General Consideration

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% (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 havea 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).

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 (*Oncorhynchus*

*mykiss)*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 toxico-dynamic 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 insecticide 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 *Oreochromis niloticus* (Ansoar-rodríguez et al., 2015,) 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) have reported the biochemical, Behavioural and Histological alterations on exposure of Curzate. However, to our knowledge there is a lacunae as far as *in vitro* studies of CZ is concerned.

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 and 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 (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* and *Oreochromis mossambicus s*(Sadekarpawar, et al, 2010, 2015) However, till date there are no reports on *In Vitro* study of MN 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, *Oreochromis mossambicus*has proved the cytotoxic potential of pyrezonsulfuron ethyl (PE) with reference to biochemical, behavioural and histological alterations (Upadhyay et al.,2014). Further, an attempt has made to throw an insight on the Neuroendocrine response on exposure to PE (Patel, et al .,2016) 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,CZand PE by reporting the alteration of Hematological, Histological, blood biochemical parameters, behavior 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.

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 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 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). 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.

Expression of the proliferative marker genes such as pcna, cyclin A and cyclin E showed different expression. There was a significant dose dependent decrease 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(Hreljac et al., 2008; Anbarkeh et al., 2019).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. Most likely, pesticide exposure changed this process by preventing cell cycle progression from G1 to DNA synthetic S phase, where certain endogenous anti-mitogenic signals might have been working through CDK inhibitors to decrease cyclin-CDK complex activity and impede G1/S transitionapoptosis(Duffy et al., 2005; Burke et al., 2017).

Furthermore, microscopic observation also revealed presence of many abnormal cells associated with missing normal cell morphology: increase of cell granularity. In addition, the cells were seen to get detached, floating and were almost seen to be dead. All the alterations were dose dependent and were maximum at the high dose of IMI. So to have an insight whether the cells are undergoing any stress the enzymatic and non-enzymatic antioxidants parameters were checked. Level of non-enzymatic antioxidant GSH and activity of antioxidant enzyme SOD and catalase have always been considered as important biomarkers to study antioxidant defense system in animals. Dose dependent depletion of GSH and decrease in activity of antioxidant enzymes has been observed in the present study. Severe oxidative stress causes a decrease in the level of these antioxidant enzymes and hence in total antioxidant potential. The results of enzymatic

and non-enzymatic antioxidative parameters clearly revealed the failure of the defence mechanism system of the ICG cells. The overall decrease in SOD and catalase thus is suggestive of the failure of the defence system against the oxidative stress of the AGs and the generated ROS is perhaps promoting apoptosis(Abdel-Halim & Osman, 2020).

Apoptosis is a type of regulated programmed cell death that controls the development by eliminating physiologically redundant, physical damaged and abnormal cells. The DCFDA staining and Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining concluded the apoptosis-associated changes of cell membranes during the process of apoptosis. This method can also accurately distinguish cells in different stages of apoptosis. We speculate that AO penetrated normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA. EB only entered cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies(Ribble et al., 2005). Furthermore, dual AO/EB staining is able to detect mild DNA injuries (Li & Darzynkiewicz, 1995). Therefore, we could distinguish normal, early apoptotic, late apoptotic cells. Apart from these the result of FACs analysis also revealed the similar results where it was reported revealed that a significantly elevated rate of apoptosis in dose-dependent manner. The outcome of the AO/EB and FACs analysis revealed that most of the cells were in the early and late apoptosis, of all the AGs exposed to ICG cells, maximum alterations were observed with IMI and CZ compared to MN and PE exposed cells.

The expression of the bax, bcl2. caspase-3, tnfα and nfķb genes confirm that the AGs are effective through intrinsic mechanistic pathway of Apoptosis. 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$_2$familythat 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 (Kratz et al., 2006).

The result of the present study is suggestive of the similar mechanism by which IMI is probably influencing in its genotoxic expression. However, to illustrate and confirm the mechanism the generation studies will throw more light to interpret role of IMI in epigenetics. Overall, putting all the results it can be concluded that: The exposure of AGs has led to formation of nuclear abnormalities in which micronucleus formation, bi-nucleated and lobed nucleated cells were highest at HD of IMI followed by CZ and PE suggesting its genotoxic potential of AGs in ICG cells. The significant alteration in expression and sequences of p450 and dnmt was reported in ICG cells exposed to HD of IMI suggest the probability of its role in toxicity leading to epigenetic alterations.

Imidacloprid exhibited binding in diversified protein classes including nuclear receptor, cytochromes, enzymes, proteases, Kinases, GPCRs, and transporters. Whereas rest compounds like pyrazosulfuron ethyl, cymoxanil, and Mancozeb exhibited very little binding probability with proteins. A total of 396 genes were found to be in close association with the candidate genes whose gene expression was studied. Out of which 18 were found to function as controlling state change of other genes, while seven were found to be involved in controlling the expression and the remaining 371 were designated as state change genes. The interaction showed that casp3 had controlling state change with bcl2 and bax, while the expression pattern control was only found between bcl2 and

casp3. The interaction also revealed that casp9, tnf (Tumor Necrosis Factor), fasl (Fas Ligand), ptk2 (protein tyrosine kinase 2) had a control state change. In addition, tnf with this interaction showed that it was controlling the expression of nfkb. The interaction further revealed that it showed the controlling the state change of hdac2 (histone deacetylase 2) hence regulating the epigenetic changes.

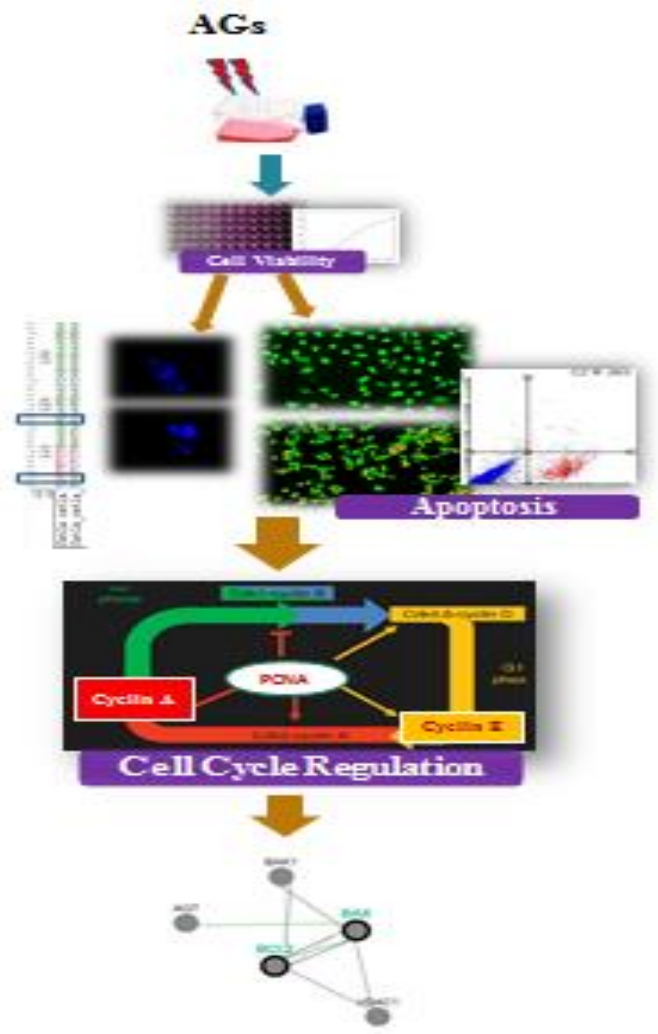

Figure GC I : Graphical Abstract

Future prospects/Recommendation

- The present study concluded that of all the agrochemicals tested; IMI to be highly toxic compared to CZ, MN, and PE. However, the toxic study needs to be conducted using different combinations of agrochemicals to throw more light in overall orchestration.
- The study also reported a dose dependent alteration in cell viability and proliferation markers in ICG cell line exposed to agrochemicals which ultimately leading towards the dysregulation of cell cycle. Further to check the toxic effect of agrochemicals the FACs studies are recommended for validating the cell cycle arrest.
- The exposure of all AGs in general have altered the morphology of the ICG cells further to check the detailed structure of tissues, cells, organelles and macromolecular complexes electron microscopy is recommended for high resolution images of cells.
- AO/EB double staining along with FACS analysis and the expression of the bax, bcl2. Caspase-3, tnfα and nfķb genes confirm that the AGs are effective through intrinsic mechanistic pathway of Apoptosis. The Next-gen sequencing will further help in authentication and will throw more light on its genome characteristics and toxicity of all agrochemicals.
- The acetylation, methylation and histon modification may help to interpret environmental as well epigenetic modulation occurred by the toxicity of AGs.
- The future holds promise for continued development as seen in recent work on cytotoxicity, proliferation, apoptosis and signalling mechanism and several other aspects of ecotoxicology. We hope that present work will provide the foundation for future toxicologist to address questions in ecotoxicology, xenobiotic metabolism and epigenetic studies**.**