Materials & Methods
MATERIALS:

Glutathione (GSH) and GST affinity matrix (Glutathione-CL Agarose) were purchased from Genei, Bangalore. GST substrates: 1-chloro-2, 4-dinitrobenzene (CDNB), 1, 2-dichloro-4-nitrobenzene (DCNB), bromosulfophthalein (BSP), P-Nitro benzyl chloride (p-NBC), P-Nitro phenyl acetate (p-NPA), 1,2-Epoxy-3-(P-nitrophenoxy) propane (EPNP), cumene hydro peroxide (CHP), Triton X-100. Paracetamol was purchased from Sigma Chemical Co., St-Louis, U.S.A. Acrylamide (99.9%), N, N'-methylene-bis-acrylamide, N, N, N', N'-tetra methyl - ethylenediamine (TEMED), 2-mercaptoethanol, sodium dodecyl sulphate (SDS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Nitro blue tetrazolium (NBT), IPG strips, comassie brilliant blue R-250, bromophenol blue and adjuvants were purchased from Bio-Rad laboratories (Richmond, USA) and also from Genei, Bangalore (India.). Low molecular weight markers for SDS-PAGE and PVDF membranes for immunoblotting were procured from Amersham (England, UK). The formalin, Haematoxylin, Eosin, Ethylene diamine tetra acetic acid disodium salt (Na₂EDTA), Tris – HCl, Sodium hydroxide (NaOH), Sodium chloride (NaCl), Dimethyl sulphoxide (DMSO), Sodium N – Lauryl Sarcosinate and Silver nitrate were of indigenous.

Sodium carbonate, Ammonium nitrates were purchased from BDH, chemical company, Mumbai. All other chemicals procured from the local companies were of Analytical grade. The albino male mice weighing about 25±50gms and 3 months old were purchased from Sri Venkateswara Enterprises, Bangalore.

Plant material:

The plant materials (Hybanthus enneapermus) leaves were collected from the surroundings of Sri Venkateswara university campus, Tirupati, Andhra Pradesh, India and identified by comparison with a voucher specimen.
depotted in the herbarium of the department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Preparation of the extracts:
The collected plant leaves were air-dried in the shade and powdered. About 100g of leaf powder was soxhlated with 500ml of ethanol for more than six hours and the extracts were then concentrated invacuo to yield dense residues. The samples were transferred to glass vials and stored in refrigerator for further studies.

Phytochemical – Qualitative Analysis of ethanolic extract of *Hybanthus enneaspermus*

Qualitative Analysis was performed according to the presence (Gibbs, 1974; Dey and Harborne, 1989; Evans, 1989; Harbone, 1998 and Kabir Akinyemi *et al.*, 2005.

Tests for alkaloids

*Iodine test:* 1ml of KI in iodine solution was added to the 2ml of test solution. A brown precipitate formation indicates the presence of alkaloids.

*Dragendorff's test:* 2ml of Dragendorff's reagent (Potassium bismuth iodide solution) and 2ml of diluted HCl were added to the test solution. A reddish brown precipitate indicates the presence of alkaloids.

*Wagner's test:* 2ml of Wagner's reagent (solution of iodine in potassium iodide) was added to 2ml of test solution. The formation of orange-red coloured precipitate indicates the presence of alkaloids.

*Mayer's test:* To a little of test solution added a few drops of Mayer's reagent (Potassium mercuric iodide solution). White precipitate formed indicates the presence of alkaloids. Some alkaloids are soluble in excess of the reagent. If no precipitate forms with the addition of few drops more reagent gives positive test.
Tests for flavonoids

Pew's test (Zn/HCl): A pinch of zinc powder and about 5 drops of 5N HCl were added to the test solution. It results in deep purple red colour is flavonoids are present.

Shinoda test (Mg/HCl): A pinch of magnesium powder and 5N HCl were added to the test solution. It results in deep red or magenta colour.

NaOH test: 1ml of 1N NaOH solution was added to the 1ml of test solution formation of yellow colour indicates the presence of flavonoids.

Tests for glycosides

Killer-Kiliani test: 1ml of glacial acetic acid was carefully added to 2ml of test solution of the extract and mixed well. Further, 2 drops of ferric chloride solution was added after cooling. These contents were transferred carefully to a test tube containing 2ml of concentrated H₂SO₄. A reddish brown ring was observed at the junction of two layers.

Concentrated H₂SO₄ test: 1ml of conc. H₂SO₄ was added to 1ml of test solution and allowed to stand for 2 min. The formation of reddish colour indicates the presence of glycosides.

Tests for phenols

Ellagic acid test: The test solution was treated with few drops of 5%(V/V) glacial acetic acid and 5% (W/V) NaNO₂ solution. The solution turns muddy yellow, olive brown, niger brown, deep chocolate colours depending on the amount of ellagic acid present.

Phenol test: When 0.5ml of FeCl₃.6H₂O (W/V) solution was added to 2ml of test solution, formation of an intense colour indicates the presence of phenols.
Test for saponins

*Foam test:* 0.1 g of crude extract was shaken vigorously in 2 ml distilled water. Formation of honey comb like froth persists for a few minutes indicate the presence of saponins.

Test for Sterols:

*Libermann-Buchard test:* A green colour was formed, when the Libermann-Buchard reagent (chloroform, acetic anhydride and sulphuric acid) was added to the test solution, indicated the presence of sterols.

*Salkowski test:* A wine red colour was developed when chloroform and conc. H₂SO₄ were added to the test solution. It indicates the presence of steroidal nucleus.

Test for tannins:

*Gelatin test:* The test solution was evaporated to dryness and the resulted residue was dissolved in 1% (W/V) liquefied gelatine. To this 10% (W/V) NaCl solution was added. White precipitate was obtained which indicate the presence of tannins.

Test for Triterpinoids:

Both *Salkowski test* and *Libermann-Buchard tests* were also given positive results for triterpinoids.

*Tschugajiu test:* Excess of acetyl chloride and pinch of ZnCl₂ are added to the chloroform solution kept aside and warmed on water bath, eosin red is produced.

Free amino acids test:

*Ninhydrin Test:* Add about 2 mg of the sample to 1 mL of a solution of 0.2 g of ninhydrin (1,2, 3indanetrione monohydrate) in 50 mL of water. The test mixture is heated to boiling for 15-20 sec. Purple color was not formed, absence of aminoacids
Test for peptides:

**Biuret test:** The Biuret Reagent is added to the sample. The blue color don't turns to pink colour, absence of polypeptides

**Sodium Bicarbonate Test for Carboxylic Acids:** A few drops or a few crystals of the sample are dissolved in 1mL of methanol and slowly added to 1 mL of a saturated solution of sodium bicarbonate. Evolution of a carbon dioxide gas is a positive test for the presence of the carboxylic acid

**Bromine CCl₄ Test:** Add few drops of bromine to the sample the decolourisation of bromine confirms presence of 1,3 butadienes

In vivo studies

**Animals:**

Experiments were carried out in albino male mice weighing 25±50g. They were obtained from Sri Venkateswara Enterprises, Bangalore and maintained in standard laboratory conditions, fed with ad libitum and water.

**Preparation of the tissue homogenate:**

The liver of mice were homogenized in 50 mM Tris-HCl buffer (pH 8.0), and then centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for assaying antioxidant enzymes, lipid peroxidation and liver marker enzymes.

For lipid peroxidation, 10% tissue homogenate was prepared in 1.15% KCl and for estimating total sulfhydryl content the tissue was homogenized in 0.2 M EDTA.

**Protein Estimation:**

Protein content of all samples was determined by the method of Lowry et al., 1951, using bovine serum albumin (BSA) as the standard, and also by measuring the change in absorbance at 260 and 280nm spectrophotometrically (Warburg and Christian, 1941).
Isolation and Purification of GST

The purification of GSTs was carried out by affinity chromatography (Frangioi et al., 1993; Simons et al., 1977). The following buffers were used for purification of GSTs and prepared in double distilled water.

Buffer A: 50mM Tris-HCl pH8.0
Buffer B: Buffer A+ 0.2M KCl
Buffer C: Buffer B + 5mM GSH

All purification steps were carried out at 4°C. The tissue for purification was cut in to small pieces and homogenized with buffer A containing 0.25 M sucrose and PMSF proteinase inhibitor in a glass homogenizer. The homogenate was centrifuged at 36,000Xg for 20 min. The supernatant fraction was collected and the pellet was discarded. The filtrate was centrifuged at 105,000 x g for 45 min. and the cytosolic fraction was passed through glass wool to remove floating lipid material. The filtrate was dialyzed overnight against buffer A with four changes (Reddy et al, 1983).

Affinity column chromatography

The Glutathione S-transferase affinity column has cross-linked with 4%beaded agarose as the matrix to which Glutathione is coupled by epoxy method and hexyl chain as a spacer arm. The ten ml affinity column was equilibrated with ten volumes of buffer A and the flow rate was adjusted to 10 ml/hr .The crude extract was applied on to the column and the elutes were collected in 5 ml fractions after the application of buffer A. The fraction, which was unbound, was collected as flow through. To remove the non-specifically bound proteins buffer B was applied to the column until A280 for the fraction reaches 0.0005. The bound protein was eluted by using buffer C. The fractions were collected until the absorbance reached to 0.0005. The active fractions with maximum protein absorbance at 280 nm and high enzyme activity with CDNB at 340 nm were pooled and dialyzed against buffer A for 24 hrs with
four changes to remove glutathione and KCl. The dialyzed protein was concentrated by freeze drying in a lyophilizer.

**SDS-PAGE:**

SDS-PAGE was conducted to observe the GST subunit profile of purified proteins.

**Solutions**

1. Acryl amide and bisacrylamide in a ratio of 29:1
2. 1.5 M Tris-HCl, pH 8.8
3. 0.5 M Tris-HCl, pH 6.5
4. 10%SDS
5. 10%APS (Ammonium persulphate)
6. TEMED
7. Tank buffer: 25 mM Tris base, 192 mm glycine and 0.1% SDS, pH 8.3

Polyacrylamide gel electrophoresis (PAGE) was conducted according to the method of Laemmli 1970. The denaturing gel electrophoresis was carried out with 12% resolving gels and 5% stacking gels containing 0.1% SDS. Samples with 20μgms protein concentration was boiled at 100°C for 5 min in the presence of 1x loading dye and was loaded into the wells. The separation of protein bands will be carried out at a voltage of 50 for stacking gel and 80 for resolving gel. The separated proteins on the gel were stained with coomassie brilliant blue solution, for overnight and destained in destaining solution till the background completely reaches transparent with good visibility of protein bands.
Preparation of Antisera against affinity purified GSTs:

In male New Zealand white rabbits antibodies were produced to 100μgms of affinity purified GST protein per ml emulsified with an equal volume of Freund's complete adjuvant. The emulsified mixture injected subcutaneously to the rabbit at 6-10 sites. The booster doses were given with incomplete adjuvant with an interval of a week for four times. The titer of the antibodies was tested after booster dose. After the fourth week of booster dose the immune response was high and the animals were bled and the serum was collected after centrifugation at 6000Xg.

Enzyme activity and substrate specificity assays:

Glutathione S-transferase activity in rat tissue extracts was measured spectrophotometrically at 340nm by the method of Habig et al., (1974) with CDNB as substrate. The reaction mixture containing 1ml of 0.3 M PO₄ buffer, pH6.5, 100μl of enzyme, 30mM CDNB and 30mM GSH were added and the total volume was made up to 3ml with distilled water and the increase in absorbance was read at 340nm. The blank value will be measured without the enzyme and it will be subtracted from the experimental value. The activities were calculated using molar extinction coefficient of 9.6. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one micromole of 2, 4 Dinitrophenol GSH adduct formed per minute. Specific activity was expressed as micromoles of GSH conjugate formed per milligram of protein. The GST activity with several substrates such as BSP, EPNP, P-NPA, P-NBC and DCNB was determined to the purified protein by the method of Habig and Jakoby (1981). The reactions were carried out using different substrates, buffers and initiators in a total volume of 3ml reaction mixture, the blank reaction without enzyme were subtracted from the test value and the activities were calculated using molar extinction coefficient as mentioned in Table. All the enzyme assays were carried out at room temperature and the specific activities will be expressed as micromoles or nmoles conjugate formed/min/milligram protein.
Table: Assay conditions for spectrophotometric analysis of GSTs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Contents of reaction mixture</th>
<th>Molar extinction coefficient</th>
<th>$\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-nitrophenyl acetate</td>
<td>125mM$\text{Mg}^+$ Buffer-$\text{pH}$7.0 0.3mM P-NPA 5mM GSH</td>
<td>$8.79 \times 10^3 \text{ cm}^{-1}$</td>
<td>400</td>
</tr>
<tr>
<td>P-nitrobenyl acetate</td>
<td>100mM$\text{Mg}^+$ Buffer-$\text{pH}$6.5 1.0Mm P-NBC 5mM GSH</td>
<td>$1.9 \times 10^3 \text{ cm}^{-1}$</td>
<td>310</td>
</tr>
<tr>
<td>1,2epoxy-3 (P-nitrophenoxy) propane</td>
<td>125mM$\text{Mg}^+$ Buffer-$\text{pH}$6.5 1.0Mm EPNP 5mM GSH</td>
<td>$0.5 \times 10^3 \text{ cm}^{-1}$</td>
<td>360</td>
</tr>
<tr>
<td>Bromosulfophthalein</td>
<td>125mM$\text{Mg}^+$ Buffer-$\text{pH}$7.5 0.03mM BSP 5mM GSH</td>
<td>$4.5 \times 10^3 \text{ cm}^{-1}$</td>
<td>330</td>
</tr>
<tr>
<td>DCNB</td>
<td>125mM$\text{Mg}^+$ Buffer-$\text{pH}$7.5 0.03Mm DCNB 5mM GSH</td>
<td>$8.5 \times 10^3 \text{ cm}^{-1}$</td>
<td>344</td>
</tr>
</tbody>
</table>
Antioxidant Assays:

Estimation of superoxide dismutase activity (E.C.1.15.1.1)

Superoxide dismutase was measured according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine transition by the enzyme.

Reagents:

1. 50 mM Sodium carbonate-bicarbonate buffer, pH 10.2 containing 0.1 mM of ethylene diamine tetra acetic acid (EDTA).
2. 0.6 mM epinephrine (Adrenaline).

Procedure:

To 0.5ml of the homogenate 2ml of carbonate buffer and 0.5mL of 0.6mM epinephrine was added. Epinephrine was the last component to be added and the adrenochrome formed in the next 4 min was recorded at 470 nm in spectrophotometer. SOD activity is expressed in units/min/mg protein. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of epinephrine auto-oxidation.

Estimation of catalase activity (E.C.1.11.1.6)

Catalase assay was carried out by the method of Aebi (1984). The decomposition of H₂O₂ was followed directly by measuring the decrease in absorbance at 240 nm.

Reagents:

1. 50 mM Phosphate buffer, pH 7.0
2. 30 mM H₂O₂: 340 μL of 30% (v/v) H₂O₂ was dissolved in 100 mL of phosphate buffer (pH 7.0).
Procedure:

0.5 mL of the tissue homogenate was mixed with 1.5 mL of phosphate buffer. Then 1 mL of H₂O₂ was added and change in absorbance was recorded after every 15 seconds up to 1 min. The activity of catalase was expressed as µmoles of H₂O₂ utilized/min/mg protein.

Estimation of Glutathione peroxidase activity, (E.C.1.11.1.9)

Assay of glutathione peroxidase was carried out by continuous monitoring of NADPH oxidation in a recycling assay as described by Wendel (1981). Total GPx was measured by using cumene hydroperoxide as a substrate.

Reagents:

1. 0.25 M phosphate buffer, pH 7.0 containing 2.5 mM disodium ethylene diamine tetra acetic acid and 2.5 mM sodium azide.
2. Glutathione reductase: 0.3 U/mL.
3. 10 mM reduced glutathione (GSH).
4. 2.5 mM NADPH in 0.1% sodium carbonate.
5. 12.5 mM cumene hydroperoxide.

Procedure:

The reaction mixture contained 0.1 mL of phosphate buffer, 0.1 mL glutathione reductase, 0.1 mL reduced glutathione and 0.1 mL of NADPH. To this 0.5 mL of homogenate was added and incubated at 37°C for 10 min. The reaction was started by the addition of 100 µL of cumene hydroperoxide. The linear decrease in absorption was recorded at 340 nm. The spontaneous reaction was assayed without enzyme and was subtracted from the samples. Activity of GPx was expressed as µmoles of GSH oxidized/min/ mg protein.
Estimation of glutathione reductase activity, (E.C.1.6.4.2)

Glutathione reductase was assayed by the method of Stahl et al., (1969).

Reagents:

1. 0.3 M sodium phosphate buffer (pH 6.8).
2. 250 mM EDTA
3. 12.5 mM glutathione oxidized (GSSG).
4. 3 mM Nicotinamide adenine dinucleotide phosphate reduced (NADPH).

Procedure:

The reaction mixture containing 1 mL of phosphate buffer, 0.5 mL EDTA, 0.5 mL of glutathione oxidized and 0.2 mL of NADPH was made up to 3 mL with distilled water. After the addition of 0.1 mL of tissue homogenate, the change in optical density at 340 nm was monitored every 30 seconds for 2 min. The enzyme activity is expressed as μmoles of GSH utilized/min/mg protein.

Estimation of lipid peroxidation

Lipid peroxidation in tissues was carried out by the method of Okhawa et al., (1979). Estimation of lipid peroxides was based on the reduction of thiobarbituric acid to give a pink coloured complex, MDA which is measured at 532 nm. MDA formed as an end product of the peroxidation of lipids served as an index of the intensity of oxidative stress.

Reagents:

1. 1.15% KCl.
2. 8.1% Sodium dodecyl sulphate(SDS)
3. 0.8% Thiobarbituric acid.
4. n-butanol: pyridine mixture (15:1 v/v).
5. Standard: 1, 1, 2, 2-tetraethoxy propane (TEP)

Procedure:

The assay mixture contained 0.1 mL of 10% tissue homogenate (prepared in 1.15% KCl), 0.2 mL of SDS and 1.5 mL of TBA. The mixture was finally made up to 4 mL with distilled water and boiled at 95°C for 1 hr. After cooling, 1 mL of distilled water and 5 mL of n-butanol: pyridine mixture were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. Then the absorbance of the organic layer was measured at 532 nm. Amount of lipid peroxidation is expressed as nmoles of MDA produced/mg protein.

Estimation of Serum and liver marker enzymes:

Estimation of Serum glutamate oxaloacetate transaminase/ Aspartate aminotransferase (SGOT/AST) activity.

SGOT/AST was assayed by the method of King (1965a).

The reaction catalyzed by this enzyme involves the formation of glutamate and oxaloacetate from the substrate containing aspartic acid and 2-oxoglutaric acid. The oxaloacetate thus formed was allowed to react with DNPH reagent. The colour developed was measured at 540 nm after the addition of NaOH. A set of standard pyruvate was also run in a similar manner.

Reagents:

1. Phosphate buffer, 0.1 M, pH 7.5.
2. Substrate: 1.33 g weight of aspartic acid and 1.5 mg of 2-oxoglutarate were dissolved in 20.5 mL of 1N NaOH and made up to 100 mL with distilled water.
3. 2, 4-dinitro phenyl hydrazine (DNPH) 0.002%: 20 mg of DNPH was dissolved in 100 mL of 1N HCl.
4. Sodium hydroxide, 0.4 N.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 mL of phosphate buffer (pH 7.5). This contains 1 µmol pyruvate/mL.

Procedure:

1 mL of substrate was incubated at 37°C for 10 min. Then, 0.2 mL of enzyme was added and the mixture was incubated at 37°C for 1 hr. To the control tubes the enzyme was added after the reaction, and it was arrested by the addition of 1 mL of DNPH reagent. The tubes were kept at room temperature for 30 min. Then 5 mL of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The colour developed was read against the blank at 540 nm using a colorimeter. The enzyme activity was expressed as units/L (One unit corresponds to enzyme that liberates one micro mole of pyruvate/min).

Estimation of Serum glutamate pyruvate transaminase/Alanine amino transferase (SGPT/ALT) activity.

SGPT was assayed by the method of King (1965a).

This enzyme catalyzes the formation of pyruvate and glutamate from alanine and 2-oxoglutaric acid. The pyruvate formed was made to react with DNPH reagent and the colour developed was measured at 540 nm after the addition of NaOH. A set of standard pyruvate was also run simultaneously.

Reagents:

1. Phosphate buffer, 0.1 M, pH 7.5.

2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 mL of phosphate buffer (pH 7.5). 0.5 mL of 1N NaOH was added and made up to 100 mL with distilled water.

3. 2, 4- dinitro phenyl hydrazine (DNPH) 0.002%: 20 mg of DNPH was dissolved in 100 mL of 1N HCl.
4. Sodium hydroxide, 0.4 N.

5. Standard: 11 mg of sodium pyruvate was dissolved in 100 mL of phosphate buffer (pH 7.5). This contains 1 μmol pyruvate/mL.

Procedure:

1 mL of substrate was incubated at 37°C for 10 min. Then, 0.2 mL of enzyme was added and the mixture was incubated at 37°C for 30 min. To the control tubes the enzyme was added after the reaction, and it was arrested by the addition of 1 mL of DNPH reagent. The tubes were kept at room temperature for 20 min. Then 5 mL of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The colour developed was read at 540 nm using a colorimeter. The enzyme activity was expressed as units/L. (One unit corresponds to enzyme that liberates one micro mole of pyruvate/min).

Estimation of Alkaline phosphatase (ALP) activity.

The activity of alkaline phosphatase was assayed by the method of King (1965b) using disodium phenyl phosphate as substrate. The colour developed is read at 640 nm.

Reagents:

1. Carbonate-bicarbonate buffer, 0.1 M, pH 9.8.

2. Disodium phenyl phosphate, 0.1 M: 218 mg of disodium phenyl phosphate was dissolved in 100 mL of distilled water.

3. Magnesium chloride, 0.1 M: 952.2 mg of magnesium chloride was dissolved in 100 mL of distilled water.


5. Standard: 10 mg of phenol was dissolved in 100 mL of distilled water. The standard solution contained 100 μg phenol/mL.
**Procedure:**

The incubation mixture containing 1.5 mL buffer, 1 mL substrate, 0.1 mL magnesium chloride were preincubated at 37°C for 10 min. Then 0.1 mL of enzyme was added and incubated at 37°C for the 15 min. The reaction was arrested by the addition of 1 mL of Folin-phenol reagent. The control tubes received enzyme after the addition of Folin-phenol reagent. Then 1 mL of sodium carbonate was added and the tubes were incubated at 37°C for 10 min. The colour developed was read at 640 nm in a colorimeter. Standards and blank were treated in a similar manner. The activity is expressed in terms of units/L (One unit corresponds to enzyme that liberates one micro mole of phenol/min/mg protein under incubation conditions).

Detection of DNA damage by Alkaline single cell gel electrophoresis (Comet Assay)

Possible DNA damage induced by over dosage of paracetamol at different doses was detected using the alkaline single cell gel electrophoresis (Comet Assay) following a simplified protocol with slight modifications (Endoh et al., 2002; Singh et al., 1988).

**Reagents:**

1. 1% Normal melting agarose.
2. 1% Low melting agarose.
3. 0.5% Low melting agarose.
4. Lysing solution: 36ml of lysing buffer (73.5g of NaCl, 18.612g of EDTA, 5G OF Sodium Isuryl sarcosinate, 0.6g of Tris dissolved in 500ml of distilled water and adjust the pH to 10.0), 4ml DMSO and 400µl TritonX-100.
5. Electrophoresis buffer: 30ml of 10N NaOH and 5ml of 0.5M EDTA made up to 1000ml with distilled water and adjust the pH to 10.0.
6. Neutralizing buffer: 24.228g of Tris dissolved in 500ml of distilled water and pH was adjusted to 7.5.

Procedure:

At the end of the treatment with paracetamol, blood was collected freshly from the retro-orbital plexus and used for the assay. Half frosted microscope slides were coated with 1% normal melting agarose in physiological buffer saline (PBS). The slides were then allowed to dry at room temperature protected from dust and other particles. An aliquot of 10μl of fresh blood was mixed with 140 μl of 1% low melting point agarose in Milli-Q water. This mixture was then layered on the top of the pre-coated slide and covered with a 24 X 50mm cover slip and kept on ice to allow the agarose to solidify. After the agarose had solidified on ice for at least 10-15min, the cover slip was gently removed and a third layer of 0.5% low melting agarose was layered on the top of the second layer and covered with a cover slip and kept on ice for 5-10 min. After the agarose had solidified, the cover slip was gently removed and the slides were carefully immersed in a freshly prepared ice-cold lysing solution. After lysis overnight at 4°C the slides were placed in an electrophoresis unit and the buffer reservoirs were gently filled with fresh electrophoresis buffer to level of 0.25 cm above the microscope slides, and incubated for 20min at 4°C to allow the unwinding of DNA. Keeping the same temperature, the slides were, subjected to electrophoresis, (25 V, 400 mA) for another 25min. After electrophoresis, the slides were placed on a tray to remove alkali and detergents and neutralized with neutralizing buffer for 10min. Excess liquid was carefully removed from each slide using a paper towel. The microscope slides were carefully dried at room temperature avoiding dust and other particles and then stored in a sealed container until the day of image analysis. The dried microscope slides were stained with ethidium bromide in water (20μg Ml⁻¹; 50 μl/ slide). The slides with a cover slip were examined at 400X magnification under a fluorescence microscope and the photomicrographs of cells were taken. 150-200 randomly selected cells (5-7
zones/slide) in each slide were counted (4 slides/animals in each group) to determine the number of damaged cells and then the percentage of damaged cells were calculated using the formula: \( \% \text{ damage} = \frac{\text{Number of damaged cells}}{\text{Total number of cells counted}} \times 100. \)

The length of the comet tail was determined by using an occulometer affixed in the eye piece of the microscope. The comet tail length was measured between the edge of the comet head and the end of the comet tail, calculated in micrometers (Sebastien et al., 2003). The results were expressed as:

1. percentage of cells with tail(tailed cells) in each group was scored and
2. Average tail length due to DNA migration in each group.

**Histopathology:**

Fixation and staining control and treated samples. The tissues were isolated from control and treated mice very gently rinsed with physiological saline to remove blood and debris if any adhering to them. They were fixed in bovins solution until processing (in case of liver 5% formalin). The tissues were washed running tap water, to remove bovin’s solution. After dehydrating through a graded series of alcohols, the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut at 6\( \mu \) thickness and stained with haematoxylin (Harris, 1900) and counter stained with eosin dissolved in 95% alcohol. After dehydration and cleaning, sections were mounted in Canada balsam. Histological examinations of these tissues were observed under the light microscope (10x X 40x).