Unravelling the Genotoxic Potential of Agrochemicals on Fish Cell Line

Research Synopsis for Ph.D.

Submitted to

Department of Zoology

The Maharaja Sayajirao University of Baroda, Vadodara

By

Salunke Ankita Yashwant

Guide

Prof. P. H. Parikh

Department of Zoology, Faculty of Science,

The Maharaja Sayajirao University of Baroda,

Vadodara - 390002

Gujarat, India

JUNE -2021

Introduction

India is world's second largest nation with a population of 1.3 billion which is approximately 18% of the global population. The global population is expected to cross 10 billion by 2050. Rising population has led to increase food demand. To meet the food and nutrition needs of a growing population, a country requires a sustainable approach that put thrust on increasing productivity against the background of lower yields in a definite land. However, increase in food production faces with the ever-growing challenges especially the new area that can be increased for cultivation purposes is limited (Soheil et al., 2011). The Food and Agricultural Organization (FAO) of the United Nations has in-fact issued a sobering forecast that in order to keep pace with the demand of growing population, world food production needs to increase by 70% (H. K. Gill & Garg, 2014); Riggs et al., 2018). The increasing world population has therefore put a tremendous of pressure on the existing agricultural system to meet food needs from the same current resources like land, water etc. India comprises nearly 16% of the total world's population, but has just less than 2% of the total landmass, whose economy primarily depends on agriculture. A high emphasis on achieving food grain self-sufficiency along with rapid population growth has compelled Indian farmers to resort to the substantial use of pesticides. Pesticides are widely used to guarantee increased crop production and meeting the constantly escalating global food demand (Juraske et al., 2009). Approximately 25% of global crop output is lost due to attacks by pest, weeds and diseases which doesn't favour for farming, given the critical challenges ahead and thus agrochemicals have a vital role to play. In order to increase crop production, herbicides, insecticides, fungicides, nematicides, fertilizers and soil amendments are now being used in higher quantities than in the past (Juraske et al., 2009; Gill & Garg, 2014).

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 abundant use of these chemicals, under the adage, "if little is

good, a lot more will be better" has played important role in increasing the consumption. 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). Fishes are most important and highest interacted species of aquatic ecosystem and have become a bridge between aquatic and terrestrial ecosystem as consumed as primary source of food.

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. Therefore, REACh (Registration, Evaluation, Authorization and Restriction of Chemicals) supports development of alternative methods. The EURLECVAM (The European Union Reference Laboratory for alternatives to animal testing, former European Centre for Validation of Alternative Methods) is actively working on their development, according to the 3R strategy, Reduce, Refine, Replace, concept which was coined by Russel and Burch in 1959. 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 are known to develop new ways to detect the toxicity using various cell lines.

The application of in vitro techniques for questions related to fish toxicology started as early as ecotoxicology emerged as scientific discipline. Rachlin & Perlmutter, (1968) published a very first study using an in vitro assay with fish cells to assess metal toxicity to fish. From the middle of the 1990s, fish cell systems became a commonly used tool for ecotoxicological research. Babich & Borenfreund, (1991) are considered to be pioneers for evaluating the cytotoxic potential of various toxicants on fish cells. Later on, it was the laboratory of Niels Bols succeeded in establishing diverse fish cell lines such as the RTL-W1 from liver and the RTgill-W1 from gills of rainbow trout (*Oncorhynchusmykiss)* which was then used to detect specific toxicant responses. (Clemons et al., 1996; Behrens et al., 2001; Bols & Dayeh, 2005). In addition, fish cell lines were also used for purposes like the assessment of genotoxic or immunotoxic activities of chemicals or for the toxicity screening of complex environmental samples such as water effluents or sediment extracts. (Bols & Dayeh, 2005); Rehberger et al., 2018). Earlier fish hepatocytes cell lines were preferred due to its central role in toxicokinetic and toxicodynamic processes and xenobiotic biotransformation. (Segner & Cravedi, 2001). Toxic potential of fluoroacetate pesticide was studied for the first time on two fish cell lines- RTG 2 and PLHC1. (Zurita et al., 2007). Later on number of scientist have explored the toxic potential in fish muscle cell line Wallago attu muscle (WAM) in In- vitro system (Nagpure et al., 2016). However, there is a dearth of

information with regards to different classes of agrochemicals for *In-Vitro* studies compared to *In-Vivo* condition. In the present study an attempt is made to prove the advantage of *In-Vitro* assays for toxicity studies.

Over the last 2 decades, a new class of insecticide, the neonicotinoids, has become the most important and fastest growing classes of insecticides on the global market; (Tomizawa & Casida, 2011; Wang et al., 2020). **Imidacloprid** 1-(6-chloro-3-pyridylmethyl)-N- nitroimidazolidin-2-ylideneamine **(IMI)**, a new class of neonicotinoid insectide acts by binding to pharmacologically diverse nicotinic acetylcholine receptor (nAChR)**.** IMI is a potential groundwater and surface water contaminant (PAN Pesticides database, 2012), because it can leach and runoff from soil and crops (Armbrust & Peeler, 2002; Fossen, 2006) Additionally, it enters water bodies from spray drift or accidental spills, leading to local point-source contaminations. *In-Vivo* and *In-Vitro* studies have been reported to misbalance the antioxidants on exposure of IMI (X. Wang et al., 2018). Further, the genotoxic Potential of the IMI has been well explored in *Oreochromisniloticus* (Ansoar-rodríguez et al., 2015), where they have proved primary DNA damage at the chromosomal level confirming the potential risk of IMI. Feng et al., (2006) have also reported the cell growth inhibition inn FG cell line by IMI.

Curzate (CZ) fungicide was discovered by Dupont and is primarily used on grapes, potatoes and tomatoes. It is commercial use in over 50 countries on more than 15 crops. It is formulated as a 72% wettable powder: 8% cymoxanil and 64% Mancozeb.Chemical name of the substance: Mancozeb is Manganese ethylenebisdithiocarbamate polymeric complex with zinc salt and that of Cymoxanil is 1-(2- Cyano-2-methoxyiminoacetyl)-3-ethylurea. Cymoxanil belongs to the class of aliphatic nitrogenfungicides. It acts as a foliar fungicide with protective and curative action. It has contact and local systemic activity, and also inhibits sporulation (FAO, 2005). Cymoxanil is slightly toxic to fish and other estuarine and marine organisms (Guida et al., 2008) Mancozeb is "moderately to highly toxic to fish and aquatic invertebrate animals (Grisolia et al., 2004; Mellish & Specialist, 2013). Earlier studies have been individually studied with respect to Mancozeb and Cymoxanilon various animal models and found to be mild to moderately toxic (Marques et al., 2016; Tzanova et al., 2017; Simakani et al., 2018). In addition studies conducted by Patel et al, (2016) where they have reported the biochemical, Behavioural and Histological alterations on

exposure of Curzate. Apart from this no studies have been recorded on CZ with reference to *in vitro* studies.

Fertilizers containing trace elements (such as boron, copper, manganese, zinc, and cobalt) — in small quantities are called as micronutrient fertilizers. It is called micronutrients as they are needed only in minuscule amounts, these substances are the "magic wands" that enable the plants to produce enzymes, hormones and other substances essential for proper growth and development (Yoshida, 2008). Micronutrient (**MN**) fertilizers like Librel^{TM}are specially formulated for delivering micronutrients with maximum bioavailability, tolerability, & safety. Indian agriculture is now in an era of multiple plant nutrient deficiencies. Nutrients like N, P, K, Zn, Mn, Mg, Mo, B, S and Cu are now of widespread practical importance from an application point of view. To meet this deficiency, application of trace elements in the form of fertilizers micronutrients have been used rampantly whereas remediation of soils contaminated with metals is not addressed (Z. L. He et al., 2005). Repeated use of such metal-enriched chemicals, fertilizers, and organic moieties contaminate aquatic ecosystem by surface runoff leading to toxic effect to no- target organisms specially the fish which has been well explored with reference to the biochemical, histological and behavioural alterations on exposure of a plant nutrient Libre^{TM} on two edible fresh water fishes : *Labeo rohita* ns *Oreochromis mossambicus* (Sadekarpawar, et al, 2010, 2015) However, there has been a lacuna as far as *In Vitro* study is concerned.

Herbicides are the most commonly used pesticides, and are the most often detected in surface waters (Tanneberger et al., 2013). Numerous commercial formulations containing different herbicides (glyphosate, paraquat, sulfonolureaetc) have become popular around the world due to their effective action and low toxicity to mammals (Ali et al., 2014; Bren et al., 2017). Because of its its widespread use, it has become a potential water pollutant and presents environmental risk, especially for aquatic organisms, and thus, proved to be harmful to the environment. Toxicity of tri-sulfuron on aquatic organisms has been reported earlier (Seeland et al., 2012). Sub-acute studies of herbicide PE on fresh water fish, *Oreochromi smossambicus* has proved the cytotoxic potential of pyrezonsulfuron ethyl (PE) with reference to biochemical, behavioural and histological alterations (Upadhyay et al., 2014) . Further, Patel, et al (2016) have made an attempt to throw an insight on the Neuroendocrine response on exposure to PE and have opined that PE does imbalance the hormonal titres in the freshwater Teleost fish *Oreochromis mossambicus*.

Literature survey done till date has plethora of references for screening the toxic potential of agrochemicals which are limited to *In-Vivo* conditions. That too with either single or in combination of the pesticides. Baring the previous *In-Vivo* studies from our lab which has well established the toxic potential of all the classes of agrochemicals viz: IMI, CZ, MN and PE by reporting the alteration of Haematological, Histological, blood biochemical parameters, behaviour alteration and neuroendocrine response as well. (Sadekarpawar, et al, 2010, 2015; Upadhyay et al., 2014; Pandya et al., 2016). However, there is a gap in our understanding with regards to the molecular mechanism. Thus to fill the gap the present study was undertaken. to unravel the genotoxic potential of agrochemicals (PE, CZ, MN and IMI) in *In-Vitro system*. To evaluate these obscure aspects of the loss of normal cell orchestration, cell death, cell proliferation and other genetic markers which will make us to understand the disturbed machinery by taking up the following objectives:

Objectives:

1. To check the rate of proliferation in fish cell line exposed to agrochemicals

a) To determine the IC_{50} value of different classes of agrochemicals.

b) To check the proliferation by performing gene expression study of pcna, Cyclin genes in fish cells exposed to agrochemicals.

2. To validate the mechanism behind cell death upon exposure to agrochemicals in fish cell line

a) Gene expression study of caspase3, bax, bcl, tnf α, nfκb

b) To study the cell death by annexin PI staining FACS.

3. To investigate the genotypic variations of agrochemicals on fish cell line

a) To detect the presence of micronucleus in DNA

b) To study gene expression of dnmt and cyp 450 Oxygenase

Material and methods

Culturing of ICG cells:

ICG (*Catla catla*) gill cell line was procured from Indian Council of Agricultural Research-National Bureau of Fish Genetic Resources, Lucknow (ICAR-NBFGR). *Catla* gill cell line (ICG) was established from gill tissue of Indian major carp (*Catla catla*), a freshwater fish cultivated in India. (Taju et al., 2013). The cell line was maintained in Leibovitz's L-15 (Himedia AL0011A) supplemented with 10% fetal

bovine serum (FBS). The flasks were incubated at 28 ºC in a normal atmosphere incubator and half of the medium was changed every fourth day. Upon reaching 95% confluence, the cells were sub-cultured at a ratio of 1:2 following the standard trypsinization method using trypsin–EDTA solution (trypsin 0.25%, EDTA 0.02%) in phosphate buffered saline (pH 7.4).

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 96hrs 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, $1/10^{th}$ and $1/20^{th}$ does 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 x 10^4 cells per well and incubated overnight at 28 ºC. After incubation, the medium was removed and the cells were 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 4 h at 20 ºC, 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 and Cell viability and inhibition were obtained using the following formula.

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

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

Calculation of IC_{50}: After treating the cells with the test chemicals, their mortality rate was determined from the formula given above. 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 sub further sub-acute studies for low dose (LD), medium dose (MD) and high dose (HD) respectively.

Gene Expression Study:

Total RNA Extraction (Trizol method)

Total RNA was extracted isolated from ICG cells from control and treated cells for all agrochemicals. 500 µlTRIzol 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 at 260nm using NanodropC 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.

	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 Reaction mix for cDNA synthesis

After setting up reaction mix, samples were kept in thermocycler in following conditions:

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 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 and Schmittgen, 2001).

Component	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 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/exxend	60 ºC	30 seconds	

Table 4 Real Time PCR condition

	Gene Name	Primer Type	Sequence	Tm
1	GAPDH	Forward	CTCACACCAAGTGTCAGGACGAACAG	66.38
		Reverse	GTCAAGAAAGCAGCACGGGTCACC	66.13
2	P_{450}	Forward	CCCAATTTCTGTGTCTGAGAGCCTTG	64.8
		Reverse	CAGCTTCTGGAGCCCTTCAGGAATC	66.26
3	bax	Forward	GGGAGCTGCACTTCTCAACAACTTTG	64.8
		Reverse	ATTTCATCTCCAGCATCCCGTAACAC	64.21
4	bcl2	Forward	TCCAAACTCTGACAGGAGGCTTCAGG	66.38
		Reverse	ATTTCATCTCCAGCATCCCGTAACAC	63.22
5	pcna	Forward	GCACGTCTGGTTCAGGGATCTATCC	66.26
		Reverse	TGCAGAGAAATGCCCGACGAGC	63.98
6	caspase 3	Forward	GATGCCAAGCCTCAATCCCATG	62.12
		Reverse	GGTTCATGCCTGTCCTGCGATC	63.98
7	cyclin A	Forward	CTCAAGCCCGGCCAAAGAGTTG	63.98
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38
8	cyclin E	Forward	CGTGAAACCAAAGGGTGAAGACACTG	64.80
		Reverse	GCATCCATCTGAACGAGTCCAGGATC	66.38
9	nfk b	Forward	GTACCTTGGAGGGAACTGGC	61.40
		Reverse	CATCCACAGGACCACCACTC	61.40
10	dnmt	Forward	TCAGCCTTCGTCAAAGACCC	59.35
		Reverse	TTCGCCTTCTTCTCTGCCTC	59.35

Table 5 PCR real time PCR primer sequences

AO/EB staining:

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 nucleic acid selective dye that emits green fluorescence

when bound to ss DNA or RNA (at 520 nm) and red fluorescence when bound to ss DNA 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 centred at 590 nm.

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 agrochemicals for 7 days. 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^{o}C 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).

DAPI staining

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. ICG 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 all agrochemicals (IMI, CZ, MN and PE) for 7 days. The cells were washed twice with PBS, stained in DAPI dye (D9542, Sigma-aldrich, USA) liquor at a final concentration of 1 mg/ml, and incubated in dark for 15 min at 28 ^{o}C. After washing with PBS, cells were observed using a fluorescence microscope (Floid cell imaging station (Invitrogen, USA).

Results

Objective 1

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

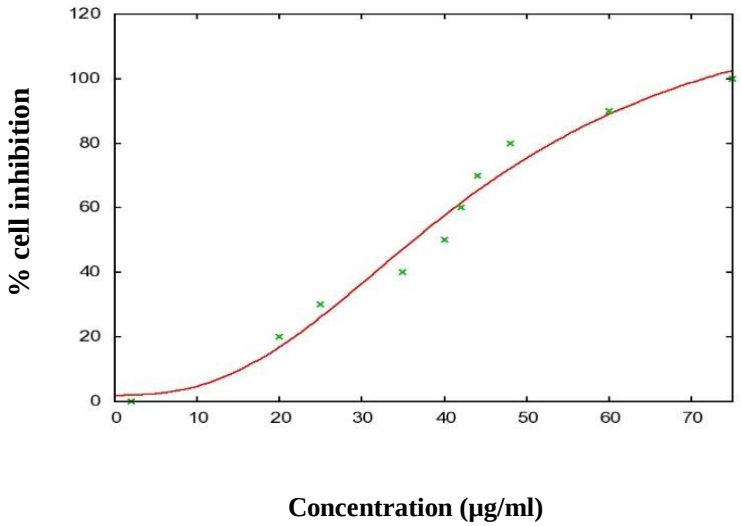

Figure 1: ICG cell mortality against different concentration of IMI

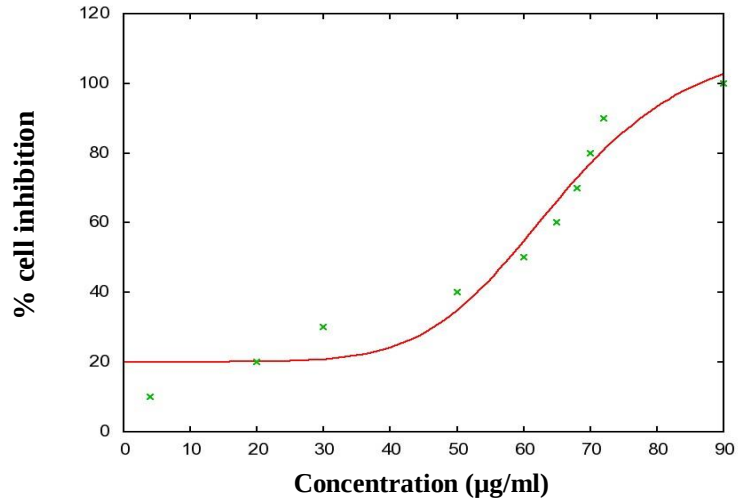

Figure 2 ICG cell mortality against different concentration of CZ

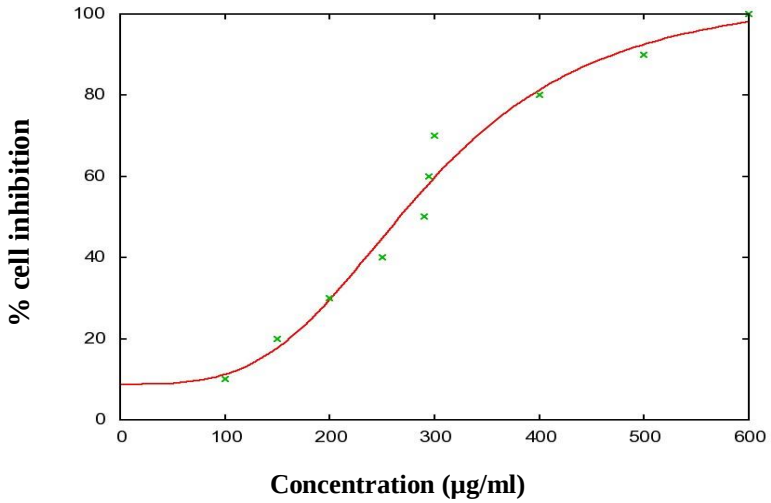

Figure 3 ICG cell mortality against different concentration of MN

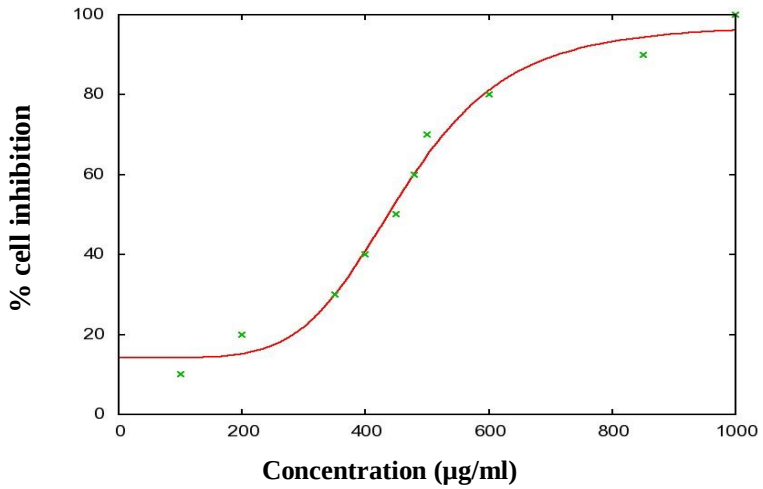

Figure 4 ICG cell mortality against different concentration of PE

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	460.85 µg/mL	23.04 µg/mL	46.08 µg/mL	92.17 µg/ml

Table 6: IC_{50} values and their Sub lethal doses for IMI, CZ,MN and PE for ICG cell line.

RNA isolation

After treating the ICG cells with all the agrochemicals (CZ, IMI, PE, MN) for 7 days, RNA was isolated from each group and and quantified for checking the purity using Nanodrop C. All the values of the nanodrop were found to be in the range of 1.8 to 2.00. **Table 7 depicts** the quantities values of total RNA and A260/A280 ratio obtained by nanodrop.

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	1.84
MN HD	267.23	1.99
PE LD	330.43	2.01
PE MD	347.21	1.88
PE HD	179.26	2.01

Table 7 Depicts the quantified values of total RNA and A260/A280 ratio obtained by nanodrop

Quantitative PCR:

Subacute exposure of agrochemicals resulted into a differential expression of the proliferative as well the apoptotic markers. Expression of the proliferative marker genes such as pcna and cyclin A showed different expression, where pcna was found to significantly decreasing ($p<0.05$) in a dose dependent manner (**Figure 5**), while cyclin A was found to be significantly decreasing ($p<0.05$) at MD and HD of IMI, CZ and MN, and PE exposure resulted an insignificant alteration at LD and MD compared to control. However, at HD there was a significant ($p<0.05$) decrease **(Figure 6).** On the other hand, the apoptotic marker genes like bax and caspase 3 found to be significantly upregulated($p<0.05$). Sub-acute exposure of agrochemicals with respect to bcl2 gene was found to be significantly decreasing ($p<0.05$) at LD, MD and HD of IMI, CZ, and PE but sub-acute exposure showed significant decrease only at HD (**Figure 7**). Expression of caspase3 expression was found to increase significantly ($p<0.05$) at MD and HD with respect to IMI and PE and at LD it showed non-significant increase. Sub-acute exposure of CZ and MN resulted into and significant ($p<0.05$) increase only at HD (**Figure 8**). The expression of nfκb did not show any significant alteration at LD, but at MD and HD it showed a significant ($p<0.05$) decrease. Agrochemical PE too did not show significant alterations in the expression of nfκb at LD and MD, but a significant ($p<0.05$) decrease was recorded at HD (**Figure 9**). On the other hand expression of bcl_2 were down regulated significantly ($p<0.05$) at the HD of IMI, CZ. and MN exposure of agrochemicals **(Figure 10**).

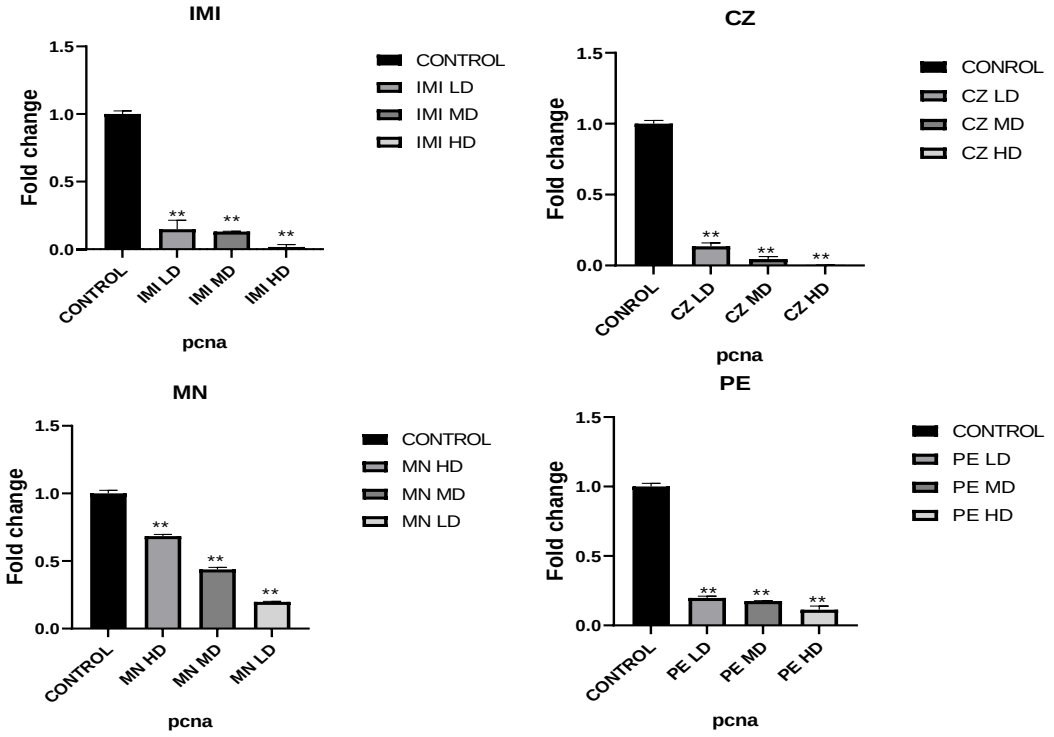

Figure 5 Depicts the level of pcna (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

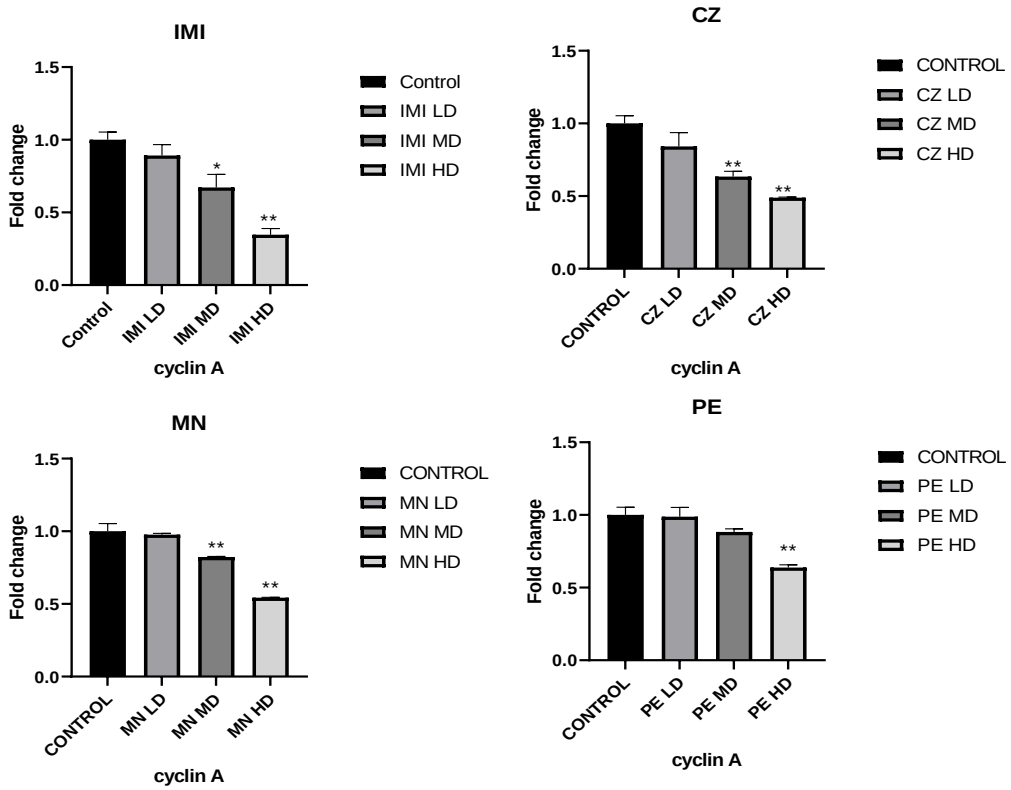

Figure 6 Depicts the level of cyclin A (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

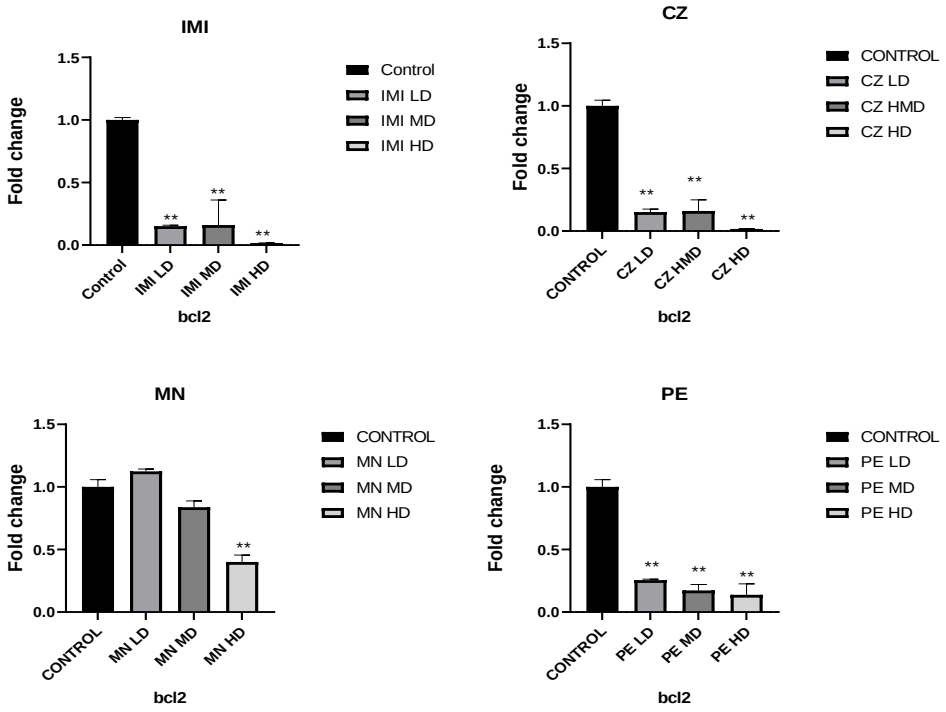

Figure 7 Depicts the level of bcl_2 (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3). Significant level indicated by *p<0.05; **p<0.01

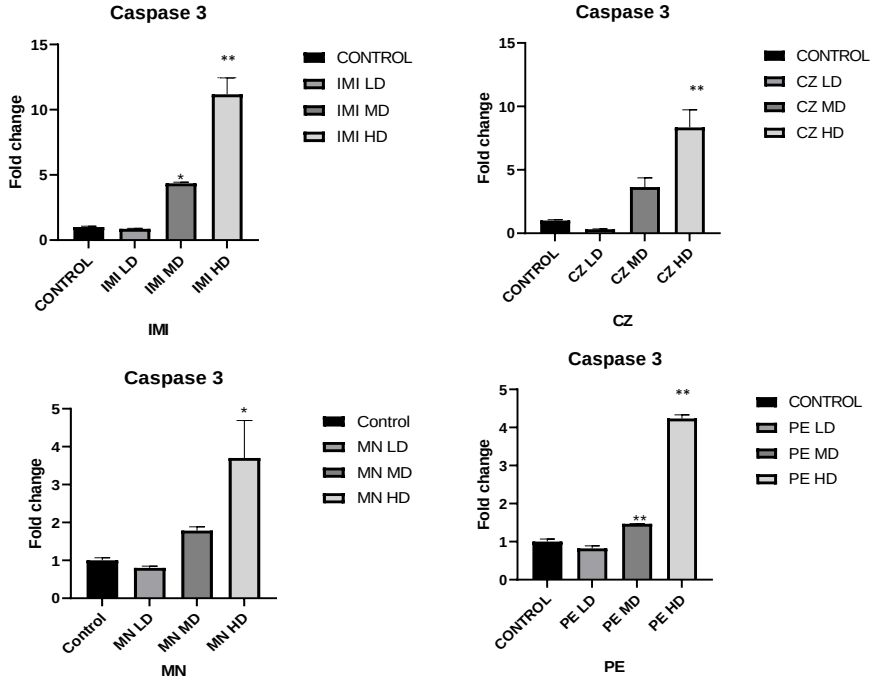

Figure 8 Depicts the level of caspase 3 (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

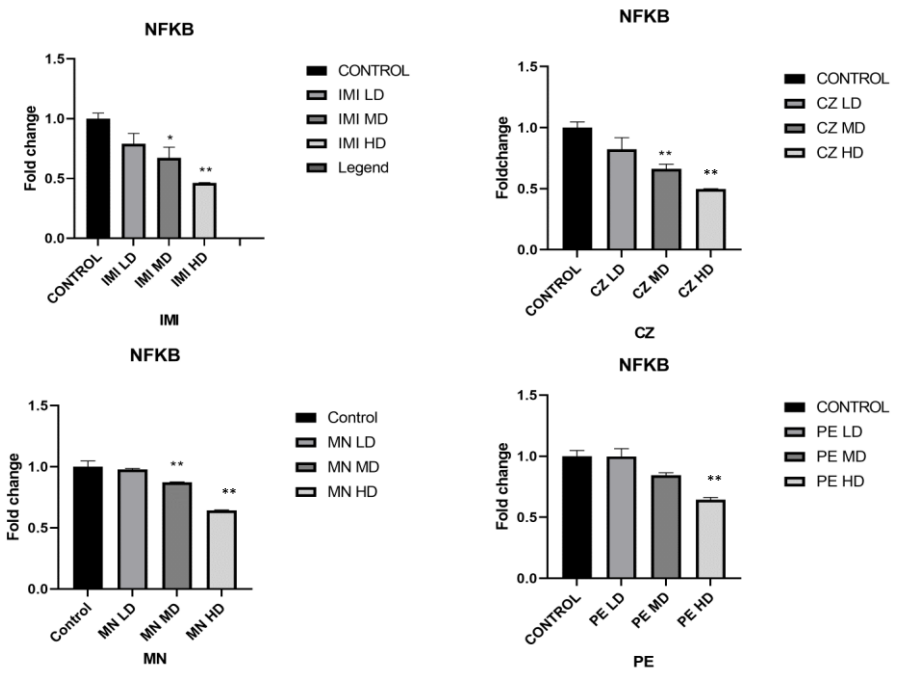

Figure 9 Depicts the level of nfκb (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01

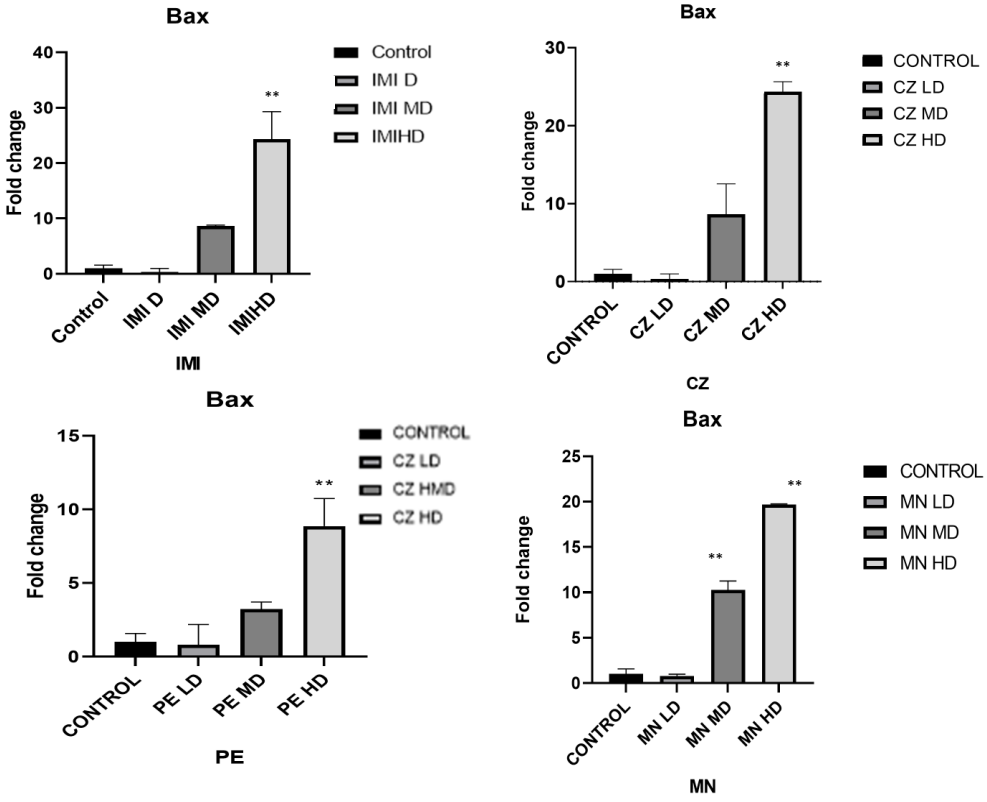

Figure 10 Depicts the level of bax (in folds) in ICG cells treated with sub-lethal doses of IMI, CZ, MN and PE. Each value represents the mean ± SEM. (n=3), Significant level indicated by *p<0.05; **p<0.01.

AO/EB Staining in cells exposed to all agrochemicals- The AO/ EtBr double staining tests early apoptotic cells appeared in greenish yellow; cells stained in orange indicated late apoptosis, and necrotic cells were stained in red. In the present study a dose dependent apoptotic body formation was observed with all the agrochemicals. However, it was IMI which revealed the highest toxicity as seen by the apoptotic body formed. On a comparative ground MN revealed the least formation of the apoptotic bodies. **Figure 11** depicts the results obtained for AO/EB staining.

DAPI Staining in cells exposed to all agrochemicals – Sub acute exposure of the agrochemicals resulted into an appreciable number of micronuclei induced by agrochemicals in the ICG cell line were analysed using DAPI. A dose dependent micronucleus formation was observed with all the agrochemicals. However, cells exposed to IMI revealed the highest toxicity by appearance of maximum micronuclei formation compared to CZ, MN and PE. **Figure 12** depicts dose dependent formation of micronucleus exposed to all agrochemicals.

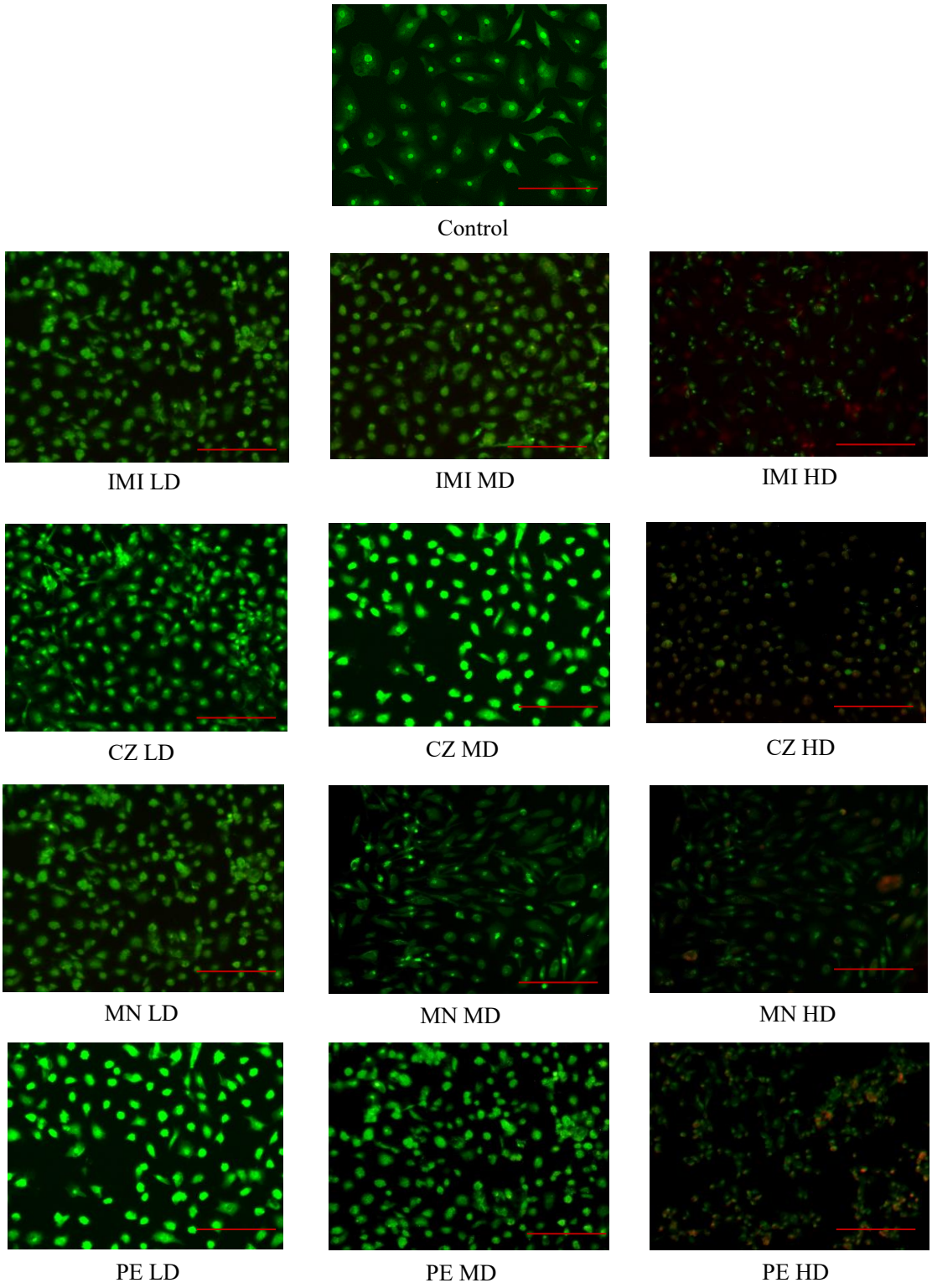

Figure 11 Results of Acridine orange/Ethidium bromide assay for ICG cells after 7days of treatment with sub-lethal concentrations of all agrochemicals (IMI, CZ,MN and PE) Bar =50µm

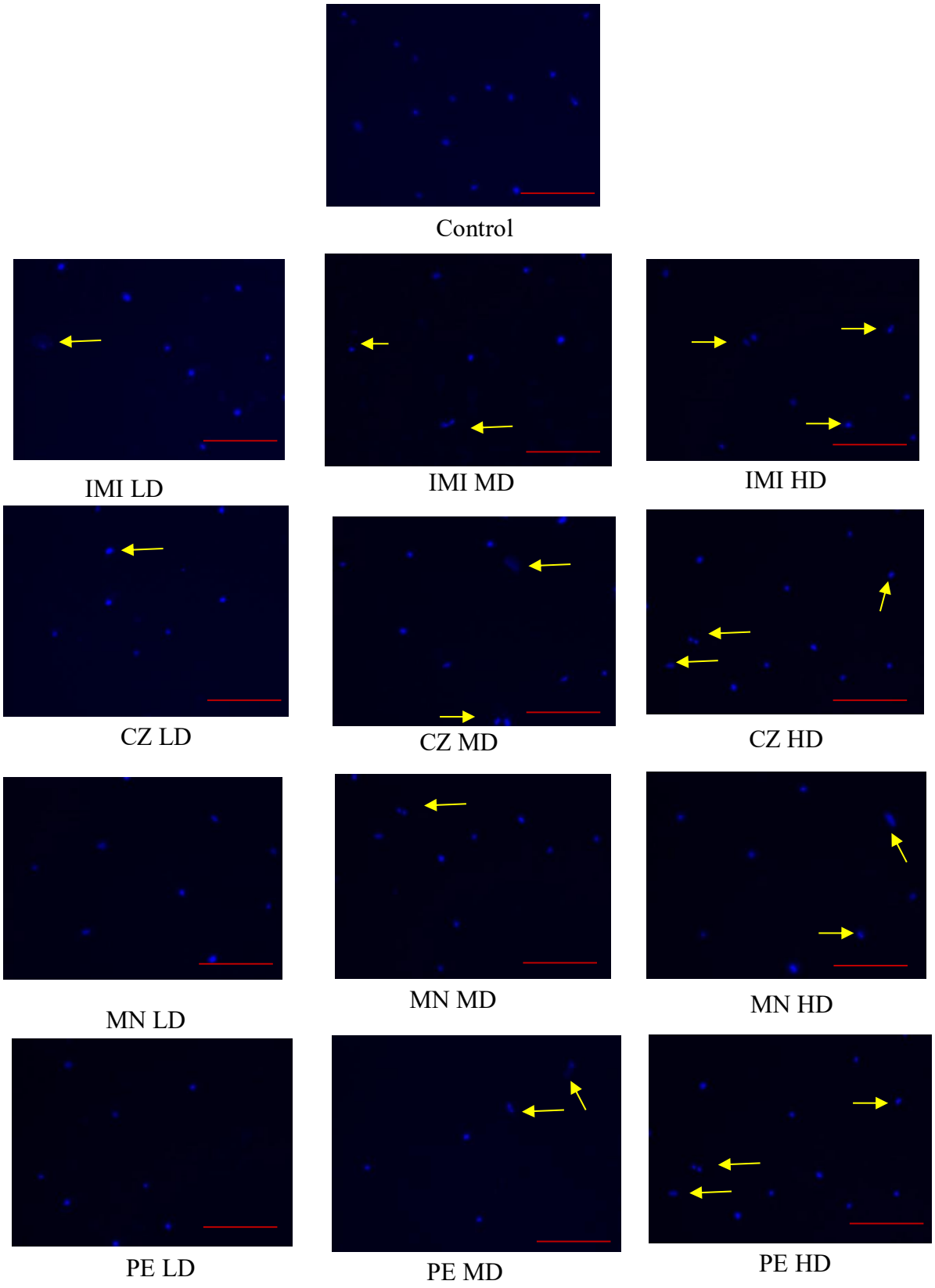

Figure 12 Results of DAPI staining for ICG cells after 7days of treatment with sub-lethal concentrations of all agrochemicals (IMI, CZ, MN and PE) Bar =50µm

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. (R. J. 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 such 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 half maximal inhibitory concentration (IC_{50}) is a measure of the potency of a chemical in inhibiting a specific biological or biochemical function. (Yilmaz et al., 2012). IC_{50} is a quantitative measure that indicates how much of a particular inhibitory substance (agrochemicals) is needed to inhibit, *In-Vitro*, a biological component by 50%.

The MTT assay is a quantitative, colorimetric and sensitive detection, widely used in assessment of cytotoxicity and cell viability as assessed by the mitochondrial ability to metabolize MTT (Vellonen et al., 2004). MTT assay also use to detect the % cell inhibition for the toxic substance to check its effects in mitochondrial activity that influence the cell death caused by exposure of agrochemicals (Rai et al., 2018). Of all the agrochemicals tested IMI was reported to be highly toxic (IC_{50} =43.95µg/mL) compared to CZ (IC_{50}=65.34µg/mL), MN (IC_{50}= 290.87µg/mL) and the least toxic was PE (IC_{50}= 460.85µg/mL), Earlier studies have been reported that the LC_{50} values individually and have proved that the neonicotinoids in general are the most toxic to the non-target organisms in *In-Vivo* conditions (Patel et al, 2016). Furthermore, *In Vitro* studies have also suggested that the neonicotinoids are more toxic compared to other agrochemicals therefore the sub-lethal concentrations were selected for further experiments.

Cell proliferation is a key monitoring parameter to evaluate the toxicity caused by xenobiotics for which we have assessed cell proliferation markers like pcna

and cyclin genes to confirm whether the agrochemical induces the cell proliferation or cell arrest (Ateeq et al., 2006; Venkatesan et al., 2017). Hence, relative quantification of proliferative markers (pcna and cyclin genes) were investigated for all agrochemicals (IMI, CZ, MN and PE). 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 and is generally considered as a universal marker for cell proliferation (Leung et al., 2005). In the present study a significant dose dependent decrease was observed on exposure of all the agrochemicals, suggesting that the decrease in the pcna mRNA has probable lead to impaired repair mechanism leading to a decreased replication process in the S-phase of the cells. Further, the results also indicate that cells may have undergone stress condition heading them to cell death(Sanden & Olsvik, 2009). Our results are in agreement with the earlier report. However, the order of the toxicity expressed by the agrochemicals was found to be IMI>CZ>MN>PE.

Control of cell cycle progression is central not only in maintaining homeostasis but its alteration may also lead to imbalances in proliferation, cell death that is governed by Cyclins and Cyclin dependent kinases. Cyclin D and E regulates transition from G_1 to S phase, cyclin A is active in S phase, cyclin B regulates progression from G_2 to M phase (Duffy et al., 2005). The result of the present study showed a decrease in the dose dependent manner in the expression of Cyclin A on exposure of IMI, CZ and MN, however, with reference to PE the pattern was a not the same and a significant decrease was noted only at high dose. A decreased in Cyclin A thus is suggestive of an arrest happening at S phase, however the results of Cyclin E which are underway will throw a clear understanding of the state of cell arrest. Probably the agrochemical exposure has altered this process by inhibiting cell cycle progression from G1 into DNA synthetic S phase where some endogenous anti-mitogenic signals could have been acting via CDK inhibitors to reduce cyclin-CDK complex activity and prevent G1/S transition (Cunningham & Roussel, 2001). Studies of Cyclin E are underway which will help us in understanding the a role of agrochemicals in cell cycle arrest (Burke et al., 2006)

Apoptosis is a type of genetically regulated programmed cell death that controls the development of multicellular organisms and tissues by eliminating physiologically

redundant, physical damaged, and abnormal cells (Danial & Korsmeyer, 2004). Studies focusing on the genes and signals regulating apoptosis have played an important role to determine the cell death pathway (Liu & Levine, 2015). Cell death is discriminated into two main forms: apoptosis and necrosis. In contrast to necrosis, apoptosis is a regulated, energy-dependent form of cell death leading to phagocytosis of cellular remnants by neighbouring cells. Cells undergoing apoptosis activated numerous proteins in a temporally as well as spatially tightly regulated sequence. Initiation is induced by various stimuli, including the binding of ligands to cell surface receptors of the tumor necrosis factor family, damage of DNA integrity by various stress factors like toxicants or major changes of the homeostasis of cells. A main theme in transduction of many of these signals further downstream is the oligomerization and interaction of proteins with death effector domains. These proteins with conserved structural modules like death and death effector domains have a number of different functions in the cell, including connecting membrane-bound receptors to cytosolic effector caspases (Ziegler & Groscurth, 2004; Fábio et al., 2021)

Intracellular stress induces apoptosis through the intrinsic cell death pathway, while extrinsic apoptosis is initiated through transmembrane death receptors. Initiation and execution of these processes are regulated by the BCL-2 and caspase families of proteins (Danial & Korsmeyer, 2004; Galluzzi et al., 2012). Activation of the BCL-2 family members Bax and Bak results in mitochondrial outer membrane permeabilization and the release of pro-apoptotic proteins, including cytochrome c, from the inter-membrane space into the cytosol (Eskes et al., 2000; Wei et al., 2012). Bax is a member of bcl_2familythat forms heterodimer with BCL_2 and functions as an apoptotic activator. It interacts and open mitochondrial voltage dependent anion channel (VDAC) and leads to loss in membrane potential leads to release of cytochrome c (Kratz et al., 2006). Cytochrome c can then bind Apaf-1 forming the apoptosome and activating caspase-9. Once active, caspase-9 can directly cleave and activate caspase-3. Caspase3 interacts with caspase-8 and caspase-9 and is encoded by the cas3 gene. It is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Caspase 3 is widely used as apoptotic marker to detect apoptosis in

progress (Yabu et al., 2001). In the present study we have obtained a dose dependent increase in caspase 3 expression which was significant ($p<0.05$) at MD and HD of IMI, while for CZ, MN and PE it was only found at HD. However, the results were not same with reference to expression of bax, and the alterations were observed only at high dose for all the agrochemicals, suggesting that the elevated caspase-3 and bax activates death protease, catalysing the specific cleavage of many key cellular proteins associated with the dismantling of the cell and the formation of apoptotic bodies which leads to program cell death (He et al., 2018).

Bcl-2which is localized at the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. The pro-apoptotic proteins in the BCL-2 family, including Bax and Bak, normally act on the mitochondrial membrane to promote permeabilization and release of cytochrome C and ROS, that are important signals in the apoptosis cascade. These pro-apoptotic proteins are in turn activated by BH3-only proteins, and are inhibited by the function of BCL-2. (Kratz et al., 2006). In the present study a significant dose dependent decrease in the bcl_2 expression was observed for IMI, CZ and PE, while for MN it was only found at HD. BAX/BAK oligomerization is prevented byBCL_2 which lead to the release of several apoptogenic molecules from the mitochondria. Decrease expression of m RNA of bcl2 suggests a decrease anti-apoptotic nature of cells which ultimately leads to decrease in cell survival and cause cell death (Kratz et al., 2006).

NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in immune and inflammatory responses, cell survival, cell adhesion, differentiation, and growth. Given that NF-κB transcribes genes that generally control both innate and acquired immune response and genes that play a positive effect on cell survival and proliferation, disregulation of the mechanisms controlling its activation often results in immunoproliferative and inflammatory phenotypes (Mazzone et al., 2015). In the present study a significant decrease in the expression of NF-κBwas observed at MD and HD of IMI, CZ and MN while for PE it was only found in HD. This suggest, that the decrease in the NF-κB mRNA has probably lead to impaired cell survival mechanism and that the cells may have undergone program cell death due to the toxicity exerted by the exposure of agrochemicals in ICG cell line.

Apoptosis is a well-controlled and vital process characterized mainly by cytoplasmic shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and apoptotic bodies (Ateeq et al., 2006). Cytological characteristics of apoptosis are generally different from those seen in cell death resulting from cell injury. Morphological criteria like apoptotic cell rounding and shrinkage, and appearance of membrane bubbles in early necrosis, allow to distinguish these cell death mechanisms, and also show that, independently of the damaging agents, the necrotic process occurs in a characteristic sequence like coalescence of membrane bubbles in a single big one that detaches from cells remaining on the substrate (Rello et al., 2005).

The AO/EtBr double staining test was employed to analyse the morphological changes of apoptosis. Early apoptosis cells appeared in greenish yellow; cells stained in orange indicated late apoptosis, and necrotic cells were stained in red. In the present study the AO/EB staining on the exposure of agrochemicals demonstrated the appearance /formation of the apoptotic bodies proposing its toxic potential (Mahajan et al., 2018). The occurrence of the morphological alterations in the ICG cell line exposed to agrochemicals revealed that of all the agrochemicals it was IMI which proved to be toxic by visualizing the number of the apoptotic bodies which were seen to be dose dependent and was maximally evident at the HD. Thus, morphological changes, like cell shrinkage, cell fragmentation, nuclear condensation, cytoplasmic disintegration were clearly observed. The result obtained in the present study are in agreement with the earlier studies conducted by Radoševićy et al., (2013) where they have done cytotoxicity assessment of imidazolium in fish Channel Cat fish Ovary (CCO) cell line, as well as by Abdul Majeed et al., (2013) who have reported the toxicity assessment of endosulfan by EB/AO staining in the gill cell line from air breathing fish *Channa striatus* (Bloch 1793). However, the FACS study which are undergoing will further confirm the quantitative toxic potential of agrochemicals.

The micronucleus assay, developed by Schmidt [1975], is an *In Vivo* and *In Vitro* short-time screening method, is sensitive, and is an extensively used tool for detecting mutagenic and genotoxic effects of toxic chemicals in the environment. Micronuclei analysis has been used as an index of cytogenetic damage for many years, is well-established method that is useful in the evaluation of genotoxic effects of xenobiotic compounds, in fishes and other species (Grisolia et al., 2004). In the present study nuclear abnormalities were assessed using DAPI staining to assess the genotoxic

potential of the agrochemicals. Formation of micronuclei induced by agrochemicals in the ICG cell line were generally dot shaped and were close to the main nucleus with varied size and shape as well as with regards to numbers, providing evidence of DNA breakage, spindle, or other parts of the mitotic apparatus dysfunction caused by agrochemicals (Naqvi et al., 2016). Our results are parallel with the earlier *In Vivo* studies (Upadhyay et al., 2014; Patel et al, 2016) with reference IMI and PE respectively. Assay with the permanent rainbow trout liver cell line RTL-W1 have also been reported on exposure of the xenobiotics (Hallare et al., 2011; Ermler et al., 2013). The micronuclei result thus have confirmed the genotoxic potential of the agrochemicals where the range of toxicity was found to be different with all the four agrochemicals and also proves the application of the micronuclei assay in the toxicological studies.

Conclusion-

The present study has unravelled the molecular mechanism involved in the toxicity of the agrochemicals in *In-Vitro* conditions. The specific markers of proliferation as well as apoptosis were found to be deregulated and were dose dependent. Of all the agrochemicals it was IMI which resulted into maximum alterations. The work is still under progress where epigenetic regulators like dnmt, p450 will be carried out along with the tnfα and cyclin E cell cycle regulators which will throw more light to understand and prove the exact molecular mechanism through which the agrochemicals are exerting Ggenotoxicity. Further, FACS analysis in support of the morphological alterations obtained by AO/EB and DAPI will help us in better understanding of precise mechanistic pathways involved n apoptosis induced by the agrochemicals.

References

Abdul Majeed, S., Nambi, K. S. N., Taju, G., Sundar Raj, N., Madan, N., & Sahul Hameed, A. S. (2013). Establishment and characterization of permanent cell line from gill tissue of Labeo rohita (Hamilton) and its application in gene expression and toxicology. *Cell Biology and Toxicology*, *29*(1), 59–73. https://doi.org/10.1007/s10565-012-9237-7

Ali, U., Syed, J. H., Malik, R. N., Katsoyiannis, A., Li, J., Zhang, G., & Jones, K. C. (2014). Organochlorine pesticides (OCPs) in South Asian region: A review. *Science of the Total Environment*, *476–477*, 705–717. https://doi.org/10.1016/j.scitotenv.2013.12.107

Ansoar-rodríguez, Y., Christofoletti, C. A., Marcato, A. C., Correia, J. E., Bueno, O. C., Malaspina, O., & Fontanetti, C. S. (2015). *Genotoxic Potential of the Insecticide Imidacloprid in a Non-Target Organism (Oreochromis niloticus -Pisces) *. December*, 1360–1367.

Armbrust, K. L., & Peeler, H. B. (2002). Effects of formulation on the run-off of imidacloprid from turf. *Pest Management Science*, *58*(7), 702–706. https://doi.org/10.1002/ps.518

Arora, V. K., Nath, J. C., & Singh, C. B. (2013). Analyzing potato response to irrigation and nitrogen regimes in a sub-tropical environment using SUBSTOR-Potato model. *Agricultural Water Management*, *124*, 69–76. https://doi.org/10.1016/j.agwat.2013.03.021

Ateeq, B., Farah, M. A., & Ahmad, W. (2006). Evidence of apoptotic effects of 2,4-D and butachlor on walking catfish, Clarias batrachus, by transmission electron microscopy and DNA degradation studies. *Life Sciences*, *78*(9), 977–986. https://doi.org/10.1016/j.lfs.2005.06.008

Babich, H., & Borenfreund, E. (1991). Cytotoxicity and genotoxicity assays with cultured fish cells: A review. *Toxicology in Vitro : An International Journal Published in Association with BIBRA*, *5*(1), 91–100. https://doi.org/10.1016/0887-2333(91)90052-f

Behrens, A., Schirmer, K., Bols, N. C., & Segner, H. (2001). Polycyclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1, and in primary cultures of rainbow trout hepatocytes. *Environmental Toxicology and Chemistry*, *20*(3), 632–643.

Bols, N. C., & Dayeh, V. R. (2005). *Use of fish cell lines in the toxicology and ecotoxicology of fish . Piscine cell lines in environmental toxicology*. *6*.

Bren, C., Reitsma, F., Baiocchi, G., Barthel, S., Güneralp, B., & Erb, K. (2017). *Future urban land expansion and implications for global croplands*. *114*(34). https://doi.org/10.1073/pnas.1606036114

Burke, K., Cheng, Y., Li, B., Petrov, A., Joshi, P., Berman, R. F., Reuhl, K. R., & DiCicco-Bloom, E. (2006). Methylmercury elicits rapid inhibition of cell proliferation in the developing brain and decreases cell cycle regulator, cyclin E. *NeuroToxicology*, *27*(6), 970–981. https://doi.org/10.1016/j.neuro.2006.09.001

Clemons, J. H., Lee, L. E. J., Myers, C. R., Dixon, D. G., & Bols, N. C. (1996). Cytochrome P4501A1 induction by polychlorinated biphenyls (PCBs) in liver cell lines from rat and trout and the derivation of toxic equivalency factors. *Canadian Journal of Fisheries and Aquatic Sciences*, *53*(5), 1177–1185. https://doi.org/10.1139/f96-039

Cunningham, J. J., & Roussel, M. F. (2001). Cyclin-dependent kinase inhibitors in the development of the central nervous system. *Cell Growth and Differentiation*, *12*(8), 387–396.

Danial, N. N., & Korsmeyer, S. J. (2004). Cell Death: Critical Control Points. *Cell*, *116*(2), 205–219. https://doi.org/10.1016/S0092-8674(04)00046-7

Duffy, K. T., McAleer, M. F., Davidson, W. R., Kari, L., Kari, C., Liu, C. G., Farber, S. A., Cheng, K. C., Mest, J. R., Wickstrom, E., Dicker, A. P., & Rodeck, U. (2005). Coordinate control of cell cycle regulatory genes in zebrafish development tested by cyclin D1 knockdown with morpholino phosphorodiamidates and hydroxyprolyl-phosphono peptide nucleic acids. *Nucleic Acids Research*, *33*(15), 4914–4921. https://doi.org/10.1093/nar/gki799

Ermler, M. E., Yerukhim, E., Schriewer, J., Schattgen, S., Traylor, Z., Wespiser, A. R., Caffrey, D. R., Chen, Z. J., King, C. H., Gale, M., Colonna, M., Fitzgerald, K. A., Buller, R. M. L., & Hise, A. G. (2013). RNA Helicase Signaling Is Critical for Type I Interferon Production and Protection against Rift Valley Fever Virus during Mucosal Challenge. *Journal of Virology*, *87*(9), 4846–4860. https://doi.org/10.1128/jvi.01997-12

Eskes, R., Desagher, S., Antonsson, B., & Martinou, J.-C. (2000). Bid Induces the Oligomerization and Insertion of Bax into the Outer Mitochondrial Membrane. *Molecular and Cellular Biology*, *20*(3), 929–935. https://doi.org/10.1128/mcb.20.3.929-935.2000

Fábio, J., Ferreira, L., & Amorim, M. T. (2021). *SARS-CoV-2 Triggers Inflammatory and Cell Death in Experimental Infection by Activating Human Cells Caspases*. *10*(04), 853–859.

Feng, Q., Baird, D., Peng, X., Wang, J., Ly, T., Guan, J. L., & Cerione, R. A. (2006). Cool-1 functions as an essential regulatory node for EGF receptor- and Src-mediated cell growth. *Nature Cell Biology*, *8*(9), 945–956. https://doi.org/10.1038/ncb1453

Fossen, M., & Ph, D. (2006). *Environmental Fate of Imidacloprid*.

Galluzzi, L., Kepp, O., Trojel-Hansen, C., & Kroemer, G. (2012). Non-apoptotic functions of apoptosis-regulatory proteins. *EMBO Reports*, *13*(4), 322–330. https://doi.org/10.1038/embor.2012.19

Gill, H. K., & Garg, H. (2014). *Pesticides : Environmental Impacts and Management Strategies Pesticides : Environmental Impacts and Management Strategies*. *February*. https://doi.org/10.5772/57399

Gill, R. J., & Raine, N. E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology*, *28*(6), 1459–1471. https://doi.org/10.1111/1365-2435.12292

Grisolia, C. K., Bilich, M. R., & Formigli, L. M. (2004). A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. *Ecotoxicology and Environmental Safety*, *59*(1), 123–126. https://doi.org/10.1016/j.ecoenv.2004.01.014

Guida, M., Inglese, M., & Meric, S. (2008). A multi-battery toxicity investigation on fungicides. *Desalination*, *226*, 262–270. https://doi.org/10.1016/j.desal.2007.04.096

Hallare, A. V., Seiler, T. B., & Hollert, H. (2011). The versatile, changing, and advancing roles of fish in sediment toxicity assessment-a review. *Journal of Soils and Sediments*, *11*(1), 141–173. https://doi.org/10.1007/s11368-010-0302-7

He, Y. F., Huang, H., Li, L. H., Yang, X. Q., Hao, S. X., & Zhao, Y. Q. (2018). Changes in apoptosis factors and activation of caspase-3 in tilapia muscle during storage. *International Journal of Food Properties*, *21*(1), 1800–1810. https://doi.org/10.1080/10942912.2018.1494199

He, Z. L., Yang, X. E., & Stoffella, P. J. (2005). Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine and Biology*, *19*(2–3), 125–140. https://doi.org/10.1016/j.jtemb.2005.02.010

Jacquin, L., Petitjean, Q., Côte, J., Laffaille, P., & Jean, S. (2020). Effects of Pollution on Fish Behavior, Personality, and Cognition: Some Research Perspectives. *Frontiers in Ecology and Evolution*, *8*, 86. https://doi.org/10.3389/fevo.2020.00086

Joseph, B., & Raj, S. J. (2010). PHARMACOGNOSTIC AND PHYTOCHEMICAL PROPERTIES OF ALOE VERA LINN – AN OVERVIEW Taxonomy : Enzymes : *October*, *4*(2), 106–110.

Juraske, R., Mutel, C. L., Stoessel, F., & Hellweg, S. (2009). Life cycle human toxicity assessment of pesticides: comparing fruit and vegetable diets in Switzerland and the United States. *Chemosphere*, *77*(7), 939–945. https://doi.org/10.1016/j.chemosphere.2009.08.006

Kasi Elumalai, L. (2012). Synergistic Effect of Seaweed Manure and Bacillus sp. on Growth and Biochemical Constituents of Vigna radiata L. *Journal of Biofertilizers & Biopesticides*, *03*(03). https://doi.org/10.4172/2155-6202.1000121

Kratz, E., Eimon, P. M., Mukhyala, K., Stern, H., Zha, J., Strasser, A., Hart, R., & Ashkenazi,

A. (2006). Functional characterization of the Bcl-2 gene family in the zebrafish. *Cell Death and Differentiation*, *13*(10), 1631–1640. https://doi.org/10.1038/sj.cdd.4402016

Leung, A. Y. H., Leung, J. C. K., Chan, L. Y. Y., Ma, E. S. K., Kwan, T. T. F., Lai, K. N., Meng, A., & Liang, R. (2005). Proliferating cell nuclear antigen (PCNA) as a proliferative marker during embryonic and adult zebrafish hematopoiesis. *Histochemistry and Cell Biology*, *124*(2), 105–111. https://doi.org/10.1007/s00418-005-0003-2

Liu, Y., & Levine, B. (2015). Autosis and autophagic cell death: The dark side of autophagy. *Cell Death and Differentiation*, *22*(3), 367–376. https://doi.org/10.1038/cdd.2014.143

Mahajan, N. P., Coppola, D., Kim, J., Lawrence, H. R., Lawrence, N. J., & Mahajan, K. (2018). Blockade of ACK1/TNK2 to Squelch the Survival of Prostate Cancer Stem-like Cells. *Scientific Reports*, *8*(1), 1–10. https://doi.org/10.1038/s41598-018-20172-z

Marques, A., Rego, A., Guilherme, S., Gaivão, I., Santos, M. A., & Pacheco, M. (2016). Evidences of DNA and chromosomal damage induced by the mancozeb-based fungicide Mancozan(®) in fish (Anguilla anguilla L.). *Pesticide Biochemistry and Physiology*, *133*, 52–58. https://doi.org/10.1016/j.pestbp.2016.03.004

Mazzone, P., Scudiero, I., Ferravante, A., Paolucci, M., D'Andrea, L. E., Varricchio, E., Telesio, G., De Maio, C., Pizzulo, M., Zotti, T., Reale, C., Vito, P., & Stilo, R. (2015). Functional characterization of zebrafish (Danio rerio) Bcl10. *PLoS ONE*, *10*(4), 1–14. https://doi.org/10.1371/journal.pone.0122365

Mellish, S., & Specialist, A. (2013). *Acute Toxicity Hazard of Pesticides to Freshwater Fish*. *September*.

Nagpure, N. S., Mishra, A. K., Ninawe, A. S., Rasal, A., Dubey, A., Kumar, A., Goswami, M., Kumar, R., & Jena, J. K. (2016). Molecular and Cytogenetic Characterization of Fish Cell Lines and its Application in Aquatic Research. *National Academy Science Letters*, *39*(1), 11–16. https://doi.org/10.1007/s40009-015-0365-5

Naqvi, G. E. Z., Shoaib, N., & Ali, A. M. (2016). Genotoxic potential of pesticides in the peripheral blood erythrocytes of fish (Oreochromis mossambicus). *Pakistan Journal of Zoology*, *48*(6), 1643–1648.

Pandey, S., Kumar, R., Sharma, S., Nagpure, N. S., Srivastava, S. K., & Verma, M. S. (2005). Acute toxicity bioassays of mercuric chloride and malathion on air-breathing fish Channa punctatus (Bloch). *Ecotoxicology and Environmental Safety*, *61*(1), 114–120. https://doi.org/10.1016/j.ecoenv.2004.08.004

Patel B, P. P. and P. P. H. (2016). Effect of agrochemicals on antioxidant enzymes and lipid peroxidation in Oreochromis mossambicus and Labeorohita. *Journal Of Zoology And Applied Biosciences*, *1*(3), 163–172.

Rachlin, J. W., & Perlmutter, A. (1968). Fish cells in culture for study of aquatic toxicants. *Water Research*, *2*(6), 409–414. https://doi.org/https://doi.org/10.1016/0043-1354(68)90060-2

Rai, Y., Pathak, R., Kumari, N., Sah, D. K., Pandey, S., Kalra, N., Soni, R., Dwarakanath, B. S., & Bhatt, A. N. (2018). Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. *Scientific Reports*, *8*(1), 1–15. https://doi.org/10.1038/s41598-018-19930-w

Reddy, P. V. L., & Kim, K. H. (2015). A review of photochemical approaches for the treatment of a wide range of pesticides. *Journal of Hazardous Materials*, *285*, 325–335. https://doi.org/10.1016/j.jhazmat.2014.11.036

Rehberger, K., Kropf, C., & Segner, H. (2018). In vitro or not in vitro : a short journey through a long history. *Environmental Sciences Europe*. https://doi.org/10.1186/s12302-018-0151-3

Rello, S., Stockert, J. C., Moreno, V., & G, A. (2005). *<morphologydeadcell_Stockert2005.pdf>*. 201–208.

Riggs, P. K., Fields, M. J., & Cross, H. R. (2018). *Food and nutrient security for a growing population*. *8*(3), 3–4. https://doi.org/10.17226/24883

Sadekarpawar, S., Desai, B. and Parikh, P. (2010). Acute Toxicity and Behavioural responses

of Oreochromismossmbicus (Peters, 1852) to Insecticide, Fungicide and Plant Nutrient. *Biohelica*, *1*(2), 16–21.

Sadekarpawar, S., Pandya P., Upadhyay, A. and P. P. (2015). A comparative assessment of trace metal accumulation in Oreochromis mossambicus and labeorohitaexposed to plant nutrient librel TM. *International Journal of Current Advanced Research.*, *4*(10), 441-449.

Sanden, M., & Olsvik, P. A. (2009). Intestinal cellular localization of PCNA protein and CYP1A mRNA in Atlantic salmon Salmo salar L. exposed to a model toxicant. *BMC Physiology*, *9*(1), 1–11. https://doi.org/10.1186/1472-6793-9-3

Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology*, *224*(3), 163–183. https://doi.org/10.1016/j.tox.2006.04.042

Schug, H., Maner, J., Hülskamp, M., Begnaud, F., Debonneville, C., Berthaud, F., Gimeno, S., & Schirmer, K. (2020). Extending the concept of predicting fish acute toxicity in vitro to the intestinal cell line rtgutgc. *Altex*, *37*(1). https://doi.org/10.14573/altex.1905032

Seeland, A., Oetken, M., Kiss, A., Fries, E., & Oehlmann, J. (2012). Acute and chronic toxicity of benzotriazoles to aquatic organisms. *Environmental Science and Pollution Research International*, *19*(5), 1781–1790. https://doi.org/10.1007/s11356-011-0705-z

Segner, H., & Cravedi, J. P. (2001). Metabolic activity in primary cultures of fish hepatocytes. *Alternatives to Laboratory Animals : ATLA*, *29*(3), 251–257. https://doi.org/10.1177/026119290102900321

Simakani, P., Abolhasani, M. H., & Hoseini, S. M. (2018). Original Article Determination of mancozeb toxicity and biochemical effects in common carp (Cyprinus carpio). *International Journal of Aquatic Biology*, *6*(3), 157–161.

Soheil, S., Saravi, S., & Shokrzadeh, M. (2011). *Role of Pesticides in Human Life in the Modern Age : A Review Role of Pesticides in Human Life in the Modern Age : A Review*. *October 2014*, 2–12. https://doi.org/10.5772/18827

Taju, G., Abdul Majeed, S., Nambi, K. S. N., & Sahul Hameed, A. S. (2013). Development and characterization of cell line from the gill tissue of Catla catla (Hamilton, 1822) for toxicological studies. *Chemosphere*, *90*(7), 2172–2180. https://doi.org/10.1016/j.chemosphere.2012.11.027

Taju, G., Abdul Majeed, S., Nambi, K. S. N., & Sahul Hameed, A. S. (2014). In vitro assay for the toxicity of silver nanoparticles using heart and gill cell lines of Catla catla and gill cell line of Labeo rohita. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *161*(1), 41–52. https://doi.org/10.1016/j.cbpc.2014.01.007

Taju, G., Abdul Majeed, S., Nambi, K. S. N., & Sahul Hameed, A. S. (2017). Application of fish cell lines for evaluating the chromium induced cytotoxicity, genotoxicity and oxidative stress. *Chemosphere*, *184*, 1–12. https://doi.org/10.1016/j.chemosphere.2017.05.151

Tanneberger, K., Knöbel, M., Busser, F. J. M., Sinnige, T. L., Hermens, J. L. M., & Schirmer, K. (2013). Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environmental Science and Technology*, *47*(2), 1110–1119. https://doi.org/10.1021/es303505z

Tomizawa, M., & Casida, J. E. (2011). Neonicotinoid insecticides: Highlights of a symposium on strategic molecular designs. *Journal of Agricultural and Food Chemistry*, *59*(7), 2883–2886. https://doi.org/10.1021/jf103856c

Upadhyay A, Pandya P, Parikh, P. H. (n.d.). Acute exposure of pyrazosulfuron ethyl induced haematological and blood biochemical changes in the freshwater teleost fish oreochromismossambicus.. *Int.J.Adv.Res.Biol.Scie*, *1*(2), 179–186.

Vellonen, K. S., Honkakoski, P., & Urtti, A. (2004). Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity. *European Journal of Pharmaceutical Sciences : Official Journal of the European Federation for Pharmaceutical Sciences*, *23*(2), 181–188. https://doi.org/10.1016/j.ejps.2004.07.006

Venkatesan, R., Park, Y. U., Ji, E., Yeo, E. J., & Kim, S. Y. (2017). Malathion increases apoptotic cell death by inducing lysosomal membrane permeabilization in N2a neuroblastoma cells: A model for neurodegeneration in Alzheimer's disease. *Cell Death Discovery*, *3*(January). https://doi.org/10.1038/cddiscovery.2017.7

Wang, A., Mahai, G., Wan, Y., Yang, Z., He, Z., & Xu, S. (2020). Assessment of imidacloprid related exposure using imidacloprid-ole fi n and desnitro-imidacloprid : Neonicotinoid insecticides in human urine in Wuhan , China. *Environment International*, *141*(February), 105785. https://doi.org/10.1016/j.envint.2020.105785

Wang, X., Anadón, A., Wu, Q., Qiao, F., Ares, I., Martínez-Larrañaga, M. R., Yuan, Z., & Martínez, M. A. (2018). Mechanism of Neonicotinoid Toxicity: Impact on Oxidative Stress and Metabolism. *Annual Review of Pharmacology and Toxicology*, *58*, 471–507. https://doi.org/10.1146/annurev-pharmtox-010617-052429

Wei, Q., Li, Y. X., Liu, M., Li, X., & Tang, H. (2012). MiR-17-5p targets TP53INP1 and regulates cell proliferation and apoptosis of cervical cancer cells. *IUBMB Life*, *64*(8), 697–704. https://doi.org/10.1002/iub.1051

Yabu, T., Kishi, S., Okazaki, T., & Yamashita, M. (2001). Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo. *Biochemical Journal*, *360*(1), 39–47. https://doi.org/10.1042/0264-6021:3600039

Yilmaz, A. D., Coban, T., & Suzen, S. (2012). Synthesis and antioxidant activity evaluations of melatonin-based analogue indole-hydrazide/hydrazone derivatives. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *27*(3), 428–436. https://doi.org/10.3109/14756366.2011.594048

Yoshida, T. (2008). Micronutrients and Health Research. *Nova Publishers*, *978*(1).

Ziegler, U., & Groscurth, P. (2004). Morphological features of cell death. *News in Physiological Sciences*, *19*(3), 124–128. https://doi.org/10.1152/nips.01519.2004

Zurita, J. L., Jos, Á., Cameán, A. M., Salguero, M., López-Artíguez, M., & Repetto, G. (2007). Ecotoxicological evaluation of sodium fluoroacetate on aquatic organisms and investigation of the effects on two fish cell lines. *Chemosphere*, *67*(1), 1–12. https://doi.org/10.1016/j.chemosphere.2006.10.027

A-Y. Salunke

PhD Candidate Sign

Guide Sign

DR. (Mrs.) P. H. PARIKH
PROFESSOR
DEPARTMENT OF ZOOLOGY
FACULTY OF SCIENCE
THE MAHARAJA SAYAJIRAO
UNIVERSITY OF BARODA
VADODARA - 390002.