Chapter 1

Agrochemicals induced alteration in proliferation on ICG cell line

1.1 Introduction

The discovery of pesticide residues in various sections of the environment has raised serious alarms regarding their use; concerns of which have outweighed the overall benefits derived from them (Ali et al., 2014). The potentially deleterious effect on various components in the natural environment has elevated a great deal of concern in scientific community for pesticide management (Reddy & Kim, 2015). Due to low cost and broad-spectrum toxicity, it is estimated that more than 100,000 tons of pesticides have been applied in India alone, primarily for agricultural pest control (Arora et al., 2013). The annual application of agricultural fertilizers and pesticides is over 140 billion kilograms which is a massive source of pollutant through agricultural runoff (Arora et al., 2013). Agricultural pollution is the biotic and abiotic waste products of agriculture that contribute to pollution, degradation, and/or injuries to human beings and their economic interests, of the environment and surrounding ecosystems. Food and drinking water may be polluted by agrochemicals, and human health may be at risk (Taju et al., 2017). Application of such agrochemicals directs towards potential health hazards and has becomes a major concern for aquatic habitat due to their toxicity, persistency and tendency to accumulate in the organisms (Joseph & Raj, 2010).

Every day, about 15,000 new substances are registered with the Chemical Abstracts Service (CAS, 2014), and of the ~100 million chemicals thus far registered, very few are being regulated, and even fewer are assessed

for their safety (Stadnicka-Michalak et al., 2014). Safety assessment of chemicals is a daunting task. Only about 10 high-production volume chemicals (that is, >1000 tons/ year) were tested per year in the past, and an ~300-fold increase in throughput is required to comply with the European legislation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (Rudén & Hansson, 2010). The main goals of assessing the risk of chemicals are to prevent environmental pollution and to ensure safe and sustainable use of chemicals by keeping a balance between the benefits to humanity and dangers of introducing synthetic compounds into Earth's ecosystems. Every year, more than a million fish are used for experimental and other scientific purposes in the European Union (Taylor & Alvarez, 2019). Between 3 and 6 million fish per year are currently used for whole effluent testing in the United States (Ermler et al., 2013). Moreover, at least 400 fish are used per one Fish Early Life Stage (FELS) test (OECD, 2021) which is often required by environmental agencies because it covers different developmental stages from fertilization to juvenile stage, at which fish are more sensitive to chemicals than adult fish (Stadnicka-Michalak et al., 2014). Thus, there is consensus among scientists, regulators, and industry that a paradigm shift in risk assessment is needed because the current approach is too slow and expensive and consumes millions of animals per year, which is ethically questionable and therefore controversial. For these reasons, methods other than in vivo fish toxicity testing are urgently sought to be included in an integrated testing framework.

The concern of toxicity with reference to pesticides has been one of the key dilemmas in human health in the past few decades. However, pesticides were developed to control pests for more and safe production, but several pesticides have presented prospective risks to human health and the environment. Human beings are exposed to numerous toxic insults every day.

Fortunately, the body has several defense mechanisms to combat toxicants. Some toxicants are prevented from entering the body by virtue of their particle size. Toxicants that do enter the body are metabolized or conjugated in an attempt to safely carry out their excretion. When these first lines of defense are overcome, toxic substances may cause severe cell injury or even cell death. At this point, the tissue may respond by stimulating its healthy cells to divide and restore tissue structure and function. The ability of the tissue to undergo repair depends on the type of tissue damaged and the extent of the damage. The process of tissue repair stops at a precise, preordained point. At low to moderate doses of a particular toxicant, the process functions well, and repair is usually adequate. At high doses of a toxicant, however, the ability of the cells to progress through the cell cycle is inhibited, leading to two consequences. First, dead cells are not replaced, which may lead to organ failure and death. Second, in the absence of compensatory cell division, which normally serves to contain the toxic injury, tissue injury can progress in an unrestrained manner (Yang et al., 2014). Cells within a tissue exert an inhibitory effect on each other's growth. This restraining force is called social control of cell division, and it is mediated by a set of genes called social control genes. An understanding of the mechanisms in control of cell proliferation is critical in the development of tissue restoration therapies. Drug overdoses or chemical poisoning are aimed primarily at preventing additional injury, either by blocking further formation of toxic metabolites or by increasing clearance of the toxin from the body. While these strategies are useful, the survival of the tissue is heavily dependent on tissue repair, the success of which is in turn contingent on the ability of cells to proliferate.

Toxic exposure tissue repair is delayed, either due to the massivity of the exposure because the damage compromises the regenerating ability of the cells, thereby paving the way for unrestrained progression of injury. Animal experiments provide concrete examples of how modifications of tissue repair

directly influence survival. Animals given ordinarily lethal doses of toxins are able to survive – even when there is massive liver injury – when tissue repair in the liver is stimulated. Conversely, animals receiving otherwise nonlethal doses of toxins develop liver failure and die if cell division is blocked by antimitotic agents. Perhaps carefully induced suppression of pathways involved in cell death and stimulation of pathways involved in cell division stops the progression of toxic injury and restore organ structure and function. Environmental toxicants (metals and non-metals) originated from either air, water or soil has been reported to inhibit cell proliferation by DNA damage or by their interaction with DNA metabolism (Chatterjee & Walker, 2017). Pesticides such as DDTs and PCBs Increases cell proliferation by increasing ER (Plísková et al., 2005) or by chromosomal alterations (Thompson & Compton, 2011).

The environmental risk assessment of chemicals in traditional toxicity testing is mostly based on *in vivo* single compound experiments and has been well explored on all representatives of the tropic levels viz. producer and consumer level. However, *In-vivo* testing is extremely time-consuming and costly, requiring much maintenance and a high number of animals, which is ethically debated. Thus, interest in *In-vitro* methods has been growing greatly in the recent years for economical, practical and ethical reasons, and the use of cell lines as alternatives to in vivo testing is being seriously considered (Kasi Elumalai, 2012; Nagpure et al., 2016, Schug et al., 2020). The use of cell lines has many advantages. It avoids the testing of contaminants on living animals or even the regular sampling of cells for primary cultures. Their maintenance is less demanding since the only requirements are cell medium and an incubator at the right temperature and CO_2 concentration which is even unnecessary in the case of piscine cell lines. These methods are cost affecting and non-invasive, and the testing in itself uses very limited amounts of the test

chemicals and creating little toxic waste. Results present little variability since the cell lines are relatively homogeneous and used in a very controlled environment, the complex interactions happening in a whole organism being avoided.

In-vitro fish cell assays are considered to be a promising alternative to fish bioassays to replace or reduce the use of fish in toxicological testing. Chemicals or water samples can be applied to fish cells at temperatures more typical of the temperatures to which fish would be exposed. Moreover, fish cells are largely easier to maintain and more tolerant to simple culture conditions. A large number of research has been done for toxic chemicals to compare *In-vitro* cytotoxicity in fish cell lines with *In-vivo* fish toxicity and confirmed its widespread applicability. Schirmer, (2006) proposed several routes for advancing fish cell line-based toxicity assays to overcome the hurdle like selecting cell lines derived from tissues that reflect the specific mode of action of a particular chemical; increasing sensitivity of the cellular response by modification of the culture environment to more closely resemble the *In-vivo* exposure; and by accounting for the chemical fraction available to the cells.

Many scientists have developed novel ways to detect the toxicity using various cell lines, which are maximally focused on human cell lines, however, there are few on fish cell line. Genotoxicity of Organophosphates (Methyl parathion, Methyl paraoxon and Dimefox on HepG2 cells (Hreljac et al., 2008), where they have reported a differential effect on the rate of proliferation with the same group of pesticide. Colle et al., (2018) in their studies on herbicide Paraquat and fungicide- Maneb exposure have reported oxidative stress induces alteration in the rat neural stem cell proliferation. Further, epidemiological and molecular studies also provide substantial evidence for pesticides used either in agricultural, commercial as well as domestic applications to be associated with excess cancer risk (Alavanja et al.,

2013).Processing of pesticides depending on their properties, dose, and routes of entry can significantly affect the organism. Many pesticides as endocrine distruptors cause endocrine disturbances, neurological disturbances, influence immune system, reproduction, development (Maxmen, 2009; Caron-Beaudoin et al., 2016; Meyer A, et al. 2017; Gaylord, et al., 2020).

PCNA (Proliferating cell nuclear antigen) is a DNA clamp that acts as a progression factor for DNA polymerase δ and is essential for replication. The protein is a homotrimer and achieves its processivity by encircling the DNA, where it acts as a scaffold to recruit specific proteins involved in DNA replication, DNA repair, chromatin remodeling and epigenetics. It is considered as a universal marker for cell proliferation (Leung et al., 2005). The control of DNA replication is a key element in the proper functioning of a cell, and it influences genome stability. Duplication of the genetic material that occurs in S phase of the cell cycle has to be coordinated with other cellular processes like mitosis. DNA replication is regulated mainly at the initiation step as a result of cooperation between different signalling pathways controlling the cell cycle.

In addition to pcna, Cyclin-Dependent kinases (CDKs) are yet another universal marker which are known to control cell cycle transitions. Structurally these enzymes contain two subunits, a catalytic Cdk subunit and a regulatory cyclin subunit that activates the Cdk (Angelini et al., 2015; Hwang & Clurman 2005). The activity of cyclin-Cdk complexes is tightly regulated by a complex network of other proteins that function as activators and inhibitors as well as influencing their transcription, sub-cellular localization and degradation. Several classes of cyclins have been described of which Cyclin E binds to G1 phase Cdk2, which is required for the transition from G1 to S phase of the cell cycle that determines initiation of DNA duplication. The Cyclin E/CDK2 complex phosphorylates p27Kip1 (an

inhibitor of Cyclin D), tagging it for degradation, promoting expression of Cyclin A (Ekholm-reed et al., 2004). Cyclin A resides in the nucleus during S phase where it is involved in the initiation and completion of DNA replication (Bendriz, 2011). Quantification of proliferative markers (pcna and cyclin genes) can thus be crucial in understanding its role of xenobiotics in cell cycle.

In the present study and attempt is made to understand the alterations in the expressions of the universal proliferative markers when exposed to diverse class of agrochemicals (IMI, CZ, MN and PE).

1.2 Materials and Methodology

Chemicals:

Agrochemicals insecticide IMI- Imidacloprid (TATAMIDA), fungicide CZ Curzate (DuPont ^{TM} Curzate M8), herbicide Pyrazosulfuron ethyl (Saathi, UPL) and micronutrients MN (Librel^{TM} , Ciba) were purchased from the local vendors and they were dissolved (individually) in water for the further experimentation.

Culturing of ICG cells:

ICG gill cell line of *Catla catla* was procured from National Repository of Fish Cell Line (NRFC), Indian Council of Agricultural Research-National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow. The cell line was cultured in Leibovitz's L-15 (AL0011A, HiMedia, India) supplemented with 10% FBS (RM9955, HiMedia, India) (Taju et al., 2013).The flasks were incubated at 28 ºC in a biological incubator (LabTech) and the medium was changed every fourth day. Upon reaching 80-85% confluence, the cells were sub-cultured in the ratio of 1:2 by using trypsin–EDTA solution (TC007, HiMedia, India).

Determination of IC_{50} value of different classes of agrochemicals:

For determination of $IC_{50}$the acute study was carried out in which, cell viability assay was performed for 96 hrs using MTT assay and an inhibition concentration of all Agrochemicals (IMI, CZ, PE, MN) were analysed using probit analysis using GraphPad Prism 9 software. After obtaining the Inhibition concentration (IC_{50}), sub lethal (1/5 (HD), $1/10^{th}$ (MD) and $1/20^{th}$ (LD) doses- of IC_{50}) concentrations were selected for further studies.

MTT Assay:

MTT assay described by Borenfreund et al. (1988) is based on inhibition by chemical injury of the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase. ICG cells were diluted to a concentration of 10^4 cells per mL in Leibo-vitz's L-15 medium with 10% FBS. After agitation, the cells were added to each well of 96-well tissue culture plates at the concentration of 2×10^4 cells per well and incubated overnight at 28 ºC. After incubation, the medium was removed and the cells were refiled with medium containing 0 (control), 25, 50, 75, 100, 125, 150, 175, 200, 225, 250,300, 325, 350 ,400, 425, 450, 500, 525, 550, 575, 600, 625, 650, 675, 700 µg /mL of Agrochemicals (CZ, IMI, PE, MN) for 96 h IC_{50} analysis. After a 96-h exposure period, the test medium was replaced by 10 µl of 5 mg/mL MTT in PBS. After incubation for 4h, the solution was removed carefully, and the cells were rinsed twice with PBS rapidly. Then dimethyl sulfoxide (DMSO) was added at the amount of 100 µl per well to solubilize the purple formazan crystals produced. Absorbance of each well was measured at 490 nm (Synergy HTX Multimode Reader) and Cell viability and inhibition were obtained using the following formula.

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

$$\% \, Cell \, Inhibition = 100 - \frac{\text{Average OD of test x 100}}{\text{Average OD of control}}$$

Cell Viability Assay-

Cell viability is one of the most important indicators for biological evaluation in *In Vitro* studies. Chemicals such as pesticides have different cytotoxicity mechanisms through which it alters the viability and survival of the cells. In order to determine the cell death caused by these damages cytotoxicity and cell viability assays are recommended**.** Cell viability is a measure of the proportion of live, healthy cells within a population and also used to determine the overall health of cells. The proportion of viable cells in a cell population can be estimated in various methods, the most common is dye exclusion method by which determination of membrane integrity is possible. Trypan blue is most used dye, where viable cells exclude dyes, but dead cells not exclude them.

Trypan blue assay was performed to understand the effect of agrochemicals on the viability of ICG cells. The cells were seeded at a density of 1×10^5 cells/ml in a complete L-15 medium. Following 24 hrs of cell growth, different concentrations of agrochemicals (LD, MD and HD) were added to the cells. After 7 days, cells were trypsinized, washed and re-suspended in PBS containing 0.4% trypan blue (TCL046, HiMedia, India). The number of viable cells were counted using haemocytometer (GW088, HiMedia, India) as per standard protocol. Each experiment was done with three replicates (n=3) for each group for statistical analysis.

$$\% \, Cell \, Viability = \frac{\text{No of viable cells } \times 100}{\text{Total no of cells}}$$

Total RNA Extraction (Trizol method)

Total RNA was extracted isolated from ICG cells from control and treated cells for all agrochemicals. 500 µl TRIzol reagent (Invitrogen) was added in each well and scraped out in 1. 5 ml RNAse free tubes. For complete dissociation of nucleoprotein complexes, samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of chloroform and was vigorously shaken for effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes till the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000x g for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. An aliquot of upper aqueous phase was then transferred into a new 1.5 ml micro centrifuge tube. Precipitation was done by adding 500 µl of isopropanol to the supernatant that was transferred. The samples were kept in room temperature for 10 minutes, centrifuged at 12,000x G for 15 minutes at 4°C. After precipitation the supernatant was discarded without disturbing the pellet and was washed in 500 µl of 75% ethanol and then 500 µl absolute ethanol was added to the pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 x g for 5 minutes at 4°C. The pellet was resuspended by adding 40 µl of DEPC water (Diethylpyrocarbonate), was quantified spectrophotometrically using Nanodrop C and was stored in -20º C.

cDNA Synthesis:

First strand of cDNA was synthesized from each sample using Thermo Scientific Verso cDNA Synthesis Kit (AB-1453/A). Verso Reverse Transcriptase Verso is an RNA-dependent DNA polymerase with a significantly attenuated RNase H activity. Verso can synthesize long cDNA

strands, up to 11 kb, at a temperature range of 42 °C to 57 °C. In reaction, 1 µg RNA was used as a template for cDNA synthesis using oligo dT primers. The volume of each component is for a 20 µl final reaction. The Reaction mix is mentioned in Table given below.

Reagents	Volume
5X cDNA synthesis buffer	4 µl
dNTP Mix	2 µl
anchored oligo dT /random hexamers	1 µl
RT Enhancer	1 µl
Verso Enzyme Mix	1 µl
Template (RNA)	1-5 µl
Molecular grade nuclease-free Water	To 20 µl
Total Volume	20 µl

Table 1.1 Reaction mix for cDNA synthesis

Reverse transcription cycling program:

	Temperature	Time	Number of cycles
cDNA synthesis	42 °C	30 min	1 cycle
Inactivation	95 °C	2 min	1 cycle

Table 1. 2: Reverse transcription cycling program for cDNA synthesis

RT-PCR Amplification

Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (A25741, Applied Biosystems, USA) in Quant Studio 12K (Life technology) FAST 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. GAPDH 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 & Schmittgen, 2001).

Statistical Analysis:

Experiments were performed in triplicate (n=3) for each exposure concentration. Data were analyzed with GraphPad Prism 9 (GraphPad Software) and a one-way analysis of variance ($p \leq 0.05$) was performed. The post hoc test was carried out by Dunnett's multiple comparison test to further understand the level of significance ($p \leq 0.05$; $p \leq 0.01$)

Reagents	Volume (20 µL/well)
PowerUp SYBR Green Master Mix (2X)	10 µl
Forward Primer (10uM)	1 µl
Reverse Primer (10uM)	1 µl
DNA Template	2µl
Molecular grade Nuclease free water	6 µl
Total	20 µl

Table 1.3: Real Time PCR Reaction mix

Step	Temperature	Duration	Cycles
UDG activation	50ºC	2 minutes	Hold
Dual- Lock DNA polymerase	95ºC	2 minutes	Hold
Denature	95ºC	3 seconds	40
Anneal/extend	60 ºC	30 seconds	

Table1.4: Real Time PCR condition

	Gene Name	Primer Type	Sequence	Tm ºC
1	gapdh	Forward	CTCACACCAAGTGTCAGGACGAACAG	66.38
		Reverse	GTCAAGAAAGCAGCACGGGTCACC	66.13
5	pcna	Forward	GCACGTCTGGTTCAGGGATCTATCC	66.26
		Reverse	TGCAGAGAAATGCCCGACGAGC	63.98
7	cyclin a	Forward	CTCAAGCCCGGCCAAAGAGTTG	63.98
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38
8	cyclin e	Forward	CGTGAAACCAAAGGGTGAAGACACTG	64.80
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38

Table 1.5: PCR real time PCR primer sequences

1.3 Results:

IC_{50} values of different classes of agrochemicals obtained are presented in Table I and Figure 1-4. Of all the agrochemicals IMI which was found to be highly toxic followed by CZ and MN and the PE was the least toxic.

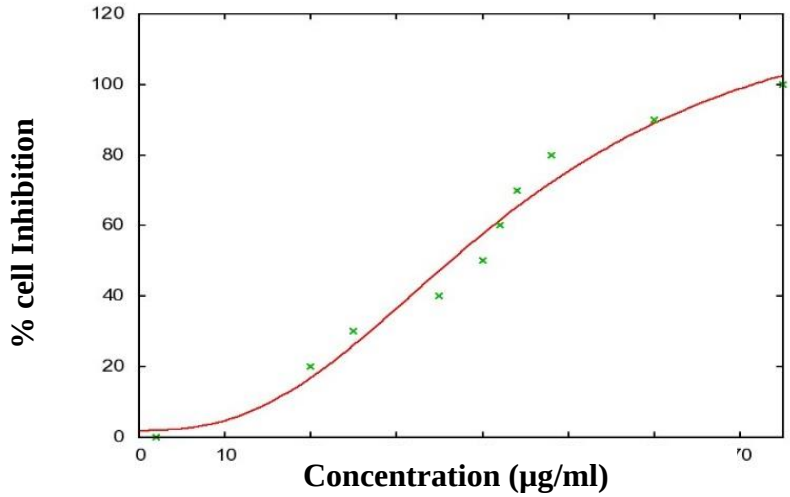

Figure1.1: ICG cell mortality against different concentration of IMI

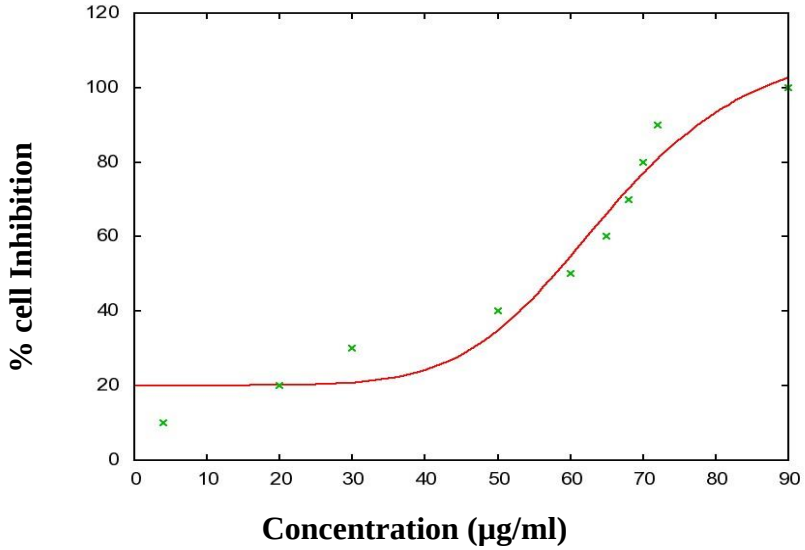

Figure1.2: ICG cell mortality against different concentration of CZ

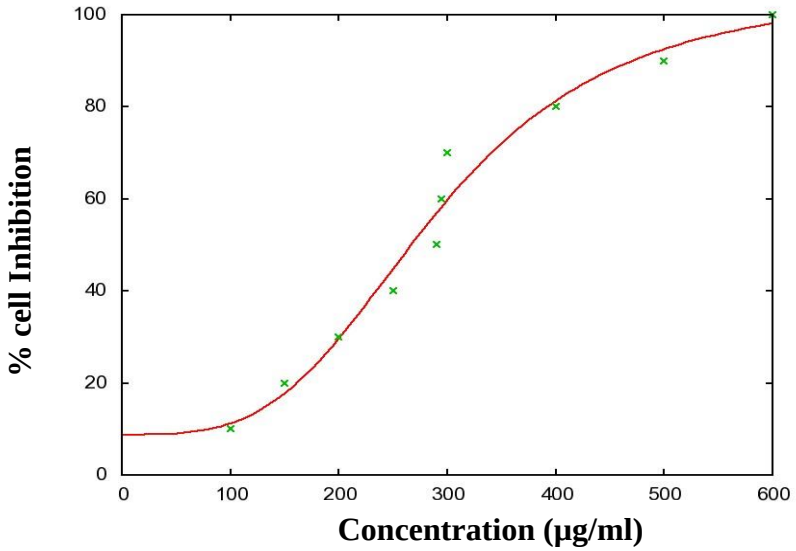

Figure1.3: ICG cell mortality against different concentration of MN

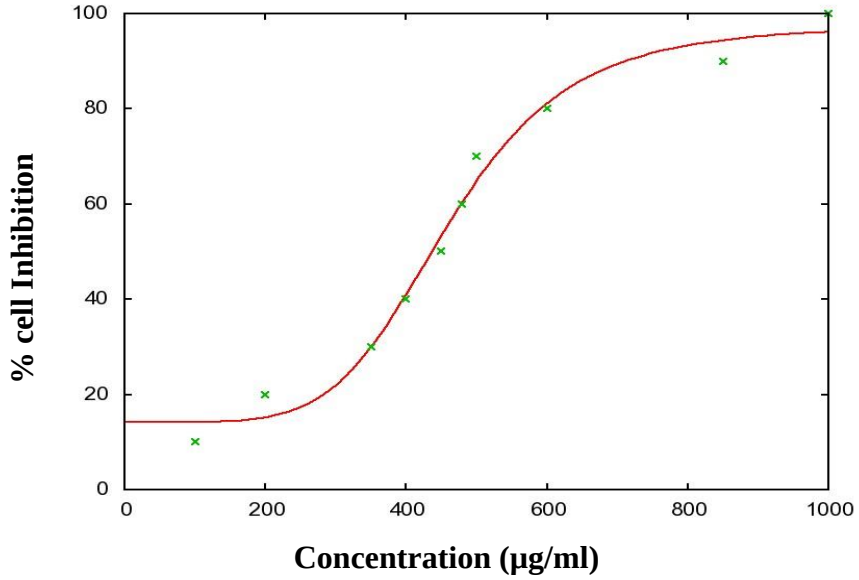

Figure1.4: ICG cell mortality against different concentration of PE

Agrochemical	IC_{50} Value	LD ($1/20^{th}$ IC_{50})	MD ($1/10^{th}$ IC_{50})	HD ($1/5^{th}$ IC_{50})
IMI	43.95 µg/ml	2.19 µg/ml	4.39 µg/ml	8.7 µg/ml
CZ	65.34 µg/ml	3. 26 µg/ml	6.53µg/ml	13.06 µg/ml
MN	290.8 µg/ml	14.54 µg/ml	29.08 µg/ml	58.16 µg/ml
PE	460.85µg/ml	23.04 µg/ml	46.08 µg/ml	92.17µg/ml

Table 1.6: IC_{50} values and their Sub lethal doses for IMI, CZ,MN and PE for ICG cell line.

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. ICG cells were treated with LD ($1/20^{th}$), MD ($1/10^{th}$) and HD ($1/5^{th}$) of all agrochemicals IMI, CZ, MN and PE for 7 days for sub-acute cytotoxic studies. Cell proliferation refers to an increase in cell number due to cell division, which occurs as the final step of the cell cycle. Healthy cells actively proliferate whereas growth-arrested, senescent, and dead or dying cells do not. Thus, cell proliferation assays are a useful tool for assessing cell viability or cell survival by providing a readout on the number of actively dividing cells present in a sample. The cytotoxicity induced by agrochemicals on proliferation of ICG cells was further verified using Trypan blue assay. Results showed that cell proliferation was affected upon treatment with agrochemicals in a dose dependent manner. Results also showed that the cell death induced by MN and PE was much more pronounced at HD compared to untreated control, whereas IMI and CZ showed significant cell death in LD, MD and HD in compared to untreated control ICG cells.

Agrochemicals	% Cell Viability		
	LD	MD	HD
IMI	76.16 ±0.67**	68.13 ±0.67**	52.17 ± 0.70**
CZ	83.00 ±0.62**	64.57 ±0.81**	57.77 ± 1.06**
MN	94.87 ± 0.74*	90.77 ±0.98**	86.83 ± 1.03**
PE	93.77 ± 1.01*	88.40 ±0.84**	71.62 ± 0.84**

Table 1.7: Cell viability at the Sub lethal doses for IMI, CZ,MN and PE for ICG cell line. Each value represents the mean ± SEM. (n=3), Significant level indicated by **p<0.05; **p<0.01*

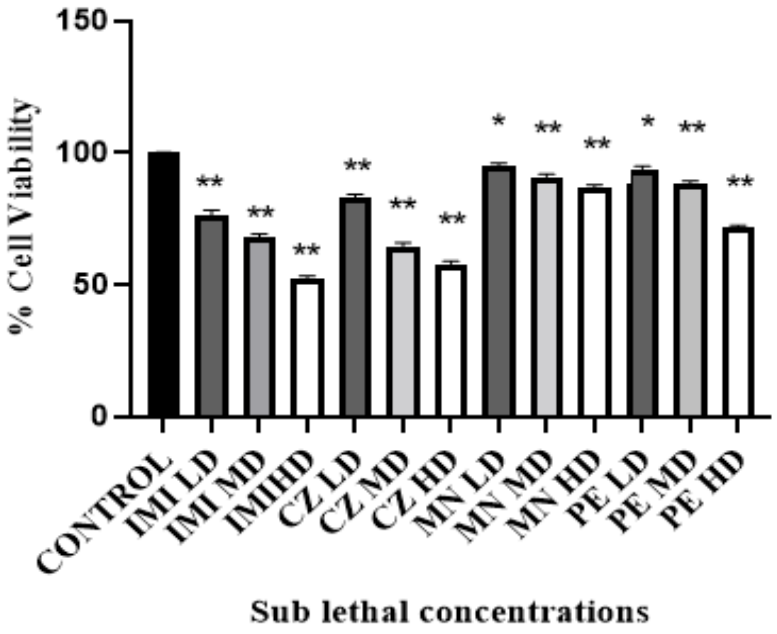

Figure 1.5: Cell viability at the Sub lethal doses for IMI, CZ,MN and PE for ICG cell line. . Each value represents the mean ± SEM. (n=3), Significant level indicated by **p<0.05; **p<0.01*

Samples	Concentration ng/µl	A_{260}/A_{280}
Control	447.17	2.04
IMI LD	264.26	2.03
IMI MD	361.02	1.96
IMI HD	129.23	2.04
CZ LD	367.37	2.01
CZ MD	231.42	2.04
CZ HD	149.67	1.91
MN LD	293.85	1.89
MN MD	234.43	1.91
MN HD	267.23	1.99
PE LD	330.43	2.01
PE MD	347.21	1.89
PE HD	179.26	2.01

Table 1.8: Depicts the quantified values of total RNA and A_{260}/A_{280} ratio obtained by nanodrop

Sub-acute exposure of agrochemicals for 7 days resulted into a differential expression of the proliferative markers. Expression of the proliferative marker genes such as pcna and cyclin A showed different expression. A significant dose dependent decrease ($p<0.05$) was seen in pcna expression (Figure 1.6), while cyclin A was found to be significantly decreasing ($p<0.05$) only at MD and HD of IMI, CZ and MN exposure compared to control. PE exposure resulted into significant ($p<0.05$) decrease only at HD (Figure 1.7). Cyclin E expression resulted into a dose-dependent decrease on exposure of IMI, CZ and PE. However, MN exposure resulted in to a decrease only at HD (Figure 1.8).

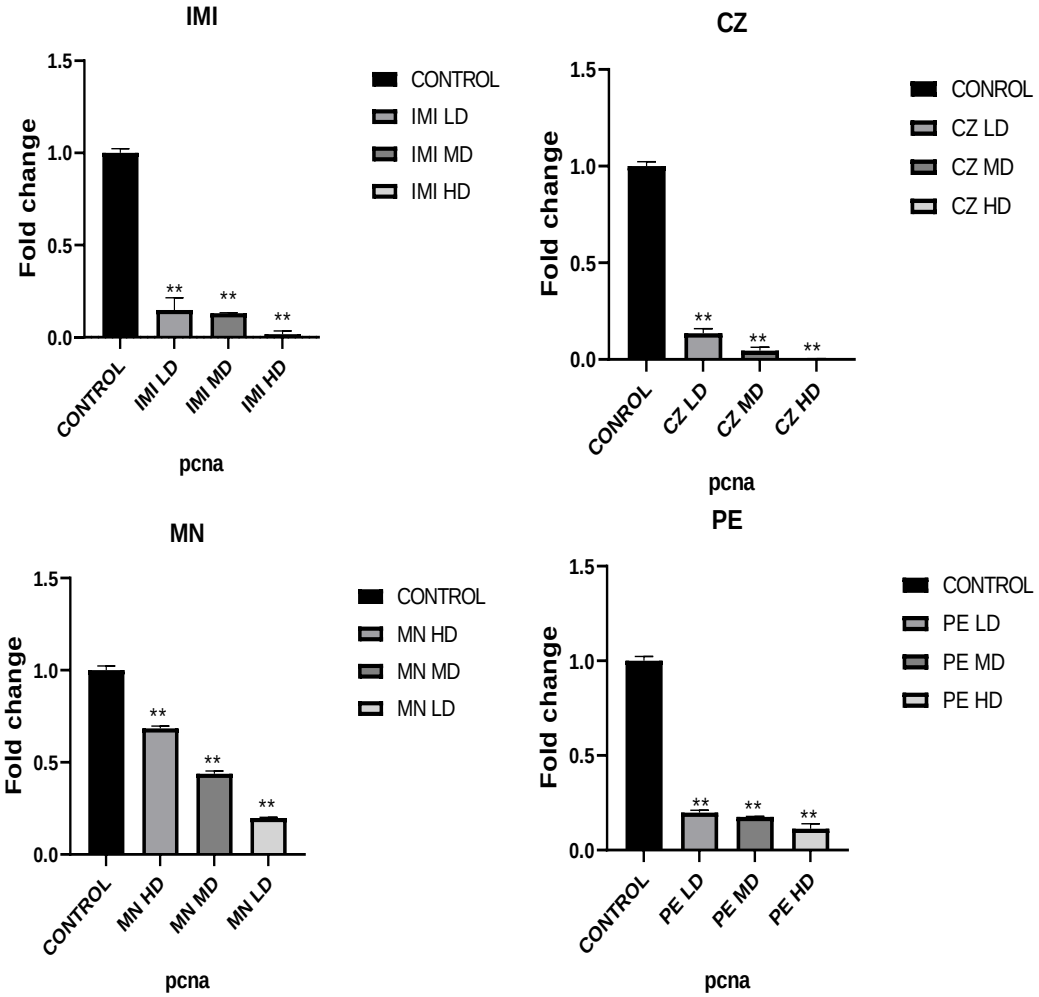

Figure1. 6: Depicts the level of pcna (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

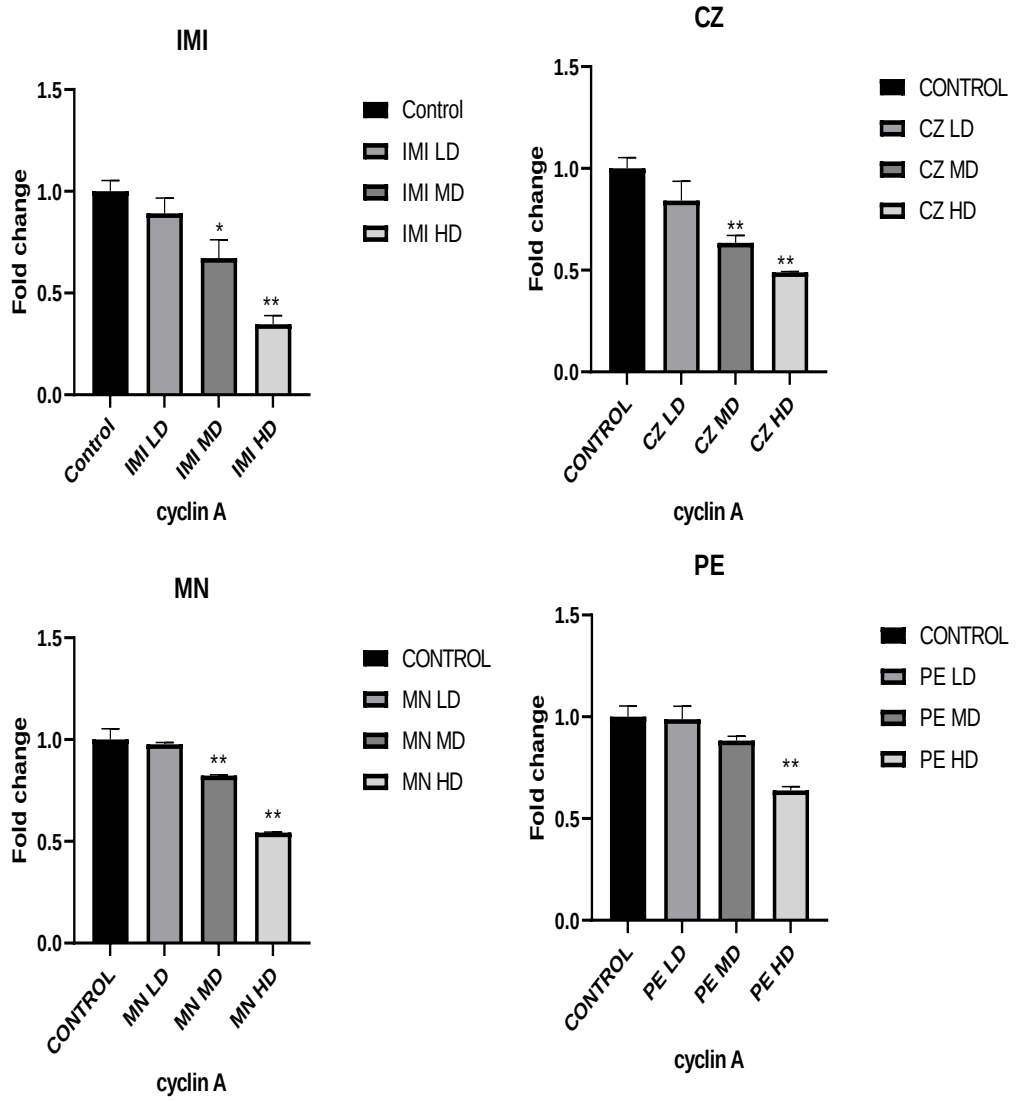

Figure1.7: Depicts the level of cyclin A (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

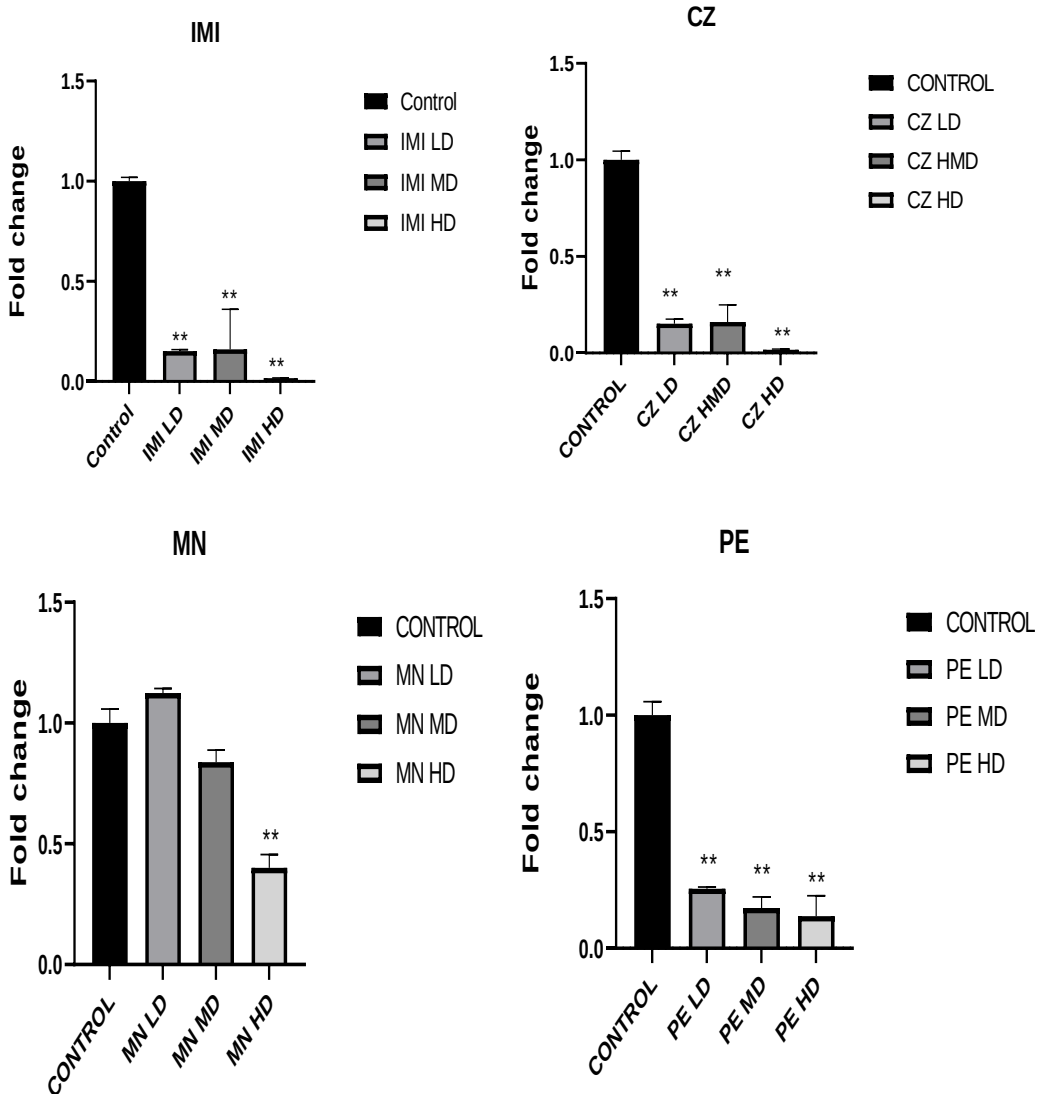

Figure1. 8: Depicts the level of cyclin E (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

Agrochemicals	pcna	cyclin a	cyclin e
Control	1±0.00	1±0.00	1±0.0
LD IMI	0.147±0.067**	0.892±0.074	0.154±0.013**
MD IMI	0.130±0.002**	0.672±0.089*	0.166±0.35**
HD IMI	0.0173±0.016**	0.346±0.043**	0.120±0.002**
LD CZ	0.1362±0.023**	0.842±0.094	0.1519±0.023**
MD CZ	0.046±0.0168**	0.634±0.036**	0.16±0.088**
HD CZ	0.0037±0.002**	0.489±0.003**	0.016±0.0029**
LD MN	0.684±0.013**	0.976±0.008	1.12±0.0182
MD MN	0.438±0.014**	0.823±0.003**	0.84±0.05
HD MN	0.198±0.034**	0.543±0.002**	0.44±0.053**
LD PE	0.0198±0.012**	0.988±0.063	0.126±0.007**
MD PE	0.176±0.016**	0.883±0.027	0.173±0.048**
HD PE	0.1128±0.027**	0.638±0.018**	0.140±0.087**

Table 1.9: Depicts the mean± SEM values of Folds change in pcna, cyclin A and cyclin E in ICG cells treated with sub-lethal doses of AGs for 7 days

1.4 Discussion:

Agrochemicals and chemical fertilizers are widely used under Green Revolution to protect the crops from pests and enhance yield, thereby increasing the productivity and economical gain of the crop yield to meet the high demand for food due to the fast growing population (Gill & Raine, 2014). Aquatic ecosystems that run through agricultural areas have high probability to get contaminated by runoff and ground water leaching by a variety of chemicals used in agricultural operations. Fish is the economically most important non-target species that are adversely affected

by severe agrochemical pollution (Pandey et al., 2005; Jacquin et al., 2020).To evaluate the toxic potential of agrochemicals many scientists have work on their toxic effect on fish in *In-vivo* and *In-vitro* system. It has been shown that ICG cells are suitable candidates for evaluating *In-vitro* acute cytotoxicity of harmful chemicals and heavy metals (Taju et al., 2014).Here we extend the use of ICG cells to evaluate *In-vitro* toxicity of agrochemicals like IMI, CZ, MN and PE.

The IC_{50} value obtained by MTT assay in the present study concluded that of all the agrochemicals tested, IMI was reported to be highly toxic compared to CZ, MN, PE. High toxicity of IMI with reference to *in-vivo* studies are well established (Dezfuli et al., 2012; Patel et al.,2016;Crayton et al., 2020). However, with regards to *in vitro* studies there are very few reports bearing the work of Abdel-Halim & Osman, (2020) where they have reported inhibition concentration of IMI to in prostate epithelial WPM-Y.1 cell line as well as Su et al., (2007) in the gill cell line of flounder and have proved IMI to be highly toxic. Furthermore, the range of IC50 of the present study is almost parallel with the earlier reported work of Abdel-Halim & Osman, (2020) and Su et al., (2007) who have reported high toxic potential of neonicotionoid IMI. The IC_{50} value obtained in the present study thus is a self-illustrative for IMI to be the most toxic of all the AGs.

Cell-based assays are often used for screening collections of compounds to determine if the toxicants have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss et al., 2004). Assays to measure cellular proliferation, cell viability and cytotoxicity are commonly used to monitor the response and health of cells in culture after treatment (Chiruvella et al., 2010). Viability and proliferation are two distinct characteristics of cells. Viability is a measure

of the number of living cells in a population whereas proliferation is a measure of cell division. Further, it is also true that not all viable cells divide.

The uptake of the dye Trypan blue in non-viable cells compared to viable cells was reported in a dose dependent manner. In continuance with the lowest IC_{50} value the maximum reduction was observed for IMI followed by CZ>PE>MN. Alteration in the cell viability has been observed by many scientists in different cell lines (keratinocyte cell line; NHBECs cells; U251 and SH-SY5Y cells) with different pesticide group (organophosphate ,fungicides, carbamates, neonicotionoidetc) and have concluded that there is decrease in the cell viability either with single or in combination of the pesticides (Coleman et al., 2012; Ilboudo et al., 2014; Abhishek et al., 2014; Angelini et al., 2015).

Cell viability is defined as the number of healthy viable cells in a system, and cell proliferation is an important indication for understanding the mechanisms behind the survival or death of cells following exposure to toxicants (Adan et al., 2016). The assessment of viability can also point to a cell's survival and, in some cases, cell multiplication. Cell cytotoxicity and proliferation are generally used for screening to detect whether the toxicants have effects on cell proliferation or display direct cytotoxic effects. Hence, our next target was to have an insight into the proliferative status of the cell on exposure of the AGs. For which we have chosen pcna and cyclin genes. Cell proliferation is an important life characteristic of living organisms, which including a series process of cell division as DNA replication, RNA transcription and protein synthesis of the complex reaction and DNA replication in nuclear is one of the most important part in the whole process.

Proliferating cell nuclear antigen (PCNA) is an evolutionarily well-conserved protein which is required for DNA synthesis during replication and other vital cellular processes such as chromatin remodelling, DNA repair, sister-chromatid cohesion and cell cycle control (Strzalka& Ziemienowicz, 2011). PCNA protein encoded by the pcna gene is used as a marker of cell proliferation because cells remain for a longer time in G1 to S phase transition(de Oliveira et al., 2008). The pcna expression has been reported in several cell types in mammalian tissues and has been reported from a number of different organs in fish (Leung et al., 2005).In the present study, a dose dependent significant decrease in the expression of pcna gene has been detected on exposure of all the AGs, however, IMI and CZ has resulted into more reduction compared to PE and MN, probably due to the pesticide stress.

The reduction in the expression of pcna is exemplifying the decrease in replication and thus the proliferation (Sun et al., 2017). The Effect of Mirex pesticide on the decreased expression of PCNA protein level in *In-Vitro* system has been reported by El-Bayomy et al., (2002). According to Sanden & Olsvik, (2009) who has reported a significant lower PCNA expression in fish cells when exposed to toxicant β-naphthoflavone, an aryl hydrocarbon receptor agonist indicating only a few cells being in the S-phase of the cell cycle and hence there is a decrease in cell multiplication. Further, studies by Anbarkeh et al., (2019) have also illustrated a significant reduction pcna and cell proliferation in germ cells of rat testis. There are reports which suggest a significant reduction in cell proliferation in liver cells when exposed to pesticides organophosphates (Hreljac et al., 2008). Our results are in agreement with the earlier reported studies performed by various group of scientist who have concluded the reduction in pcna expression due totoxicants. The overall results thus proves the toxicity of IMI and CZ, decrease in the cell proliferation also confirms their involvement in hampering the cell cycle

regulatory mechanism, however, the detailed mechanism is required to further comprehend.

The treatment of cells with toxic compounds results in multiple cell fates, including cell cycle arrest, cell cycle progression defects, or/and apoptosis (Burke et al., 2017) For further confirming the alteration in the proliferation rate, it was thought worth wile to assess the cell cycle associated genes like cyclins a and e. In the present study, a significant dose dependent decrease was observed in the expression of cyclin a on exposure of IMI, CZ and MN, however, with reference to PE a significant decrease was noted only at high dose. There was a dose dependent significant reduction observed in cyclin e expression in cells exposed to IMI, CZ and PE whereas cells exposed to MN showed decreased expression in HD only. Control of cell cycle progression is central not only in maintaining homeostasis but its alteration may also lead to imbalances in proliferation and cell death that is governed by Cyclins and Cyclin dependent kinases.

Normal cell proliferation is under strict regulation governed by checkpoints located at distinct points in the cell cycle. The deregulation of these checkpoint events and the molecules associated with them may lead to an arrest in cell cycle progression. Cyclin D and E controls transition from G_1 to S phase, cyclin A is active in S phase and the progression from G_2 to M phase is directed by cyclin B (Keyomarsi & Herliczek, 1997; Duffy et al., 2005). Cyclin E is essential for progression through the G1-phase of the cell cycle and initiation of DNA replication by interacting with and activating its catalytic partner Cdk2. Cyclin E/CDK2 regulates multiple cellular processes by phosphorylating numerous downstream proteins like p220(NPAT) to promote histone gene transcription during cell cycle progression. It also phosphorylates Retinoblastma which is critical components of cell

proliferation, Cdc6 and nucleophosmin, which are important for DNA replication (Mazumder et al., 2005). from the function in cell cycle progression, cyclin E/CDK2 plays a role in the centrosome cycle. A decreased in Cyclin A and Cyclin E is a suggestive of decrease in transition from G1 to S phase and an arrest happening at S phase through which the cell cycle regulation is getting hampered. Probably the agrochemical exposure has altered this process by inhibiting cell cycle progression where some endogenous anti-mitogenic signals could have been acting via CDK inhibitors to reduce cyclin-CDK complex activity and prevent G1/S transition (Burke et al., 2006, Burke 2017). Thus it can be summarize that of the multiple roles played by cyclins the present study S the toxic role of AGs through inhibition of the proliferation rate leading towards the cell cycle arrest.

1. 5 Conclusion:

Thus, the study on alteration in proliferation in ICG cells exposed to agrochemicals concludes:

1. The IC_{50} value obtained by MTT assay in the present study concluded that of all the agrochemicals tested; IMI to be highly toxic compared to CZ, MN, PE.
2. The study also reported a dose dependent alteration in cell viability in which the maximum reduction was observed for IMI followed by CZ>PE>MN compared to control.
3. There was a significant decrease in proliferation markers like pcna and cyclin genes indicating the toxicity of AGs is mediated by expression of proliferation-related genes and cell cycle progression genes in ICG cell line.