prvt. Ltd, Kolkata.</td>
</tr>
<tr>
<td>Hot air oven</td>
<td>Beston, India</td>
</tr>
<tr>
<td>Electronic balance</td>
<td>Contech /instrument company, Mumbai, India.</td>
</tr>
<tr>
<td>Co₂ Incubator</td>
<td>Napco, Canada.</td>
</tr>
<tr>
<td>Tissue homogenizer</td>
<td>Yorco scientific industries, Delhi.</td>
</tr>
<tr>
<td>Transiluminator</td>
<td>Vilber Lourmat, France.</td>
</tr>
<tr>
<td>Real Time-PCR</td>
<td>ABS-7300, AB Biosystems.</td>
</tr>
<tr>
<td>PCR (Master Cycler)</td>
<td>Eppendorf.</td>
</tr>
<tr>
<td>Deep Freezer, -80</td>
<td>New Brunswick, eppendorf.</td>
</tr>
<tr>
<td>ELISA Reader</td>
<td>Varioskan™ flash, Thermo scientific,</td>
</tr>
<tr>
<td>Phase contrast microscopy</td>
<td>Magnus INVI (Inverted microscopy)</td>
</tr>
</tbody>
</table>
2.1.9. Software
CaspLab, For Comet Assay analysis : University of Wroclaw, Poland.
Graph pad in Stat, For statistical analysis : GraphPad Software Inc., USA
ORGIN 9.1, For Graphing and Analysis : OriginLab Corporation, USA.

2.1.10. Reagents

(a) Phosphate buffered saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.00 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.20 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Na₂HPO₄. 2H₂O</td>
<td>1.44 g</td>
</tr>
</tbody>
</table>

Dissolved the contents in distilled water, made up to 1000 mL. pH was adjusted to 7.2 with 1N NaOH/ HCl. Sterilized by autoclaving at 15 lbs for15min.

(b) Trypsin solution

Trypsin : 200 mg
Dextrose : 20 mg

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) 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.

(d) Turk’s fluid

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>10% crystal violet</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>98 mL</td>
</tr>
</tbody>
</table>

Stirred overnight, filtered and used.

(e) Hank’s balanced salt solution (HBSS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>818.16 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>37.28 mg</td>
</tr>
<tr>
<td>Dextrose</td>
<td>180.0 mg</td>
</tr>
</tbody>
</table>
CaCl₂ : 14.70 mg  
MgCl₂ : 20.33 mg  
HEPES : 230.30 mg  
Dist. Water : 100 ml  

(f) Spizizen’s salt solution (10X)

MgSO₄.7H₂O : 0.2 g  
Sodium citrate : 1 g  
K₂HPO₄ : 14 g  
KH₂PO₄ : 6 g  
(NH₄)₂SO₄ : 2 g  

Warm distilled water : 100 ml  

This solution was sterilized by autoclaving at 121°C for 20 minutes.

(g) Nutrient broth

Nutrient broth : 1.3 g  
Distilled water : 100 ml  

Nutrient broth was dissolved in 100ml distilled water and was autoclaved before using.

(h) Minimal agar plates

Agar : 1.5g for 100 ml  
Distilled water : 85 ml  
10X Spizizen’s salt solution : 10 ml  
40% glucose : 5 ml  

The agar and water were autoclaved for 20 minutes at 121°C and 10 ml of sterile Spizizen’s salt solution and 5 ml sterile 40% glucose solution were added and this solution was poured into petriplate.

(i) Top agar

Agar : 0.6 g  
Sodium chloride : 0.5 g  
Distilled water : 100 ml  

The agar was dissolved in distilled water and autoclaved for 20 min at 121°C and 2 ml of the solution was distributed into sterile tubes.

(j) Phosphate buffer (0.2M)-pH:7.4

0.2M NaH₂O₄. H₂O (1.38g / 50 ml) : 12 ml
0.2M Na₂HPO₄ (1.42 g / 50ml) : 88 ml

(k) **Citrate buffer (0.1 M)-pH 6.5**

Solution A : 0.1M solution citric acid (2 g/100 ml)
Solution B : 0.1M trisodium citrate (1.45 g/50 ml)

7.2 ml A + 42.8 ml B then diluted to 100 ml with distilled water.

(l) **Glucose ammonium nitrate medium for *Aspergillus* culture**

<table>
<thead>
<tr>
<th>Glucose</th>
<th>50 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>2.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>10 g</td>
</tr>
<tr>
<td>MgSO₄. 7H₂O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Mineral supplements**

a) **Solution A**

| ZnSO₄. 7H₂O | 20 g |
| CuSO₄. 5H₂O | 2 g  |
| Co (NO₃). 6H₂O | 1 g |
| Distilled water | 1000 ml |

b) **Solution B**

| CaCl₂ | 50 g |
| Distilled water | 1000 ml |

10 ml of each solution (A and B) was mixed with 7.5 L of glucose- ammonium nitrate medium.

2.1.11. Stains

(a) **Trypan blue**

Trypan blue stain : 100 mg
Normal saline (0.9%NaCl) : 100 mL

Trypan blue stain was dissolved in saline followed by filtration using Whatmann No.1 filter paper.
(b) **May-Grunwald stain**

May-Grunwald powder : 250 mg  
Methanol : 100 mL

The stain was dissolved in methanol by stirring and filtered through Whatmann’s No.1 filter paper and stored at 4°C.

(c) **Leishmann’s stain**

Leishmann’s stain : 150 mg  
Methanol : 100 mL

Leishmann’s stain was dissolved in methanol, filtered and used.

(d) **Giemsa stain**

Giemsa powder : 800 mg  
Glycerol : 50 mL  
Methanol : 50 mL

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’s No.1 filter paper and stored at 4°C.

(e) **Harris haematoxylin**

Haematoxylin : 5 g  
Ethyl alcohol : 50 mL  
Potassium alum : 50 mg  
Potassium iodide : 50 mg  
Distilled water : 950 mL

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.

(f) **Eosine solution**

Eosine : 500 mg  
Ethyl alcohol : 100 mL
Eosine was dissolved in 5mL of ethyl alcohol and made up to 100 mL with ethyl alcohol.

2.2. Methods

2.2.1. Preparation of Turmeric essential oil (TEO)

To get a uniform suspension of spices oil for \textit{in vitro} studies, the TEO (1 mg/ml) was dissolved in hexane and 1 µl of Triton X-100 was added and further evaporated to dryness and finally made up to 1 ml with distilled water. For \textit{in vitro} cytotoxicity and MTT assay TEO was dissolved in DMSO (0.1%). TEO was dissolved in paraffin oil for animal studies and prepared fresh every time.

2.2.2. Tissue culture

2.2.2.1. Sterilization of glasswares

All the glass wares required for the preparation of culture media were soaked in a solution of extran (1%) overnight, cleaned using brush and washed in distilled water and dried in a hot air oven. There were then autoclaved at a pressure of 15 pounds/ square inch for 15 min dried and used for experiment.

2.2.2.2. Preparation of tissue culture medium and sterility checking

Dulbecco’s modified Eagle’s Medium (DMEM) (9.98 g/L) was prepared in autoclaved double distilled water and pH was adjusted to 7.2 using sodium bicarbonate. This was then supplemented with L-glutamine (2 mM), .1mM non-essential aminoacids, 1.5 g/L sodium bicarbonate, 1 mm sodium pyruvate and filtered under negative pressure using a 0.22 µm cellulose filter. RPMI (Rosewell Park Memorial Institute) medium with 2mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate, 10% fetal calf serum. Sterility of the medium was tested using fluid thio-glycollate medium. For this, 1 ml of the filtered medium was added to 10 ml of sterile thio-glycollate (29.96 g/L) incubated at 37°C for 1 week and checked for any visible contamination. Broad spectrum antibiotics such as penicillin (100 units/ml) and streptomycin (100 µg/ml), and also foetal serum (10%) were added to the medium prior to use.
2.2.2.3. Maintenance of cells

2.2.2.3.1. Maintenance of DLA and EAC cells in mice

DLA and EAC were maintained in the intraperitoneal cavity of mice. Briefly, 1x10^6 (100 μl) cells were injected into the intraperitoneal cavity of mice. After 15 days, the cells were aspirated using a 1 ml syringe containing PBS. The cells were washed in PBS and the number of cells was counted using a hemocytometer and made up to 10^6 cells/ml with PBS. The 0.1 ml cells (10^7 cells/mL) were injected into the intraperitoneal cavity of new mice for tumor development.

2.2.2.3.2. Maintenance of L929, HeLa, HepG2 and Vero cells in tissue culture medium

L929, HeLa and Vero cells were maintained in DMEM (Dulbecco’s modified Eagle’s Medium) and HepG2 cell was maintained in RPMI 1640 (Rosewell Park Memorial Institute). For in vitro culture of L929, HeLa, HepG2 and Vero cell lines, the spent medium of confluent bottle of cells were removed and cells were washed twice with sterile 3 mL of PBS-EDTA twice. Trypsin (0.2% -100 μL) solution containing EDTA was added and incubated for 2-3 min at 37°C and the bottles were tapped on the sides to displace the cells. Cells were dispersed to single cell suspension by repeated pipetting and 1×10^3 cells were added to fresh tissue culture bottles containing 10 mL of the medium and further incubated at 37°C. Cells were sub-cultured once in every week.

2.2.2.4. Determination of cell viability

2.2.2.4.1. Tryphan blue dye exclusion method (Short term cytotoxicity).

Cell viability was determined by trypan blue dye exclusions method as described by Gupta and Bhattacharya (1978).

**Principle**

Tryphan blue is not permeable in living cells duo to the presence of intact plasma membrane. When the cells are dead, they will take up the dye and appear in blue color. The method is an index of the dead cells in a cell population.
Procedure

Short term cytotoxic activity of TEO was done by determining the percentage of viability of Daltons Lymphoma Ascites (DLA) and Ehrlich’s Ascites Carcinoma (EAC) cells using trypan blue dye exclusion technique. EAC and DLA cells were grown in the peritoneal cavity of healthy mice weighing 25-30 g by injecting a suspension of cells (1x10^6 cells/ml). For this tumour cells were aspired from the peritoneal cavity of the mice on day 15 and washed with PBS (0.2 M, pH 7.4) and centrifuged for 15 min at 1,500 rpm. The pellet was resuspended with PBS and the process was repeated 3 times. Finally, the cells were suspended in a known quantity of PBS and the cell count was adjusted to 1 x 10^7 cells/ml. 0.1 ml of this cell suspension was dispensed in 0.8 ml of phosphate buffer and incubated with different concentrations of TEO at 37°C for 3 h. After 3 h, the trypan blue dye exclusion test was performed to determine the percentage viability and the IC_50 value was calculated. 0.1 ml of cell suspension was mixed with 0.1 ml of 1% tryphan blue, kept for 2-3 minutes and load on a haemocytometer. The number of stained and unstained cells was counted separately.

\[
\text{% Death cells} = \frac{\text{Number of death cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100
\]

2.2.2.4.2. Anti-proliferating activity of TEO in cell lines (MTT assay)

Principle

3-(4, 5-Dimethyl-2-thiazoly)-2,5-diphenyl-2-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

Cells (5 x10^4) were plated in 96-well flat bottom titer plate in appropriate volume of the medium supplemented with FCS and antibiotics. The drugs were added 24h after the plating of the cells and incubation continued (37°C). Four h before (44th h) the completion of incubation, 20 μL of MTT (5 mg/mL) was added to each well and the incubation was continued up to 48th hr. DMSO (100 μL/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 570 nm.

\[
\text{Cell growth inhibition} = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100
\]

2.2.3. Haematological parameters

2.2.3.1. Determination of haemoglobin

Principle

The haemoglobin is converted to cyanmethaemoglobin in the presence of potassium ferricyanide and potassium cyanide (Drabkin’s reagent). The absorbance of cyanmethaemoglobin is proportional to the haemoglobin concentration (Drabkin & Austin, 1932).

Procedure

The reagents used were procured from Agappe diagnostic kit. 20 \( \mu \)L of heparinised fresh blood was mixed with 5mL of Drabkin’s reagent and incubated for 5 minute 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.

\[
\text{Hb} = \frac{\text{O.D of sample}}{\text{O.D of standard}} \times N \times 0.251
\]

Where ‘N’ is concentration of standard (60 mg/dL)

2.2.3.2. 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 blood cells. All the nucleated cells become intact and are stained by crystal violet.
Procedure

Blood (20 μL) was mixed with 380 μL 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.

\[
\text{Total leucocyte counts/mm}^3 = \frac{\text{No. of cells counted} \times \text{dilution factor} \times \text{depth factor} \times \text{Area counted}}{\text{Area counted}}
\]

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

Therefore, Total leucocyte counts/mm³ = \( \frac{N \times 20 \times 10}{4} \)

2.2.3.3. 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 minutes, 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 objectives and a total of 100 cells were counted.

2.2.4. Determination of in vitro antioxidant activity

2.2.4.1. Determination of lipid peroxidation activity

Reaction mixture (0.5mL) containing rat liver homogenate (0.1mL, 25%w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbic acid (0.06 mM) and different concentrations of TEO 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 (SDS) (8.1%, 0.2mL), TBA (0.8%, 1.5 mL) and acetic acid (20%, 1.5 mL, pH3.5). The
total volume was then made up to 4 mL by adding distilled water and kept in a water bath at 100°C for 1h. After cooling, 1 mL of distilled water and 5 mL 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 TEO.

2.2.4.2. Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe³⁺/ascorbate EDTA/H₂O₂ system (Kunchandy and 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 TEO in a final volume of 1mL. The reaction mixture was measured as TBARS and percent inhibition was calculated.

2.2.4.3. Determination of superoxide radical scavenging activity

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

2.2.4.4. 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. TEO 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.

2.2.4.5. Determination of ABTS radical scavenging assay

In this method, the radical scavenging activity of TEO was determined using ferryl myoglobin/ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) protocol (Alzoreky and Nakahara, 2001). The stock solutions of 500 µM ABTS diammonium salt, 400 µM myoglobin (Mb III), 740 µM potassium ferricyanide and 450 µM H₂O₂ 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 TEO and PBS. The reaction was initiated by adding 75 mM H₂O₂ 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.

2.2.4.6. Ferric reducing antioxidant power (FRAP)

The ferric reducing ability was measured at low pH (Benzie & Strain, 1996). The FRAP reagent contained 0.3 M acetate buffer, 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) and 20 mM ferric chloride. 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 obtained from FeSO₄.7H₂O std. graph and expressed as µM/ml of ferrous chloride formed.

2.2.5. Determination of in vivo antioxidant activity

Preparation of tissue homogenate

At the end of the experiment, all animals were sacrificed. Blood was collected by heart puncture and liver was excised and washed in ice-cold Tris HCl buffer. The liver tissue
was homogenized in ice-cold Tris HCl buffer (0.1M, pH-7.4) and 10% or 25%
homogenate was prepared. The homogenate was then centrifuged at 10000 rpm for 30
minute at 4° C and the clear supernatant was taken for the assay. It was stored at -70°C till
analysis.

2.2.5.1. Determination of superoxide dismutase (SOD) activity in the blood and in
the tissue
SOD activity was determined according to the method of McCord and Fridovich (1969).

Principle
The illumination of riboflavin solution in the presence of EDTA causes a reduction of the
flavin. Then it re-oxidizes and simultaneously reduces oxygen, which is allowed to react
with a detector molecule nitro blue tetrazolium (NBT). Then NBT is reduced to a
formazan blue. The SOD in the sample inhibits the formazan production.

Procedure
The heparinized blood was centrifuged at 2500 rpm 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 2.2.3.1. Packed RBCs (100 μL) was
haemolysed by 900 μL of cold water. The haemolysate was then treated with 250 μL of
CHCl₃ 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. 100 μL of the clear
supernatant was used for the SOD assay. Supernatant (0.1 mL) was mixed with 0.1 mL of
0.1M EDTA (containing 0.0015% NaCN), 0.1 mL of 0.15 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 560 nm. Percentage 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 0.1 mL 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.

\[
\text{SOD} = \frac{100 \times 50 \times \text{mg protein (for tissue) or Hb (for blood)}}{\% \text{ inhibition} / 100 - \text{sample volume}}.
\]

2.2.5.2. Determination of catalase activity in the blood and in the tissue

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 H₂O₂. The H₂O₂ has absorption maxima at 240 nm and absorption decreases with the decomposition of H₂O₂. The difference in extinction per unit time is a measure of the catalase activity.

**Procedure**
*Catalase in the blood:* 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 H₂O₂ (30 mM in the buffer, fresh every time) to the test cuvette, mixed well and the decrease in extinction was measured at 240 nm for 1min with an interval of 15 second. Catalase activity was calculated using the formula and expressed as k/g Hb,

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

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

E₁ is E₂40 at t=0 and E₂ is E₂40 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 H₂O₂ solution in buffer, decrease in extinction was measured at 240 nm, at 1 minute 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 nmoles of H₂O₂ consumed/min/mg of protein sample (U/mg protein).

\[
\text{Catalase (U/mg protein)} = \frac{\Delta A/\text{min} \times 1000}{43.6 \times \text{mg protein in sample}}
\]

2.2.5.3. Determination of reduced glutathione (GSH) content in the blood and in the tissue

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

Principle

Reduced glutathione (GSH) forms a yellow colored complex with dithionitrobenzene (DTNB) with an absorbance at 412 nm.

Procedure

Haemolysate of heparinized blood was prepared in distilled water. Haemolysate (0.5 mL of the tissue homogenate) was mixed with 0.125 mL of 25% TCA and cooled on ice for 5 min followed by further dilution of the mixture with 0.6 mL 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 350 μL of sodium phosphate buffer (0.2M, pH 8.0) and 1.0 mL of DTNB (0.6 mM 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 nmol) 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.
2.2.5.4. Determination of glutathione peroxidase (GPx) activity in tissue

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

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

The remaining GSH was measured by its reaction with DTNB

Procedure

0.1 mL of tissue homogenate was treated with 0.1 mL of 2 mM GSH, 0.1 mL of 1.2 mM H₂O₂, 0.1 mL of 1 mM NaN₃ and phosphate buffer (0.1 M, 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 1.61% m-H₂PO₃ and the reaction mixture was centrifuged at 1800 rpm for 15 min to settle down the precipitate. Supernatant (2.0 mL) was mixed with 2.0 mL 0.4 M Na₂HPO₄ and 1 mL of 1mM 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. A sample without the haemolysate was processed in the same way and was kept as the blank.

\[
\text{GPx (units/mg protein)} = \frac{\text{O. D of blank} - \text{O. D of sample} \times 1}{0.001 \times \text{g Hb or mg protein} \times 10} \times 1000
\]

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 non enzymatic reaction and expressed as units/mg protein.

2.2.5.5. 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 GSSG + NADPH+H⁺ \( \rightarrow \) 2GSH + NADP⁺
**Procedure**

The reaction mixture contains 100μL EDTA (10 mM), 100 μL GSSG (10mM), 50 μL of serum and buffer (1M, pH 7.0) in a total volume of 900 μL. The reaction mixture was incubated for 5min. at 37°C, followed by the addition of 100 μL NADPH (2 mM) 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 co-efficient of 6.22 mm⁻¹cm⁻¹ and expressed as nmoles of NADPH consumed /min/mg protein.

**2.2.5.6 Determination of glutathione-S-transferase (GST) activity in liver tissue**

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 (100 μl) in ethanol and 1 mM GSH (100 μl). 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 min with 1 min interval. The activity of GST was calculated from the following formula and it is expressed as μM or nM of CDNB-GSH conjugate formed/min/mg protein.

\[
\text{GST} = \frac{\Delta \text{min} \times 3 \times 1000}{9.6 \times \text{mg protein}}
\]

**2.2.5.7. Determination of lipid peroxidation in tissue**

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

- Tris buffer : 0.2M (pH 7.4)
- SDS : 8%
- TBA : 0.8%
- Acetic acid : 20% (pH 3.5)
- Tris buffer (for tissue homogenase) : (0.1M)

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 100 μL of the tissue homogenate, 1.5 mL of 0.8% TBA, 1.5 mL of acetic acid (20%, pH 3.5) and distilled water were kept for 1 hr 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 pyridine (15:1) mixed 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 (1-10 nmol) 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.

2.2.5.8. Determination of the total protein in the tissue

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 phosphomolybdic acid and phosphotungstic acid components of the Folin-Ciocalteau reagent in an alkaline medium to give a bluish purple color with absorbance at 660 nm.
**Procedure**

10 μL of the homogenate was mixed with 990 μL 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. 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 (2-4μm) and were stained using haematoxylin (0.5%) and eosin (1%) and mounted in DPX. Histochemical sections were evaluated by light microscopy. The sections were observed under light microscope (40X).

**2.2.7. Biochemical parameters**

**2.2.7.1. Preparation of tissue homogenate**

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

**2.2.7.2. Estimation of γ-Glutamyltransferase (γ-GT) activity in the blood and in the tissue**

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

**Principle**

Gamma-glutamyl tranferase catalyses the transfer of the γ-glutamyl moiety of a γ-glutamyl donor to the acceptor.

\[
\text{Gamma-glutamyl-p-nitroanilide + acceptor} = \gamma\text{-glutamyl acceptor} + \text{p-nitroaniline}
\]
The formation of p-nitroaniline was determined from the increase in absorbance at 410 nm.

**Procedure**

The assay solution (1 mL) contained 0.05M Tris-HCl buffer (pH 8), 75 mM NaCl, 2.5 mM L-\(\gamma\)-glutamyl-p-nitroanilide, 20mM glycyl glycine (pH 8) and enzyme (0.2 to 1 \(\mu\)g). The rate of release of p-nitroaniline was read at 410 nm.

2.2.8. Mutagenicity and antimutagenicity study using *S. typhimurium* strains

2.2.8.1. Mutagenicity study using *S. typhimurium* strains

Evaluation of the ability to induce reverse mutation at the histidine loci in four *S. typhimurium* strains of TA 98, TA 100, TA 102 and TA 1535 (Ames test) was conducted according to standard procedures (Ames et al., 1975). Mutagenicity was assayed at three doses in triplicates. Sodium azide /plate (5 \(\mu\)g) dissolved in distilled sterile water was used as positive control for strain TA 98, TA 100 and TA 102. For strain TA 1535, 20 \(\mu\)g nitro-o- phenylenediamine/plate (standard mutagen) dissolved in distilled water was used as positive control. A plate without drug or mutagens was used as negative control and 20 \(\mu\)l DMSO was used as vehicle control.

Two ml of top agar layer (0.6% agar and 0.5% NaCl) containing *S. typhimurium* strains, 0.5 mM histidine/biotin solution and different concentrations of drug were shaken well and poured on to the surface of 25 ml of minimal agar. After the overlay solidified, the plates (triplicate) were inverted and incubated for 48 h at 37\(^\circ\)C and revertant colonies counted using colony counter.

2.2.8.2. Mutagenicity study using *S. typhimurium* strains after metabolic activation by S9 mixture.

Rat liver microsomal enzyme was used for metabolic activation of mutagen *in vitro* (Jayaprakasha et al., 2002). Microsome P450 enzymes was induced in rat liver by oral administration of 0.1 % phenobarbital dissolved in water for 4 days. The animals were sacrificed on the 5\(^{th}\) day. Livers were excised aseptically and microsomal S9 fraction was prepared by centrifuging the homogenate at 9000 g for 15 min. Activation
mixture was prepared by mixing S9 mix (500 μl) with sodium phosphate buffer (0.2M, pH 7.4), NADP (0.1 M), glucose 6 phosphate (1 M), MgCl₂–KCl (10 μl) in presence of mutagen, 2-acetamidoflourene (20 μg/plate) (standard mutagen) / different concentrations of drug and bacterial strains TA 98 and TA 100 and incubated at 37°C for 45 min. Further, it was mixed with 2 ml of molten top agar supplemented with histidine and biotin (0.05 mM). The mixture was shaken well and poured on to the surface of 25 ml of minimal agar. After the overlay solidified, the plates (triplicate) were inverted and incubated for 48 h at 37°C. The mutagenic response was evaluated by counting manually the revertant colonies per plate and compared with the control groups. The test substance is considered to be mutagenic if there is a three fold increase in the tester strains when compared to the negative controls.

2.2.8.3. Evaluation of antimutagenic potential of drug

Antimutagenicity of TEO was tested against mutagens such as sodium azide, NPD and MNNG using TA 100, TA 1535, TA 98 and TA 102 strains Salmonella typhimurium by the method of Maron and Ames, (1983) and modified by Kaur et al., (1998) in triplicate. TEO was added to 2 ml of top agar at 45°C (0.5% NaCl and 0.6% agar) containing 0.5 mM histidine-biotin, bacterial culture of 1-2x10⁹ cells/ml (0.1 ml) and direct acting mutagens at concentrations mentioned above. It was mixed well and poured into minimal agar plates. After incubation at 37°C, the number of histidine independent revertant colonies were counted using colony counter. The plates with mutagen alone acted as positive control and plates without test sample and mutagen were considered as negative controls or spontaneous revertants.

In the case of mutagen needing activation, 2-acetamidoflourene (2-AAF) (20 μg/plate), Salmonella strains TA 98 or TA 100, different concentrations of TEO, 0.1 ml bacteria (1-2x10⁹ cells/ml) and 0.5 ml S9 mix were incubated for 45 min at 37°C. This mixture was then added to 2 ml of melted top agar, gently mixed and overlaid onto the minimal glucose agar plates. After solidification, the plates were inverted and incubated for 48 h at 37°C. The number of revertant colonies were counted using colony counter.
All the plates were prepared in triplicate. The percentage inhibition of mutagenicity was calculated using the formula:

\[
\text{Percentage inhibition} = \left[ \frac{(C-SR) - (T-SR)}{(C-SR)} \right] \times 100
\]

where ‘C’ is the number of revertants in the presence of mutagen alone.

‘T’ is the number of revertants in the presence of TEO with mutagens.

‘SR’ is the spontaneous revertants.

No antimutagenic effect was considered if the percentage of inhibition was smaller than 25%, a moderate effect if value is between 25% and 40% and a strong antimutagenic activity if value is greater than 40%. Untreated plates (without mutagens) and plates treated with DMSO (Vehicle control) were also kept. The plates with diagnostic mutagen acted as positive control and plates without test sample and mutagen were considered as negative controls or spontaneous revertants.

2.2.9. Genotoxicity assay

2.2.9.1. Effect of TEO on micronuclei formation

Animals were sacrificed 1 h after drug administration and both femora were removed. The proximal and distal ends of the femur were carefully drilled with needle until a small opening to the marrow canal became visible. Cells were flushed out with phosphate buffered saline (PBS) containing 10% FCS using a needle and syringe in the test-tube. Tubes were centrifuged at 3000 rpm for 10 min and the cell pellet was collected and smeared onto a clean glass slide. The smeared glass slides were then air dried and fixed in absolute methanol for 6 min. The cells were stained with May Grunwald Geimsa (MGG) and observed under oil immersion objective. Micronucleated Polychromatic erythrocytes (MNPCE), Micronucleated norchromatic erythrocytes (MNNCE) and corresponding polychromatic erythrocytes (PCE) normochromatic erythrocytes (NCE) were recorded based on the observation of 2000 polychromatic erythrocytes and 2000 normochromatic erythrocytes from each animal (Schmid, 1975).
2.2.9.2. Effect of TEO on chromosomal aberrations

Animals were injected intra peritoneally with colchicine (2mg/kg b.wt, in saline) 90 min before sacrifice to cause mitotic arrest. Bone marrow cell from each femur were flushed into phosphate buffered saline containing 10% FCS. Cell button was separated by centrifugation and 5 ml of 0.075 M KCL (37°C) was added to each tube and incubated for 30 min. Tubes were centrifuged again and 5 ml (3 ml drop wise and 2 ml at a stretch) of ice cold methanol-acetic acid mixture (3:1) was added and centrifuged again and cell pellet was separated. The cell pellet was dropped into a chilled clean glass slide (4 drops/slide) from a height of 60 cm and slides were air dried and kept in the dark for 5 days for maturation. Four slides were prepared from each animal and stained with Geimsa and observed under oil immersion and screened for metaphase chromosomes. A minimum of 100 metaphase spread were scored for aberrations (Savage, 1983).

2.2.9.3. Analysis of genotoxic effect of TEO by comet assay

The DNA damages in the bone marrow and intestine cells were measured as single strand-breaks using alkaline single cell gel electrophoresis (comet assay) (Singh, 2000). A small portion of intestinal tissue in 1 ml cold HBSS containing 20 mM EDTA/10% DMSO and mince into fine pieces after settled the cells can collect. The proximal and distal ends of the femur were carefully drilled with needle until a small opening to the marrow canal became visible. The cells from bone marrow and small intestine were flushed into Hank’s balanced salt solution (HBSS) using a needle and syringe in the test-tube. The cells from bone marrow and small intestine were flushed into Hank’s balanced salt solution (HBSS) and detected as 50000-100000 cells/ml. The mixture of 10 µl of cells and 200 µl (0.8%) of low melting agarose was layered on 1% normal melting agarose recoated slides and then covered with a cover slip. The slides were placed in the chilled lysing solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris HCL-pH 10, 1% DMSO and 1% Triton X 100) for at least 1.5 h at 4°C. Prior to electrophoresis the slides are incubated in alkaline electrophoresis buffer for 2 min to produce single strand DNA (pH>13). After that the single strand DNA in gel is subjected to electrophoresis under alkaline buffer for 20 min, at 25 V and 300 mA to produce comets.
After electrophoresis, the alkali in the gels is neutralized by washing with neutralizing buffer for 5 min. The slides were stained with DNA binding stain such as ethidium bromide (20 µg/ml, 50 µl) and visualized using fluorescence microscope. Images were captured and a minimum of 50 comets per slide, in triplicates for a group were analyzed using the software ‘CASP’ which gives Olive tail moment (OTM) directly. DNA damage, as reflected by percentage DNA in tail (tail intensity), and olive tail moment of the stored images.

2.2.10. Statistical analysis

The values are expressed as mean ± SD. The statistical significance was compared between control and experimental groups by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey analysis) using Graph pad in Stat software (version 3.05). P-value considered as significant are indicated by “*” “**” and “***” for p<0.05, p<0.01 and p<0.001 respectively.