ANIMAL AND MAINTENANCE
Male Sprague Dawley (SD) rats procured from CPCSEA approved animal breeders were kept in the departmental animal house (827/ac/04/CPCSEA) at controlled temperature (24±2°C) and light-dark schedule (12:12) and were provided laboratory rat food (Pranav Agrochemicals) and water ad libitum. They were acclimatized for 10 days before starting the experimental procedure. Efforts were made to minimize the number of animals used and care was taken that they were subjected to minimal suffering. Dosing was performed on 7 weeks old rats weighing an approximate 225-275gms. All experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India (Form B No. ZL/IAEC/13-2010). All the experiments were conducted in strict adherence to the procedures of the Drugs and Cosmetics rules 1945, Appendix - III animal care standard.

TEST CHEMICAL AND DOSAGE
A widely used formulated product, Thiacloprid (Alanto 240, 21.7% SC) manufactured by Bayer CropScience Ltd. India, was selected as the test chemical for the current study and was procured from the local market (Batch No. PGSC000002). Thiacloprid was diluted in distilled water for dose preparation according to desired concentration for oral exposure. Dose of thiacloprid was calculated based on the LD$_{50}$ value given in the fact sheet of manufacturer (EPA, 2005). This was further validated in the current laboratory condition for Alanto 240, 21.7% SC through a dose range study. Subsequently, two doses (1/15 and 1/30 of median lethal dose) were selected as per standard regulatory norms for further repeated dose studies (OECD, 1998). Rats were divided into three groups viz., control group, low dose group and high dose group with 5 animals in each group. Treated groups of animals were given dosage for 28 days for subacute and 90 days for subchronic evaluation of thiacloprid toxicity. Rats were given oral exposure to thiacloprid once in a day at the concentration of 50mg/kg body weight for low dose (LTD) and 100mg/kg body weight for high dose (HTD) group. Every day food intake, morphological and behavioral changes were observed and recorded.

EXPERIMENTAL PROCEDURES
At the end of the treatment period, rats were kept for overnight fasting and sacrificed.
neurotoxicity study, rats were perfused and brains were removed. Target organs of study were removed blotted free of blood or tissue fluid and weighed. Blood was collected from orbital sinus and serum was separated for evaluating various biochemical parameters. Tissues were weighed and homogenized in chilled PBS (pH 7.2) for various biochemical estimations.

1. Preparation of chemicals

**Acetylthiocholine (ATC) solution**
21.67mg of acetylthiocholine was dissolved in 1ml of distilled water.

**Agarose**
For comet assay: 1g agarose in 100ml of PBS
For electrophoresis: 1.4g in 100ml of TAE buffer

**Carnoy’s fixative**
3:1 methanol: glacial acetic acid

**Cholesterol Reaction Reagent**
Horse radish peroxidase (1:50), Cholesterol oxidase (1:100) and Cholesterol esterase (1:200) were prepared in 10 ml of phosphate buffer (prepared freshly and used within 30 minutes).

**0.1% Cresyl violet solution**
Cresyl violet (0.1g) was dissolved in distilled water (100ml). 0.1ml of glacial acetic acid was added just before use and solution was filtered.

**1-chloro-2, 4-dinitrobenzene (CDNB) reagent**
30mM CDNB in 95% ethanol

**Dichromate acetic acid reagent**
Potassium dichromate (5%) and glacial acetic acid were mixed in the ratio of 1:3. From this 1ml was diluted with 4ml of acetic acid.

**Direct bilirubin reagent**
Sulfanilic acid 32mM was prepared with 0.01N hydrochloric acid.

**5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent**
For AChE (Acetylcholinesterase) – 39.6mg of DTNB with 1mg NaHCO₃ was dissolved in 10ml of 0.1M phosphate buffer (pH 7).
For reduced glutathione - 40mg of DTNB was prepared in 100ml of 1% sodium citrate.

**Folin-Ciocalteu reagent**
5ml 2N Folin and ciocalteu’s phenol reagent + 6ml distilled water (prepared freshly in amber coloured bottle).

**GSH Standard solution**
10mg of reduced glutathione was dissolved in 100ml distilled water

**Geimsa Stain**
3.8g of Giemsa powder was dissolved in 250ml of methanol, heated at 60°C for 45 minutes in a water bath. This was stirred continuously, and then 250ml of glycerin was added to it. The stain was filtered and stored as the stock solution.
**Incubation medium for AChE localization**
5mg of substrate (i.e. acetylthiocholine iodide) was dissolved in 7.5ml of 0.1M phosphate buffer (pH 7.2). Then following items were added in order while stirring - 0.5ml of 0.1M Trisodium citrate, 1 ml of 0.03M Copper sulfate and 1 ml of 0.005M Potassium ferricyanide 0.005M. (Total volume 10ml).

**Low melting agarose (LMA)**
1g of LMA was dissolved in 100ml of phosphate buffer.

**Lowry solution**
*Solution A* (alkaline solution) (500ml)
2.86g NaOH
14.31g Na$_2$CO$_3$

*Solution B* (100ml)
1.423g CuSO$_4$·5(H$_2$O) in 100ml distilled water

*Solution C* (100ml)
2.853g Na$_2$Tartarate.2(H$_2$O) in 100ml distilled water
(Freshly prepared by mixing 50ml solution A+1ml of solution B + 1ml of solution C)

**Lysis buffer (Comet assay)**
2.5M NaCl (58.4g/mol), 100mM Tetra-sodium EDTA (416g/mol), 10mM Tris base (121.1g/mol), 1% sodium dodecyl sulfate. Solution diluted with ddH$_2$O while mixing to achieve a volume of 1L. The pH was adjusted to 10 and solution stored at room temperature.

**Neutralization Buffer**
1M Ammonium acetate (77.1g/mol) is prepared by dissolving 7.7g ammonium acetate in distilled water and made up to 100ml.

**10% Neutral buffered formalin**
Sodium phosphate monobasic 4.0g, Sodium phosphate dibasic 6.5g, 100ml Formaldehyde (37%) and made up to 1000 ml with distilled water.

**Phosphate buffer**
*Stock solution A* - 0.2M monobasic sodium phosphate monohydrate (27.6g/L).
*Stock solution B* - 0.2M dibasic sodium phosphate (28.4g/L).
51ml of A and 49ml of B solutions were mixed and diluted to a total volume of 200ml to make a 0.1M phosphate buffer of pH 6.8 at room temperature.

**Phosphate buffer saline (0.1M, pH 7.2)**
8g of NaCl, 0.2g of KCl, 1.44g of Na$_2$HPO$_4$·2H$_2$O and 0.24g of KH$_2$PO$_4$ were dissolved in 1L of double distilled water. The pH was adjusted to 7.2 and solution was stored at 4ºC.

**Phosphate solution (reduced glutathione)**
0.3 M Na$_2$HPO$_4$ was dissolved in 1L of distilled water.

**Precipitating reagent (reduced glutathione)**
1.67g of metaphosphoric acid, 0.2g of EDTA disodium salt, 30g NaCl were dissolved in 1L of distilled water.

**Pyrogallol solution**
25.2mg of pyrogallol was dissolved in 1ml of 0.05M Tris-HCl buffer (pH 7.4) in an amber
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colored vial. At the time of assay 0.5 ml of this stock was diluted to 50 ml with 0.05 M Tris-HCl buffer (pH 7.4) to give 2 mM solution

Reagent 1 (HDL-C)
α-cyclodextrin 0.5 mM, dextran sulfate 0.5 g/L, magnesium chloride 2 mM, HSDA 0.3 g/L, phosphate buffer pH 7.0 ± 0.1

Reagent 2 (HDL-C)
POD 15,000 U/L, PEG-CO 5,000 U/L, PEG-CE 800 U/L, 4-aminoantipyrine 0.5 g/L, phosphate buffer pH 7.0 ± 0.1, Triton X-100

Sodium azide solution
10 mM is prepared by dissolving 6.5 mg NaN₃ in 1 ml of distilled water.

Sodium nitrite reagent
Sodium nitrite 60 mM was prepared in 25 ml of phosphate buffer.

TAE buffer
4.84 g of Tris base [tris (hydroxymethyl) aminomethane], 1.14 ml of glacial acetic acid (17.4 M) and 0.37 g of EDTA, disodium salt were added to 1 L of deionized water.

2% Thiobarbituric acid (TBA) reagent
2 g of TBA was dissolved in 100 ml distilled water

Total bilirubin reagent
16 mM Sulfanilic acid, 164 mM hydrochloric acid, 4.4 M dimethyl sulfoxide, 2.1 mM caffeine were added in 50 ml of phosphate buffer.

10% trichloro acetic acid (TCA)
10 g of TCA was dissolved in 100 ml of distilled water.

Unwinding/Electrophoresis Buffer (Comet assay)
0.3 M NaOH (40.0 g/mol), 10 mM Tetra-sodium EDTA (416 g/mol), 0.1% (w/v) 8-hydroxyquinoline, mixed with 700 ml ddH₂O and 2% (v/v) DMSO (20 ml) added while mixing. Once dissolved, additional ddH₂O was added to achieve a final volume of 1 L. The pH was adjusted to 13.1 with concentrated NaOH or HCl.

2. Protocols for hepatotoxicity evaluation
2.1 Enzyme parameters
2.1.1. Estimation of Transaminase
Alanine aminotransferase (ALAT/ALT), formerly called Glutamic Pyruvic Transaminase (GPT) and Aspartate aminotransferase (ASAT/AST), formerly called Glutamic Oxalacetic Transaminase (GOT) are the most important representatives of a group of enzymes, the aminotransferases or transaminases, which catalyze the conversion of α-keto acids into amino acids by transfer of amino groups.
Alanine Transaminase (ALT) (E.C. 2.6.1.2)
The ALT level was estimated by the IFCC (International federation of clinical chemistry) (Bergmeyer and Horder, 1980) method. In the presence of alanine transaminase in the sample, L-alanine is converted to pyruvate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD. The ALT activity in the sample is proportional to the decrease in rate of absorbance of NADH which is measured at 340nm kinetically for 2 minutes.

\[
L - \text{Alanine} + 2 - \text{Oxoglutarate} \xrightarrow{\text{ALT}} L - \text{Glutamate} + \text{Pyruvate} \\
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} D - \text{Lactate} + \text{NAD}^+
\]

0.1ml of sample and 1ml of freshly prepared reagent (L-alanine 500mM, α-ketoglutaric acid 15mM, LDH 10mM, NADH 0.18mM in 0.1M phosphate buffer pH 7.5) were mixed and incubated for 1 minute at room temperature. Optical density (OD) was measured at 340nm for 2 minutes at the interval for every 30 seconds and ΔA was calculated.

Serum ALT activity(IU/L) = \(\frac{\Delta A \times \text{Total volume of assay} \times 1000}{\text{Sample volume} \times 6.22}\)

Where, 6.22 = Millimolar absorptivity of NADH

Aspartate Transaminase (AST) (E.C. 2.6.1.1)
The AST level was estimated by the International federation of clinical chemistry (Bergmeyer et al., 1978) method. In the presence of AST in the sample, L-aspartate is converted to Oxaloacetate. Oxaloacetate is reduced by malate dehydrogenase to yield malate with the oxidation of NADH to NAD. The AST activity in the sample is proportional to the decrease in rate of absorbance of NADH which was measured at 340nm kinetically for 2 minutes.

\[
L - \text{Aspartate} + \alpha - \text{Ketoglutarate} \xrightarrow{\text{ALT}} \text{Oxaloacetate} + L - \text{Glutamate} \\
\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} L - \text{Malate} + \text{NAD}^+ + H_2O
\]

0.1ml of sample and 1ml of freshly prepared reagent (L-aspartate 500mM, Oxaloacetate 15mM, MDH 10mM and NADH 0.18mM in 0.1M phosphate buffer pH 7.5) were mixed and incubated for 1 minute at room temperature. OD was measured at 340nm for 2 minutes at the interval of every 30 seconds and ΔA was calculated.

Serum AST activity(IU/L) = \(\frac{\Delta A \times \text{Total volume of assay} \times 1000}{\text{Sample volume} \times 6.22}\)

Where, 6.22 = Millimolar absorptivity of NADH
2.1.2. Estimation of Alkaline Phosphatase (ALP) (E.C. 3.1.3.1)
Activity of alkaline phosphatase was estimated by the protocol of Tietz et al. (1983) approved by IFCC. ALP catalyzes the hydrolysis of phosphate esters bond in an alkaline environment (pH 10) and at an optimum temperature of 37°C, resulting in the formation of an organic radical and inorganic phosphate. p-Nitrophenyl Phosphate (PNPP) is a non-proteinaceous, non-specific substrate for alkaline phosphatases. p-nitrophenyl phosphate is hydrolyzed by ALP into p-nitrophenol, which is yellow at alkaline pH and its concentration can be measured at 405nm.

\[
p - \text{Nitrophenyl Phosphate} \xrightarrow{\text{ALP}} p - \text{Nitrophenol} + P_i
\]

0.3ml of buffered substrate (0.01M PNPP in 0.1M phosphate Buffer) and 0.1ml of sample were mixed and incubated for 15 minutes in water bath at 40°C. Then 0.5ml of 0.5N NaOH was added to develop colour and intensity of colour was measured at 405nm spectrophotometrically.

\[
\text{Enzyme Activity} = \frac{\text{OD} \times \text{Conversion factor} \times \text{dilution} \times 100}{\text{Volume of Aliquote} \times \text{Tissue Weight}}
\]

Unit - μ moles pNP released/15 minutes/mg tissue

2.1.3. Gamma Glutamyl Transpeptidase (GGT) (E.C. 2.3.2.2)
Calibrated method of Szasz (1969) was used to measure the activity of GGT. Gamma-glutamyl 3 carboxy-p-nitroanilide (GLUPA-c) and glycylglycine are converted by the action of GGT to p-nitroaniline and L-gamma glutamylglycine. The rate of increase in absorbance at 405nm due to the release of p-nitroaniline is directly proportional to the GGT activity.

\[
L - \gamma \text{glutamylglycine} + \text{Glycylglycine} \xrightarrow{\text{GGT}} L - \gamma \text{glutamylglycylglycine} + 5 \text{ amino 2 nitrobenzoate}
\]

1ml of regent buffer (glycylglycine 126mM, GLUPA-C 3.3mM and 0.095% sodium azide in Tris buffer (pH 8.1)) and 100µl of sample were mixed and incubated for 1 minute at room temperature. Absorbance of test was read first exactly at 60seconds and then, second, third, fourth readings were taken at an interval of 30 seconds at 405nm. The mean change in absorbance per minute (Δabs/min) was determined and results were calculated.

\[
\text{Serum GGT activity (IU/L)} = \frac{(A_2 - A_1) \times \text{Total volume of assay} \times 1000}{9.5 \times \text{Sample volume}}
\]

Where, 9.5 = millimolar absorptivity of 5-amino-2-nitrobenzoate

2.1.4. Lactate Dehydrogenase (LDH) (E.C. 1.1.1.27)
Lactate dehydrogenase activity was measured according to the protocol of Buhl et al. (1977).
LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD at 340nm.

\[
\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}
\]

50µl of sample was mixed with 1ml of reagent buffer containing 55mM L-lactate and 5.8mM NAD in Tris buffer (pH 8.9). OD was recorded after 30 seconds (A₁) and again noted exactly after 90 seconds (A₂) at 340nm. The change in absorbance (A₂-A₁) multiplied by the factor 3376 (if volume of assay remains constant) which yield results in U/L.

\[
\text{Serum LDH activity (IU/L) } = \frac{(A_2 - A_1) \times \text{Total volume of assay} \times 1000}{6.22 \times \text{Sample volume}}
\]

Where, 6.22 = Millimolar absorptivity of NADH

2.2. Estimation of Biomolecules

Total Protein
The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured at 660nm in the method described by Lowry et al. (1951).

Standard graph of protein was prepared by 20mg/ml BSA. 0.5ml of Lowry reagent was mixed with sample and standard and then incubated for 15 minutes at room temperature. 0.2ml of Folin reagent was added to each test tube and incubated again at room temperature for 30 minutes in dark. OD was measured at 660nm against CuSO₄ blank.

Unit – mg protein/g of tissue weight or g/dl for serum/plasma

Glucose
Glucose was estimated by GOD/POD method (Glucose oxidase/Peroxidase) as described by Trinder (1969). Glucose oxidase oxidises the aldehyde group of specific substrate, β-D-glucose, to gluconic acid and generates hydrogen peroxide. Hydrogen peroxide thus produced is acted upon by peroxidase which transfers oxygen to the chromogen system, 4-aminoantipyrine and phenolic compound. The chromogen system gets oxidized to a red quinoneimine dye. The intensity of colour is directly proportional to the concentration of glucose and is measured photometrically at 505nm.

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + \text{H}_2\text{O}_2
\]
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1. Material and Methods

2. Experimental Procedures

2.1. Glucose Measurement

Glucose measurement was performed using the Folin-Denis method with modifications. A 1 ml reagent buffer (2000IU/L glucose peroxidase, 1200IU/L peroxidase and 0.246mM 4-amino antipyrine) was incubated at room temperature with 10µl of sample and standard. Absorbance was measured at 505nm against reagent blank.

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Concentration of standard}
\]

Unit = mg/dl in serum or mg/g of tissue

2.2. Estimation of Metabolite

2.2.1. Bilirubin

Bilirubin was estimated by the method described by Jendrassik and Grof (1938). Bilirubin is estimated by reacting it with diazotised sulfanilic acid obtained from sodium nitrite and sulfanilic acid solutions. Bilirubin when reacted with diazotised sulfanilic acid forms a pink coloured Azocompound. The unconjugated or free bilirubin takes longer time to react and requires caffeine as accelerator.

1 ml of direct bilirubin reagent or 1 ml of total bilirubin reagent was added to 100µl of sample or standard as well as blanks of respective studies. 100µl of Na nitrite reagent was mixed in all sample and standard tubes but not the blank tubes. OD was read at 555nm using water as a blank.

\[
\text{Bilirubin (mg/dl)} = \frac{\text{Abs. of Unk.} - \text{Abs. of Blank}}{\text{Abs. of Std.} - \text{Abs. of Std. Blank}} \times \text{Concentration of standard}
\]

2.3. Parameters for Neurotoxicity study

2.3.1. Acetylcholinesterase (AChE) (E.C. 3.1.1.7)

Acetylcholinesterase activity was evaluated as per the method described by Ellman et al. (1961). Activity of AChE was measured in whole blood, plasma, various regions of brain and neuroblastoma IMR cell line. The assay uses the thiol ester acetylthiocholine instead of the oxy ester acetylcholine. AChE hydrolyses the acetylthiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the DTNB liberating nitrobenzoate, which absorbs at 412nm.

\[
\text{Acetylthiocholine} \xrightarrow{\text{AChE}} \text{Thiocholine + acetate}
\]

\[
\text{Thiocholine + dithiobisnitrobenzoate(DTNB)} \rightarrow \text{Yellow color}
\]
For blood AChE activity
200µl of blood was mixed with 5ml of DTNB reagent. 2ml of this was used for estimating whole blood enzyme activity and 3ml was centrifuged at 2000rpm for 10 minutes at 10ºC for plasma AChE activity. In each tube 0.1ml of acetylthiocholine substrate was added and mixed thoroughly. Change in absorbance was recorded for a period of 5 minutes at an interval of 1 minute at 412nm.

\[ R = \frac{\Delta A \times \text{Total volume of assay} \times 1000 \times 5.74 \times 10^{-4}}{\text{Sample volume}} \]

Where, \( R = \text{Rate in micromoles of acetylthiocholine hydrolyzed /minute / dl} \)

For brain AChE activity
The rats were decapitated; brains were removed quickly and placed in ice-cold PBS. Various regions of brain were quickly dissected out on chilled petri dish placed on crushed ice. The tissues were weighed and homogenized in 0.01% Triton X-100 in 0.1M phosphate buffer (pH 8). 0.4ml of aliquot of homogenate was added in tube containing 100µl of DTNB and contents were mixed properly. In each tube 0.1ml of acetylthiocholine substrate was added and mixed thoroughly. Change in absorbance was recorded for a period of 5 minutes at an interval of 1 minute at 412nm.

\[ R = \frac{\Delta A \times \text{Total volume of assay} \times \text{dilution factor} \times 5.74 \times 10^{-4}}{\text{Weight of tissue} \times \text{Sample volume}} \]

Where, \( R = \text{Rate in micromoles of acetylthiocholine hydrolyzed /minute / g tissue weight} \)

For cell line AChE activity
After removing the medium from the neuroblastoma cells, the cells were washed three times with Phosphate buffered saline (PBS). The cultured cells were extracted by adding 4ml of High ionic strength buffer (10mM NaHPO₄, pH 7.5, 1M NaCl, 10% Triton X-100 and 1mM EDTA) and incubated for 5 minutes at room temperature. Cells were then centrifuged at 12,000rpm in a 15 ml tube for 20 minutes. Supernatant was removed. 20µl of supernatant was mixed with 50µl of DTNB. 10µl of acetylthiocholine was added and change of absorbance was measured for 2 minutes at 30 seconds time interval at 412nm.

3.2. Neurotoxic Esterase (NTE) (E.C. 3.1.1.5)
The procedure is a modification for rat brain of a standard method for hen brain (Johnson, 1977) and for IMR cell line (Correll and Ehrich, 1991). Brain homogenate (50µl) was added to Tris-EDTA buffer (950µl). Phenyl valerate was introduced in 0.03% Triton X-100 in Tris-
EDTA buffer (1ml) and incubated for 15 min. The reaction was stopped with 1% SDS and 0.025% 4-aminoantipyrine in distilled water (1ml). Addition of 0.04% potassium ferricyanide in water (0.5 ml) allowed spectrophotometric determination at 490nm of phenol liberated by NTE.

\[
\text{NTE activity} = \frac{\text{OD} \times \text{Volume of assay} \times \text{dilution factor} \times 1000}{\text{Weight of tissue} \times \text{Sample Volume}}
\]

Unit – mM phenol liberated/15minutes/g of tissue weight

3.3. Localization of enzyme

**Acetylcholine esterase**

Direct colouring method described by Karnovsky and Roots (1964) was adapted for the localization of acetylcholine esterase. 15μm thick cryosections of various regions of brain were taken. Sections were incubated at 37°C for 2 hours in the incubating medium for AChE localization and then rinsed with distilled water. Slides were observed under microscope (Leica DM2500) at10X for localization of AChE and pictures captured using EC3 Camera (utilizing LEICA LAS EZ (V 1.6.0) software).

3.4. Functional observation battery (FOB)

**Forced swimming test**

According to the protocol described by Cryan et al. (2002), rats were placed in glass cylinder containing water. The water level in the glasses was high enough to prevent the rat from touching the bottom of the cylinder with its paws or tail, and low enough to avoid an escape through the top opening of the cylinder. The rats were thus forced to swim in the cylinders for 10 minutes. This activity was repeated every month. Parameters like active swimming, floating and dipping were recorded with time.

**Rotarod Test**

The rotarod test is used to assess motor coordination and balance in rodents. This is done through examining the balance of the rat on a rotating rod (rotarod). One can investigate brain injury or neuromuscular damage if the rat fails to keep its balance on the rotarod. The method described by Hamm et al. (1994) was used to assess the motor activity of experimental animals. Time (latency) taken by the rat to fall off the rotating rod at different speeds or under continuous acceleration (e.g. from 4 to 40rpm) was recorded. Each animal was observed for 5 minutes and each fall was noted.
3.5. Nissl body stain

Nissl substance (rough endoplasmic reticulum of neuron) is lost after cell injury and if the axon degenerates, the myelin covering also breaks down. Nissl body staining method was used for the detection of neuronal damage. 15μm thick cryosections of brain were stained with warmed (50°C in oven) 0.1% cresyl violet for 8 to 10 minutes and rinsed immediately with distilled water. Differentiation was done with 95% ethyl alcohol for 2-10 minutes and sections were checked under the microscope (Leica DM2500) at 10X magnification for examining Nissl body which are stained pink-violet.

4. Analysis of Biochemical parameters for Thyroid toxicity study

4.1. Estimation of biomolecules

**Cholesterol**

Cholesterol concentration in serum was estimated by method given by Allain et al. (1974). The cholesterol esters are hydrolysed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidised by cholesterol oxidase (CO) to cholesterol 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505nm. The intensity of colour produced is directly proportional to the concentration of total cholesterol in the sample.

\[
\text{Cholesterol ester} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{free fatty acid}
\]

\[
\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol} - 4 - \text{en} - 3 - \text{one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4 - \text{aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + \text{H}_2\text{O}
\]

1ml of cholesterol reaction reagent was mixed with 10μl of standard (10mg/ml) and test sample. 50μl of 16mM 4-aminoantipyrine was added to it and then incubated for 30 minutes at room temperature. OD was measured at 505nm against reagent blank.

\[
\text{Cholesterol (mg/dl)} = \frac{\text{OD of sample} \times \text{Volume of assay} \times \text{Conc. of Std} \times 100}{\text{OD of standard} \times \text{Volume of Sample}}
\]

**HDL Cholesterol (HDL-C)**

Serum HDL-C levels were estimated using a kit (Reckon Diagnostics Pvt. Ltd., India) based on the method described by Grundy et al. (1993). In this method, pretreatment or centrifugation steps are not required. The method involves a two-reagent format. The first reagent contains α-cyclodextrin and dextran sulphate to stabilize LDL, VLDL, and chylomicrons. The second
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The reagent contains PEG modified enzymes that selectively react with the cholesterol present in the HDL particles. Consequently, only the HDL cholesterol is subject to cholesterol measurement at 690nm.

10µl of serum sample or standard were incubated with 300µl reagent 1 for 10 minutes at room temperature. 100µl of reagent 2 was added to it and again incubated for 10 minutes. Absorption was measured at 690nm.

\[
\text{HDL-C Concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Calibrator Concentration}
\]

**LDL-C (LDL-C)**

The LDL cholesterol assay was also done using a kit (Reckon Diagnostics Pvt. Ltd., India) based on the method described by Grundy et al. (1993) for measuring LDL cholesterol levels from serum or plasma. This kit uses a specific detergent formulation to selectively dissolve non-LDL lipoprotein particles (HDL, VLDL and chylomicrons) while leaving LDL particles intact. The dissolved cholesterol is degraded by the cholesterol esterase and cholesterol oxidase enzymes. Subsequently a second detergent is added to the sample to solubilize the remaining LDL particles. The soluble cholesterol and cholesterol esters are oxidized by cholesterol oxidase to produce hydrogen peroxide. The hydrogen peroxide product then reacts with bis (4-sulfobutyl)-m-toluidine and 4-aminoantipyrine to form a coloured product. The resulting colour change is measured at 550nm and is proportional to the amount of LDL cholesterol originally present in the sample.

10µl of serum sample or standard were incubated with 750µl reagent 1 for 10 minutes at room temperature. 250µl of reagent 2 was added to it and again incubated for 10 minutes. Absorption was measured at 550nm.

\[
\text{LDL-C Concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Calibrator Concentration}
\]

**Triglyceride**

The method given by Fossati and Lorenzo (1982) was adopted for triglyceride estimation in serum. Lipase hydrolyses triglycerides sequentially to Di and Monoglycerides and finally to glycerol. Glycerol Kinase (GK) using ATP as PO₄ source converts Glycerol liberated to Glycerol-3-Phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidises G-3-Phosphate and forms Dihydroxy acetone phosphate and hydrogen peroxide. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidise 4- Aminoantipyrine and TOOS (N-ethyl-N-Sulphohydroxy propyl-m Toluidine) to a purple coloured complex. The absorbance of the
A coloured complex is measured at 546nm (530-570nm or with yellow filter) which is proportional to Triglyceride concentration.

\[
\text{Triglyceride} + \text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{fatty acid}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} - 3\text{phosphate} + \text{ADP}
\]

\[
\text{Glycerol} - 3\text{phosphate} + \text{O}_2 \xrightarrow{\text{Peroxidase}} \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{aminoantipyrene} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + \text{H}_2\text{O}
\]

10µl of standard (10mg/ml) or sample was mixed with freshly prepared triglyceride reaction reagent and incubated at 37°C for 15 minutes. 50 µl of 16mM 4-aminoantipyrene was added to the mixture and absorbance was taken at 550nm against reagent blank.

\[
\text{Triglyceride (mg/dl)} = \frac{\text{OD of sample} \times \text{Volume of assay} \times \text{Conc. of Std} \times 100}{\text{OD of standard} \times \text{Volume of Sample}}
\]

4.2. Assessment of Thyroid hormone and TSH

For the quantitative determination of thyroid stimulating hormone (TSH), Triiodothyronine (T3) and thyroxin in serum, ELISA kit of TSH, T3 and T4 was used (GenWay Biotech Inc., USA). The kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the intact TSH, T3 and T4 molecules. Mouse monoclonal anti-TSH, anti T3 and anti T4 antibody was used for solid phase immobilization (microtiter wells), and goat anti-TSH, anti T3 and anti T4 antibody was present in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the TSH, T3 and T4 molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 60-minute or overnight incubation at room temperature, the solid phase was washed with water to remove unbound labelled antibodies. A solution of 3,3’,5,5’-Tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development was stopped with the addition of 1N HCl, and the resulting yellow colour was measured at 450nm with a microplate reader (Metertech Σ960). The concentration of TSH, T3 and T4 is directly proportional to the colour intensity of the test sample.

5. Parameters for Genotoxicity Study

5.1. Micronucleus (MN) assay

Incidence of micronuclei serves as an index of genetic damage. A micronucleus is formed during the metaphase or anaphase transition of mitosis or meiosis (cell division). MN test is a
rapid and reliable assay for genotoxic assessment of test substance. MN test was performed according to prescribed protocol of Romagna and Staniforth (1989). Blood was collected in 2ml EDTA coated vacutainer and blood smear was prepared on a glass slide and fixed with methanol. Immediately after sacrifice of rat, femur bone was dissected out and then bone marrow was removed by injection of RPMI 1460 medium. The collected cells were centrifuged at 3000rpm for 5 minutes. Pellet of bone marrow cells was resuspended in RPMI medium, of which a small drop was smeared on glass slide and fixed in absolute methanol. The smear was air dried and stained for 5 minutes with 10µg/ml acridine orange, a fluorescent dye and then washed with PBS twice. Slides were observed under fluorescent microscope (Leica DM2500) to score micronucleus. 1000 cells were counted per rat.

5.2. Polychromatic erythrocyte (PCE)/Normochromatic erythrocyte (NCE)
Bone marrow smear was stained with 2% Geimsa stain for 5 minutes and then washed with phosphate buffer. 200 bone marrow cells were scored for PCE (immature or intermediate erythrocyte with ribosome) and NCE (mature erythrocyte without ribosome). PCE/NCE ratio was calculated for analysis of cytotoxicity of test chemical. Micronucleated PCE (MNPCE) and micronucleated NCE (MNNCE) were also scored with Geimsa staining.

5.3. Chromosomal aberration
Standard method for preparation of metaphase chromosome was followed (Evans et al., 1964). Rats were weighed and colchicine (4mg/Kg body weight) was administered intraperitoneally 2½ hrs before harvesting bone marrow cells. Immediately after sacrifice of rat, femur bone was dissected out and then bone marrow was removed by injection of RPMI 1460 medium. The collected cells were centrifuged at 3000rpm for 5 minutes. Pellet of bone marrow cells was resuspended in 0.6% KCl and then agitated for a minute. Sample was incubated at 50ºC in water bath for 45 minutes and centrifuged for 10 minutes at 3000rpm. Methanol:Glacial acetic acid fixative (3:1) was added to the pellet and agitated properly for a minute and then centrifuged at 3000rpm for 10 minutes to collect the pellet. This step was repeated twice. Cells were spread on a chilled glass slide which was then kept on a hot plate at 60ºC for 10 seconds. Finally slides were stained with 2% Geimsa for 10 minutes.

5.4. Comet Assay
Comet assay was performed in rat bone marrow cells, according to the in vivo comet assay guidelines of Tice et al. (2000), as described by Saquib et al. (2009). Briefly, 100µl bone marrow cell suspensions of rats were mixed with 100µl of 1% Low Melting Agarose (LMA)
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The cell suspension (100μl) was then layered onto frosted slides, pre-coated with agarose (1% in PBS) and kept at 4°C for 10 min. After gelling, a layer of 100μl of LMA (1% in PBS) was added. Cells were lysed overnight in a lysis buffer and then rinsed in PBS twice before subjecting to DNA denaturation in cold electrophoretic buffer at 4°C for 20 minutes. Electrophoresis was performed at 25V (300mA) at 4°C for 30 minutes. Slides were then washed three times with neutralization buffer. Each slide was stained with 20μg/ml ethidium bromide solution for 2 minutes. Slides were analyzed at 40X magnification (excitation wavelength of 515-560nm and emission wavelength of 590nm) using a fluorescence microscope (Leica DM2500). Images from 100 cells (50 from each replicate slide) were randomly selected and subjected to image analysis with CometScore software (TriTek Corporation, Virginia). Mean values of the Olive tail movement (OTM), tail length (μm) and tail intensity (%) were separately analyzed for statistical significance. To quantify the DNA damage, tail length (TL) and tail movement (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometres. It was calculated from the centre of the cell. Tail movement was calculated as the product of the tail length and the fraction of DNA in the comet tail.

5.5. DNA ladder assay

DNA fragmentation in the form of a ladder due to the endonucleolytic attack is reportedly considered as a characteristic of apoptosis (Basnakian and James, 1994). Bone marrow cells were lysed in lysis buffer with 10% sodium dodecyl sulfate (SDS) and 50μg/ml proteinase K for 2 hrs at 45°C. The cell lysates were first extracted with Tris-saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), then with chloroform–isoamyl alcohol (24:1), precipitated overnight at -20°C in two volumes of absolute ethanol in the presence of 0.3M Na acetate, and recovered by centrifugation. Pellets were air-dried, resuspended in TAE buffer. Electrophoresis was carried out in a 1.4% agarose gel containing 5μg/ml ethidium bromide. The gel was examined and photographed under a Gel Documentation system to visualize intra-nucleosomal DNA fragmentation (laddering), a characteristic of apoptosis or smearing of DNA due to necrosis.

5.6. Assessment of cell morphology for apoptosis

Bone marrow smear was fixed followed by staining with acridine orange:ethidium bromide (1:1) solution, slides were incubated for 2 minutes with the stain and washed with PBS twice. At least 1000 bone marrow cells of each group were examined under fluorescent microscope (Leica DM2500) using a fluorescein filter and 40X objective. Green fluorescence is observed
for viable cells and red fluorescence for necrotic cells, while the apoptotic cells show red and green fluorescence with apoptotic characteristics.

6. Oxidative stress parameters

6.1. Lipid Peroxidation product (Malondialdehyde (MDA))

The lipid peroxidation product present in the tissues was estimated by thiobarbituric acid (TBA) method (Janero, 1998). Malondialdehyde (MDA) is a low-molecular-weight end product formed via the decomposition of certain primary and secondary lipid peroxidation products. At low pH and high temperature, MDA readily participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red fluorescent MDA-TBA adduct that absorbs at 532nm.

To 0.2ml of tissue homogenate, 0.2ml of 8% SDS, 1ml of 20% acetic acid and 1ml of 2% TBA were added. The mixture was made up to 4ml with phosphate buffer and then incubated in a water bath at 95°C for 60 minutes. After cooling, 3ml of 10% TCA was added and shaken vigorously. After centrifugation at 3000rpm for 10 minutes, the supernatant was taken and its absorbance was read at 532nm. The level of lipid peroxidase was expressed as nanomoles of MDA released/hour/g tissue.

$$\text{MDA liberated} = \frac{\text{OD of sample} \times \text{dilution factor} \times 10^9 \text{ moles}}{E \times \text{Tissue weight}}$$

$$E = \text{Extinction coefficient of MDA (1.56} \times 10^5)$$

6.2 Estimation of antioxidant enzymes

Catalase (E.C. 1.11.1.6)

Catalase activity was assayed by the method described by Sinha (1972). Dichromate in acetic acid is reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed is measured at 590nm. Catalase was allowed to split H$_2$O$_2$ for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H$_2$O$_2$ was determined by measuring chromic acetate spectrophotometrically after heating the reaction mixture.

The assay mixture consisted of 0.5ml of tissue homogenate, 0.5ml phosphate buffer (0.1M, pH 7.0), 0.5ml H$_2$O$_2$ (0.2M) in a final volume of 1.5ml. About 2ml dichromate acetic acid reagent was added in reaction mixture at the interval of 15, 30, and 45 and 60 seconds, incubated in boiling water bath for 10 minutes then cooled. Absorbance was recorded at 590nm.
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Catalase activity = \( \frac{OD \times \text{total volume of assay}}{\text{Volume of sample} \times \text{Tissue weight} \times \frac{1}{\text{mg protein concentration}}} \)

Unit = \( \mu \text{mole H}_2\text{O}_2 \text{ liberated/ minute/ mg protein} \)

**Superoxide dismutase (E.C. 1.15.1.1)**

The activity of superoxide dismutase (SOD) was assessed by method described by Marklund and Marklund (1974). Superoxide anion is involved in auto-oxidation of pyrogallol at alkaline pH. The SOD inhibits the auto-oxidation of pyrogallol, which can be determined as an increase in absorbance per two minutes at 420nm on a spectrophotometer. The SOD activity was measured as unit/ml of homogenate. One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

The reaction mixture for auto-oxidation consisted of 2ml of Tris-HCl buffer, 0.5ml of 2mM pyrogallol and 1.5ml water. Initially, the rate of autoxidation of pyrogallol was noted at an interval of 30 seconds for two minutes. The assay mixture for the enzyme contained 2ml of 0.05M Tris-HCl buffer, 0.5ml pyrogallol, aliquots of the homogenate and water to give a final volume of 4ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted.

The enzyme activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

Unit = \% inhibition of pyragallol/min/mg tissue

**Glutathione peroxidase (GPx, E.C. 1.11.1.9)**

The activity of glutathione peroxidase in the samples was determined by the method of Rotruck et al. (1973). Enzyme preparation was allowed to react with \( \text{H}_2\text{O}_2 \) in presence of GSH for a specific time period. The GSH content remaining after the reaction was measured by the method of Ellman et al. (1961).

The reaction mixture in a total volume of 1ml contained 0.2ml of phosphate buffer, 0.2ml EDTA, 0.1ml sodium azide and 0.5ml of the enzyme source (tissue homogenate/ plasma/ hemolysate). 0.2ml of glutathione and 0.1ml of \( \text{H}_2\text{O}_2 \) were added to reaction mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5ml of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH by adding phosphate buffer and Ellman’s reagent (DTNB) into the supernatant. A blank was treated similarly to which 0.2ml of enzyme was added after incubation and absorbance was recorded at 412nm. The activity of glutathione peroxidase was expressed as mM of GSH consumed/ mg tissue.
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GPx activity = \( \frac{OD \text{ of sample} \times \text{volume of assay} \times \text{dilution}}{CF \times \text{Volume of sample} \times \text{Tissue weight}} \)

Where, \( CF = 0.00373 \)

**Glutathione-S-transferase (GST, E.C. 2.5.1.18)**

The method described by Habig et al. (1974) was adopted for estimation of GST activity which was measured by following the increase in absorbance at 340nm using 1-chloro 2,4 dinitrobenzene (CDNB) as a substrate.

1ml of phosphate buffer, 0.1ml of CDNB and 1ml of tissue homogenate were mixed. The volume was adjusted to 2.9ml with water. The reaction mixture was pre-incubated at 37°C for 5 minutes and the reaction started by the addition of 0.1ml of 30mM glutathione. The absorbance was recorded for 5 minutes at 340nm. A system devoid of enzyme served as the blank. The specific activity of GST was expressed as µmoles of CDNB-GSH conjugate formed/ minute/ mg protein).

\[
\text{GST activity} = \frac{OD \text{ of sample} \times \text{volume of assay}}{9.6 \times \text{Volume of sample} \times \text{mg of protein}}
\]

Where 9.6 = mM extinction coefficient of CDNB-GSH conjugate

**6.3. Reduced glutathione (Non-Enzymatic antioxidants)**

The reduced glutathione level was determined by the method of Beutler et al. (1963). This method was based on the development of yellow colour when thiol reagent, 5,5'- dithio-bis-2-nitrobenzoic (DTNB) reacts with GSH present in tissue sample forming 5-thio nitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm. The level of TNB and GS-TNB is equivalent to the GSH present in the tissue.

0.2ml of sample was mixed with 0.8ml of EDTA solution. To this 2ml of precipitating reagent was added, mixed thoroughly and kept for 5 minutes before centrifugation. Tubes were centrifuged at 3000rpm for 10 minutes. 1ml of the filtrate, 1ml of 0.3 M phosphate solution and 1ml of DTNB reagent were added and the colour developed was read at 412nm with a spectrophotometer. A set of standard solutions containing 20-100µg of reduced glutathione was treated similarly.

\[
\text{GSH concentration (µg/g tissue)} = \frac{OD \text{ of sample} \times \text{volume of assay} \times \text{dilution}}{\text{Volume of sample} \times \text{tissue weight}}
\]
7. Histopathology
Rats were sacrificed and target organs were removed and washed with PBS. Tissues were cut and fixed in 10% neutral buffered formalin. Tissue samples were dehydrated in upgrading alcohol series (50%, 70%, 90% and 100%; 2 hours in each). Xylene was used as a clearing agent. Paraffin wax was used as the embedding medium. Tissues were processed for 2.5hrs in paraffin: xylene series (25:75, 50:50 and 75:25) and then finally kept in 100% paraffin. Paraffin block was prepared and tissue sections were taken using a microtome. Tissue section was deparaffinized in xylene and rehydrated in down grade of alcohol series. Haematoxylin was used to stain nuclei of cells for 5 to 10 minutes. Differentiation was done by dipping the slide in ammonia solution, followed by a wash with water. Sections were covered with eosin for cytoplasmic staining. Slides were rinsed and dehydrated in increasing concentrations of alcohol, then xylene and mounted in DPX. Slides were observed under the microscope (Leica DM2500) for analysis of histopathological changes.

8. Transmission electron microscopy (TEM)
Method described by Glauert (1974) was adopted for TEM. Tissues were cut into pieces of 2-3mm size and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 hrs at 4°C. The tissues were rinsed with buffer and osmicated in 2% osmium tetroxide. Tissues were rinsed again and dehydrated using increasing alcohol concentrations, followed by rinsing in propylene oxide twice for 15 minutes on the rotator for each rinse. The specimen was put in a mixture of 50% propylene oxide and 50% resin for at least 2 hours and then in 100% resin as embedding medium. Resin blocks were prepared. Sections were cut on ultra microtome with glass knife and collected on gold grid. Sections were stained in uranyl acetate for 20 minutes and then rinsed. The sections were exposed to NaOH for 10 minutes and again rinsed. Sections were observed under a transmission electron microscope.

9. In vitro study
IMR 32, a neuroblastoma cell line was procured from the National Chemical Laboratory, Cell culture facility, Pune. Culture was maintained as an adherent cell line in T25 flask by providing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 100µl of antimycotic solution at 37°C in a 5% CO₂:95% air-humidified atmosphere. All the cell culture solutions were purchased from HiMedia Chemicals Ltd., USA. Cells were passaged as needed using 0.5% trypsin-EDTA.
**Cell Viability test**

Cells were exposed to 0.5% trypsin EDTA solution for 3 to 5 minutes and reaction was stopped by adding chilled phosphate buffer. Cells were collected in 15ml centrifuge tube and centrifuged for 10 minutes at 3000rpm. Cell pellet was resuspended in 1ml of phosphate buffer. 1 part of 0.4% trypan blue and 1 part of cell aliquot were taken and incubated for 2 minutes. Cells were observed under the microscope using hemocytometer. The unstained (viable) and stained (nonviable) cells were counted separately in the hemocytometer.

\[
\% \text{ Cell Viability} = \frac{\text{Total Viable cells (Unstained)}}{\text{Total cells (Viable + Dead)}} \times 100
\]

\[
\text{Viable Cells/ml} = \text{Average viable cell count per square} \times \text{Dilution Factor} \times 10^4
\]

**Cytotoxicity test**

The reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase occurs in viable cells. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. Isopropanol, DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells (Mosmann, 1983).

Cell viability was checked and equal number of cells were distributed in 96 well plate supplemented by 200µl DMEM medium with 10% FBS and 100µl antimycotic solution for 18 to 20 hrs. First line of wells was kept blank without the cells. The culture medium from each well was removed and cells were exposed to different concentrations of thiacloprid except for the second row of wells, into which 200µl of culture medium was added. Plate was incubated for different time intervals like 12 hrs and 24 hrs. Again culture medium with thiacloprid was removed and cells were washed with PBS. Cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, HiMedia, USA) at a final concentration of 0.5 mg/ml in RPMI 1640 medium for 5 hours at room temperature in dark. After incubation, medium was removed and crystals of the insoluble formazan product were dissolved in 100µl of DMSO. The OD was read at 570nm using a microplate reader (Metertech Σ960). IC (inhibition concentration) 50 (IC50) value of thiacloprid was calculated.

\[
\% \text{ inhibition} = 100 - \left( \frac{\text{OD of test}}{\text{OD of control}} \right) \times 100
\]
**Acridine orange/ Ethidium bromide (AO/EtBr) staining**
Cells adhered on cover slips in 6-well plate after exposure to thiacloprid (12 hours) were stained with acridine orange and ethidium bromide stain for 60 seconds and observed under the fluorescent microscope (Leica DM2500). Live and dead cells can be identified easily with this stain on the basis of their membrane integrity. Dead cells show red fluorescence, live cells show green fluorescence and apoptotic cells show red and green fluorescence.

**10. Statistical analyses**
All quantitative variables are summarized and presented as mean and standard error around mean. In order to confirm the variances of the observations in the individual groups are equal Bartlett’s test for homogeneity was done. The statistical significance of the differences between the mean values of control and experimental groups was evaluated through one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test. Statistical analyses were performed using SPSS-PC Statistical Analysis Package (SPSS 12.0, SPSS Inc, Chicago, IL) and GraphPad Prism (version 6) software. Differences between groups were considered statistically significant at probability (p) ≤ 0.05.