MATERIAL AND METHODS
3.1 *Drosophila melanogaster* CULTURE MAINTENANCE

Wild type Oregon R *D. melanogaster* culture was maintained in a light/dark cycle of 12:12h at 25±2°C in glass bottles containing a standard cornmeal agar diet with yeast granules. Diet was prepared according to a standard protocol (Murlidhara, 2009).

3.2 PREPARATION OF DIFFERENT CONCENTRATIONS OF PESTICIDES USED

Paraquat was a kind gift from Dr. S.Z. Imam, Department of Medicine, UT Health Science Centre. Five doses of paraquat viz., 50µm, 100µm, 150µm, 200µm and 500µm were prepared from 5mM/ml stock solution which was prepared from the pure paraquat grade. From the stock, desired doses were obtained by putting molar formula. These doses were prepared in tissue culture grade water.

Six doses of malathion viz., 50µm, 100µm, 150µm, 200µm, 500µm and 1mM were prepared from 5mM/ml stock solution which was prepared from the pure malathion grade. From the stock desired doses were obtained by putting molar formula. These doses were prepared in tissue culture grade water.

3.3 DRUG TREATMENT

From the stock culture, flies were transferred to glass vials containing 50µl of test food (sucrose+paraquat) and (sucrose+malathion). The flies were held in groups of 20 per vial and incubated at 25°C for 7 days at different doses with a solution containing 0-500µM paraquat and 0-1mM malathion. The flies were allowed to feed on the test food mixed with different concentrations. It was made sure that freshly made solution were transparent.
3.4 LOCOMOTOR ASSAY

Locomotory assay with negative geotaxis was determined as by (Le Bourg and Lints, 1992). Test flies were anesthetized and placed in a vertical glass column (standard length, 25cm; diameter, 1.5cm). After a brief recovery period, flies were gently tapped to bottom of the column. After 1 min, flies that reached the top of the column and flies that remained at the bottom were counted separately. Three trials were performed in each experiment at 1 min intervals. The scores are the mean of the numbers of flies at the top (ntop) and at the bottom (nbot), expressed as percentages of the total number of flies (ntot). Results are presented as mean±SEM of the scores obtained in three independent experiments, which was calculated as 1/2[(ntot +ntop-nbot)/ntot] (Coulom, 2004). The one-way anova was performed to compare proportion of percentage between independent groups. Differences were considered statistically significant at P<0.05.

3.5 HISTOLOGICAL DETECTION

A group of 20 wild type Oregon R D. melanogaster was selected from the stock for each doses viz: 50μM, 100μM, 150μM, 200μM, 500μM of paraquat and 100μM, 150μM, 200μM, 500μM, 1mM of malathion mixed with food medium and incubated at 25°C for 7days. After 7 days of incubation flies were anesthetized and fixed in 8% w/v paraformaldehyde for 8hrs. After fixation, flies were rinsed in PBS five times, each time for 10 min at room temperature. The tissues were dehydrated through an ethanol series to xylene. It was then kept in xylene+paraffin at 60°C in an oven. Tissues were finally embedded in paraffin wax and stored at 0°C for at least 24hr. The sections were cut at 6μm thickness on a Yorco rotary microtome and slides containing sections were stored upright overnight at 37°C. Slides were processed in xylene for 10 min, followed by descending ethanol series (100-30%). The sections
were stained with Ehrlich’s Haemotoxylin for 2 min followed by 0.5% hydrochloric solution (1 dip), 0.5% sodium bicarbonate (5 min) and Scott’s solution (5 min). After that slides were stained with eosin for 15 min, rinsed with upgrade ethanol series (30-100%), followed by xylene for 10 min. Finally the slides were mounted with DPX and observed under Leica microscope.

3.6 SILVER NITRATE STAINING

Silver nitrate staining was done by Belchowsky (1902) method with slight modifications. Sections were deparaaffnize and wash with distill water three times. Slides were kept in pre warmed 10% silver nitrate solution and stained for 15 minutes and then washed three times with distill water. Slides were incubated in ammonium silver solution in 40°C oven for 30 minutes after that slides were directly placed in developer working solution for less than 1 min. Then slides were placed in 1% ammonium hydroxide solution for 1 minute and then washed with distill water three times. After washing with distill water slides were placed in 5% sodium thiosulphate for 5 minutes and then again washed with distill water three times. After washing slides were dehydrated with ethanol series followed by xylene. Finally the slides were mounted with resinous medium and observed under Magnus Microscope (Model No-MLX-DX).

3.7 COMET ASSAY (SINGLE CELL ALKALINE ELECTROPHORESIS)

In the experimental study 20 adult *D. melanogaster* in each group were kept at 25± 1°C and fed on different concentration of paraquat and malathion mixed with food for 24 hrs. The control received normal food medium. All the experiments were conducted in triplicate.
3.7.1 PREPARATION OF CELLULAR SUSPENSION FOR COMET ASSAY

After 24h of treatment of both treated and control adult *D. melanogaster* from each group were removed from food and anesthetized with diethyl ether and washed thoroughly with 50mM of phosphate buffer. The brains were explanted from the fly in modified PBS and collected separately in microcentrifuge tube. The single cell suspensions of the tissues were prepared by the method as modified by Mukhopadhyay et al. (2003). PBS in microcentrifuge tube was replaced by 300μl of collagenase (0.5mg/ml in PBS, pH 7.4) and kept for 15min at 25± 1°C in environmental chamber. Then cells were then passed through nylon mesh and their filtrate were kept in separate microcentrifuge as cell suspension of each organ.

3.7.2 VIABILITY ASSESSMENT OF THE CELLS FOR COMET ASSAY

The viability of the cells in the suspensions was checked by standard trypan blue exclusion method (Pool-Zobel et al., 1993). For this, 100μl of the cell suspensions was mixed with same volume of 0.4% trypan blue solution. The percentage of stained cells to the total number of the cells was calculated. The method is based on the principle that the non-viable cells have degenerated membrane and hence, they take-up the dye while viable cells do not show any staining.

3.7.3 COMET ASSAY PROCEDURE

The assay was performed in alkaline condition in accordance with protocol of Singh et al., (1988) with few modifications. First of all, fully frosted slides precoated with 1% NMA (normal melting agarose) as base layer at 60°C were prepared on a day before dissection. In brief 75μl of a mixture containing 80μl of cell suspension and 80μl of 1% of LMPA to form the working cell suspensions separately. Such suspension was pipetted over the base layer at 37°C followed by covering with cover slips immediately. After solidification of second layer on ice packs, the cover slips
were removed and a third layer of 0.5% LMPA (80μl) was pipetted over followed by placing of cover slips on it. This layer was also kept on ice packs for solidification. Then, the coverslips were removed and the slides were immersed in cold lysing solution (2.5M NaCl+100mM EDTA+10mM tris-base+ 1% triton X-100 with pH 10). The lysis was allowed to proceed for 3h followed by unwinding for 30 minutes in alkaline electrophoretic running buffer (300mM NaOH+1mM EDTA at pH 13) in electrophoretic tank. Then electrophoresis was performed for 35 minutes at 4°C with constant field strength of 0.74volts/cm and variable current strength under the range of 295-310 mA. The slides were washed with cold saline and were subjected to neutralization by 0.4 M tris-buffer of pH 7.5 followed by washing with cold saline and this process was repeated twice. After that, they were stained with 75μl EtBr (20μg/ml) for 5 minutes. Finally, the slides were washed with chilled saline again and cover slips were placed on them and were kept in humidified slide box in refrigerator followed by analysis on the next day. The slides were scored with the help of CX41 fluorescent microscope (Olympus, Japan) coupled with an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, U.K.) attached with an integrated CC camera COHU 4910 (equipped with 510-560nm excitation and 590nm barrier filters). The comets were scored at the magnification of 100X and images from 50 cells (25 from each replicate slide) were scored. Comet tail-length (migration of DNA from its nucleus in μm) was chosen as the parameter to assess the cellular DNA damage that was automatically generated by Komet 5.5 image analyzing system.
3.8 HISTOLOGICAL PREPARATION OF *D. melanogaster* BRAIN TISSUE FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

The whole head was explanted from control and treated *D. melanogaster* and were primarily fixed for 4-6 hours in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH= 7.4). After that the tissue was kept in PB (pH= 7.4) at 4° C for 2 hours or overnight. The secondary fixation of the tissue was done in 1% osmium tetraoxide in distill water for 1h at room temperature and then washed twice with distill water followed by upgraded ethanol series (50%-100%) for 15 minutes each. After washing tissue were kept in propylene oxide (2 changes) for 10 min, followed by propylene oxide: resin (1:1 mixture) for 1-2 hrs. The tissue was infiltrated with a resin before being placed in an embedding mould, which was then polymerized in an oven at 60°C. The sections were cut at 0.5-1.0μm thickness and were transferred on the slide. After drying slides were stained with toluidine blue for 2-5 min. The sections were observed under microscope for precise location to cut for ultrathin sections. Ultrathin sections were cut at 60-90 nm thickness (silver-yellow colour) (Ultra-microtome- Model-UC6, Reichet) and the sections were collected on to the grids. The sections were dried overnight before staining and finally the grids were stained with uranyl acetate for 15 minutes and with lead acetate for 5 minutes. After staining the sections were observed under transmission electron microscope (Model=Morgagni, 268D, Fei, Netherland) in Sophisticated Analytical Instrument Facility For Electron Microscopy, Department of Anatomy, AIIMS, New Delhi, INDIA.

3.9 CYTOTOXICITY ASSESSMENT:

*Drosophila* cell lines were maintained for in vitro cytotoxicity assessment of pesticides. *Drosophila* S2 cell lines were obtained from National Centre For Cell
Sciences, Pune. Cells will be maintained in Schneider’s Medium+10µl/ml insulin medium (Invitrogen, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc. CA, USA) and were grown in a refrigerated incubator at 25°C. Cells were treated with different doses of Paraquat and Malathion to assess the cell viability:

**3.9.1 MTT ASSAY:**

The cytotoxic effect of paraquat and malathion was assessed in D-Mel-2 cell lines by MTT assay.

\[
\text{MTT assay - \% of viable cells} = \frac{A_T - A_B}{A_C - A_B} \times 100
\]

Where \( A_c \) = absorbance of the control (mean value); \( A_T \) = absorbance of treated cells (mean value), \( A_B \) = absorbance of blank (mean value).

Briefly, cells were seeded at a number of \( 2 \times 10^4 \) per well onto 96well plates (200µl/well) in triplicates, allowed to attach and grow for 24h and subsequently exposed to different Paraquat and Malathion (100µM to 1mM) concentrations for 24h,48h and 72h. At the end of the treatment, the medium was removed and cells were incubated with 20µl of MTT (5mg/ml in PBS) in fresh medium for 4h at 27°C. After four hours, formazon crystals formed by mitochondrial reduction of MTT, were solubilized in DMSO (150µl/well) and the absorbance was read at 570 nm after 10min incubation on the Multiskan EX microplate reader (Thermo scientific, Germany). Percent inhibition of cytotoxicity was calculated as a fraction of control.

**3.9.2 LDH ASSAY:**

LDH assay is a colorimetric assay which measures lactate dehydrogenase (LDH), a relatively stable cytosolic enzyme released by cells when they undergo significant membrane damage or cytolysis (Korzeniewski et al., 1983). The amount of
LDH released is proportional to the number of cells damaged or lysed. The assay were performed using all concentrations of paraquat and malathion.

\[
\% \text{ treatment-induced cytotoxicity} = \frac{\text{Abs}_{\text{Exp}} - \text{Abs}_{\text{Con}}}{\text{Abs}_{\text{Trit}} - \text{Abs}_{\text{Con}}} \times 100
\]

Where: \( \text{Abs}_{\text{Exp}} \) = mean absorbance from the treated cells, \( \text{Abs}_{\text{Con}} \) = mean absorbance from controls (untreated cells), \( \text{Abs}_{\text{Trit}} \) = mean absorbance from Triton X-100 treated cells (standard/maximum LDH release, a positive control).

Cytotoxicity induced by paraquat and malathion was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Cell were exposed to different concentrations of paraquat and malathion for 24h, 48h and 72h.

**3.10 STATISTICAL ANALYSIS:**

The data obtained was statistically analyzed by the application of the following methods and formulae.

**3.10.1 MEAN:**

It is obtained by summing up all the observations and dividing it by the total number of observations.

\[
\text{Mean} (\overline{X}) = \left( \frac{\Sigma X}{N} \right)
\]

where, \( X \) = sum of the observations

\( N \) = number of observations

**3.10.2 STANDARD DEVIATION:**

It is used as a measure of dispersion and defined as “Root mean square deviation from mean”.

\[
\text{Standard deviation (SD)} = \sqrt{\frac{\Sigma (X - \overline{X})^2}{N}}
\]

where, \( X \) = value of variables
\[ \bar{X} = \text{arithematic mean} \]
\[ N = \text{number of observations} \]

### 3.10.3 STANDARD ERROR:

It is the ratio of standard deviation of the sample divided by the square root of the total number of observations.

\[
\text{Standard error (SE)} = \frac{SD}{\sqrt{N}}
\]

where, SD = standard deviation
\[ N = \text{number of observations} \]

### 3.10.4 TEST OF SIGNIFICANCE:

All statistical were performed by using Graph Prism and SPSS (17.0 version).