Chapter 3

Screening of insecticides: Fenitrothion and Malathion on SoMG primary cell line and Sf9 cell line

3.1 Introduction

Pests are any unwanted organisms that affect human life negatively; these organisms can be insects, plants, animals or microorganisms. In order to reduce the damages caused by these pests, they are controlled by using pesticides (Hill, 2008; Gray *et al.,* 2009). A pesticide is any chemical or biological substance that is used against these pests (Goel and Aggarwal, 2007). A pesticide is classified according to the chemical composition, the mode of action, and the targeted pest species (Drum, 1980). The exploding, growing human population has led to a higher demand for food. The Green Revolution began with the primary goal of boosting grain yields in the world to meet such demand. The success of this revolution led to a widespread movement around the world. However, the success of this revolution required the intensive use of pesticides to control pests because high-yielding varieties of grains were not widely resistant to pests and diseases (Johnson *et al.,* 2017). Pests destroy approximately 30-48% of the world's production of food every year (Hellar, 2002). The use of pesticides in agriculture has led to a significant improvement in crop yields per hectare of land. Studies have established a possible correlation between the number of pesticides used per hectare and the number of crop yields per hectare (Hellar, 2002). However, as the world anticipates feeding nine billion people as sustainably as possible by 2050, crop protection against pest insects, diseases and weeds have a vital role in maintaining and improving crop yields (Godfray *et al.,* 2010).

Frequent use of the same class of pesticides to control the pest causes u ndesirable changes in the plant gene pool leading to another form of artificial

pesticide resistance. When a pesticide is first used, due to its distinct genetic makeup, a small proportion of the pest population may survive exposure to the material. These individuals move by the use of the genes for resistance to the next generation. Subsequent uses of the pesticide increase the proportion of less-susceptible individuals in the population.

The population is gradually developing resistance to the pesticide throu gh this selection process. Worldwide, more than 500 species of insects, mites, and spiders have developed some level of pesticide resistance (Epstein *et al.*, 2002). Multistep pesticide resistance has been developing slowly over many years in the field. Rather than having distinct groups of sensitive and resistant individuals, the population consists of individuals with a range of pesticide sensitivities. With each pesticide application, these individuals live and replicate at the more tolerant end of the spectrum. Over the years, the proportion of the population that can survive a pesticide spray increases until that pesticide eventually becomes ineffective (Oerke, 2006). Hence, Just as the effectiveness of antibiotics in the prevention of human disease is threatened by the evolution of resistant strains of bacteria (World Health Organization, 2014), the control of agricultural pests and crop diseases is threatened by the evolution of pesticide resistance, affecting insecticides (Bass *et al.*, 2015), herbicides (Powles and Yu, 2010) and fungicides. (Lucas *et al.*, 2015). Thus, Monitoring resistance in pest populations is thus crucial and requires accurate, sensitive, and reliable methods.

In-vivo studies on insecticides' activities are carried to evaluate the insecticides' actual effects by simulating the natural environment, however, some of these assays are labour intensive and time-consuming (Huang *et al.*, 2003). In contrast, *in-vitro* studies are becoming a useful assay method in the screening of insecticides, not only because they can greatly reduce the screening time but also provide more useful information, such as insecticides mechanisms of action rapidly and inexpensively (Andersen and Krewski,

2009; Zhang *et al.*, 2012). Over the past decades, different industries have demonstrated an increasing interest in the development of *in-vitro* methods for the studying of insecticides effect. Sf9, Sf21, Tn-368, and High-Five are the cell lines most widely used in industrial applications and the number of established insect cell lines has expanded, along with the number of tissue sources. The utility of insect cell lines for protein production has grown from laboratory-scale experimental work to industrial applications (Elias *et al.*, 2007; Drugmand *et al.*, 2011). The commercially available Sf9 insect cell line derived from pupal ovarian tissue of *Spodoptera frugiperda* is used for *in-vitro* assays to estimate the effect of different insecticides (Lynn, 2002; Ikonomou, 2003; Saito *et al.*, 2005; 2006; Taniai *et al.*, 2006; Szewczyk, 2009; Tebourbi *et al.*, 2011; Cox, 2012; Ilboudo *et al.*, 2014; Sihler e*t al.*, 2018; Real *et al.*, 2019).

Organophosphates are agricultural insecticides and are among the most commonly used insecticides in the world (Lerche *et al.*, 2002, 2003). They are employed widely as pesticides in residential settings and in agricultural practices to increase crop yield. Because the use of organophosphate pesticides has been and remains pervasive in both developed and developing nations, concerns are increasing regarding the relative safety of these chemicals for the environment, humans, and animals (Pal *et al.*, 2016). Number of organophosphates such as malathion and fenitrothion insecticides have been and are being used against stored grains pests which includes lesser grain weevil, *Sitophilous oryzae* (CABI, 2007), lesser grain borer *Rhyzopertha dominica* (Nighat *et al.*, 2007; Hooper *et al.*, 2003; Rajendran and Muralidharan, 2005); red flour beetle *Tribolium castaneum*, (Khawaja *et al.*, 2012; Bajracharya *et al.*, 2013).

Malathion is a nonsystemic, wide-spectrum organophosphate insecticide. It inhibits acetylcholinesterase activity of most eukaryotes. Malathion is toxic to aquatic organisms but has relatively low toxicity for

birds and mammals. Fenitrothion is a broad-spectrum organophosphate insecticide with low mammalian toxicity. Fenitrothion is an insecticide, acetylcholinesterase inhibitor, used in agriculture, horticulture, and sucking insects on rice, cereals, fruits, vegetables, stored grains, cotton, till date the applications of organophosphates have thus been used directly on the pests in warehouses where grains are stored in large quantities. However, as the present work is focused on the establishment of a new cell line from the stored grain pest, *S. oryzae* and according to our knowledge there exist lacunae as far as screening of organophosphate on newly developed cell line is concerned.

Keeping in view the above mentioned facts the present work is an attempt where screening of two organophosphates: Malathion and Fenitrothion has been undertaken in newly developed SoMG cell line and Sf9 cell line has been taken as a positive control. In an attempt to fulfill the aim, first, the IC_{50} of both the organophosphates was checked and then after the sub-acute dose was taken into consideration for further studies where, ROS generation, DNA damage, and cell death was checked.

3.2 Materials and Methodology

Experimental Design: (Toxicity Studies)

Two insecticides (i.e. Fenitrothion (CAS no. 122-14-5, Sigma-Aldrich, USA) and Malathion, (CAS no. 121-75-5, Sigma-Aldrich, USA)) were selected according to their usage in warehouses. The insecticide was dissolved in the complete media before the treatment. An inhibition concentration of insecticide was performed and analyzed using probit analysis (SPSS, version 15). After obtaining the Inhibition concentration (IC_{50}), sub lethal (1/5, $1/10^{th}$ and $1/20^{th}$ does of IC_{50}) concentrations were selected for further studies. The acute study were carried out in which, cell viability assay were performed for 24, 48, 72 and 96 hrs using MTT. Furthermore, annexin V /Pi staining was

performed to test whether the cells are undergoing apoptotic or necrotic death. Simultaneously, Oxidative status for ROS parameters was carried using standard protocol.

Cell viability Assay

Cell density was determined by Haemocytometer counts and Cell viability was evaluated by Trypan blue (0. 4% W/V) (TCL046, HiMedia, India) staining.

Principle

Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology.

Materials

- Haemocytometer
- Coverslips
- Sf9 cells
- PBS
- Trypan blue stain (0.4%)

Method

A cell suspension of 2 x 10^4 cells was prepared (by centrifugation). Then 0.1 ml of Trypan blue (0.4%) dissolved in 0.8 ml PBS was added to 0.1 ml of cell suspension. It was allowed to stand for two minutes after mixing it thoroughly. Further, the surface of haemocytometer and coverslip was cleaned with 70% ethanol and air dried. Wet edges of the coverslip were layered over the groove, central area of haemocytometer. Then using a pipette, cell suspension was transferred to the edges of coverslip to fill haemocytometer chambers. The haemocytometer slide was placed under the microscope (Metzler M, India) and cells were observed using 10x objective. Non-viable cells were stained blue whereas viable cells remain unstained. If the cell

density was too high, the cell suspension was diluted. Count the cells in all the 4 corner squares.

To determine the average of cells in the 4 squares,

Average cell count per square = (Total number of cells in 4 squares)/4

For cell count, cell count (cells/ml) = Average cell count x 10^4

If the original cell suspension was diluted, multiply by the dilution factor to obtain the number of cells/ml.

For the cell viability,

% cell viability= (Number of viable cells)/ (total number of cells) x 100

MTT Assay

Principle

MTT is an assay to measure cell viability in which yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. Mitochondrial dehydrogenase of the viable cells cleaves the tetrazoliumring, yielding purple solution which is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells.

Materials

- Cell Culture
- Cell Culture media
- 12mM MTT (TC191, HiMedia, India) solution (5mg/ml in sterile PBS)
- DMSO
- Microtiter plate (flat-bottomed)
- Sterile pipettes
- Sterile pipette tips

SoMG cell suspensions (2 x 10^4 cells/ml) were seeded onto a 96-well culture plates (100 µL per well). After 24 h of incubation, varying concentration of Fenitrothion was added at final concentration of 25, 50, 100, 125, 150, 175, 200, 225 and 250 µg/ml for 96 hrs. For malathion different concentration i.e. 100, 200, 300, 400, 500 and 1000 µg/ml were kept for 96 hrs. Complete insect culture medium was used as control. Four hours prior to the assay, 10 µl of MTT (TC191, HiMedia, India) solution (5mg/ml in incubation media) was added in to each well. To dissolve formazon crystals, the media in each well was replaced with 100 µL of DMSO. The optical density was measured using a microplate reader at 570 nm with a reference wavelength at 630 nm. Cell viability was obtained using the following formula.

$$\% \, \boldsymbol{Cell\ Viability} = \frac{\textbf{Average OD of test x 100}}{\textbf{Average OD of control}}$$

IC_{50} value determination: After treating the cells with the test chemicals, their mortality rate was determined from the formula given above. The results were plotted and the dose response curve and the IC_{50} value were obtained using the SPSS version 15 software.

Toxicity studies using the sub-lethal concentrations

ROS generation, DNA damage by DAPI staining , Cell death by Acridine Orange and Ethidium Bromide (AO/EB) staining and by Annexin/PI staining was observed by time and dose dependent manner. Effects of the test chemical on the insect cell culture were studied using the sublethal concentrations: $1/5^{th}$ (HD), $1/10^{th}$ (MD), $1/20^{th}$ (LD) of the obtained IC_{50} value.

AO/EB staining: (Yu *et al.,* 2016)

Principle

Acridine Orange/Ethidium Bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Acridine Orange is a cell permeable nuleic acid selective dye that emits green fluorescence when bound to ssDNA or RNA(at 520 nm) and red fluorescence when bound to ssDNA or RNA(at 650 nm). Since it is a cationic dye, it also enters acidic compartments such as lysosomes which in low pH conditions will emit orange light. The most commonly used stain for detecting DNA/RNA is ethidium bromide. Ethidium bromide is a DNA interchelator, inserting itself into the spaces between the base pairs of the double helix. Ethidium bromide possesses UV absorbance maximal at 300 and 360 nm. Additionally, it can absorb energy from nucleotides excited by absorbance of 260 nm radiation. Ethidium re-emits this energy as yellow/orange light centered at 590 nm.

Materials

- Acridine Orange (AO)
- Ethidium Bromide (EB)
- Sterile PBS
- 6 well culture plate
- Fluorescence microscope
- Acridine Orange (AO)

Method

The dual staining of acridine orange (MB116, Himedia, India) and ethidium bromide (MB071, Himedia, India) was used to measure live cells from apoptotic and necrotic cells (Li, 2013). The cells were harvested and washed three times with PBS (pH 7.4) after being incubated with sub lethal concentration-LD, MD, HD of the two chemicals for 48 h. Then the cells were stained with 20 µl of AO and EB (to a final concentration of 100µg/ml for both) and incubated for 15 minutes at 37 ºC for 15 min in dark and washed three times with PBS (pH 7.4). The morphology of the treated cells was examined by fluorescence microscopy (Floid cell imaging station (Invitrogen, USA)).

H_2DCF-DA Staining: (Yu *et al.*, 2016)

Principle

The 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) fluorescent probe reacts with several ROS including hydrogen peroxide, hydrogen radicals and peroxynitrite. The cell-permanent lipophilic. H_2DCF-DA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterase. Upon Oxidation by ROS, the non-fluorescent H_2DCF-DA is converted to the highly fluorescent hydrophilic 2', 7'-dichlorofluorescence (DCF). The fluorescence intensity of DCF is proportional to the amount of ROS produced by the cells.

Materials

- H_2DCF-DA dye (D6883, Sigma-Aldrich, USA)
- PBS
- 24 well culture plate

Method

The production of intracellular ROS was measured by oxidation of DCF-DA. Prior to the use, H_2DCF-DA stock solution was prepared in DMSO under sterile condition in laminar flow hood and 2 x 10^4 cells were plated in the 6 well plate. Cells were then treated with the sub lethal concentration – HD, MD, LD for Fenitrothion. After interval of 24 hrs i.e. 24, 48, 72 and 96 hrs of treatment, the cells were centrifuged at 1000xg for 5 minutes. Then the cells were washed twice with PBS (pH-7.4) and were incubated with DCFH-DA dye (D6883, Sigma-Aldrich, USA) at 30 ^oC for 30 minutes to allow the diffusion of the fluorescent probe into the cells and its subsequent hydrolysis to non-fluorescence dichloro fluorescein (DCFH) under the action of intracellular esterase. Intracellular ROS generation was measured by fluorescence microscopy (Floid cell imaging station (Invitrogen, USA)) with the excitation and emission wavelengths set as 488 and 528 nm, respectively.

DAPI staining

Principle

DAPI (4′, 6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that exhibits ~20-fold enhancement of fluorescence upon binding to AT regions of dsDNA. It is excited by the violet (405 nm) laser line and is commonly used as a nuclear counter stain in fluorescence microscopy.

Materials

- DAPI dye (D9542, Sigma-aldrich, USA)
- PBS
- 24 well culture plate

Method

Cells were seeded in 12-well plates and exposed to Low dose (IC_{50} / 20), moderate dose (IC_{50} / 10) and high dose (IC_{50} / 5) of Fenitrothion for 0, 24, 48, 72 and 96 hrs for SoMG. The cells were washed twice with PBS, stained in DAPI dye liquor at a final concentration of 1 mg/ml, and incubated in dark for 15 min at 28 ºC. After washing with PBS, samples were observed using a fluorescence microscope.

ROS gene expression

The cells were harvested and cell count as well as cell viability were checked by cell viability assay by using Hemocytometer. 2 $x10^4$ Cells/ ml were seeded in to 6 well plates (HiMedia, India). After 24, 48, 72 and 96 hrs RNA was isolated from the treated cells. RNA isolation and cDNA preparation was carried out by the method as described earlier in the chapter 2. RT-PCR was carried out to check the gene expression by using the ROS candidate specific primers. Primers were designed using NCBI Primer 3 designing software and the optimum conditions that were taken into consideration as described in chapter 2.

RT-PCR Amplification

Quantitative RT-PCR was performed using SYBR select Master Mix (Applied Biosystems, USA) in Quant Studio 12K (Life technology) real-time PCR machine with primers to detect selected messenger RNA (mRNA) targets. The melting curve of each sample was measured to ensure the specificity of the products. β-actin was used as an internal control to normalize the variability in the expression levels and data was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 3.1 shows the set of primers pairs used for qPCR

Accession No.	Gene Name	Sequence	Tm	Amplicon size (bp)
XM_022975529.1	β –actin F	5'-GTATCGTGCTGGACTCCGGT-3'	61.0	154
	β–actin R	5'-GTGAAGGAGTAGCCACGCT-3'	59.0	
MH177591.1	GST F	5'-AATCCCCAGCATACAGTGCC-3'	59.0	160
	GST R	5'- CGAAGTGGAGTCGTTGGTCA-3'	59.0	
XM_022976902.1	Catalase F	5'– TGGCATTGAGCCATCTCCTG-3'	59.0	109
	Catalase R	5'- ACAATTGACGGGTATGTGGAGA-3'	58.0	
XM_022958483.1	SOD F	5'-ATGTCGGTGACCTCGGAAAC-3'	59.0	150
	SOD R	5'- AGCCTAGGTCATCCTCGTGT-3'	59.0	

Table 3.1: Depicts the primer sequence with its annealing temperature and product size for the ROS gene expression of SoMG cell line

Apoptosis Detection Assay (APOAF, Sigma-aldrich, USA)

Principle

Annexin V-FITC is a fluorescent probe which binds to phosphatidylserine in the presence of calcium. Apoptosis, or programmed cell death, is a mechanism of cells used to negatively select cells that are deleterious to the host. The cellular changes involved in the process include loss of phospholipid asymmetry during the early stages of apoptosis. In living cells, phosphatidylserine is transported to the inside of the lipid bilayer by the Mg-ATP dependent enzyme, aminophospholipid translocase. At the onset of apoptosis, phosphatidylserine, which is normally found on the internal part of the plasma membrane, becomes translocated to the external portion of the

membrane. The phosphatidylserine becomes available to bind to the annexin V-FITC conjugate in the presence of calcium. The procedure consists of the binding of annexin V FITC to phosphatidylserine in the membrane of cells, which are beginning the apoptotic process, and the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been totally compromised. The cells are incubated with annexin V-FITC and propidium iodide. After a 10 minute incubation period at room temperature the cells are analyzed by flow cytometry.

Method

The SoMG cells treated with the LD, MD and HD of fenitrothion in 1x 10^5 cells/ml suspension were incubated at 24, 48, 72 and 96 hrs. The control of non-treated SoMG cells at 1 x 10^5 cells/ml was considered at a zero time data point. The cell were washed twice with distill PBS. The cells were re-suspended in Binding Buffer at a concentration of 1 x 10^5 cells/ml and 500 ml of the apoptotic cell suspension was added to a plastic 12 x 75 mm test tube. 500 ml of the non-induced cell suspension was added to a second plastic 12 x 75 mm test tube. Further, 5 µl of Annexin V FITC Conjugate and 10 µl of Propidium Iodide solution was added to each cell suspension and was incubated at room temperature for 10 minutes in darkness. The tubes were placed in the flow cytometer (BD FACSDIVA 6.1.2, USA) and fluorescence was determined immediately. Three characteristic of cells were noted 1. Cells with an early apoptotic process- stained with Annexin V FITC Conjugate alone, 2. Live cells- with no staining by either Propidium Iodide solution or Annexin V FITC Conjugate and 3. Necrotic cells- stained with both Propidium Iodide Solution and Annexin V FITC Conjugate.

Statistical analysis

The computed data was analyzed using PRISM 6 software. One and two way ANOVA followed by DUNNETs multiple comparison test were used to the test for significant differences among the individual treatment combinations. Statistical significance was accepted at $p<0.05$ for all tests.

3.3 Results

Cell viability Assay

To have a comparative analysis, Sf9 was selected as a positive control. The viability of Sf9 cells and SoMG treated with increasing concentrations of organophosphates (malathion and fenitrothion) for 24, 48, 72 and 96 hrs was assessed by MTT assay. The results showed that organophosphates inhibited cell vitality in a dose and time dependent manner at concentrations ranging from 25 to 250 µg/ml (Fig 3.1) and that for malathion in a ranged from 50 to 1000 µg/ml (Fig 3.2) in both the cell lines. The IC_{50} value for fenitrothion treatments at 48 h for SoMG cells was 100 µg/ml (Fig. 3.1), and that for malathion was 331.76 µg/ml (Fig 3.2), similarly the IC_{50} results for Sf9 cells was 179 µg/ml for fenitrothion and 415 µg/ml for malathion respectively (Fig. 3.3 and Fig. 3.4).

Toxicity bioassays showed that fenitrothion was more toxic for SoMG (IC_{50} =100 µg/ml) and for Sf9 (IC_{50}= 179 µg/ml) compared to malathion for SoMG was the most toxic (IC_{50}=331.76 µg/ml) and for Sf9 (IC_{50}= 415 µg/ml). However, to observe the alterations, three sub-lethal concentrations low, medium and high doses for SoMG i.e. 16.5(LD), 33.1(MD), and 66.3 (HD) and 5(LD), 10 (MD) and 20 (HD) were selected for fenitrothion and malathion respectively. Likewise the sub-lethal concentrations for Sf9 cells was 8.9 (LD), 17.93(MD) and 35.8(HD) µg/ml for fenitrothion and 20.7(LD), 41.5(MD) and 83(HD) µg/ml was for malathion (Table 3.2).

	Fenitrothion				Malathion			
	IC_{50} (µg/ml)	LD (µg/ml)	MD (µg/ml)	HD (µg/ml)	IC_{50} (µg/ml)	LD (µg/ml)	MD (µg/ml)	HD (µg/ml)
SoMG	100	5	10	15	331	8.9	17.93	35.8
Sf9	179	16.5	33.1	66.3	415	20.7	41.5	83

Table 3.2: Shows the IC_{50} values And their Sub lethal doses LD(IC_{50}/20), MD (IC_{50}/10) and HD (IC_{50}/5) for the fenitrothion and malathion on SoMG cell line as well as Sf9 cell line

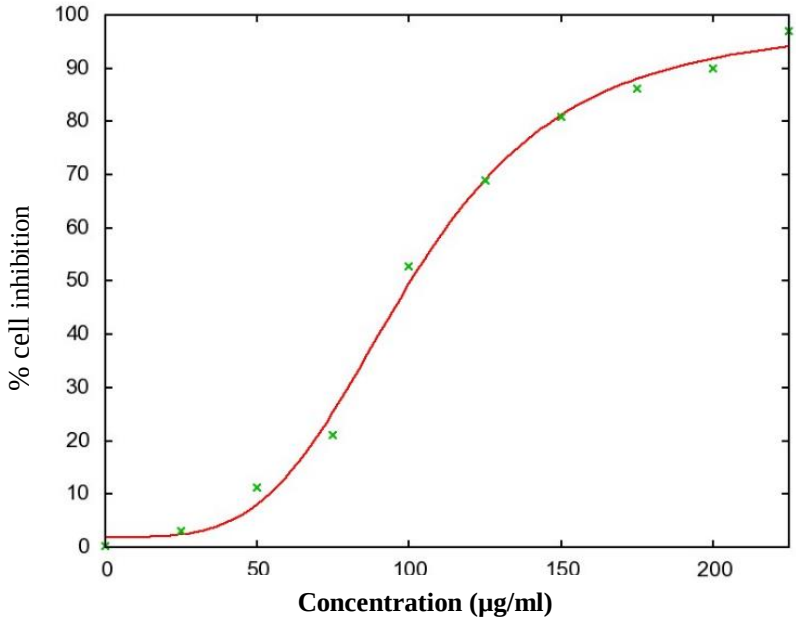

Figure 3.1: SoMG cell mortality against different concentration of fenitrothion

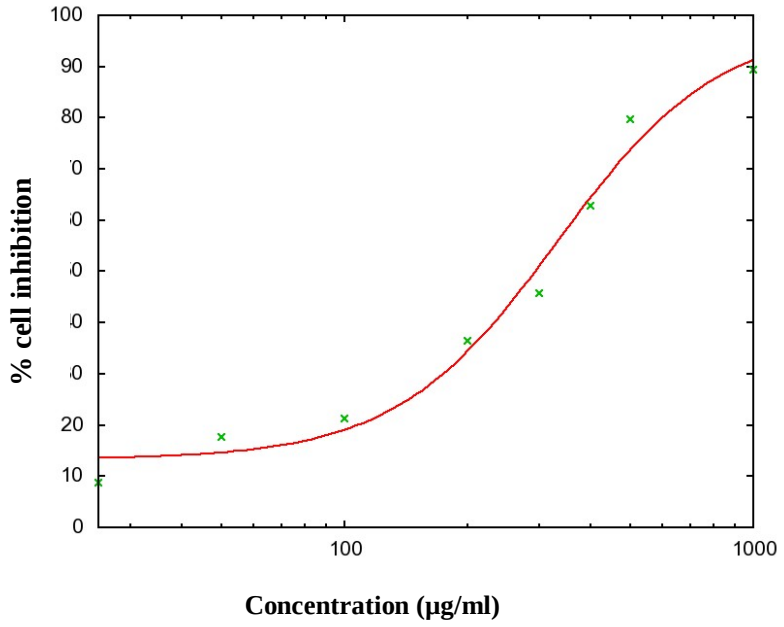

Figure 3.2: SoMG cell mortality against different concentration of malathion

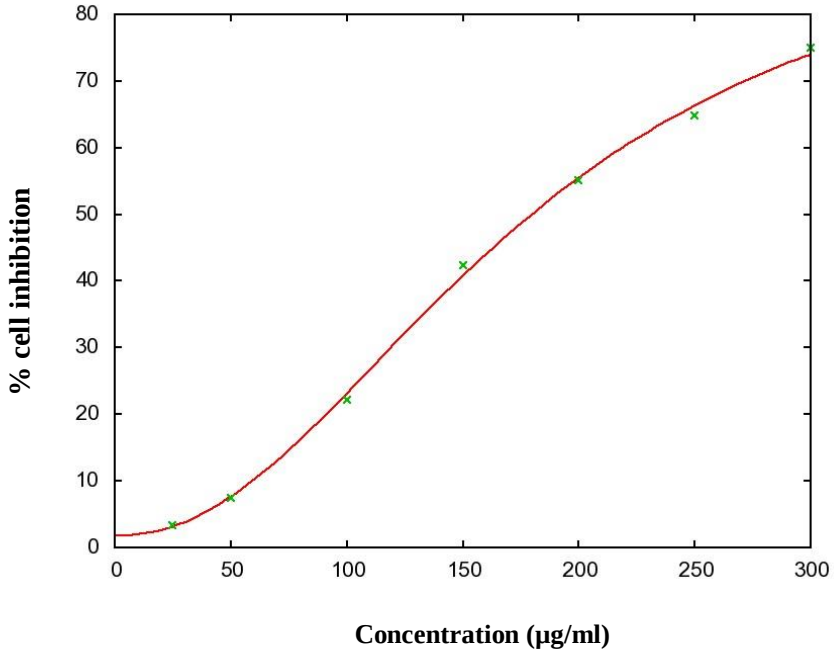

Figure 3.3: Sf9 cell mortality against different concentration of fenitrothion

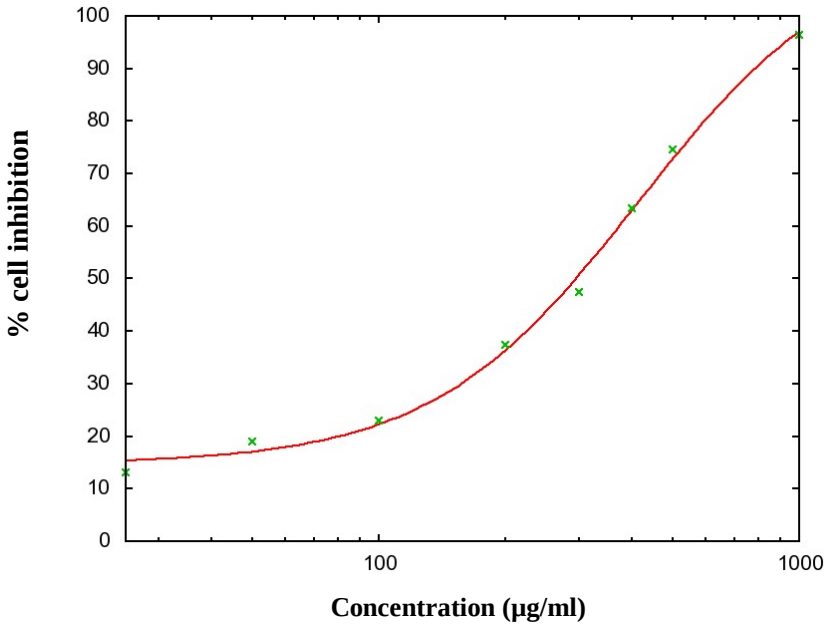

Figure 3.4: Sf9 cell mortality against different concentration of malathion

The effects of the fenitrothion and malathion on apoptosis of the SoMG cells as well as Sf9 cells were detected by qualitative as well as quantitative analysis, using AO/EB dual staining fluorescent DNA binding dyes. The cell stained in green represented AO staining while that in red/orange exhibited EB staining. The stained cells were characterized to viable (light green), early apoptotic (bright green fluorescence and condensed chromatin), late apoptotic (orange fluorescence) and nonviable cells (red coloured fluorescence) (Fig. 3.5 to fig 3.8). fenitrothion and malathion treated cells showed condensed nuclei, membrane blebbing and apoptotic bodies in an increasing trend from 24 hrs to 96 hrs compared to the control cells which showed intact nuclear architecture. A significant ($p<0.001$) dose and time dependant morphological changes as well as the induction of apoptosis in these cells were detected after treatment with fenitrothion (Fig 3.5). However, malathion treated cells exhibited less significant alterations in contrast to fenitrothion (Table 3.3 and 3.4). Hence, the further experiments were then continued only for fenitrothion as it was proved to be more toxic by MTT assay as well as AO/EB staining compared to malathion.

Acridine orange/ Ethidium bromide staining

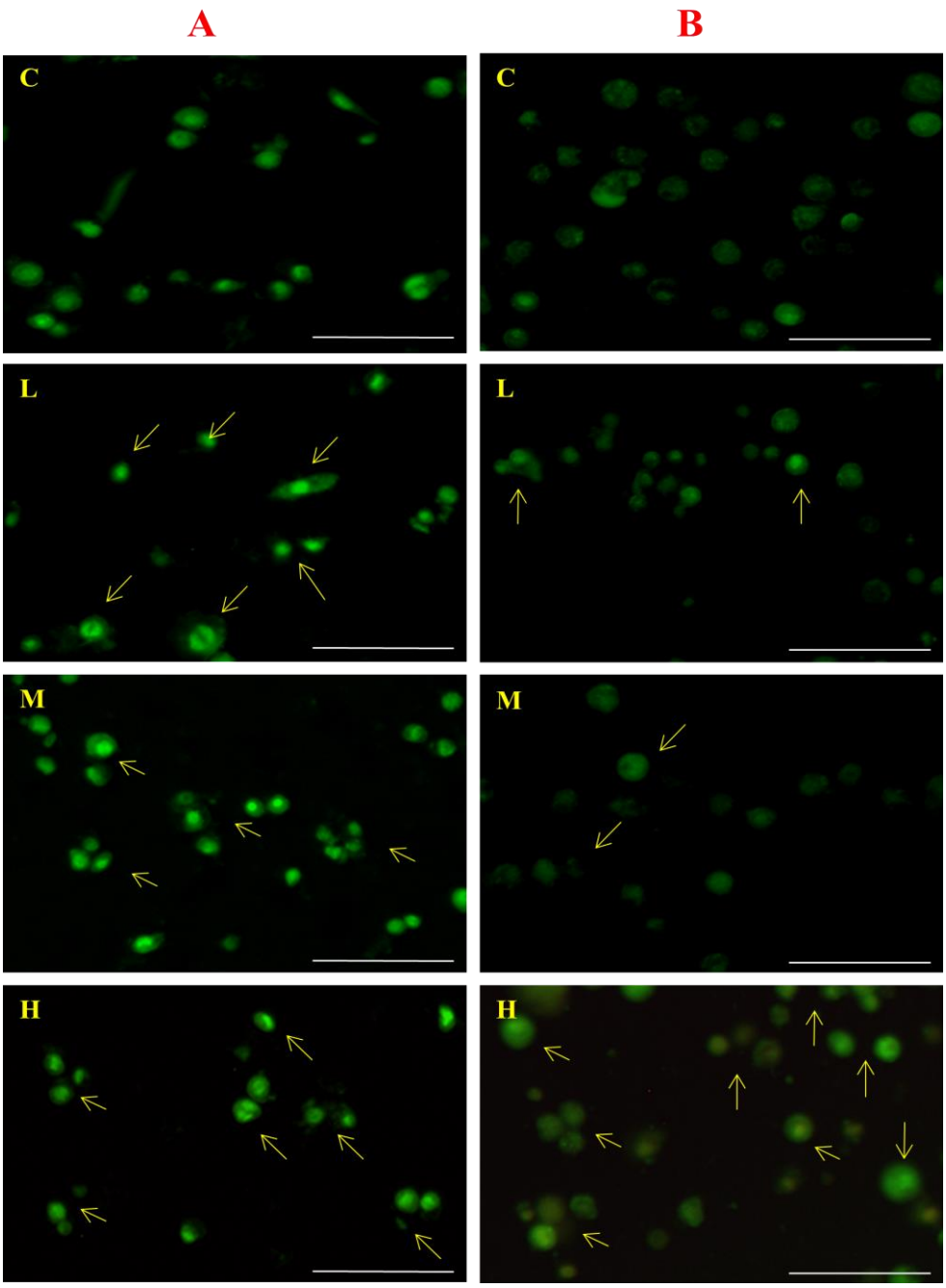

Figure 3.5: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 24 hrs of treatment with sublethal concentrations (LD, MD and HD) of fenitrothion in an increasing concentrations of 1)5, 2)10 and 3)20 µg/ml. (B) Sf9 cells treated with fenitrothion in an increasing concentration of 1)16.5, 2) 33.1 and 3)66.3 µg/ml are given. Bar =50µM

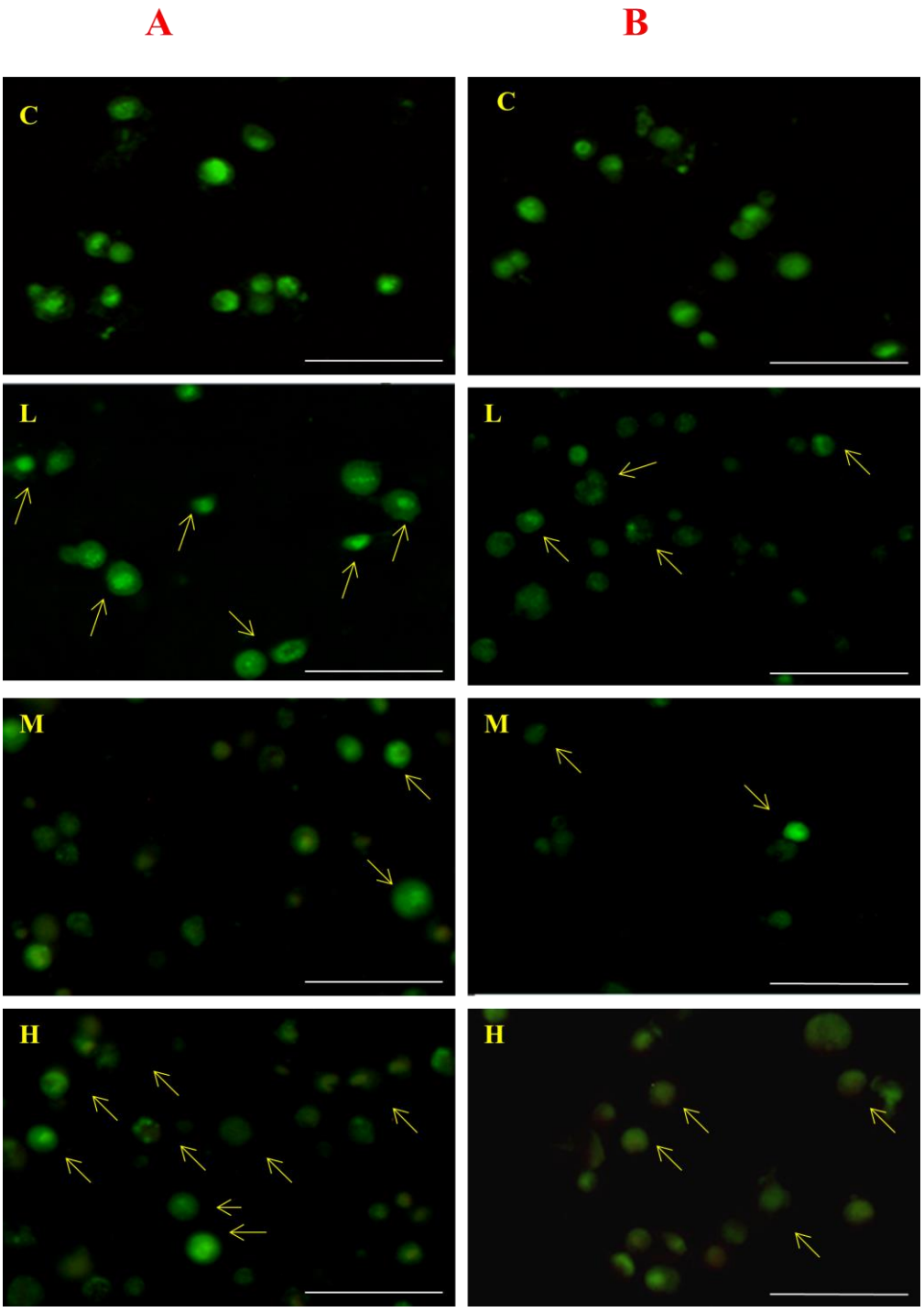

Figure 3.6: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 48 hrs of treatment with sublethal concentrations (LD, MD and HD) of fenitrothion in an increasing concentrations of 1)5, 2)10 and 3)20 µg/ml. (B) Sf9 cells treated with fenitrothion in an increasing concentration of 1)16.5, 2) 33.1 and 3)66.3 µg/ml are given. Bar =50µM

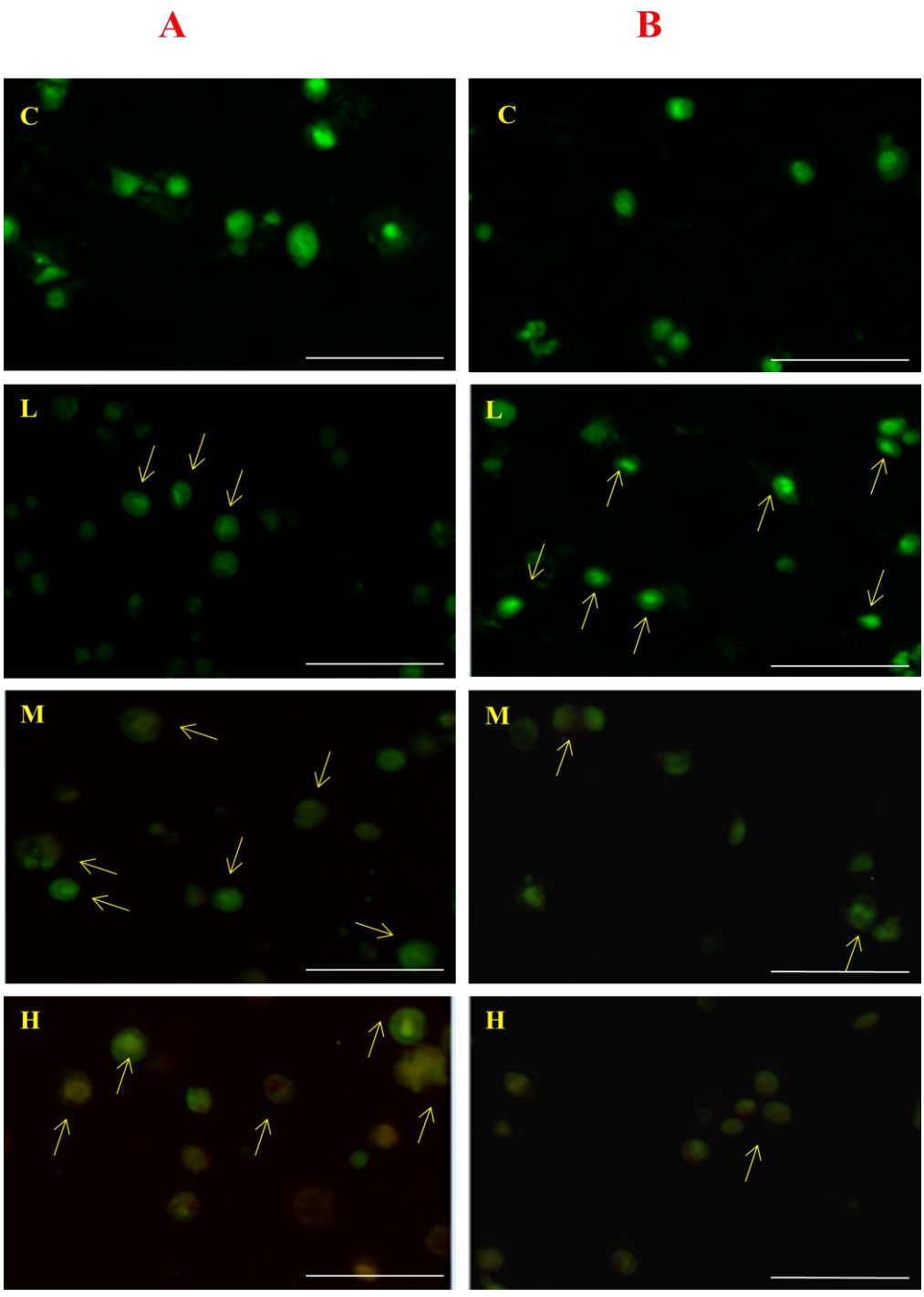

Figure 3.7: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 72 hrs of treatment with sublethal concentrations (LD, MD and HD) of fenitrothion in an increasing concentrations of 1) 5, 2) 10 and 3) 20 µg/ml. (B) Sf9 cells treated with fenitrothion in an increasing concentration of 1)16.5, 2)33.1 and 3)66.3 µg/ml are given. Bar =50µM

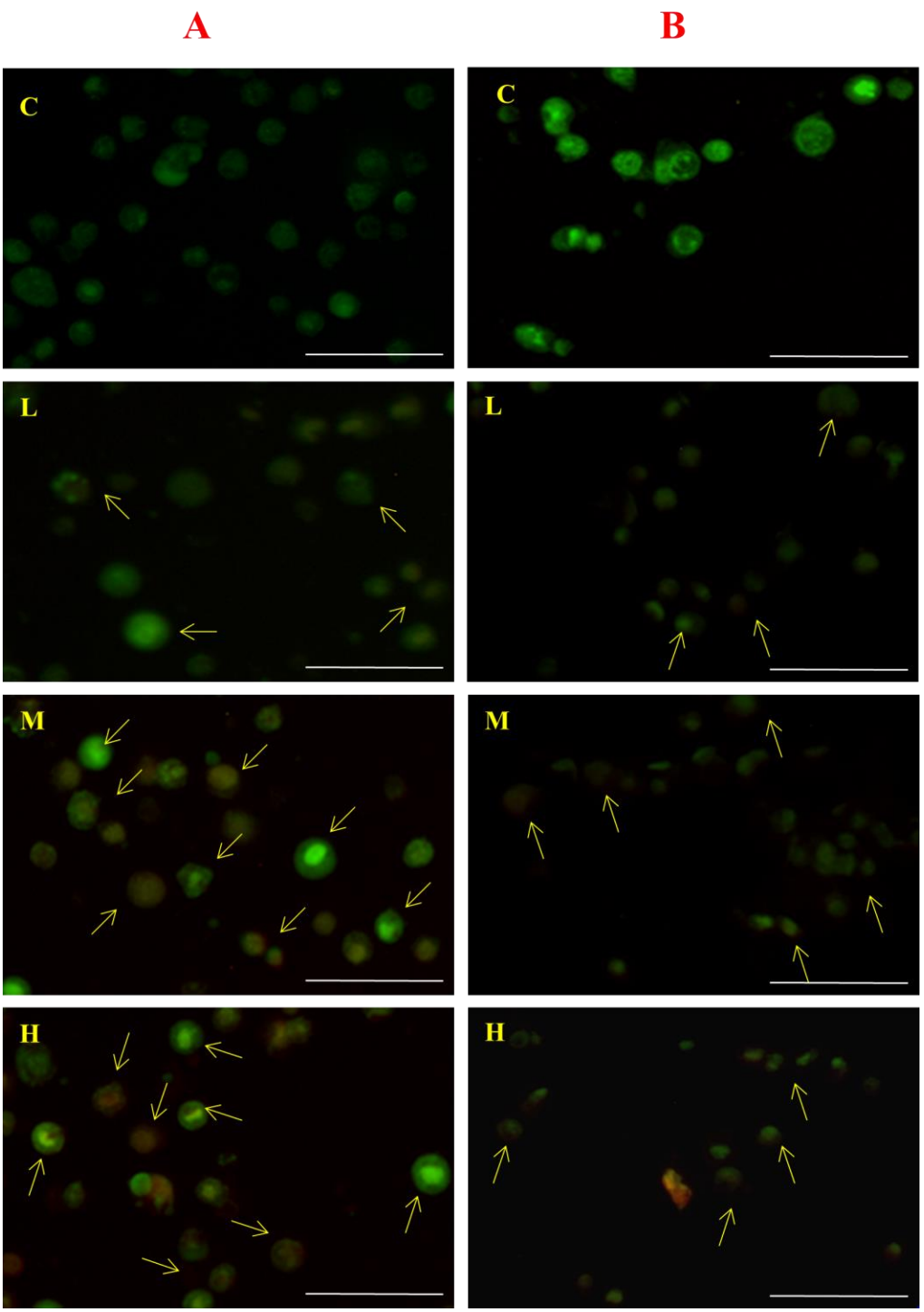

Figure 3.8: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 96 hrs of treatment with sublethal concentrations(LD, MD and HD) of fenitrothion in an increasing concentrations of 5,10 and 20 µg/ml. (B) Sf9 cells treated with fenitrothion in an increasing concentration of 16.5, 33.1 and 66.3 µg/ml are given. Bar =50µM

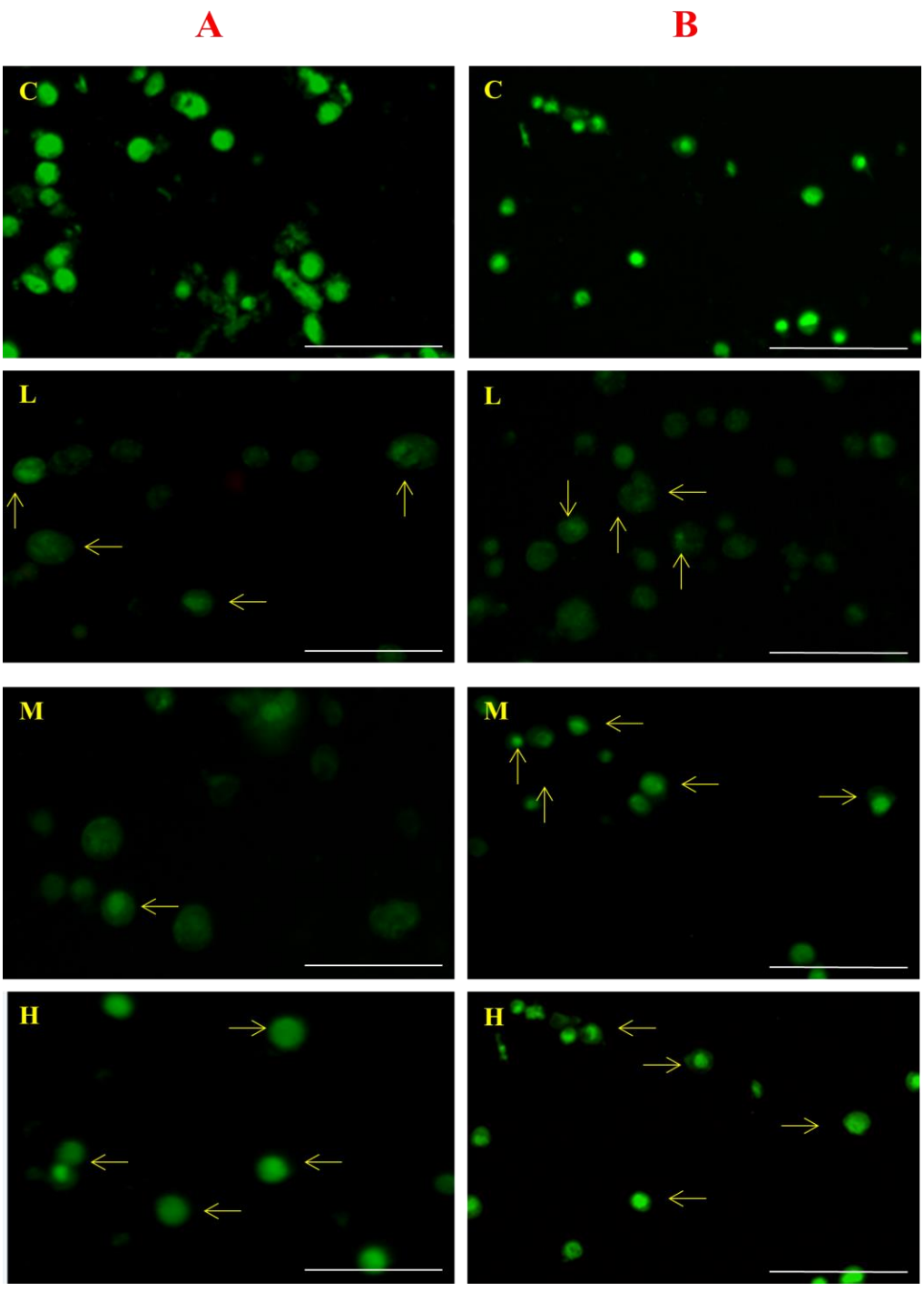

Figure 3.9: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 24 hrs of treatment with sublethal concentrations(LD, MD and HD) of malathion in an increasing concentrations of 8.9, 17.93,35.8 µg/ml. (B) Sf9 cells treated with malathion in an increasing concentration of 20.7, 41.5, 83 µg/ml are given. Bar =50µM

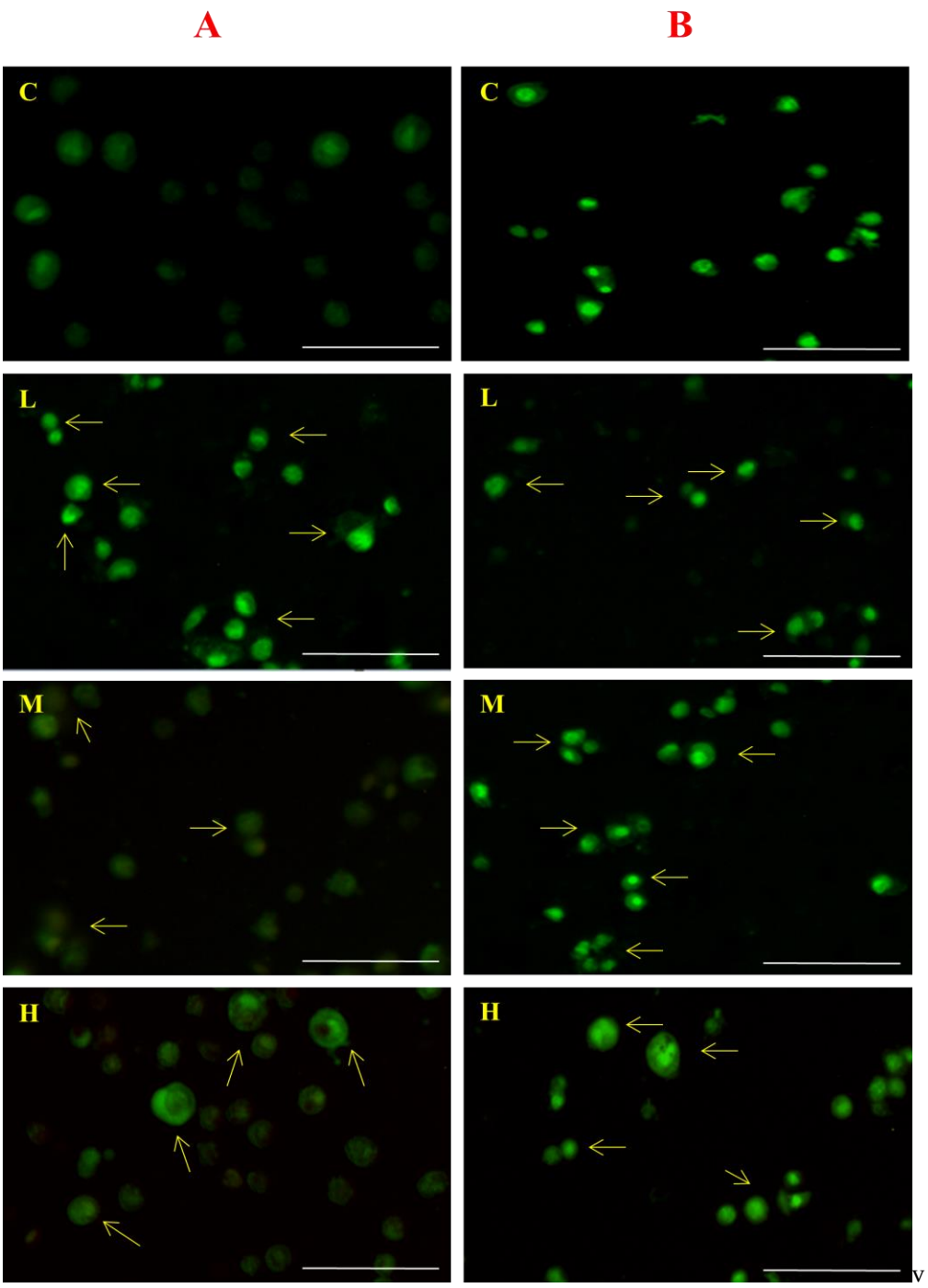

Figure 3.10: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 48 hrs of treatment with sublethal concentrations(LD, MD and HD) of malathion in an increasing concentrations of 8.9, 17.93,35.8 µg/ml. (B) Sf9 cells treated with malathion in an increasing concentration of 20.7, 41.5, 83 µg/ml are given. Bar =50µM

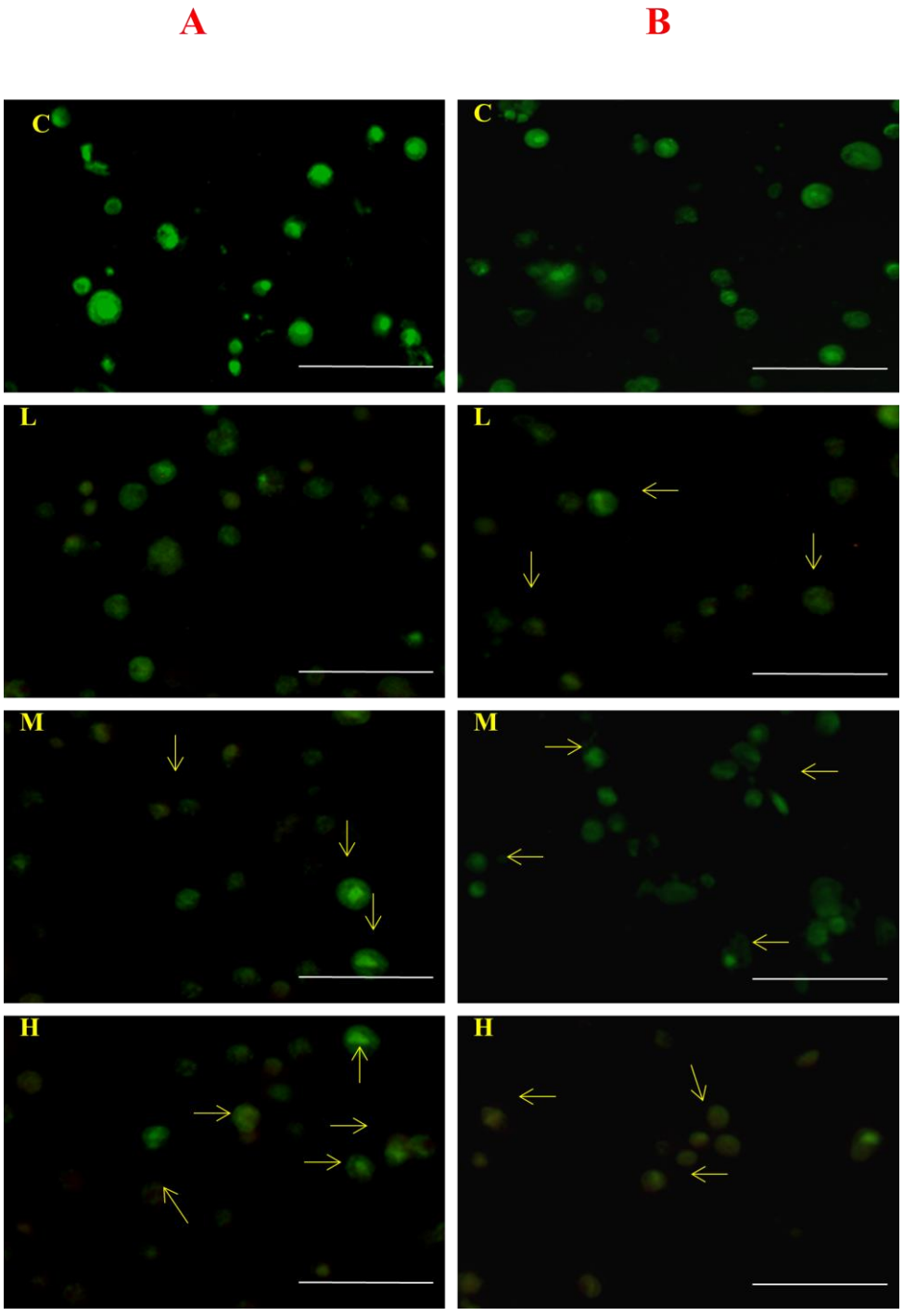

Figure 3.11: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 72 hrs of treatment with sublethal concentrations(LD, MD and HD) of malathion in an increasing concentrations of 8.9, 17.93,35.8 µg/ml. (B) Sf9 cells treated with malathion in an increasing concentration of 20.7, 41.5, 83 µg/ml are given. Bar =50µM

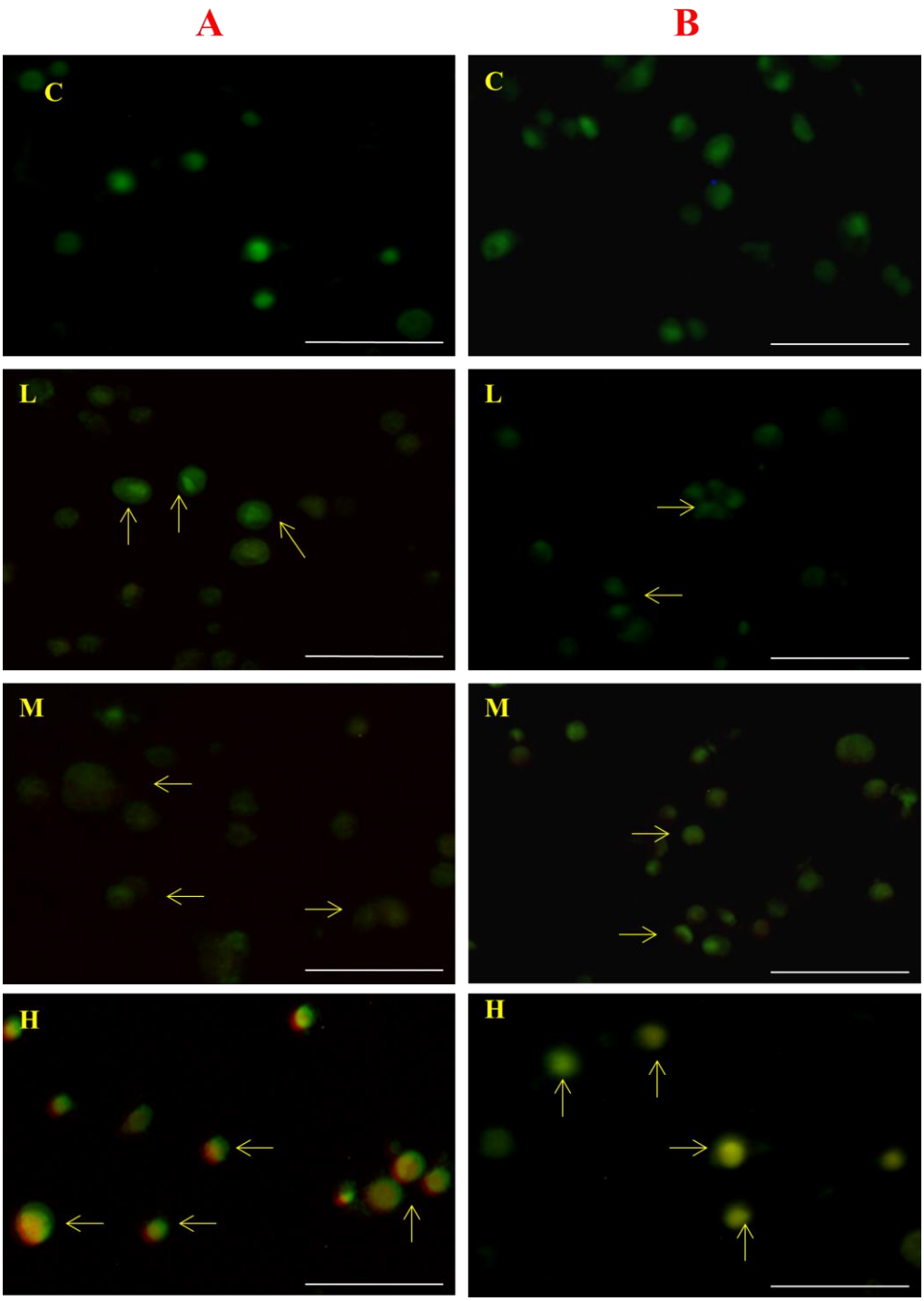

Figure 3.12: Results of Acridine orange/Ethidium bromide assay for (A) SoMG and (B) Sf9 cells after 96 hrs of treatment with sublethal concentrations(LD, MD and HD) of malathion in an increasing concentrations of 8.9, 17.93,35.8 µg/ml. (B) Sf9 cells treated with malathion in an increasing concentration of 20.7, 41.5, 83 µg/ml are given. Bar =50µM

Time (Hrs)	Sub-lethal Dose	Cell Shrinkage		Rounding of cells		Blebbing of cells		Condensation of chromatid	
		SoMG	Sf9	SoMG	Sf9	SoMG	Sf9	SoMG	Sf9
24	Control	-	-	-	-	-	-	-	-
	LD	-	-	+	-	-	-	++	+
	MD	-	-	+	-	+	-	-	+
	HD	-	-	++	+	++	-	-	+
48	Control	-	-	-	-	-	-	-	-
	LD	-	+	++	-	+	-	+	+
	MD	-	+	+	+	+	-	++	+
	HD	-	+	++	+	++	-	++	+
72	Control	-	-	-	-	-	-	-	-
	LD	-	+	++	-	+	+	++	+
	MD	-	-	+++	+	++	+	++	+
	HD	-	-	+++	+	+++	+	++	+
96	Control	-	-	-	-	-	-	-	-
	LD	-	+	+++	-	++	+	+++	++
	MD	-	+	+++	-	+++	+	+++	+
	HD	-	++	+++	-	+++	+	+++	-

Table 3.3: Effect of fenitrothion on SoMG and Sf9 Cell line in a dose and time dependant manner

Note: (-) → Normal, (+) → mild, (++) → moderate, and (+++) → maximum effect as compare to control

Time (Hrs)	Sub-lethal Dose	Cell Shrinkage		Rounding of cells		Blabbing of cells		Condensation of chromatin	
		SoMG	Sf9	SoMG	Sf9	SoMG	Sf9	SoMG	Sf9
24	Control	-	-	-	-	-	-	-	-
	LD	-	-	+	-	-	-	+	+++
	MD	-	+	++	+	++	-	+	++
	HD	-	++	-	-	+++	-	++	++
48	Control	-	-	-	-	-	-	-	-
	LD	-	+	+	-	-	-	+	+++
	MD	-	++	+	+	+	-	++	+
	HD	-	++	+++	+	+++	+	+++	++
72	Control	-	-	-	-	-	-	-	-
	LD	-	+	+++	-	++	+	++	+
	MD	-	-	+++	+	+++	+	++	+
	HD	+	-	+++	++	+++	+	++	++
96	Control	-	-	-	-	-	-	-	-
	LD	-	-	++	+	+	+	++	++
	MD	-	-	++	+	+++	++	+	++
	HD	+	-	-	-	+++	++	+	+

Table 3.4: Effect of malathion on SoMG and Sf9 Cell line

Note: (-) → Normal, (+) → mild, (++) → moderate, and (+++) → maximum effect as compare to control

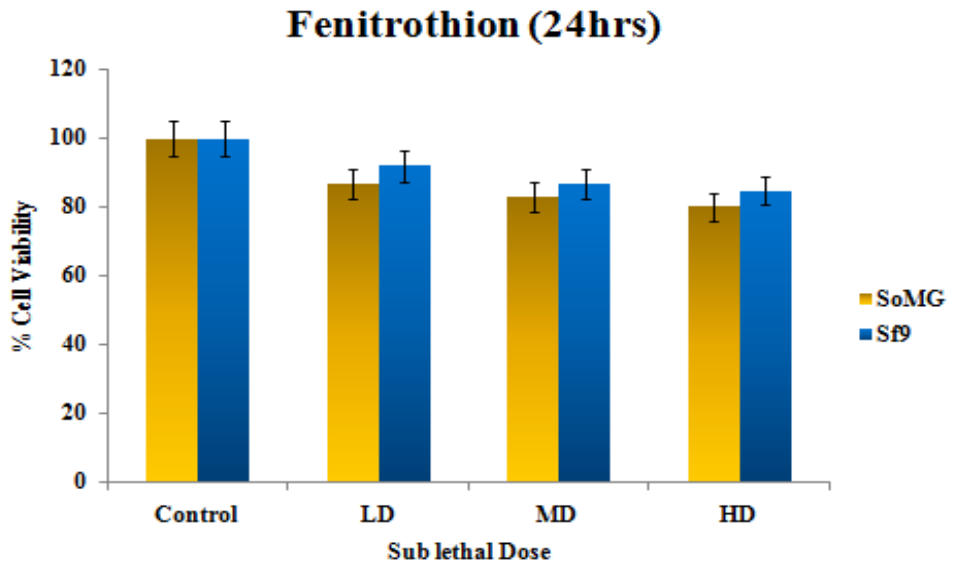

Figure 3.13: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of fenitrothion at 24 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

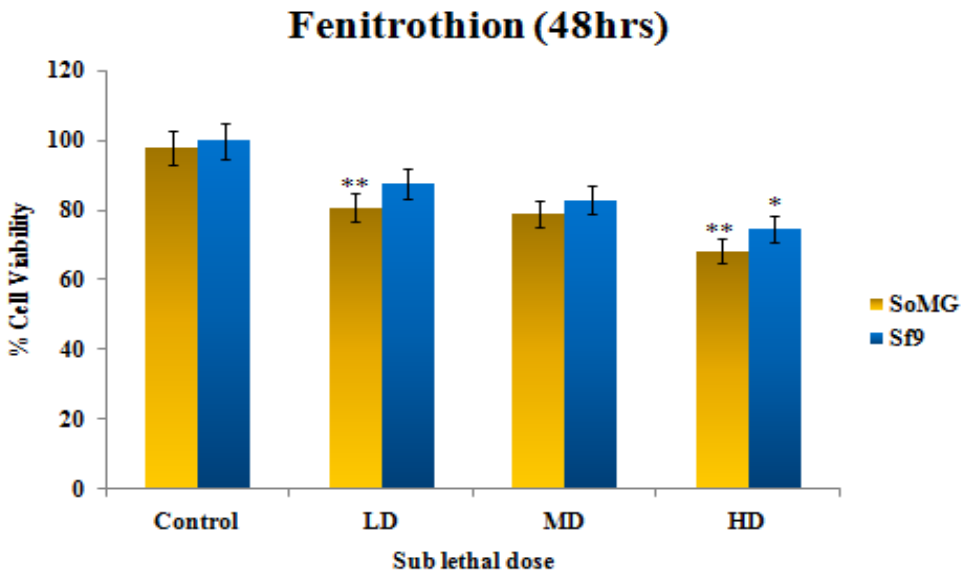

Figure 3.14: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of fenitrothion at 48 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

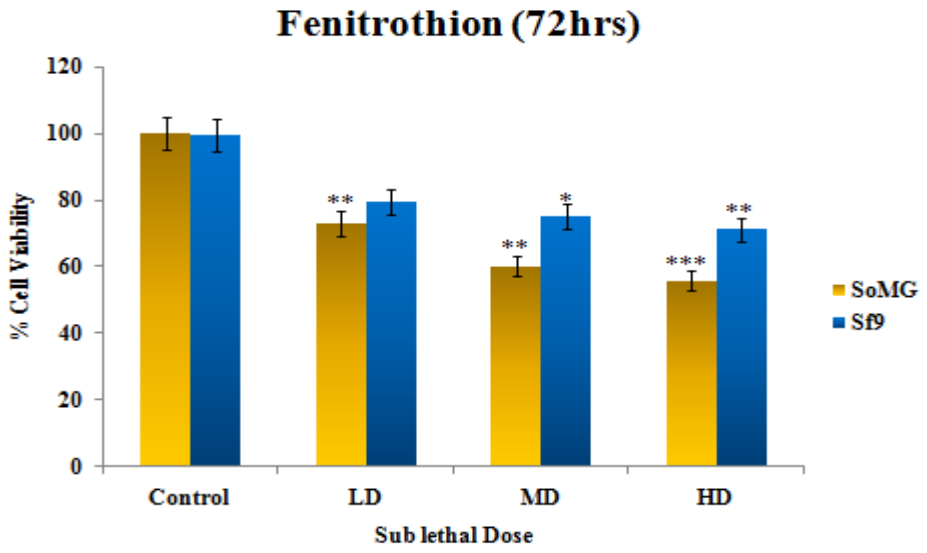

Figure 3.15: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of fenitrothion at 72 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

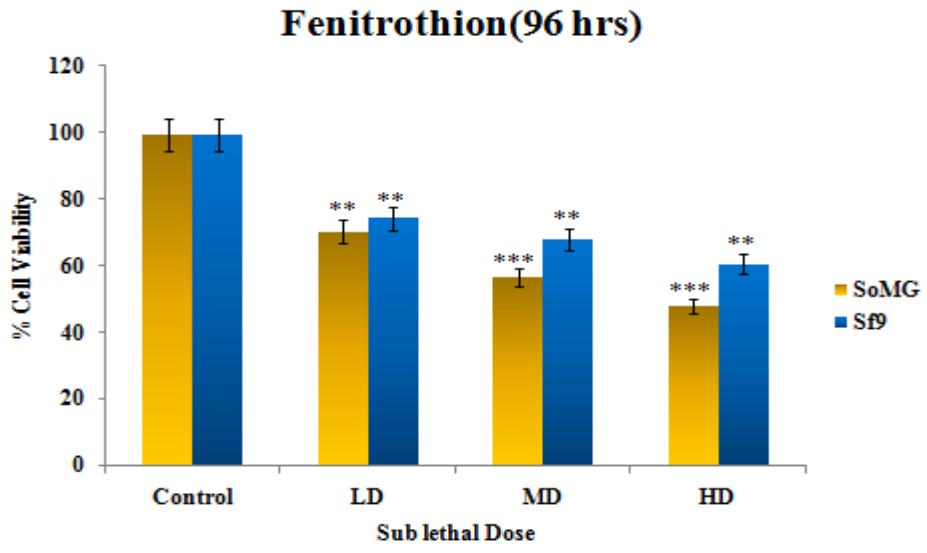

Figure 3.16: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of fenitrothion at 96 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

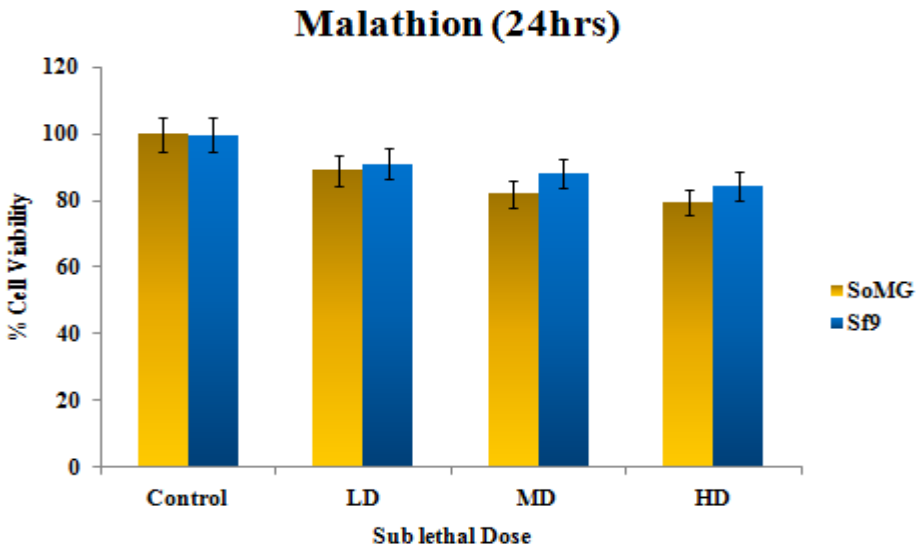

Figure 3.17: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of malathion at 24 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

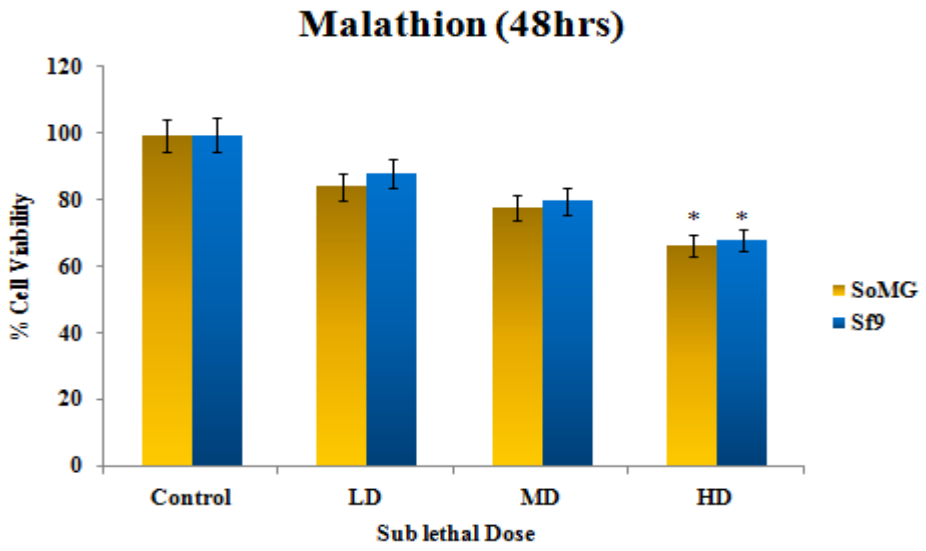

Figure 3.18: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of malathion at 48 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

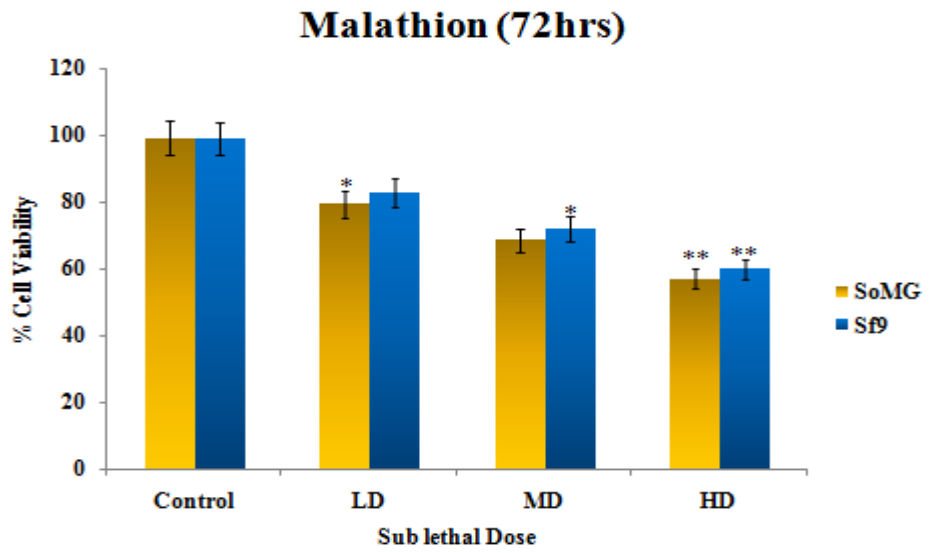

Figure 3.19: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of malathion at 72 hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

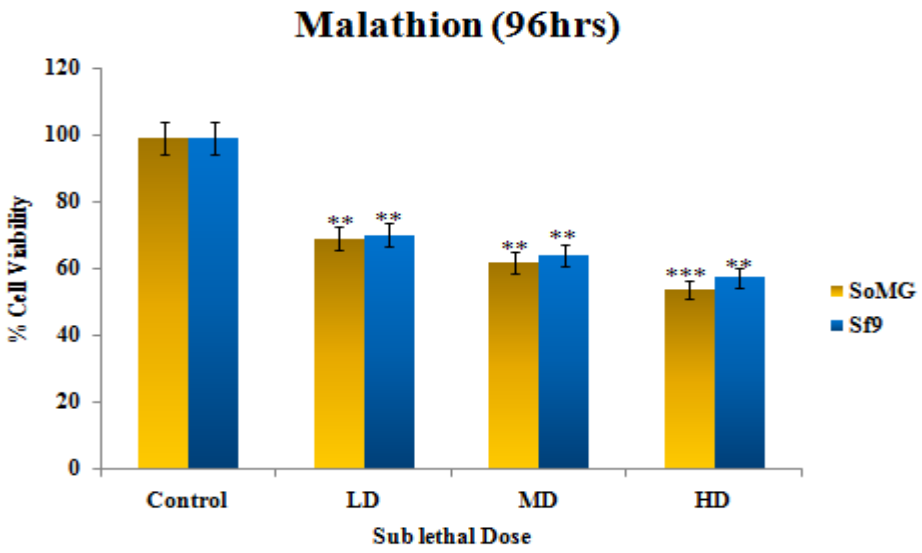

Figure 3.20: Depicts the fluorescence in SoMG and Sf9 cells treated with sub-lethal doses of malathion at 96hrs and stain with AO/EB

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Time (Hrs)	Sub-lethal Dose	Fenitrothion		Malathion	
		SoMG	Sf9	SoMG	Sf9
24	Control	99.99±0.02^{ns}	99.98±0.09^{ns}	99.89±0.66^{ns}	99.78±0.34^{ns}
	LD	86.9±0.45^{ns}	92.19±0.23^{ns}	89.08±0.23^{ns}	91.03±0.89^{ns}
	MD	83.02±0.56*	87.03±0.02^{ns}	82.04±0.79**	88.09±0.02^{ns}
	HD	80.09±0.6*	84.89±0.09**	79.47±0.89*	84.32±0.66**
48	Control	98.08±0.02^{ns}	99.94±0.23^{ns}	99.45±0.56^{ns}	99.59±0.02^{ns}
	LD	80.65±0.44*	87.5±0.56^{ns}	84.04±0.23	88.05±0.09**
	MD	79.07±0.66**	82.9±0.66^{ns}	77.67±0.66*	79.78±0.02**
	HD	68.29±0.23**	74.78±0.02*	66.56±0.02**	68.05±0.23**
72	Control	99.91±0.66^{ns}	99.34±0.44^{ns}	99.39±0.44^{ns}	99.28±0.56^{ns}
	LD	73.07±0.02^{ns}	79.45±0.66^{ns}	79.41±0.02^{ns}	82.98±0.34^{ns}
	MD	60.13±0.09*	75.04±0.44*	68.73±0.56**	72.09±0.02**
	HD	55.9±0.66*	71.28±0.02*	57.09±0.02***	60.08±0.79***
96	Control	99.24±0.34^{ns}	99.25±0.66^{ns}	99.12±0.79^{ns}	99.45±0.34^{ns}
	LD	70.29±0.02**	74.25±0.02**	69.09±0.56^{ns}	70.1±0.02**
	MD	56.56±0.79**	68.03±0.89***	61.94±0.79**	64.09±0.34***
	HD	48±0.09**	60.47±0.89***	54.03±0.56***	57.39±0.66***

Table 3.5: Depicts the mean± SD values of % cell viability in SoMG and Sf 9cells treated with sub-lethal doses of fenitrothion and malathion for 24, 48, 72 and 96 hrs and stain with AO/EB

Each value represents the mean ± SEM. (n=3)

Significant level indicated by ^{ns}= non significant, *= (p<0.05); **=(p<0.01); *=(p<0.001)**

DAPI staining

As we were interested in a more detailed characterization of the nature of fenitrothion induced cell death, we aimed to clarify whether it was accompanied by typical biochemical hallmarks of apoptotic cell death, such as changes in nuclear morphology/DNA fragmentation. Alterations in the nuclear morphology was analyzed microscopically after staining with the DNA dye 4',6-diamidino-2-phenylindole (DAPI). At 24hrs, most nuclei in untreated control cell displayed normal morphology, while Fenitrothion treated cell exhibited condensed nuclear chromatin and the percentage of apoptotic cells were identified by the abrupt nuclear membrane with reduced nuclear periphery having fragmented morphology of nuclear bodies, indicative for apoptotic cell death (Fig 3.21 A). At 48hrs, the fenitrothion exposure lead to increase in the intensity of apoptotic body formation compared to 24hrs ($p<0.001$) (Fig 3.21 A and B, Table 3.7). The results of SoMG cells under the exposure of fenitrothion at 72hrs revealed that nuclear membrane was further perturbed and the chromatin material was highly condensed ($p<0.001$) (Table 3.7). 96hrs exposure of the insecticide Fenitrothion resulted in the maximum change in the morphology of nucleus compared to control (Fig: 3.22 A and B). The results of AO/EB and DAPI staining, together suggest that apoptosis was amplified in treated group.

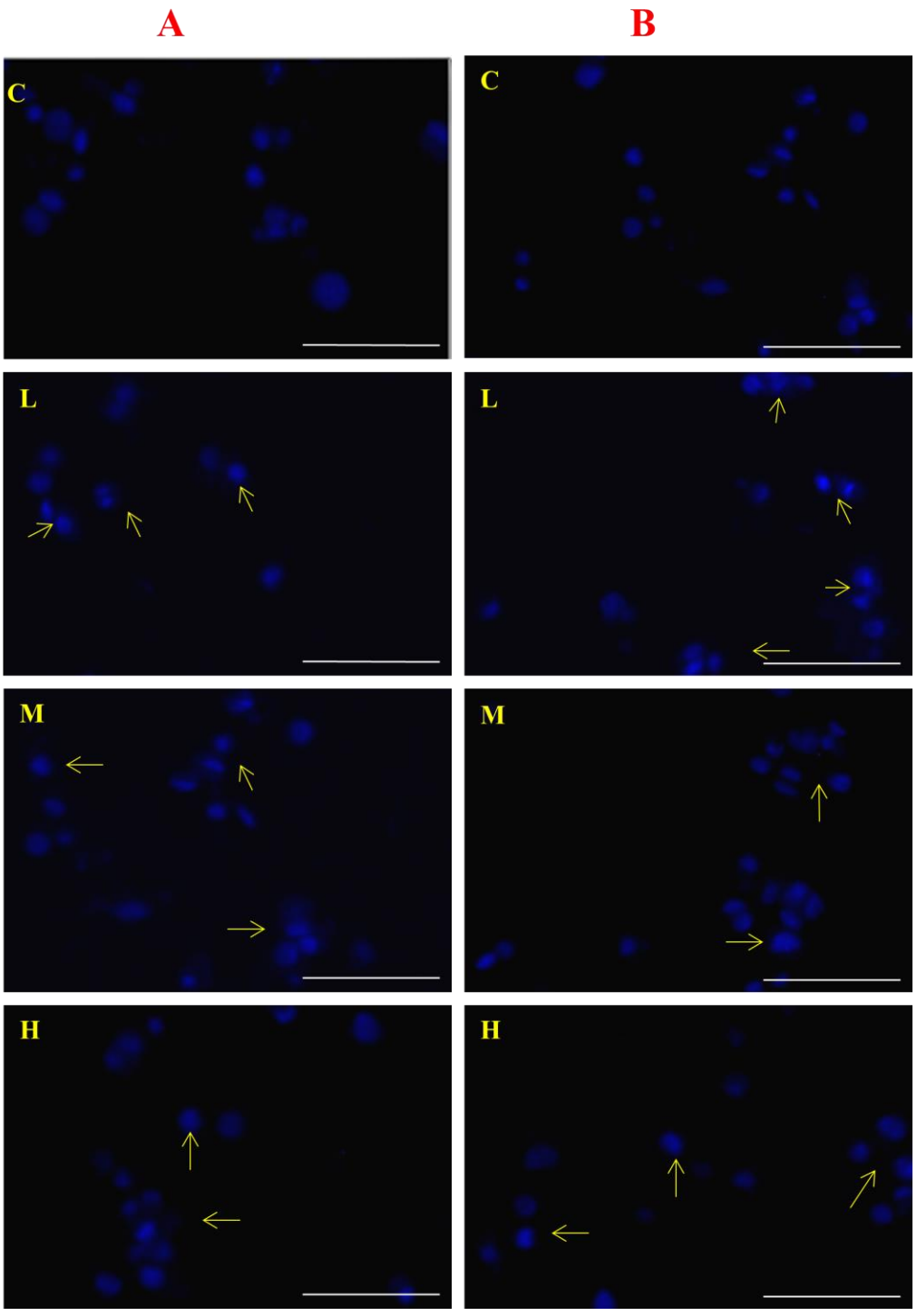

Figure 3 21: Results of DAPI staining for SoMG cell line after (A) 24 hrs and (B) 48 hrs of treatment with sublethal concentrations(LD, MD and HD) of Fenitrothion in an increasing concentrations of 5,10, 15 µg/ml. Bar =50µM

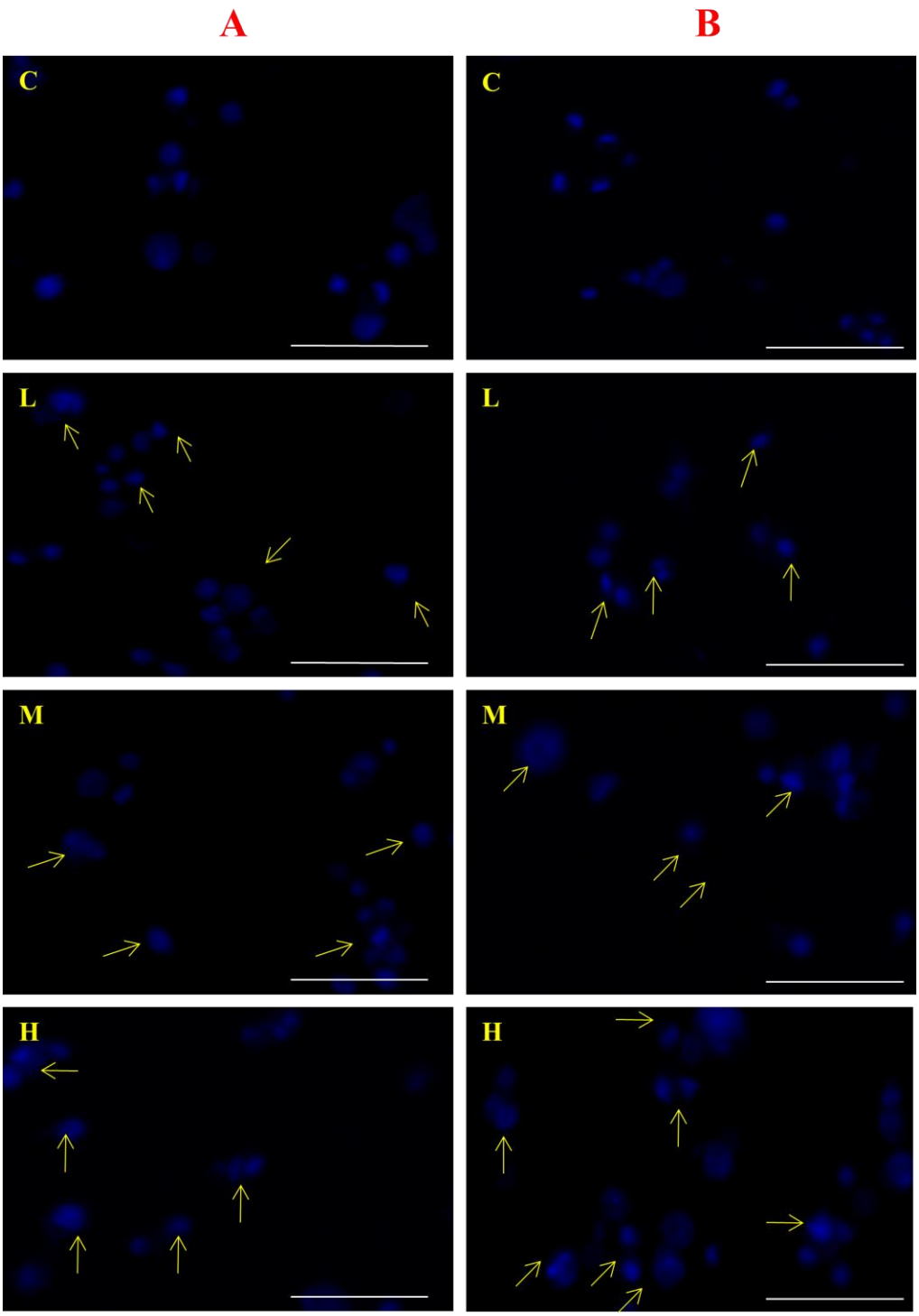

Figure 3.22: Results of DAPI staining for SoMG cell line after (A) 72 hrs and (B) 96 hrs of treatment with sublethal concentrations(LD, MD and HD) of Fenitrothion in an increasing concentrations of 5, 10,15 µg/ml. Bar =50µM

Time (Hrs)	Sub-lethal Dose	Normal cells	Cells having condensed chromatids	Cells having abrupt nucleus	Cells having nuclear membrane reduced
24	**Control**	17	0	0	0
	LD	5	6	0	0
	MD	3	6	0	3
	HD	0	8	0	0
48	**Control**	19	0	0	0
	LD	5	6	1	0
	MD	8	7	2	2
	HD	4	7	2	2
72	**Control**	9	1	0	0
	LD	6	8	2	3
	MD	0	9	1	0
	HD	2	9	3	3
96	**Control**	15	2	0	0
	LD	2	7	1	2
	MD	2	5	3	2
	HD	0	0	8	9

Table 3.6: Depicts the no of cells with alter cell morphology in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs and stain with DAPI

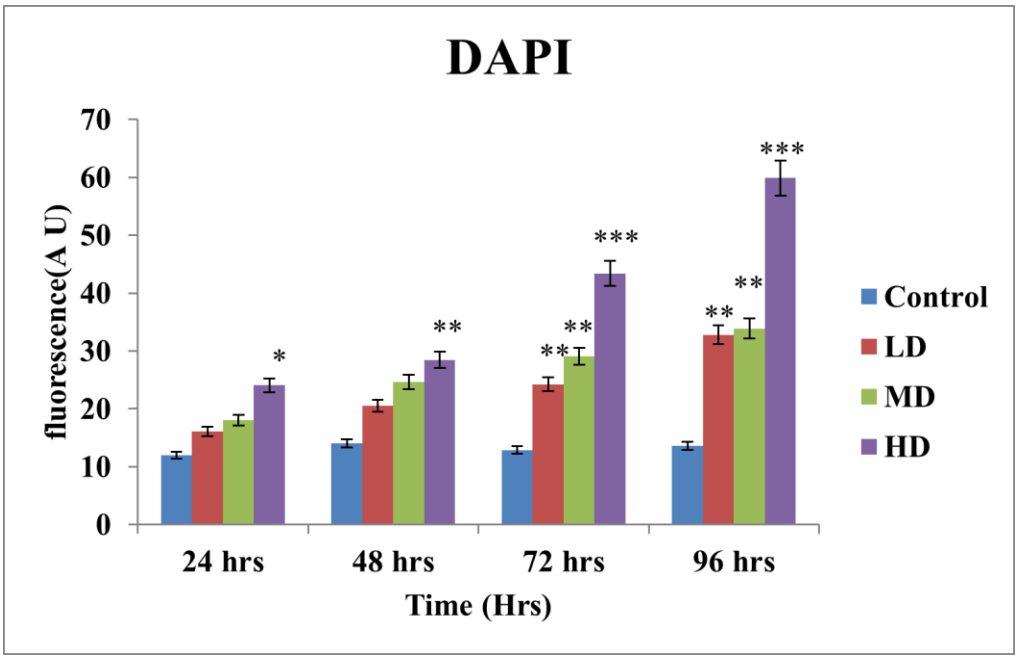

Figure 3.23: Depicts the fluorescence in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs and stain with DAPI

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

	24 hrs	48 hrs	72 hrs	96 hrs
Control	12.03±0.02^{ns}	14.09±0.02 ^{ns}	12.89±0.03 ^{ns}	13.63±0.09 ^{ns}
LD	16.07±0.08 ^{ns}	20.56±0.04 ^{ns}	24.25±0.05**	32.78±0.02**
MD	18.04±0.03 ^{ns}	24.67±0.06 ^{ns}	29.04±0.04**	33.89±0.06**
HD	24.08±0.09*	28.47±0.06**	43.37±0.07***	59.87±0.03***

Table 3.7: Depicts the mean± SEM values of fluorescence in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs and stain with DAPI

Each value represents the mean ± SEM. (n=3)

Significant level indicated by ^{ns}= non significant, *= (p<0.05); **= (p<0.01); *= (p<0.001)**

DCFH-DA Staining

Intracellular ROS level was analyzed by fluorescence microscopy after incubation of SoMG cells treated with LD, MD and HD of fenitrothion at 24, 48, 72 and 96 hrs intervals by using DCFH-DA. As shown in Fig. 3.24 and fig 3.25 the DCFH-DA fluorescence of cells increased significantly with increased fenitrothion concentrations, suggesting that fenitrothion could induce ROS accumulation in SoMG cells. These results indicated that fenitrothion can rapidly increase ROS generation in SoMG cells (Table 3.8). Excess accumulation of intracellular ROS causes oxidative stress, which can damage cellular membranes and promote mitochondrial injury. Further, gene expression analysis for SOD, GST and CAT resulted into a significant ($p<0.05$) time and dose dependent increase in all the studied genes thereby suggesting an up regulation by the exposure of fenitrothion.

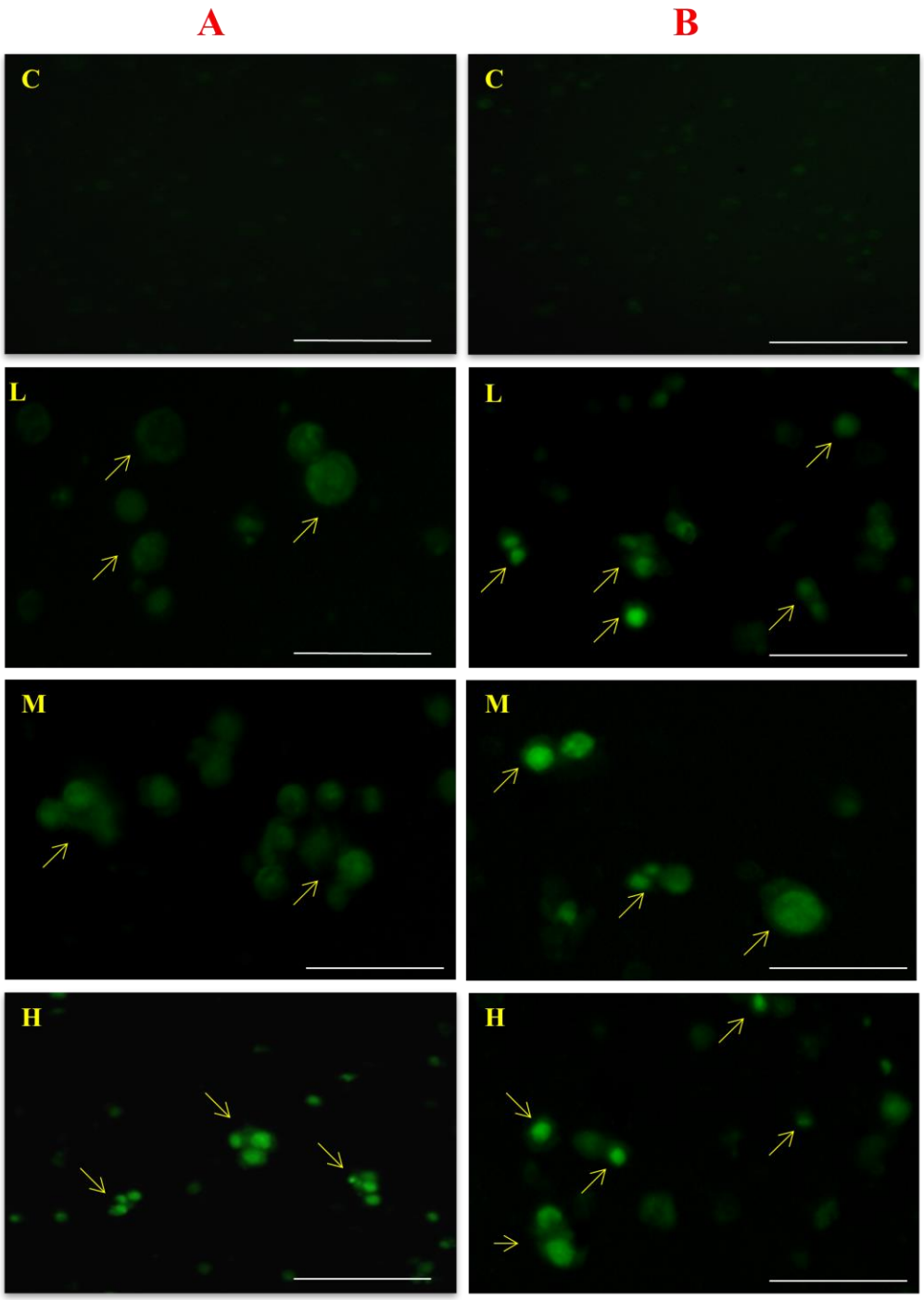

Figure 3.24: Results of DCF-DH staining for ROS Generation in SoMG cell line after (A) 24 hrs and (B) 48 hrs of treatment with sublethal concentrations(LD, MD and HD) of Fenitrothion in an increasing concentrations of 5,10, 15 µg/ml. Arrows indicates bright green nucleus with increased ROS level. Bar =50µM

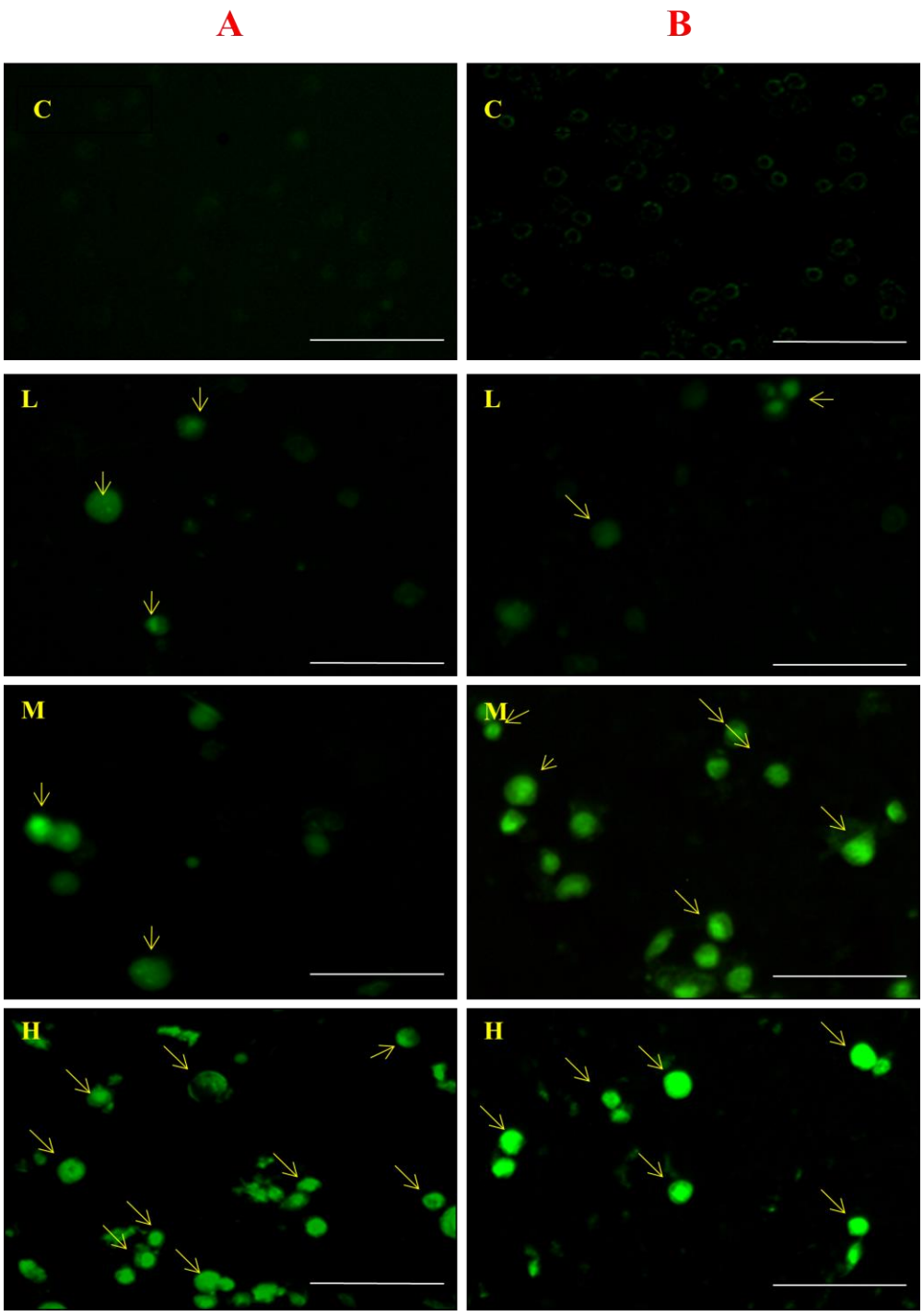

Figure 3.25: Results of DCF-DH staining for ROS Generation in SoMG cell line after (A) 72 hrs and (B) 96 hrs of treatment with sublethal concentrations(LD, MD and HD) of fenitrothion in an increasing concentrations of 5, 10, 15 µg/ml. Arrows indicates bright green nucleus with increased ROS level. Bar =50µM

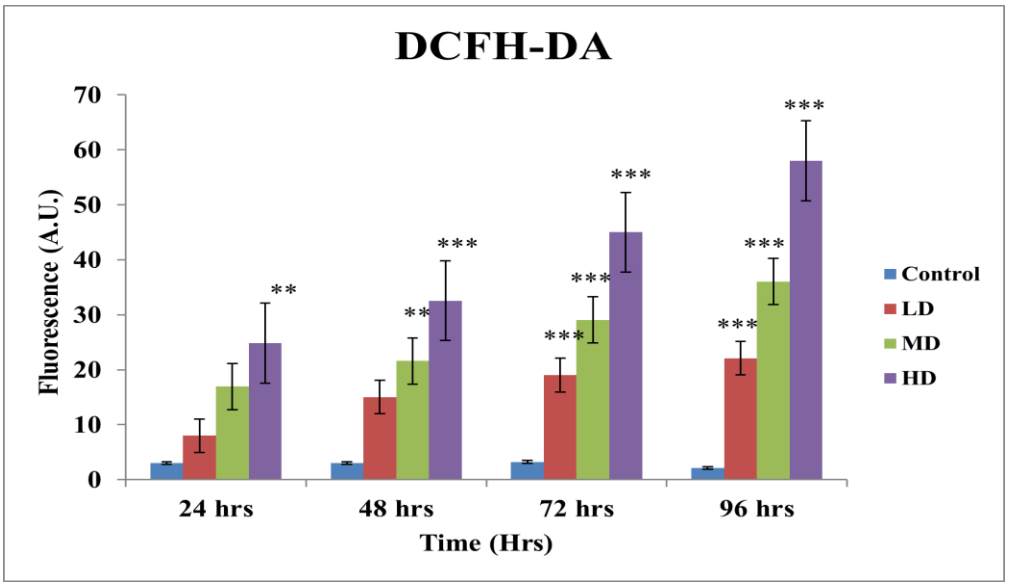

Figure 3.26: Depicts the fluorescence in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs and stain with DCFH-DA

Significant level indicated by *= (p<0.05); **= (p<0.01); *= (p<0.001)**

	24 hrs	48 hrs	72 hrs	96 hrs
Control	3.02±0.08	3.024±0.07	3.22±0.08	2.12±0.01
LD	8.01±0.07	15.03±0.06	19.02±0.07***	22.09±0.03***
MD	16.93±0.09	21.57±0.02**	29.04±0.03***	36.05±0.05***
HD	24.82±0.02**	32.56±0.01***	45.01±0.02***	57.98±0.07***

Table: 3.8: Depicts the mean± SD values of fluorescence in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs and stain with DCFH-DA

Each value represents the mean ± SEM. (n=3)

Significant level indicated by ^{ns}= non significant, *= (p<0.05); **=(p<0.01); *=(p<0.001)**

Gene expression analysis of antioxidants

After the qualitative analysis of reactive oxygen species, gene expression analyses of antioxidants (superoxide dismutase-SOD, catalase-CAT, and glutathione-s transferase-GST) were performed through real time PCR. The gene expression of SOD (Fig 3.27) was significant increased in LD, MD, HD treatment of fenitrothion in a dose dependent manner. Similarly, the results obtained for CAT and GST also illustrated a significant ($p<0.001$) increase at 24 hrs, 48 hrs, 72 hrs and 96 hrs (Fig 3.28 and Fig 3.29). For all the antioxidants, HD of fenitrothion showed the maximum levels compared to all the other doses with a mean fold change of 13.18, 13.48 and 28.76 for SOD, GST and CAT respectively compared to control (Fig 3.29).

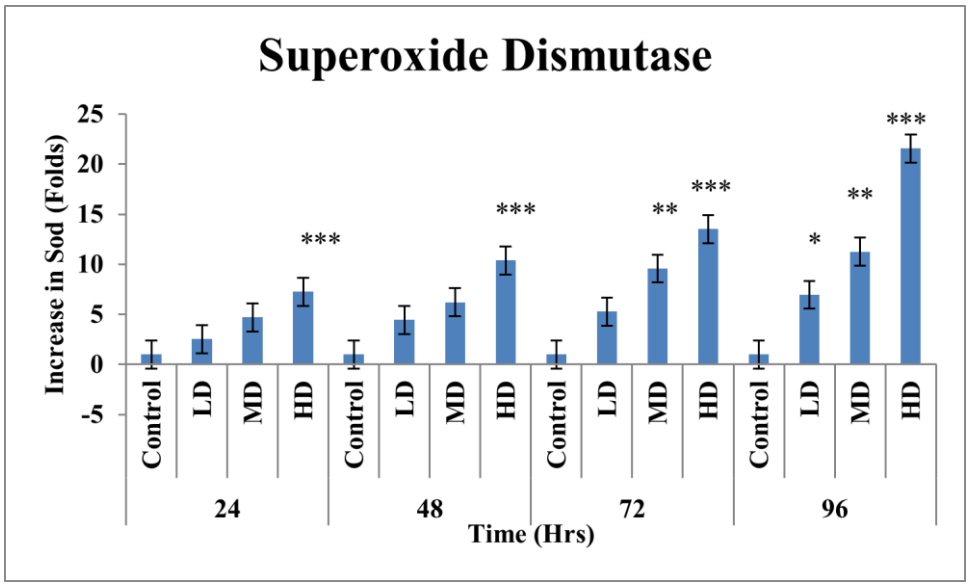

Figure 3.27: Depicts the level of SOD (in folds) in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs

Each value represents the mean ± SEM. (n=3)

Significant level indicated by *= ($p<0.05$); **= ($p<0.01$); *= ($p<0.001$)**

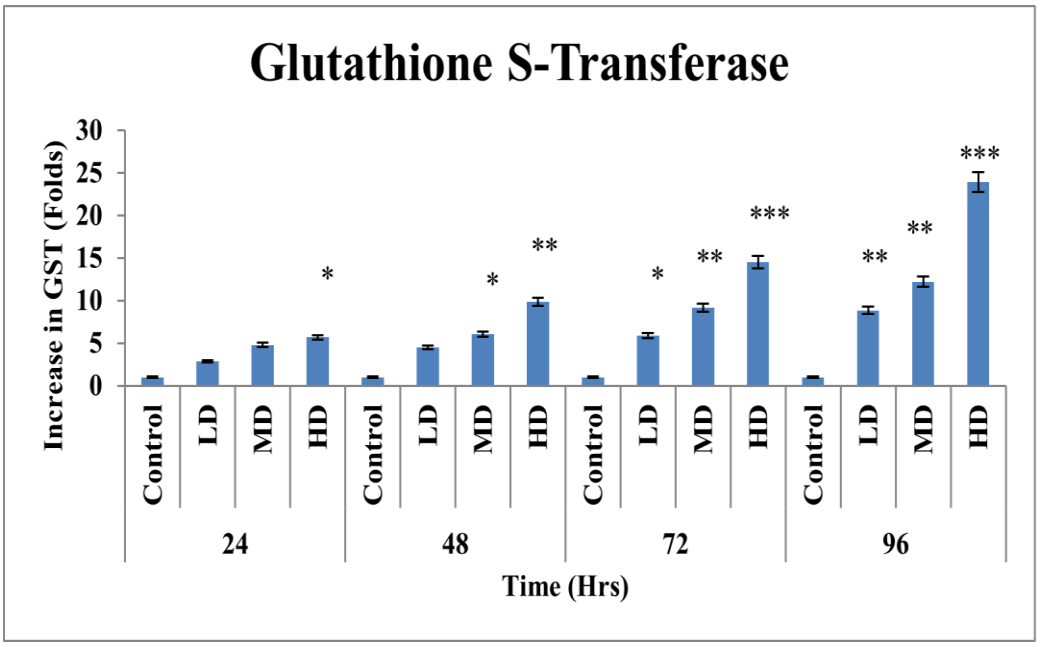

Figure 3.28: Depicts the level of GST (in folds) in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs

Each value represents the mean ± SEM. (n=3)

Significant level indicated by *= (p<0.05); **=(p<0.01); *=(p<0.001)**

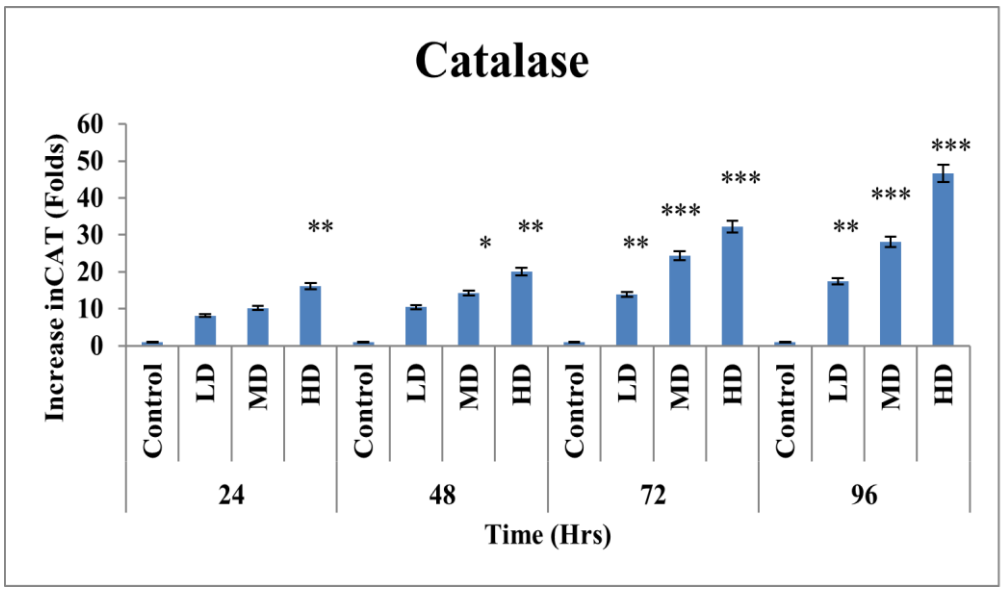

Figure 3.29: Depicts the level of CAT (in folds) in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs

Each value represents the mean ± SEM. (n=3)

Significant level indicated by *= (p<0.05); **= (p<0.01); *= (p<0.001)**

Time (Hrs)	Sub-lethal Dose	SOD	GST	CAT
24	Control	1	1	1
	LD	2.53±0.09^{ns}	2.85±0.08^{ns}	8.16±0.08^{ns}
	MD	4.69±0.5^{ns}	4.79±0.03^{ns}	10.21±0.05^{ns}
	HD	7.25±0.05***	5.67±0.02*	16.13±0.2**
48	Control	1	1	1
	LD	4.44±0.003^{ns}	4.50±0.09^{ns}	10.45±0.02^{ns}
	MD	6.22±0.02^{ns}	6.038±0.01*	14.26±0.09*
	HD	10.38±0.08***	9.83±0.02**	20.11±0.07**
72	Control	1	1	1
	LD	5.29±0.01^{ns}	5.90±0.08*	13.84±0.09**
	MD	9.57±0.02**	9.18±0.01**	24.35±0.08***
	HD	13.52±0.09***	14.49±0.02***	32.25±0.07***
96	Control	1	1	1
	LD	6.95±0.08*	8.83±0.09**	17.51±0.02**
	MD	11.26±0.02**	12.20±0.08**	28.12±0.09***
	HD	21.58±0.07***	23.91±0.01***	46.68±0.08***

Table 3.9: Depicts the mean± SEM values of Folds increase in SOD, GST and CAT in SoMG cells treated with sub-lethal doses of fenitrothion at 24, 48, 72 and 96 hrs

Each value represents the mean ± SEM. (n=3)

Significant level indicated by ^{ns}= non significant, *= (p<0.05); **= (p<0.01); *= (p<0.001)**

FACs Analysis:

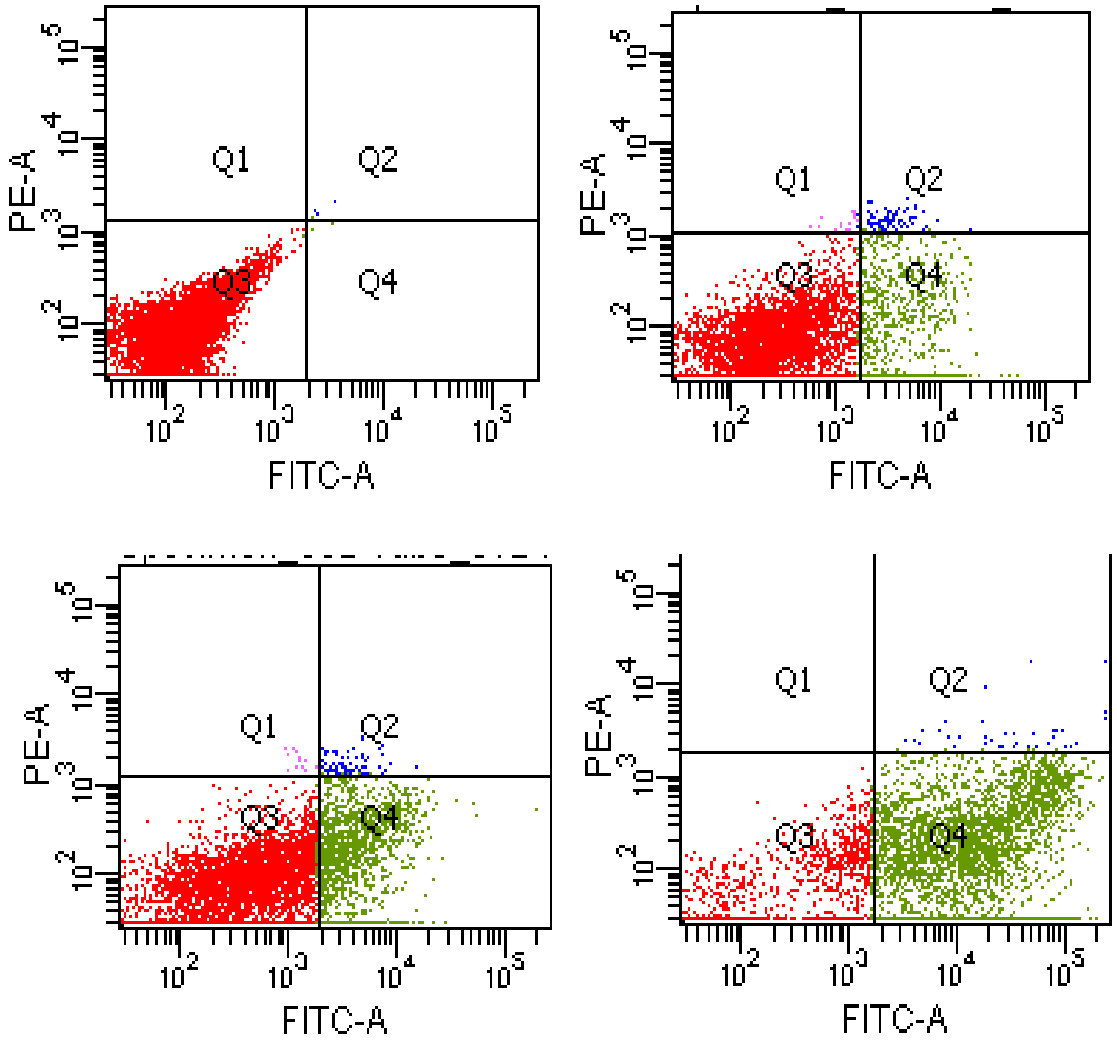

Figure 3.30: Flow cytometry dot plots with double Annexin V-FITC/PI staining for apoptosis a) control cells, b) cells exposed to LD of fenitrothion c) cells exposed to MD of fenitrothion and d) cells exposed to HD of fenitrothion at 24 hrs, Q1-necrotic cells (pink), Q2- Late Apoptotic cells (blue), Q3- Live cell (red), Q4- Early apoptotic cells (green)

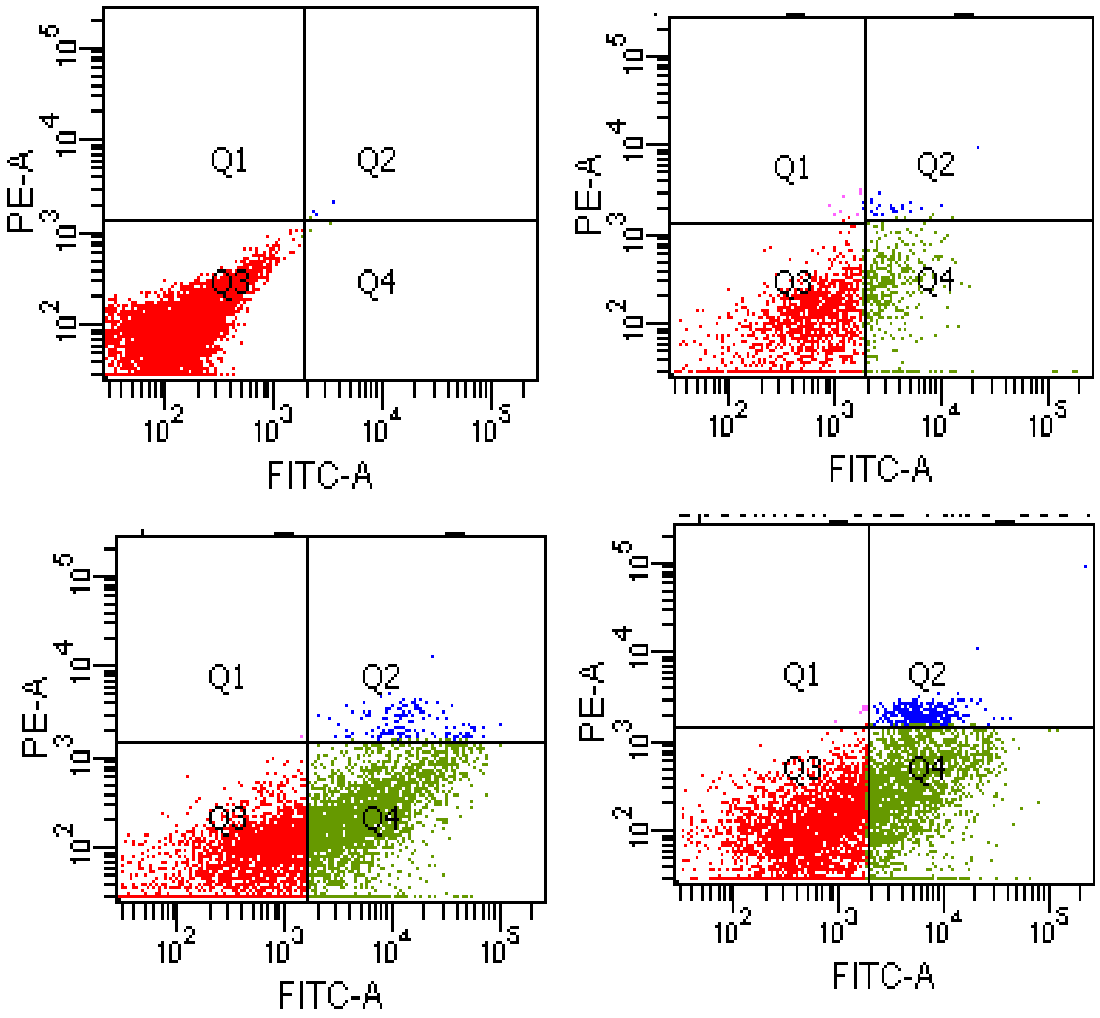

Figure 3.31: Flow cytometry dot plots with double Annexin V-FITC/PI staining for Apoptosis a) control cells, b) cells exposed to LD of fenitrothion c) cells exposed to MD of fenitrothion and d) cells exposed to HD of fenitrothion at 48 hrs, Q1-necrotic cells (pink), Q2- Late Apoptotic cells (blue), Q3- Live cell (red), Q4- Early apoptotic cells (green)

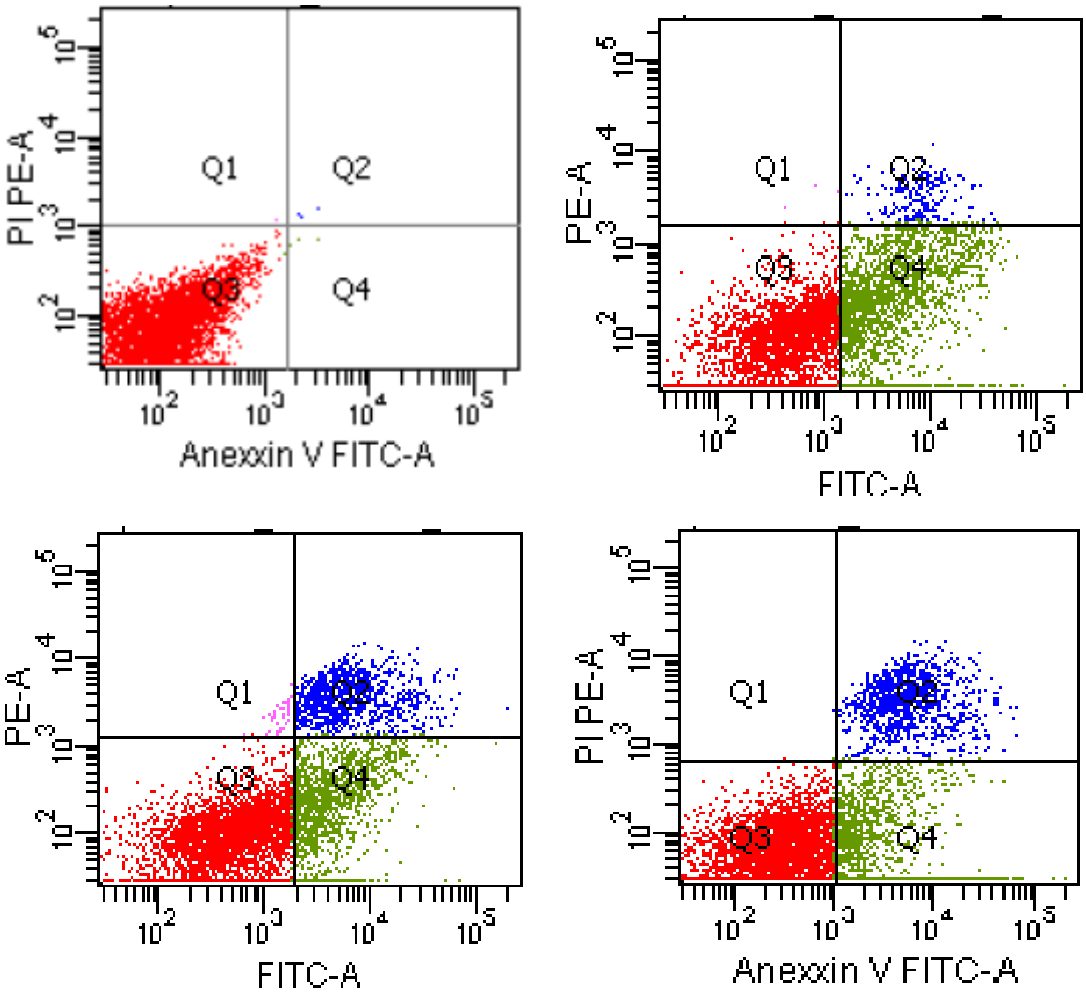

Figure 3.32: Flow cytometry dot plots with double Annexin V-FITC/PI staining for apoptosis a) control cells, b) cells exposed to LD of fenitrothion c) cells exposed to MD of fenitrothion and d) cells exposed to HD of fenitrothion at 72 hrs, Q1-necrotic cells (pink), Q2- Late Apoptotic cells (blue), Q3- Live cell (red), Q4- Early apoptotic cells (green)

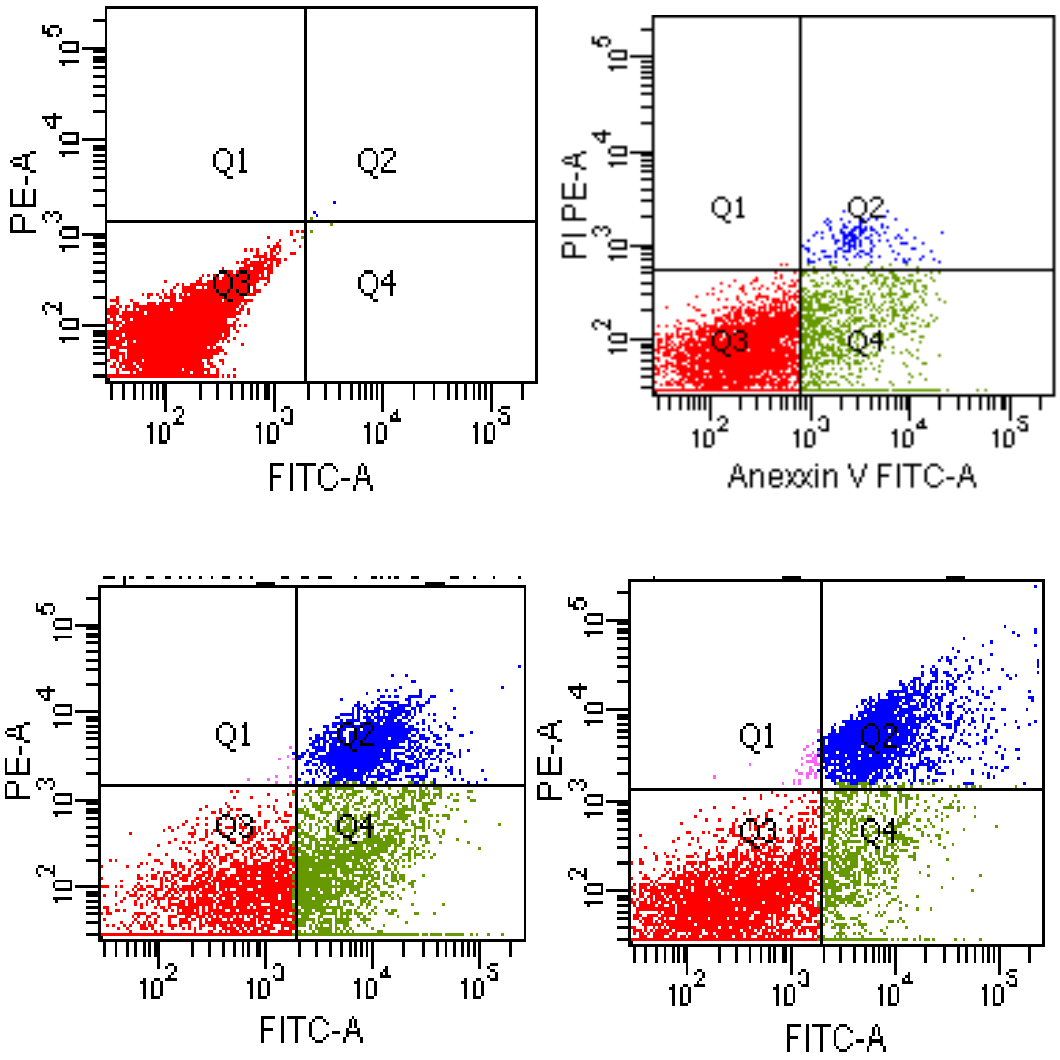

Figure 3. 33 : Flow cytometry dot plots with double Annexin V-FITC/PI staining for apoptosis a) control cells, b) cells exposed to LD of fenitrothion c) cells exposed to MD of fenitrothion and d) cells exposed to HD of fenitrothion at 96 hrs, Q1-necrotic cells (pink), Q2- Late Apoptotic cells (blue), Q3- Live cell (red), Q4- Early apoptotic cells (green)

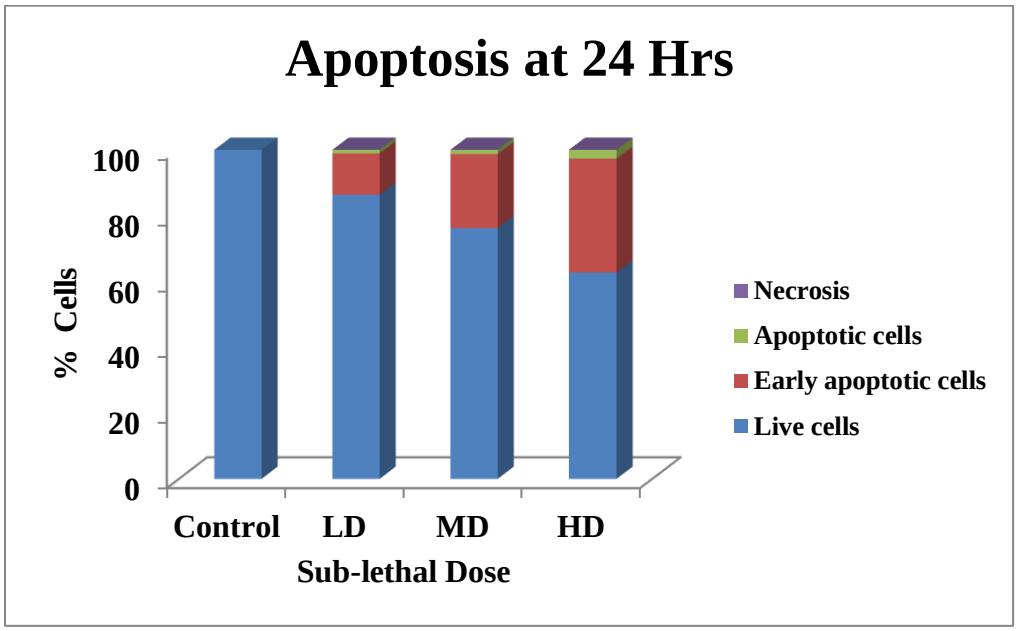

Figure 3.34: Depicts the summary data of FACs analysis of apoptosis where SoMG cells were treated with fenitrothion at 24 hrs

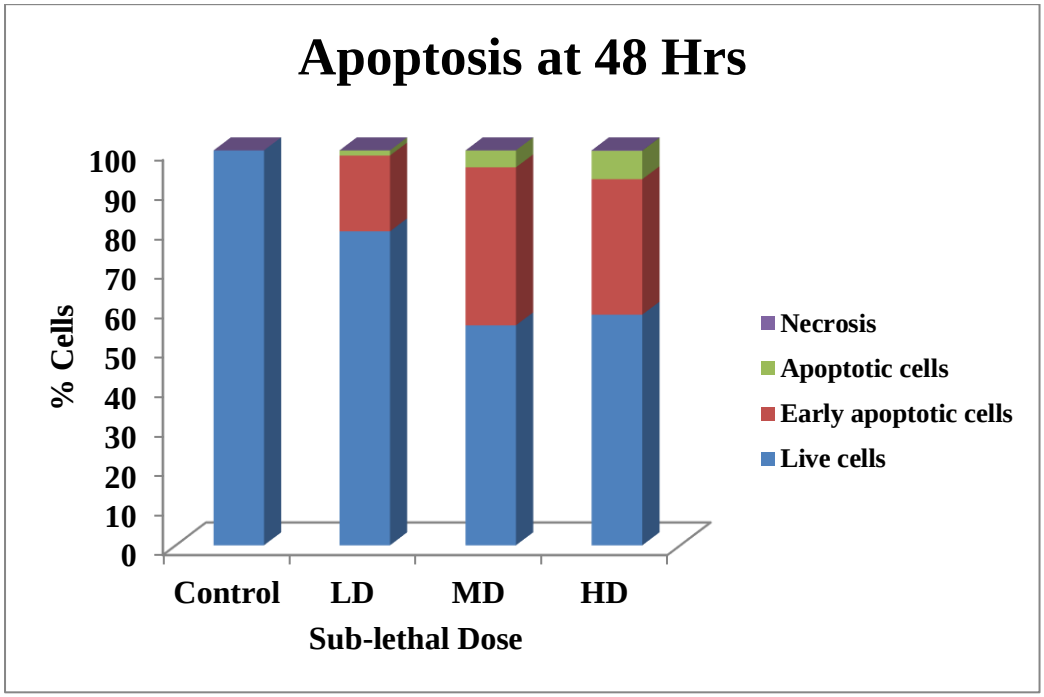

Figure 3.35: Depicts the summary data of FACs analysis of apoptosis where SoMG cells were treated with fenitrothion at 48 hrs

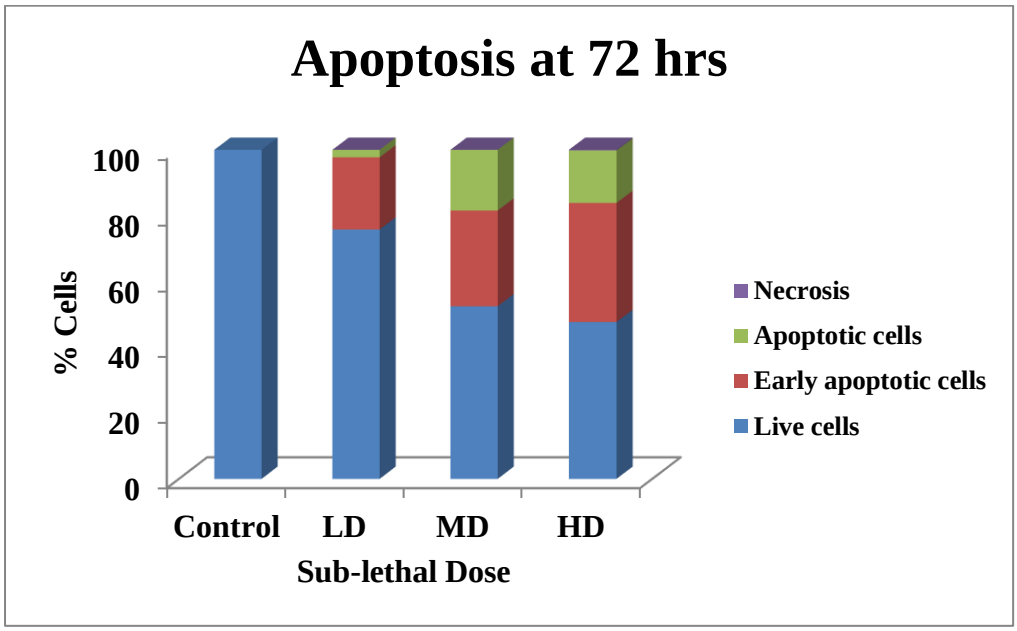

Figure 3.36: Depicts the summary data of FACs analysis of apoptosis where SoMG cells were treated with fenitrothion at 72 hrs

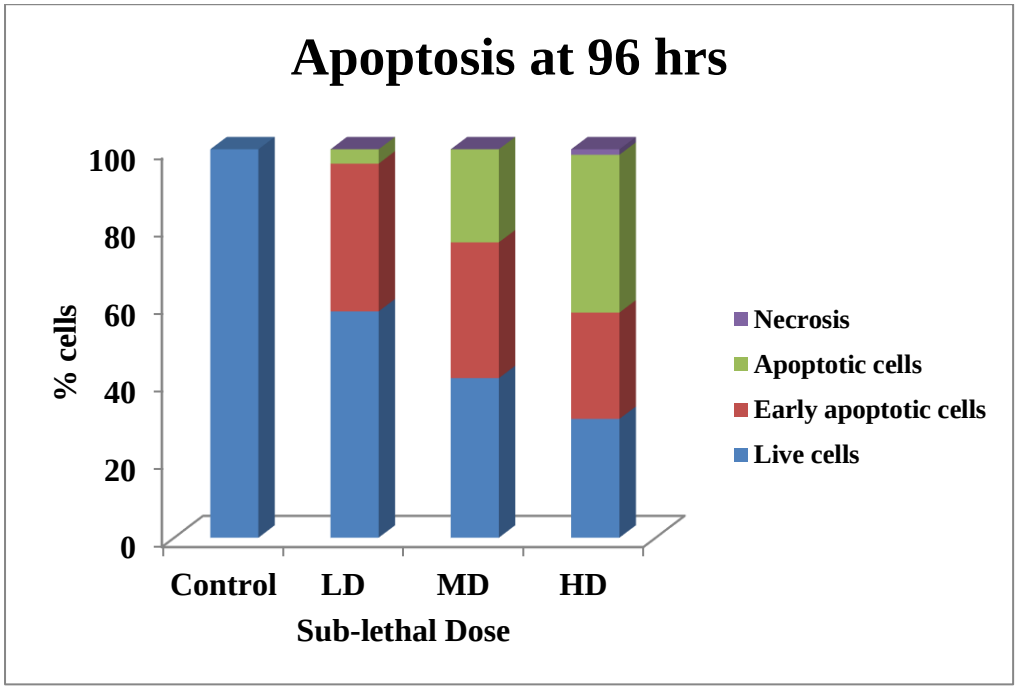

Figure 3.37: Depicts the summary data of FACs analysis of apoptosis where SoMG cells were treated with fenitrothion at 24 hrs

Time (Hrs)	Sub-lethal Dose	Live cells	Early apoptotic cells	Apoptotic cells	Necrosis
24	**Control**	100%	0%	0%	0%
	LD	86.3%	12.6%	1.1%	0%
	MD	76.1%	22.6%	1.3%	0%
	HD	62.6%	34.7%	2.7%	0%
48	**Control**	100%	0%	0%	0%
	LD	79.5%	19.2%	1.3%	0%
	MD	55.7%	40%	4.3%	0%
	HD	58.4%	34.3%	7.2%	0.15%
72	**Control**	100%	0%	0%	0%
	LD	75.7%	22%	2.3%	0%
	MD	52.3%	29.2%	18.5%	0%
	HD	47.5%	36.3%	16%	0.2%
96	**Control**	100%	0.1%	0%	0%
	LD	58.2%	38.1%	3.7%	0%
	MD	41%	35%	24%	0%
	HD	30.5%	27.4%	40.7%	1.4%

Table 3.10: Depicts the percentage status of live and dead cells of SoMG cells to sublethal dose of fenitrothion at 24 hrs, 48 hrs, 72 hrs and 96 hrs

Time (Hrs)	Sub-lethal Dose	Early apoptosis	Apoptosis
24	**Control**	0	0
	LD	17.54 ±0.45ns	1.57 ±0.06
	MD	25.36 ±0.55	4.87 ±0.07*
	HD	69.25 ±0.23	3.90 ±0.77**
48	**Control**	0	0
	LD	36.89 ±0.08^{ns}	4.47 ±0.23*
	MD	69.19 ±0.23*	10.24 ±0.00*
	HD	56.75 ±0.03**	14.45 ±0.98**
72	**Control**	00	0
	LD	40.83 ±0.56*	2.16 ±0.22*
	MD	34.63 ±0.07**	64.66 ±0.18**
	HD	41.86 ±0.09***	28.66 ±0.24***
96	**Control**	0	0
	LD	47.86 ±0.32**	4.65±0.06*
	MD	39.24 ±0.17***	69.89±0.03***
	HD	13.54 ±0.04***	213.32±0.76***

Table 3.11: Depicts the cells going in early apoptosis and apoptosis (in folds) in SoMG cells treated with sub-lethal doses of fenitrothion for 24, 48, 72 and 96 hrs

Each value represents the mean ± SEM. (n=3)

Significant level indicated by ^{ns}= non significant, *= ($p<0.05$); **= ($p<0.01$); *= ($p<0.001$)**

After the treatment (sub-lethal dose) of insecticide fenitrothion on SoMG cells, the totality number of cells was quantified with the help of FACs. The treated cells were stained with Annexin-FITC PI after 24 hrs, 48 hrs, 72 hrs and 96 hrs. The present study showed that there was significant increase ($p<0.001$) in number of cell death (apoptosis) in HD followed by MD and LD

at 48, 72 and 96 hrs that was in a range from 1.5 to 219.32 fold as shown in table. However, the cell undergoing early apoptosis was found to be significant higher with a 1.5 and 4.4 fold in LD trailed by 4.8 folds to 10.23 folds change in MD and 3.89 folds to 14.44 fold in HD after 24 hrs and 48hrs of fenitrothion treatment respectively (Table 3.11).

3.4 Discussion

Organophosphate insecticides are aimed to target the neural cells and are known to inhibit AchE; however recent *in vivo* and *in-vitro* studies have demonstrated that OPs can induce variable toxicity in non-neural cells, which may be through a novel pathway other than their action on central nervous system (Decombel *et al.*, 2004; Ojha *et al.*, 2011). The IC_{50} values obtained in this study for malathion organophosphate were relatively high compared to fenitrothion suggesting it to be having low toxicity for both SoMG and for Sf9 cell line, SoMG a newly developed cell line of the present study is from the midgut of *S. oryzae*. The high toxicity of fenitrothion in both the cell line thus confirms the induction of the toxicity in the non-neuronal cell line. In addition, the AO/EB staining on the exposure of malathion and fenitrothion of both the cell line also demonstrated the appearance /formation of the apoptotic bodies proposing its toxic potential. Qualitative assay followed by the quantitative analyses further confirmed the high toxic potential of fenitrothion in a time a dose-dependent manner compared to malathion of SoMG primary cell line by the appearance of the apoptotic bodies. Morphological changes, like cell shrinkage, cell fragmentation, nuclear condensation, cytoplasmic disintegration as well as an increase of cell granularity was clearly observed. Further, the cells undergoing apoptosis also exhibited cell shrinkage and loss of cell sphericity. Of the two chemicals used, fenitrothion unveiled relatively greater cytotoxic impacts as compared to malathion which clearly signifies it to be more toxic than the malathion. Our results are in accord with the work of Saleh *et al.* (2013) who have reported a dose dependent cell growth inhibition

for insect cell lines on the exposure of a variety of insecticides (dimethoate (OP), deltamethrin (pyrethroids) and acetamiprid). Also, the hazardous effects of insecticide spinosad on Sf9 are known which reduces cell viability and causes morphological changes, including swelling and cytoplasmic vacuolization (Yang *et al.*, 2017). The result demonstrates the toxic effects of the fenitrothion on SoMG cells.

Further studies were then done only on the SoMG primary cell line. So as to confirm the genotoxicity of fenitrothion, nuclear abnormalities were assessed using DAPI staining. Exposure to fenitrothion induced a significant concentration ($p<0.05$) and time-dependent increase in nuclear alterations inducing DNA damage and leading to Apoptosis. Thus combining the results of MTT assay as well as AO/EB staining thus illustrates that fenitrothion has resulted in overall toxicity.

Excess ROS production is an early event that triggers mitochondrial dysfunction (Doenst *et al.*, 2010). Mitochondria are considered as the power generators of the cell, converting oxygen and nutrients into adenosine triphosphate (ATP) and also known to play a critical role in several cellular processes, including energy metabolism and programmed cell death (Kumar *et al.*, 2015; Teixeira *et al.*, 2015). ROS can initiate autocatalytic reactions so that molecules to which they react, are themselves converted into free radicals to promote the signaling cascade which leads to the damage. In the present study, to determine the induced ROS generation on the exposure of fenitrothion on SoMG cells were detected using a DCFH-DA probe. The results revealed the time and dose-dependent significant increase in the ROS level which suggests the mitochondrial injury, oxidative damage which leads to an increase in ROS level and DNA damage (Sedlc *et al.*, 2010; Aldakkak *et al.*, 2011, Wu *et al.*, 2015). As a result of oxidative damage the mitochondrial membrane potential collapse, which eventually leads to the opening of mitochondrial permeability transition pore and release of proapoptotic

proteins, including Cytochrome C into the cytoplasm to initiates the caspase cascade (Youle and Strasser, 2008; Suh *et al.*, 2013; Nagy *et al.*, 2015; Orrenius *et al.*, 2015; He *et al.*, 2016; Lyakhovich *et al.*, 2016;). Hence, our results are in agreement with the earlier reports by Yang and team (2017) who investigated the effects of spinosad on ROS in Sf9 cells by using DCFH-DA as a probe, found that the cells which impart green fluorescence represent the intracellular ROS.

To further confirm the role of fenitrothion in ROS production, quantitative analysis of antioxidants (SOD, GST, and CAT) from SoMG cell was performed. The results have shown that SoMG cells were under stress and eventually significantly up-regulates the antioxidant defence system in a dose and time-dependent manner. ROS is a group of common molecules that have been found to play a key centralized role under multitudes of stressed conditions. In general, the involvement of ROS is implicated in the toxic manifestations of insecticidal chemicals. Increase of ROS develops a wide variety of physiological and cellular events; including lipid peroxidation, antioxidants' depletion, and DNA damage (Chen *et al.*, 2012; Wang, Martínez, *et al.*, 2016; Yang *et al.*, 2017). Our results are consistent with the earlier studies of Xu *et al.*, 2017 and have reported that exposure spinosad and organophosphate has resulted into excess ROS production resulting into mitochondrial dysfunction and disturbing the redox status, enhancing 8-oxoG and DNA oxidation impairment repair enzymes' production and leading to DNA strand break in Sf9 cells. The probable mechanism by which fenitrothion has expressed an increased activity is by inhibiting the activity of Na+/K+-ATPase which in turn impairs cellular respiration and leads to an enhanced level of oxygen free radicals.

An increase in ROS such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxides are known to be controlled by antioxidants such as SOD, GSH, and CAT respectively, keeping this in mind the

quantitative PCR of gene expression for GSH and CAT was also carried out.GSH and CAT are known to be a powerful antioxidant that reduces intracellular ROS and is frequently associated with a decrease in ROS induced cytotoxicity (Adamski *et al.*, 2005; Santabárbara-Ruiz *et al.*, 2018). The results revealed that there was a significant increase in the GSH along with a significant increase in CAT activity which is known to play a role against ROS and neutralizing a variety of free radicals within the cells. In the present study, fenitrothion evoked ROS production within the SoMG cell line which ultimately induces cellular damage which culminated in triggering cell death. Thus the cells undergo ROS mediated apoptosis. Therefore, we postulated that ROS might play a key role in regulating intrinsic apoptotic pathways in SoMG cells treated with SoMG cells.

Once the Cytochrome C is released into the cytosol, it activates the genes of the apoptosome, resulting in caspase activation cascade and cell death (Kim *et al.*, 2005). In the present study, FACs analysis was carried out to check the early and late apoptosis, and the analysis revealed a significantly elevated rate of apoptosis in time and dose-dependent manner. Thus, our results have suggested that the insecticide mediated toxicity in SoMG cells by the production of excess ROS can lead cells towards programmed cell death. Corroborating our results on SoMG cells, cytochrome c released into the cytosol is an earlier and important event during insect cell apoptosis induced by fenitrothion, similar studies have been performed on lepidopteron insect cell lines where apoptotic assays were employed using FACs (Huang *et al.*, 2013). In addition, the subsequent increase in ROS and the morphological and flow cytometry analysis of apoptosis had demonstrated the intrinsic programmed cell death in SoMG cells induced by fenitrothion.

Therefore, together the results of the of MTT assay, AO/EB staining, DAPI, DCFH-DA, ROS gene expression and FACs analysis suggest that out of the two pesticide screened on SoMG cell line fenitrothion was found to be

highly toxic compared to malathion and further it induces the apoptosis through the change in the mitochondrial membrane potential of cell that leads to formation of ROS (O^-, $H202^-$, NOO^- leading to the activation of the scavenger molecules like antioxidants enzymes (CAT, SOD, GST). These molecules initiate the cascades of events inside the cell inducing apoptosis. Apoptosome formation and caspase activation in insect cell extracts were found to be exclusively dependent on cytochrome c release. It seems the intrinsic apoptotic pathway of lepidopteran cells is similar to mammalian cells (Mohan *et al.,* 2003; Liu *et al.,* 2007; Kumarswamy *et al.,* 2009). Compared to Drosophila and humans, there is a lacuna in the mechanism of regulation for apoptosis in *S. oryzae* cells. More genes such as the initiator caspases, BAX and Bcl-2 family need to be identified for *S. oryzae*, and the characterizations of apoptosome formation, as well as mechanisms of caspase activation, need to be studied further. Moreover, as discovered in humans the characterization of apoptosome formation and mechanisms of caspase activation also needs to be elucidated in different representatives of insects.

3.5 Conclusion

In a nutshell, the present study summarizes that the cell line shows all the viable properties to that of a commercial cell line (Sf9) upon the treatment of an insecticide. Hence, further research should be carried out for making the cell line immortal so that it can be commercialized to promote research in the field of applied entomology.