INVESTIGATION OF COMBINED GENOTOXIC EFFECT OF PESTICIDES ON COLON CANCER CELL LINES

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By

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DECLARATION

I declare that the dissertation entitled "**INVESTIGATION OF COMBINED GENOTOXIC EFFECT OF PESTICIDES ON COLON CANCER CELL LINES.**" has been prepared by me under the guidance of Dr. Pankaj Bhardwaj (Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab) and Dr. Sandeep Singh (Co-Supervisor). No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

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ABSTRACT

INVESTIGATION OF COMBINED GENOTOXIC EFFECT OF PESTICIDES ON COLON CANCER CELL LINES

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The use of pesticides in agriculture sector is increasing widely to alleviate crop loss due to pests. To combat problem of pest, various types of pesticides are widely used, out of which insecticide are majorly used. In Malwa region of Punjab lots of insecticides are used. These pesticides when present together they may result in combined response. This combined response can be of three types such as independent, dose addition and interaction (synergism or antagonism). This combined response is very big trouble, such as sometimes even low dose of pesticide which is not toxic individually can result in toxic response in the presence of another pesticide. In the present study, Malathion and Monocrotophos (widely used organophosphorous insecticides) selected for investigation of combined effect. Out of selected pesticide Malathion also detected in water, in present study. Various parameters for genotoxicity evaluation was carried out with exposure of pesticides on HCT-116 WT/ p53 mutant (colon cancer) cell lines. Results of present study suggested that combination of pesticides resulted in more cell death, oxidative stress, membrane instability and imbalanced antioxidative system. It is also evaluated that low dose exposure of pesticides individually and in combination resulted in increased cell survival, may be due to activation of defense mechanism with prior treatment. All parameters evaluated was with short term exposure of 1-2 days, so evaluated long term exposure of ten days and found that this resulted in very less cell number and changed cell morphology. It is observed that both pesticides showed genotoxicity individually as well as in combination. The combined exposure of both pesticides deviate from there expected effect for dose addition towards more toxicity. So it is concluded that there may be some interaction between these pesticides, which may be synergistic in nature, because observed more toxicity.

Signature of student Signature of supervisor Signature of co-supervisor

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviations
1.	3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide	MTT
2.	5, 5', 6, 6'-tetrachloro-1, 1', 3, -tetraethylbenzimidazolylcarbocyanine iodide	JC-1
3.	Acetyl cholinesterase	AChE
4.	Chromosomal aberrations	CA
5.	c-Jun N-terminal kinases	JNK
6.	Cytochromes P450	CYPs
7.	Di-hydro Di-Chlorodihydro Fluorescein Di-Acetate	H2DCFDA
8.	Delbego's modified medium	DMEM
9.	Dimethyl sulfoxide	DMSO
10.	Ethylene Di-amine tetra acetic acid	EDTA
11.	Fetal bovine serum	FBS
12.	Glutathione	GSH
13.	Hydrogen peroxide	H_2O_2
14.	Insecticide Resistance Action Committee	IRAC
15.	International Agency for Research on Cancer	IARC
16.	Magnesium chloride	$MgCl_2$
17.	Malondialdehyde	MDA
18.	Micronuclei	MN
19.	Monocrotophos	MCP

20.	Nicotinamide Adenine Dinucleotide Phosphate	NADPH
21.	Organophosphate	OP
22.	Oxygen	O_2
23.	Phosphate buffer saline	PBS
24.	Reactive oxygen species	ROS
25.	Sister-chromatid exchanges	SCE
26.	Thiobarbituric acid reactive substances	TBARS
27.	International Union of Pure and Applied Chemistry	IUPAC
28.	Environmental Protection Agency	EPA
29.	Wild Type	WT

Chapter-1

Introduction

1.1 General introduction

Developing the agriculture sector has been always held as the prime objective of Indian economy. In spite of perpetually meeting the ever increasing food needs of the growing population, Indian agriculture sector has also provided employment to 58.2% of the Indian population (Abhilash and Singh, 2009). It is found that 18% of the food production is lost in India due to insects, pests, weeds, rodents, plant pathogens, birds etc. and the crop losses were 50-80% due to these plant pathogens. This posed a threat in the field of agriculture, where such a great loss of crop yield would be unable to meet the need of increasing population. To combat such problem, pesticides were widely used as a convenient method to control pests and its uses resulted in 20-30% reduction in crop losses and enhanced agricultural productivity, resulting in better yield of crops (Bolognesi, 2003). Due to this reason, use of pesticides has increased day by day. Although proving useful in increasing crop yield, pesticides are not completely innocuous, leading to numerous deleterious effects. People are exposed to pesticides through pesticide contaminated environment such as food, drinking water, air, dust, and soil resulting in disease manifestation (Gilden *et al.,* 2010). Pesticides are grouped into herbicides, fungicides, insecticides, algaecides, antimicrobials, rodenticides and various other materials used to control pests. Out of the various types of pesticides, insecticides are widely used, these insecticides are of various types such as organochlorine, carbamates, pyrethroids, organophosphates and chlorophenoxy compounds (Younes and Galal-Gorchev, 2000). Among various pesticides organophosphate compounds are the major chemical class which is used worldwide (Kwong *et al*., 2002).

Use of multiple types of pesticides by farmers results in combination of pesticides, which sometimes prove very harmful to organisms including humans. Various factors contribute towards the mixing of pesticides such as intentional mixing of multiple pesticides for crop protection, growing two or more types of crops in rotation system

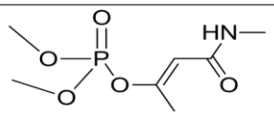
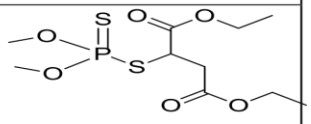
and heavy rain etc. (Tiwana *et al*., 2007). Mixtures of pesticide can take three forms that may be independent, dose addition or interaction (Wilkinson *et al*., 2000). Interaction may be synergistic (increased toxicity compared to dose addition) (Hertzberg and MacDonell, 2002) or antagonistic (decreased toxicity compared to dose addition). Interaction may be due to changes in toxicokinetic phase (changes with respect to absorption, metabolism, distribution or excretion, in the presence of other pesticide) or toxicodynamic phase (changes with respect to interaction with target site) (Tennekes and Sanchez, 2013).

In the real world, effects of single pesticide are difficult to predict because of the presence of other pesticides, which may result in combined effect on individuals. Pesticides interaction is important to evaluate as the actual dose response of individual pesticide may change during mixed exposure of pesticides. Keeping this in view, the aim of present study is to evaluate the combined effect of Monocrotophos and Malathion (two mostly used organophosphorous insecticides, in the Malwa region of Punjab).

1.2 Selected Pesticides

Out of various pesticides, insecticides are widely used (Younes and Galal-Gorchev, 2000) and out of various insecticides, organophosphorous are widely used (Jaga and Dharmani, 2003). On the basis of literature and conducted survey, Monocrotophos and Malathion were selected for study. There are some important properties of both (Table1.1).

Table1.1 Properties of Malathion and Monocrotophos

Sr. No.	Properties	Monocrotophos	Malathion
1.	Chemical structure		
2.	IUPAC Name	Dimethyl (E)-1-methyl-2-(methylcarbamoyl) vinyl phosphate	O, O-diethyl O-quinoxalin-2-yl phosphorothioate
3.	Molecular	223.2 g/mol	330.358 g/mol

	weight		
4.	Physical appearance	Reddish brown crystalline solid	Light, amber-coloured liquid
5.	Melting point	54-55^{o}C	31-32 °C
6.	Half life	96 days (pH 5), 66 days (pH 7) and 17 days (pH 9)*	4-6 days, low hydrolysis at pH 7*
7.	Solvents	Water, acetone, alcohol	Water, acetone, ethyl acetate, alcohol and other aromatic hydrocarbons
8.	Toxicity class	Class “I b” (Highly hazardous) **	Class “III” (slightly hazardous) **

* FAO, 2000; **WHO, 2009

1.3 Hypothesis

In the recent years, exposure of various pesticides to non-target species have been a main reason of concern. Since these pesticides even in a short period of time may result in unpredicted toxic effects, therefore, the cumulative toxicity assessment of pesticides in mixtures has been an enduring challenge. In this direction, in the present study, it has been planned to find out the combined toxicity of Malathion and Monocrotophos, as being the most commonly used pesticides in the Malwa region of Punjab. These are also detected in the blood sample of people of Punjab. Malathion and Monocrotophos belongs to organophosphorous insecticides. Both of these pesticides are used on some common crops and individually show some common consequences such as inhibition of acetylcholinestrase, chromosomal aberration, DNA damage, increase in white blood cells (WBC), inflammatory cell infiltration, increase in reactive oxygen species (ROS) production, cell death etc. Due to their some common factors, it is expected that, they may show combined effect on cells, when exposed in mixture. This research work has been aimed to determine their combined effect that may be independent, dose addition or interaction (i.e. synergism or antagonism).

1.4 Rationale of Research

Malathion and Monocrotophos are known to be toxic individually. The plausible mechanism of these pesticides to show toxicity have been thought to be via free radical production, that may also effect mitochondrial stability and DNA damage that leads to stimulation of apoptotic pathways. Because p53 is involved in various stress responses, so it is also expected that the combined pesticide exposure will show some relevance to p53 also. It is expected that combination of Malathion and Monocrotophos will show diverse response in cell death that may be due to imbalanced ROS production, mitochondrial stability, lipid peroxidation and antioxidative system. This combined effect may be independent, dose addition or interaction with respect to cell viability.

1.5 Objectives

The present study aims to find the pesticides commonly used in Bathinda and Mansa region of Punjab and then to evaluate the various parameters after individual as well as combined pesticide exposure on cells.

The main objectives of the study are:

- Evaluation of combined response with respect to genotoxicity and cytotoxicity in cells in response to individual as well as combined pesticides treatment.
- Measurement of oxidative stress and antioxidant defence system in cells exposed to individual and combination of pesticides.
- Assessment of nature of pesticide combined response with respect to their impact on cell survival.

Chapter-2

Review of Literature

2.1 Pesticides

Pesticides are of various nature, among which some are persistent, remaining intact rather than breaking down into safer constituents, thus showing more toxic effect on health. Different pesticides exhibit these effects to different extents; some being more aggressive than the other (Stockholm Convention on Persistent Organic Pollutants, 2001).

In Asia, India is the largest producer of pesticides and occupies the twelfth rank in the world for the use of pesticides (Abhilash and Singh, 2009). Pesticides are widely used in many states of India, especially in Punjab, which occupies only 1.54 % of the area (i.e. 50,362 square kilometre area), production being 22% wheat, 12% rice and 13% cotton of India. Such successful high productivity of Punjab is attributed to green revolution which implemented newer and advanced technologies, irrigation, fertilizers and pesticides resulting in increased yield of wheat (*Stastical abstract of Punjab*, 2005).

Malwa region of Punjab is less than 15% of the total area of Punjab, but it consumes nearly 75% of the total pesticides used in Punjab and increasing day by day (Misra, 2007). Pesticide consumption in Punjab has increased from 213 kg/hectare (in 2007-08) to 246 kg/hectare (in 2011-12) (Blaurock-Busch *et al*., 2014). Due to their widespread usage some pesticides leach into surface and groundwater, so also contaminate drinking water (Younes and Galal-Gorchev, 2000). Another factor responsible for the presence of pesticides in groundwater is soil profile of the Malwa, which is silty in nature, having less water holding capacity due to which pesticides easily leach into the groundwater (Tiwana *et al.,* 2007).

The exposure of pesticides results in various health problems via various mechanisms, such as pesticides cause an increase in the free radicles level. Free radicals are produced in the cells during normal metabolic processes such as cellular respiration, processes of biosynthesis and biodegradation, biotransformation of xenobiotics and

phagocyte activation, but increased free radical level interact with lipids, proteins and DNA, producing potentially harmful effects on cells (Yaduvanshi *et al.,* 2010). In various mutagenicity assays, most of pesticides result in gene mutation, chromosomal alteration and DNA damage. In various studies, chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) are used as biomarkers of pesticide genotoxicity (Bolognesi, 2003).

Long-term effects of pesticides have been observed to cause carcinogenic as well as genotoxic effects. Carcinogenic pesticides may increase the risk of cancer through a variety of mechanisms, including genotoxicity, hormonal action, tumor promotion, and immunotoxicity, although assumed to have no effect below the threshold level (an effective dose level) (Dich *et al*., 1997). Some studies have revealed increased risk, associated with occupational pesticide exposure, resulting in various types of cancer, such as non-Hodgkin's lymphoma, Hodgkin's syndrome, multiple myeloma, leukemia and cancers of the brain, connective tissue, prostate, abdomen and testis (Davis *et al*., 1992; Dosemeci *et al*., 1994). Organophosphorous insecticides which are widely used are one, out of various highly toxic pesticides.

2.2 Organophosphate (OP) insecticides

Organophosphate insecticides have gained popularity worldwide in preference to organochlorines, which are more persistent and damaging to the environment (Jaga and Dharmani, 2003). The effects of OP lead to various health problems, such as respiratory dilemmas, neurologic deficits, memory disorders, dermatologic conditions, cancer, miscarriages, and neonatal diseases (Arcury*et al*.,2003; Cordes and Rea,1988; Das *et al*.,2001; Strong *et al.,*2004). Organophosphate compounds show an association with Non-Hodgkin's lymphoma (McCauley *et al*., 2006; Alavanja *et al.,* 2004).

Organophosphate pesticides act on pest by phosphorylating the serine residue at the active site of acetylcholinesterase, which plays an important role in cell to cell communication in synapses and in neuromuscular junctions. They are known to be potent neurotoxicants (Kwong *et al*., 2002).

Organophosphates are being lipophilic in nature, thus interacting and perturbing phospholipid bilayer structure of most visceral organs and systems; such as liver, kidney, immune system, nervous system and reproductive system etc. (Gomes *et al*., 1999). In another way, OP damage tissues by generating oxidative stress, via the production of reactive oxygen species resulting in the activation of cellular antioxidant systems (Bagchi *et al.,*1995). OP also show cytogenetic damage such as gene mutation, chromosomal mutation and DNA Damage (Bolognesi and Morasso, 2000).

In conclusion, it may be presumed that pesticides though are essential for agriculture, may prove to be fatal to health, their long term exposure resulting in variety of diseases such as reproductive disorders or cancer via various mechanisms.

2.3 Pesticides under investigation

Mathur *et al*., (2005) observed four organophosphorous pesticides (Monocrotophos, Chlorpyrifos, Malathion and Phosphamidon) in blood sample of people of Punjab. Out of which Malathion was detected in 70% of the whole blood samples collected from Punjab at mean levels of 0.0301 mg/l and Monocrotophos was detected in 75% of the samples at mean levels of 0.0948 mg/l. So, on the basis of conducted survey and literature, in the present study, Malathion and Monocrotophos were selected for their combined effect evaluation.

2.3.1 Monocrotophos (MCP)

Monocrotophos was registered by the Central Insecticides Board and Registration Committee (CIBRC) for 14 crops, including Paddy, Maize, Bengal Gram, Green Gram, Pea, Red Gram, Sugarcane, Cotton, Castor, Mustard, Citrus Fruits, Mango, Coffee, Cardamom (Bhushan *et al.,* 2013). N-methylacetoacetamide and the O-dealkylation are primary products of the MCP hydrolysis (Lee *et al.,* 1990).

Organisms exposed to Monocrotophos exhibit various types of symptoms such as muscle weakness, blurred vision, confusion, vomiting, pain, bone marrow depression, respiratory failure and decrease in hemoglobin, red blood cells, platelets, haematocrit value and increase in white blood cell count and mutagenicity (Gupta *et al.,* 1982; Siddiqui and Mustafa, 1993). Monocrotophos is a potent neurotoxicant which inhibits

87% acetylcholinesterase in striatum, 67% in hippocampus, 58% in the cerebellum, and 53% in the cortex, which may induce oxidative stress in the brain (Kazi *et al*., 2012).

According to Yaduvanshi *et al*. (2010), MCP exposure results in increase of thiobarbituric acid reactive substances (TBARS) in liver, brain, spleen and kidney. Another effect of MCP exposure is, DNA and nuclei damage as none of the nuclei are compact, condensed, undamaged, which are properties of intact nuclei. MCP treatment also results in reduction of microsomal cytochrome P450, hepatic glutathione content and the activity of glutathione- S-transferase, brain AChE, Ca^{++} ATPase and also induces lethal mutations in male germ-line cells and somatic cells (Tripathy and Patnaik, 1992). Monocrotophos show chromosomal aberrations (CA), sister-chromatid exchange (SCE), gene mutation, DNA damage and decreased mitotic index (Saleha Banu *et al.,* 2001; Rupa *et al.,* 1989a; Rupa *et al.,* 1989b; Bolognesi and Morasso, 2000).

It is found that exposure to MCP causes enhanced ROS generation, lipid peroxidation, apoptosis and decreased glutathione (GSH) levels in PC12 cells with the involvement of response of various types of Cytochromes P450 (CYPs). It was found that MCP induced apoptosis and oxidative stress associated or regulated by specific isoforms of CYPs, the increase observed in the expression of CYPs suggested responsiveness of cells against MCP exposure, but the level decreases after long term exposure which could be attributed to significant necrotic cell death rather than apoptosis. Such induced expression of CYPs in the initial stages of exposure can be thought to play an important role in the production of reactive oxygenated molecules, known to induce c-Jun N-terminal kinases (JNK) pathway resulting in up-regulation of C-fos and C-jun gene protein expression, which induce apoptosis (Fig.2.1). JNK pathway activation which results in subsequent cell death has been thus found to have a correlation with increased expression of glutathione S transferase pi gene (GSTP1-1) and at the same time decreased GSH levels. In this research study, team concluded that CYPs are involved in the activation of caspases.

Normally this caspase cascade activation is seen to result when damaged mitochondria release the cytochrome-c which binds to procaspase-9 and Apaf-1 to form the apoptosome complex (Kashyap *et al*., 2011).

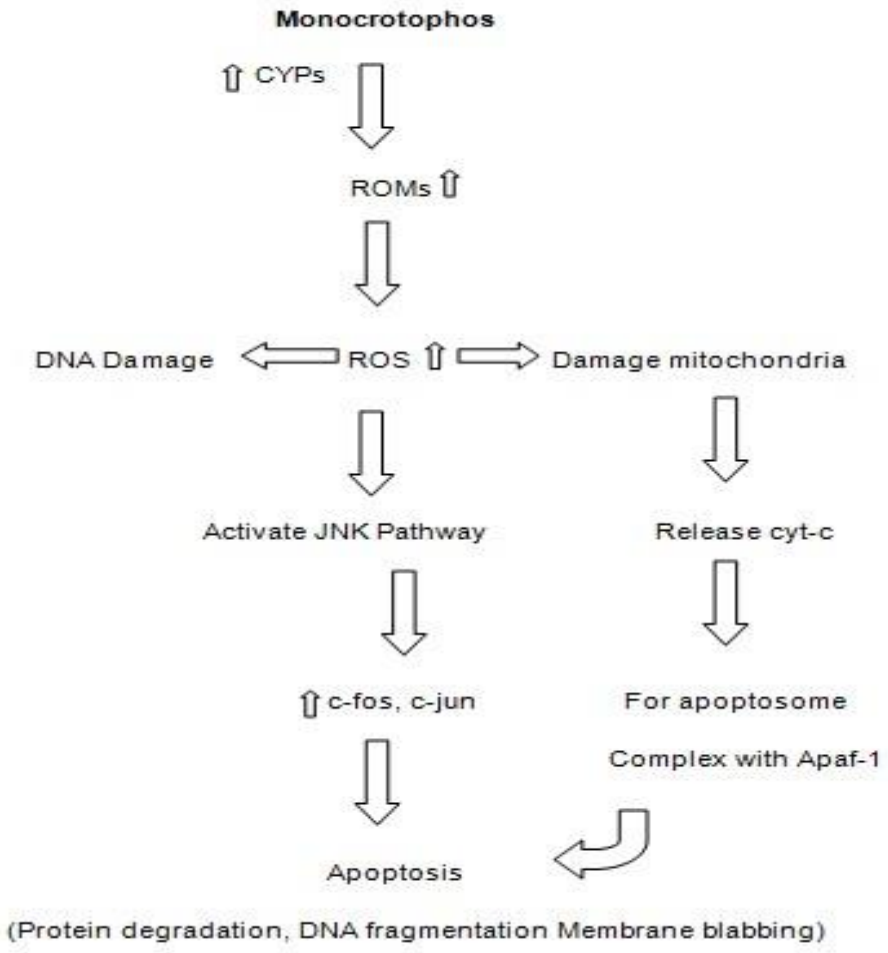


Fig.2.1 Mechanism of cell apoptosis induced via Monocrotophos.

2.3.2 Malathion

Malathion was registered for 16 crops by CIBRC which include Paddy, Sorghum, Soybean, Cotton, Castor, Groundnut, Mustard, Sunflower, Okra, Cauliflower, Radish, Turnip, Tomato, Apple, Grape, Mango (Bhushan *et al.,* 2013).

Malathion poisoning results in headache, muscles weakness, dizziness, nausea, vomiting, and difficulty in breathing (Haque *et al*., 1987). Subchronic exposure to Malathion leads to reduced Acetylcholinesterase (AChE) activity, hepatic protein and

lipid contents as well as muscular glycogen rate (Rezg *et al*., 2007).On the contrary, acute exposure causes muscle dysfunction and muscle weakness which may prove to be fatal, even causing death (Karami-Mohajeri *et al*., 2013).

Numerous studies have highlighted the genotoxic nature of Malathion. Malathion exposure in mice resulted in cytogenetic damage in the bone marrow cells, thus showing chromosome aberration, decreased mitotic index resulting in DNA damage (Moore *et al*., 2011; Ojha and Srivastava, 2014; Balaji *et al*., 1993). It is reported that low dose (6mM) of Malathion, increases cell viability, however high dose (12 mM or 24mM) of Malathion, decreases cell viability in a concentration-dependent manner. It is observed that Malathion is cytotoxic in nature and increases DNA damage at higher concentration. It increases oxidative stress and lipid peroxidation of cellular membranes through various biochemical processes (Moore *et al*., 2010; Ojha and Srivastava, 2014). Kalender *et al*., (2010) found that, the acute exposure to Malathion exhibiting disruption in the lipid metabolism, thereby elevating Low-density lipoprotein (LDL) and triglyceride levels and stimulation of glycogenolysis and gluconeogenesis by the liver.

The toxicity of Malathion is mainly owing to its most important metabolite, malaoxon. It is a neurotoxic molecule, rightly held responsible for numerous cases of acute toxicity. Malaoxon is 40 times more toxic than Malathion thus being the primary source of Malathion toxicity (Brown *et al*., 1993).

It is observed through literature, that there are some common properties in both Monocrotophos and Malathion. In the present study, since the combined effect was investigated, it becomes important to consider some common points in both (Table 2.1).

Table 2.1 Some common characteristics in Malathion and Monocrotophos

Sr. No	**Common factors in MCP and Malathion**	**Monocrotophos (Reference)**	**Malathion (Reference)**
1.	Organophosphorous insecticide	IRAC, 2014	IRAC, 2014
2.	Used on Paddy, Cotton, Castor, Mustard and Mango	Bhushan *et al.,* 2013	Bhushan *et al.,* 2013

3.	Inhibition of acetylcholinesterase	Kazi *et al*., 2012	Rezg *et al*., 2007
4.	Result in chromosomal aberration and DNA damage	Bolognesi and Morasso, 2000	Moore *et al*., 2010
5.	Result in increase in WBC's	Siddiqui and Mustafa, 1993	Kalender *et al*., 2010
6.	Result in inflammatory cell infiltration	Yaduvanshi *et al.,* 2010	Kalender *et al*., 2010

2.4 Combination of pesticides

Pesticides almost usually occur in mixture, but their toxicological effects to the human health are yet unknown. Mixtures of pesticide can be associated in three forms that may be independent, dose addition or interaction (Wilkinson *et al*., 2000).

Independent action- When the combination of pesticides results in the same effect as in individual, such type of response is known as independent response, which means that, they don't interfere with the response of each other (Wilkinson *et al*., 2000).

Dose-addition – When the combination of two or more pesticides (of concentration which don't show effect individually) result in above threshold level toxicity concentration, is known as Dose addition. Dose addition is expected for those pesticides that show the same toxic effect on the same organ by the same mechanism over all the concentrations of pesticides (Stewart and Carter, 2009). Sometimes the additive response of pesticides mixtures may fail due to involvement of secondary chemical changes that result in enhanced or reduced toxicity (Hernandez *et al*., 2013).

Interaction- When the combined effect of two or more pesticides deviate from their additive response this is known as pesticide interaction. In this combined pesticides, result may increase or decrease in the toxicity from their additive response. If the response of combination decreases from expected additive, these pesticides are known as antagonist of each other and response known as antagonistic response (Rider and LeBlanc, 2005). If combination results in more toxic effect than their

expected additive response, this interaction is known as synergistic in nature (Hertzberg and MacDonell, 2002)

The plausible mechanism behind interaction may be at toxicokinetic or toxicodynamic phases, which may take place when there is exposure to more than one pesticide. Toxicokinetics is the study related to absorption, distribution, metabolism and excretion of chemicals and Toxicodynamics is the study with respect to the receptor or target site or mechanism of chemical action (Fig.2.2).

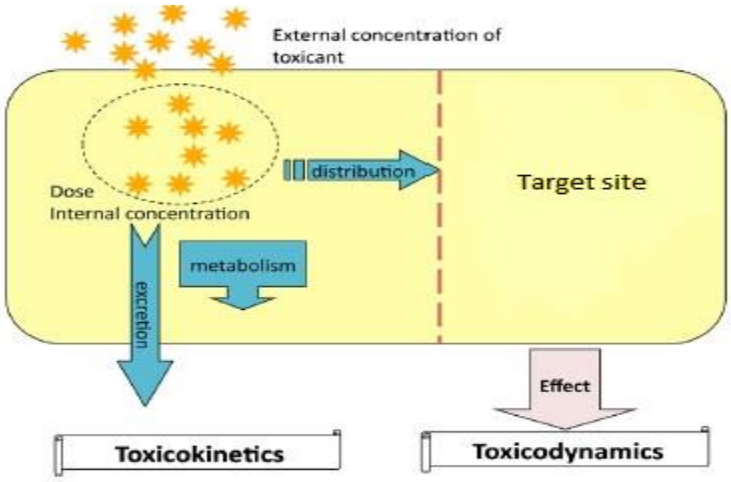


Fig.2.2 Toxicokinetic and Toxicodynamic phases at which interaction may happen in the presence of more than one pesticide (Tennekes and Sanchez, 2013).

Toxicokinetic interactions is when one pesticide changes the absorption, metabolism, delivery or exclusion of another pesticide, as a of which internal concentration change. Toxicokinetics interactions are independent from the mode of action. (1) These interactions may be due to inhibition, induction or saturation of enzymes involved in the pesticide metabolism in the presence of another pesticide. Enzyme involved in the metabolism or elimination of pesticide provide site for interaction, for example when one enzyme play role for both, one pesticide saturate enzyme active site then it will affect the metabolism of another pesticide (2) pesticides normally diffuse through cell along their concentration gradient, when one pesticide changes biological matrix or physicochemical properties of another pesticide it can change the diffusivity of pesticide (3) previous or concurrent exposure to a pesticide with another pesticide may result in

inhibition or induction of enzymes, that may leads to impact on another pesticide. Induction of enzyme leads to high level of enzyme that may be due to changes at transcription, post transcription or post translation level. Enzyme inhibition may be reversible (changes with the removal of inhibitor) or irreversible. Generally irreversible inhibition is at high dose, but in case of inhibition of cytochrome P450 via the oxons of organophosphates, even low dose results irreversible inhibition (COT, 2002; Tennekes and Sanchez, 2013).

One example of toxicokinetic interaction is change in the metabolism of Malathion in the presence of iso-malathion and other impurities. Insecticides are detoxified with the help of carboxyl esterase and various other enzymes. Malathion is also converted into various forms with the help of various enzymes, some are excreted out (decrease toxicity), and some results in increase in toxicity by more toxic metabolism. Malathion is converted into various forms such as into (a) monoacid and diacid derivatives with the help of carboxylesterase enzymes (b) malaoxon a very toxic metabolite with the help of CYP450 and (c) dimethyl dithiophosphate (DMDTP) and a non-toxic phosphoric metabolite with the help of isoforms of CYP450s. Out of these malaoxon can be further degraded into (a) malaoxon dicarboxylic acid and ethanol and (b) dimethylthiophosphate (DMTP) and diethyl succinate (a detoxification product) catalyzed with additional esterases (Hernandez *et al.,* 2012). Iso-malathion further inhibits carboxyl esterase which leads to decrease in Malathion metabolism and almost 70 fold increases in toxicity (Hathaway, 1996). Various other members of CYP family are also involved in these processes. There may be some interaction at this level also. So this shows that various compounds interfere with the toxicity of the substance.

Toxicodynamic changes mean the changes with respect to joining of toxicant molecules to the target receptors. Interaction of pesticides at toxicodyanamic results in either increase (potentiate) or decrease (antagonize) the changes in the normal tissue that is results of primary pesticide (Tennekes and Sánchez, 2013; Reffstrup *et al*., 2013). The plausible mechanism may involve the binding of one pesticide to the receptor of another or may interfere in the binding of another pesticide to their receptor. The combined effect of two compounds at same site does not result in increased response instead it

results in antagonistic response. Some compounds may interact with each other while binding to different receptors which culminates in antagonistic or synergistic response. One example of increase in response is, when the nature of one compound is to impair DNA repair and another is genotoxic in nature, the combined effect will be more (COT, 2002).

Interaction of pesticides mixture can result in toxic effect even when the individual component may be present in negligible amount. According to EPA (2002) components having a common mode of action can result in combined toxicity have been classified on the basis of (a) Chemical structure (core molecular structure, functional groups or their metabolic precursors) (b) Mechanism of pesticide action through which they cause toxicity (c) General mode or mechanism of mammalian toxicity (d) Toxic effect caused by components.

An example of increase in combined toxicity was evaluated by some researchers, wherein they investigated that the neurotoxicity of Malathion increases when exposure is combined with prochloraz (a fungicide), this was due to the induction of Malathion oxidation into its active metabolites (Johnston, 1995). In one another study, it has been investigated that the combined effects of exposure to the organophosphate insecticides ethyl p-nitrophenylbenzenethiophosphate (EPN) and Malathion displayed 10- fold synergistic toxicity effect in rats and a 50-fold synergistic effect in dogs (Frawley *et al.,* 1957).

National Institute of Hygienic Sciences in Tokyo, Japan has highlighted that non carcinogenic chemicals present in pesticides when combined with the carcinogenic chemicals present in other pesticides, resulted in cancer promotion (Kepner, 2004). In addition to promoting cancer, synergy also resulted in various other effects. Another study showed the combined effect of DEET (the active ingredient in most insect repellents) and permethrin (a pesticide), in increasing the permeability of blood-brain barrier (Abou-Donia, *et al.* 2001), increased urinary excretion of 6B-hydroxycortisol (marker for chemical poisoning) (Abu-Qare *et al.,* 2001a), release of brain mitochondrial cytochrome- c (Abu-Qare *et al.,* 2001b), neuronal cell death and cytoskeletal

abnormalities (Abdel-Rahman *et al.,* 2001). These effects were seen to be more than that seen in individual exposures.

In one study, the researchers observed the cytotoxicity and genotoxicity of Endosulfan, Carbofuran, and Monocrotophos while observing the LC_{50} values for cytotoxicity for individuals as well for binary mixtures. They observed that the LC_{50} values were 4.18, 5.76, and 7.5 µM for Endosulfan, Carbofuran, and Monocrotophos respectively, and 0.7, 0.9, and 1.0 µM for Monocrotophos & Carbofuran, Endosulfan & Monocrotophos, and Endosulfan & Carbofuran, respectively. Low amount of the binary mixtures of pesticides results in same DNA damage as with high amount of individual pesticides. Thus concluded that, these pesticides show synergy (Das *et al.,* 2007).

Another study conducted by Ojha and Srivastava (2014) explored the combined effect of Chlorpyrifos (CPF), Methyl Parathion (MPT), and Malathion and their effect on ROS production along with DNA damage. They found that the pesticide mixture caused no significant increase of superoxide anion production by the rat lymphocytes, compared to control, but showed DNA damage in a dose dependant manner. Combined exposure of mixture of these pesticides showed less increase in ROS than caused by any of the pesticide individually and these pesticides did not show any synergistic effect.

Chapter 3

Material and Methods

3.1Materials

3.1.1 Chemicals

Complete media [Culture media (DMEM- Dulbecco's Modified Eagle's Media), Fetal Bovine Serum (FBS), Antibiotic (Penicillin/streptomycin), MTT 3-(4,5- dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide, Phosphate buffer saline (PBS), DMSO (Dimethyl Sulfoxide), Agarose, NaOH, NaCl, Na_2EDTA, Sodium Dodecyl Sulphate (SDS) , Ethidium bromide, Ethylene Di-amine tetra acetic acid (EDTA), H2DCFDA dye, Triton X-100, Tris HCl, Malathion and Monocrotophos (Tradename- Monocil)

3.1.2 Cell lines used

For this research work, two cell lines HCT116 wild type and HCT116 p53 mutant type were used. These were obtained as a kind gift from Prof. Tapas Mukh Upadhyay, Former Director, National Centre for Human Genome Studies and Research (NCHGSR), Punjab University, Chandigarh.

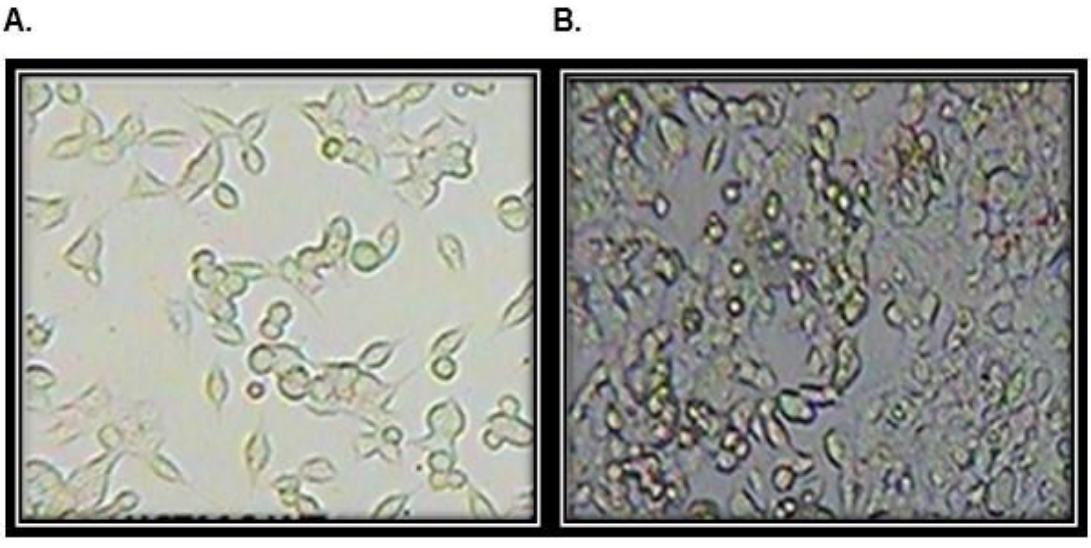


Figure 3.1 Microscopic views of A. HCT116 wild type and B. HCT116 p53 mutant colon cancer cell lines.

Table 3.1 Cell lines selected for study and their properties

Sr. No.	Properties	HCT-116 WT	HCT-116 Mutant
1.	Organism	*Homo sapiens*	*Homo sapiens*
2.	Tissue	Colon	Colon
3.	Morphology	Epithelial	Epithelial
4.	Culture Properties	Adherent	Adherent
5.	Biosafety Level	1	1
6.	Disease	Colorectal carcinoma	Colorectal carcinoma
7.	Special feature	Intact p53 genes	Mutant p53 genes

3.1.3 Instruments Used during work

Table 3.2 List of instruments

Sr. No.	Name Of Instruments	Manufacturing Company
1.	Analytical balance TE214, S	Sartorius
2.	Autoclave (vertical) NSW-227	Calton Company
3.	Co_2 incubator	New Brunswick, UK
4.	Flourescent Microscope	Olympus Magnus
5.	Inverted Microscope	Olympus Magnus
6.	Mini Centrifuge MC-02	Spinwin Daikan Scientific Co. Ltd.
7.	pH Meter	Mettle Toledo
8.	Rectangular water bath	New India
9.	Refrigerated Centrifuge 5430R	Eppendorf, Germany
10.	UV double beam Spectrophotometer	Systronics
11.	Multiplate reader	Biotek
12.	Nanodrop reader	Thermo Scientific
13.	Horizontal laminar air flow (NSW-201)	Calton
14.	Electrophoresis unit	Tarson
15.	Dancing shaker	Tarson

3.2 Methods

Experimental design and protocol used during work are as given below

Experiment 1: **Survey for commonly used pesticides from different villages of Mansa and Bathinda district.**

Experiment 2: Testing of presence of Malathion in water sample of field.

Experiment 3: Culturing cell lines and their maintenance.

Experiment 4: Assessment of cell viability

4.1: Assessment of cell viability of treated cells by MTT assay.

4.2: Assessment of cell viability of pesticide pretreated cells prior to usual treatment by MTT assay

Experiment 5: Determination of reactive oxygen species and their expected effect after pesticides treatment.

5.1: Determination of ROS (Reactive oxygen species) in treated cells

5.2: Assessment of DNA damage in treated cells

5.3: Estimation of lipid peroxidation following pesticide exposure.

5.4: Assessment of mitochondrial integrity in treated cells

Experiment 6: **Assessment of antioxidant enzymes activity**

6.1: Preparation of cell lysate for further enzyme activity evaluation assay

6.2: Assessment of Catalase enzyme activity

6.3: Assessment of Superoxide dismutase enzyme activity

6.4: Assessment of Glutathione reductase enzyme activity

Experiment 7: Long term effect on cell culture with pesticide treatment

Experiment 1: Survey for commonly used pesticides in the different villages of Mansa and Bathinda district.

A survey was conducted in different villages of Bathinda and Mansa district to find the commonly used pesticides in these regions.

Experiment 2: Testing for presence of Malathion in water sample of fields (Pandey *et al*., 2014).

The method used kinetic spectrophotometric technique, a novel and sensitive method for the determination of presence of Malathion in the water samples. This was based on the principle that Malathion get oxidized to malaoxon product in the presence of excess of N-bromosuccinimide. The unconsumed N-bromosuccinimide then reacts with safranin and form brominated safranin, which is subsequently monitored spectrophotometerically at λ_{max} 530 nm. So, Malathion pesticide presence was determined in water of fields by using this method.

Chemicals required: NBS (N-bromosuccinimide), Safranine, HCL and Malathion.

Procedure: Prepared 50 mM NBS (N-bromosuccinimide), 10mM Safranine in water and 2N HCL in water, then prepared standard of various increasing concentrations of Malathion in test tubes. In another five test tubes added 1ml of water sample (that is collected from fields of different villages). Then added 1 ml NBS and 2 ml HCL in each tube, mixed it and shake continuously for ten minutes. After ten minutes, added 1 ml of safranin. Mixed well all the test tubes and reading was taken at 530 nm.

Experiment 3: Routine assay in cell culture lab.

3(A): Culturing of cell lines

Cell lines were passaged in 25cm^2 rectangular canted cell culture flask (T-25 flasks). Adhered cells were detached from the surface by adding trypsin. After 4 minutes of trypsinization, trypsin was inactivated by addition of DMEM media containing FBS. Cells with media were taken and centrifuged at 1000 rpm (Rotation per Minute) at 4°c for 5 minutes, then discarded supernatant and resuspended pellet in 4ml fresh media. Cells were either cultured in new flasks or seeded for further various experiments.

3 (B): Maintenance and sub-culturing of cell lines

Cell lines were cultured in T-25 and T-75 flasks. When cells became confluent, these cells were sub cultured in new flasks. For sub culturing, discarded old media from flasks. Then 1 ml trypsin for T-25 flask and 2 ml trypsin for T-75 flaks was added. Subsequently, added media and centrifuged at 1200 rpm at 4°c for 5 minutes. Then discarded supernatant and the pellet obtained was resuspended in complete media containing DMEM Media, 10% FBS, 1X penicillin/streptomycin and ciprofloxacin. Cells were transferred to T-25 and T-75 flasks containing 4 ml media in T-25 Flasks and 10 ml media in T-75 flasks. Flasks were incubated in incubator at 37°C, 5% CO_2 and 95% humidity. This cell culture was maintained by adding fresh complete media after every three days.

3 (C): Cryopreservation and thawing of cell lines

Cells were preserved with freezing media (complete media containing 10% DMSO) in cryovials. The vials were thawed at 37°c and resuspended pellet in 4ml DMEM media and centrifuged at 1200 rpm for 10 minutes. Discarded supernatant and resuspended cells in 2ml media and seeded in 35mm culture dish.

Experiment 4: Assessment of cell viability (Baviskar *et al*., 2013).

4.1: MTT 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide Assay

It is a colorimetric assay used to assess viability of cells. This is based on the principle that metabolically active cells result in reduction of yellow MTT (by mitochondrial succinate dehydrogenase after entering in cell and passing mitochondria) into insoluble dark purple coloured formazan product, which is solubilized with an organic solvent like DMSO and the intensity of colour measured with the help of spectrophotometer.

Chemicals required: Malathion, Monocrotophos, DMEM media, MTT Reagent (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Phosphate buffer solution, DMSO (Dimethyl Sulfoxide).

Procedure: Two 96 well plates were seeded with 100 µL of 10, 000 cells per well, one plate seeded with HCT WT (wild type) cell line and another plate with HCT p53 mutant.

These plates were incubated in an incubator for 24 hours. After 24 hours, treatments were given to cells for duration of 48 hours. After 48 hours the media was removed from wells and the cells washed with 1X PBS. Then added 50µL of (0.5 mg/ml PBS) MTT in each well and incubated in the dark for 4 hours at 37°C. Then discarded the MTT solution and added DMSO solution to dissolve precipitate. Then after 20 minutes reading of absorbance was taken in multiplate reader at 570 nm.

4.2: Assessment of cell viability of pesticide pretreated cells prior to usual treatment by MTT assay

MTT dye is reduced into formazan by mitochondrial reductase (succinate dehydrogenase) of metabolically active cell which is further solubilized with DMSO and reading was taken of coloured product. On the basis of this principle cell survival of pre-treated cells evaluated.

Chemical required: Malathion, Monocrotophos, DMEM media, MTT Reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Phosphate buffer solution, DMSO (Dimethyl Sulfoxide).

Procedure: Two 96 well plates were seeded with 100µL of 10,000 cells per ml, one plate seeded with HCT WT (wild type) cell line and another plate with HCT p53 mutant. These plates were incubated in incubator for 24 hours. After 24 hours, pretreatment of low dose of both pesticides individually and in combination were given for 2 hours in both plates. After 2 hours replaced media of pre-treated cells with fresh complete media and incubated plates in incubator for 24 hours. This is followed by usual treatment of various doses of pesticides individually and in combination. Then after 48 hours of treatment media was removed from wells and washed cells with 1X PBS, followed by addition of 100µL of (0.5 mg/ml PBS) MTT in each well. Then plates were incubated for 4 hours at 37°C in incubator. Then discarded the MTT solution and added DMSO solution to dissolve precipitate. Then reading of absorbance was taken after 20 minutes in multiplate reader at 570 nm.

Experiment 5: Detection of reactive oxygen species and their effect

5.1: Detection of ROS (Reactive oxygen species) (Baviskar *et al*., 2013).

ROS, including the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot-}$) and hydrogen peroxide (H_2O_2) are mostly generated in mitochondria. These are mainly produced during stress conditions and can be detected with the use of fluorescence-based method, such as by using fluorogenic probe Di-hydro Di-Chlorodihydro Fluorescein Di-Acetate (H2DCFDA). Deacylation and subsequent oxidation of probe with ROS result in DCF (a fluorescent product), which can be quantified with the help of spectrophotometer.

Chemicals required: Malathion, Monocrotophos, DMEM complete media, Phosphate buffer solution, H2DCFDA Dye.

Procedure: Two 96 well plates were seeded one plate of HCT WT (wild type) cell line and another plate of HCT p53 mutant. These plates were incubated in an incubator for 24 hours. After 24 hours, cells were treated with pesticides for duration of 48 hours. After 48 hours of treatment H2DCFDA Dye was added in each well. Then plates were placed in dark for 20 minutes. Then after 20 minutes reading was taken in multiplate reader at 530nm emission and 485nm excitation.

5.2: Assessment of DNA damage in treated cells.

DNA the genetic material of cell is persistently subjected to various chemical changes. Among the various factors responsible for DNA damage, reactive oxygen species is regarded as an important contributing factor. This may results in single strand break, double strand break, oxidative DNA damage and defective DNA repair etc. which may eventually culminate in apoptosis. There are various techniques available for detection of DNA damage, out of which, in this study DNA laddering assay has been employed.

Chemicals required: Monocrotophos, Malathion, DMEM complete Media, Lysis Buffer (10 mM Tris HCl of pH-7.5, 10 mM EDTA, 10 mM NaCl, 0.5% N-Lauroylsarcosine), Phenol: Chloroform: Isoamylalcohol (P: C: I), NaCl, Ethanol, TE (Tris EDTA), Agarose.

Procedure: Two 6 well plates were seeded and incubated in an incubator for 24 hours. After 24 hours cells were treated in the prescribed concentration. After 48 hrs cells were scraped with the help of scraper and taken in eppendorfs. These are centrifuged and removed supernatant and taken pellet. Pellet was resuspended in 250 µl lysis buffer and placed in water bath for 10 min at 60°c. Added equal volume of P: C: I and the content mixed until emulsion formed followed by centrifugation at 10, 000 rpm for 5-10 minutes. Two layers were formed of which the upper layer was taken in another eppendorf. Added 30µl 5M NaCl and 1ml 70% ethanol in aqueous layer and centrifuged at 10000 rpm for 10 min, discard supernatant and added 70% chilled ethanol and again centrifuged at 10, 000 rpm for 5 min. Discarded supernatant and left pellet for air drying. Pellet was resuspended in 20 µl TE solution and determines the DNA concentration on the nanodrop instrument with a baseline correction of 350 nm. Then prepared agarose gel and load DNA sample in gel and run gel.

5.3: Estimation of lipid peroxidation following pesticide exposure (Devasagayam *et al*., 2003).

Lipid peroxidation, a common mechanism of cell or tissue injury, is also an indicator of oxidative stress. Lipid peroxides are unstable (such as polyunsaturated fatty acids) which breaks into malondialdehyde (MDA) and various other products. MDA further form adduct with Thiobarbituric acid (TBA), which is of pink colour that absorbs at 538 nm, that can be detected with the help of spectrophotometer.

Chemicals required: Monocrotophos, Malathion, DMEM complete media, HCl, Potassium Phosphate Buffer, Malondialdehyde Tetraethyl Alcohol (MTA), Thiobarbituric acid (TBA), Tri-chloro Acetic Acid (TCA) etc.

Procedure: A six well plate was seeded with HCT-116 WT cell line. Plate was left in incubator, for 24 hours. After 24 hours treatment of pesticides was given to plate and put in incubator. After 24 hours of treatment taken cells and given one washing with PBS and homogenized cells in phosphate buffer. Then centrifuged it and taken supernatant, which is used as sample. On the other hand, prepared standard of various concentrations with MDA instead of sample. Some other tubes were taken and added 500 µl of 15% TCA (prepared in 0.25 N HCl), 500 µl of 0.375% TBA (prepared in 0.25

N HCl), and 1.6ml of tris KCl and 400µl of sample respectively in each tube. Then put all the tubes in water bath at 80°c for 1 hr. After this cool the samples and centrifuged at 3000 rpm for 10 min. Taken readings of supernatant at 538 nm in spectrophotometer.

Calculation:

TBARS assay concentrations will be determined by comparison to an MDA standard curve following linear regression analysis (y = x (slope) + y int), express as MDA equivalents [A_{538}= (a [MDA] + b)]* df

Where,

[MDA] is the µM concentration of MDA in the sample

A_{538} = Net absorbance at 538 nm of the sample

a = regression coefficient (slope)

b = intercept

df = dilution factor

5.4: Assessment of mitochondrial integrity in treated cells (Wong *et al*., 2002).

This method is used to measure the electrochemical potential gradient across the inner membrane of depolarized mitochondria with the use of fluorescent plate reader. When there is accumulation of JC1 inside mitochondria, then there is a shift from green towards red. In this, two readings taken at two different wavelengths i.e. 590nm (red)/ 527nm (green), when mitochondrial depolarization occurs, red fluorescence decreases and the green fluorescence remains constant or increases which is used as indicator of mitochondrial depolarization.

Chemicals required: Monocrotophos, Malathion, DMEM complete media, Phosphate buffer solution, JC1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, -tetraethylbenzimidazolyl carbocyanine iodide) stain.

Procedure: Two 96 well plates were seeded (one plate of HCT wild type cell line and another plate of HCT p53 mutant) and incubated in incubator for 24 hours. After 24 hours treatments were given to cells. After 24 hours of treatment added 50µL JC 1 dye in all wells and left in dark for 15 minutes. Then taken reading at excitation of 490nm and emission at 527nm and 590nm.

Experiment 6: Assessment of antioxidant enzymes activity

Antioxidant enzymes are very important factors in accessing the risk of any disease in individual. The balance in antioxidant enzyme is a big player in healthy life. Some of very important antioxidant enzymes present in our body are superoxide dismutase, catalase and glutathione reductase etc. These three enzymes were evaluated in the present study. There was need to prepare cell lysate for evaluation of various antioxidant enzymes.

6.1: Preparation of cell lysate for further enzyme activity evaluation assay

Chemicals required: Monocrotophos, Malathion, DMEM complete media.

Procedure: For preparation of cell lysate seven 100 mm dishes were seeded with HCT116 WT. These dishes were incubated in incubator for 24 hours. After 24 hours treatments were given to seeded dishes. Then after 48 hours of treatment cells were harvested. These cells containing media were centrifuged at 1000rpm, for 10 min, discarded supernatant and given one washing with PBS. After that added 2ml Triton X-100 and performed freeze-thaw cycle two times to obtain lysate of cells, which were then centrifuged at 14000 rpm for 15 min. Then taken supernatant in eppendorfs and preserved at -80°c for further evaluation of various enzymes activity.

6.2: Assessment of superoxide dismutase enzyme activity (Marklund and Marklund, 1974).

Superoxide dismutase is primary antioxidant enzymes, which play an important role in maintaining the balance of free radicals in the body. There are various forms of this enzyme such as copper-zinc SOD (extracellular, tetrameric, containing compound) and Manganese (Mn) SOD (Mitochondrial). This is responsible for the catalysis of the process of dismutation the superoxide anion ($O_2^{\cdot-}$) into H_2O_2.This assay is on the principle that presence of SOD inhibits the autoxidation of pyrogallol, so activity of SOD can evaluated by taking reading of inhibition of pyragallol oxidation.

Chemicals required: Pyrogallol, EDTA, Tris HCL and sample.

Procedure: Prepared 6mM Pyrogallol, 6mM EDTA and 0.1M Tris HCL buffer of pH 8.2. Then added 1.5ml of 0.1M Tris HCL buffer, 0.5 ml of 6mM EDTA, 1ml of 6mM Pyrogallol

(light sensitive) in 100µL sample (lysate of cells) and taken reading at 420nm with respect to 1.5ml of solution containing 0.1M Tris HCL buffer, 0.5ml of 6mM EDTA, 1ml of 6mM Pyrogallol (light sensitive) as blank.

Calculation:

% inhibition= ($A_{Control}$- $A_{Treatment}$ / $A_{Control}$)*100 = X

50% inhibition= (X/50)/ mg protein = Y (U/mg)

SOD (U/mg) = Y/mg Protein

A= Absorbance

Here one unit is defined as the concentration of enzyme which inhibits the 50% autoxidation of pyrogallol.

6.3: Assessment of catalase enzyme activity (Chance and Maehly, 1955).

Catalase is an important antioxidant enzyme that converts the product formed during SOD reaction, hydrogen peroxide, into hydrogen and oxygen. Activity of catalase is monitored by detecting disappearance of H_2O_2 at 240nm in spectrophotometer.

Chemicals required: Potassium phosphate dibasic (K_2HPO_4), Potassium phosphate monobasic (KH_2PO_4), Hydrogen Peroxide (H_20_2), and sample.

Procedure: Prepared 0.1 M potassium phosphate buffer (pH-7) and fresh H_2O_2 solution with 0.1 M potassium phosphate buffer .Then taken readings of 2900 µl H_2O_2 (light sensitive) containing 100µL sample (lysate of cells) at 240nm, with respect to potassium phosphate buffer as blank.

Calculation:

Units/mg = {[(A/min (Blank) – A/min (Sample)]. d .1)/ V X 0.0436}/ mg protein

Where,

A/min = Change in Absorbance per min d = dilution

of original sample for Catalase Reaction

V = sample volume in Catalase Reaction

0.0436 = e^{mM} (extinction coefficient) for hydrogen peroxide

1 = reaction volume in ml

6.4: Assessment of glutathione reductase (GR) enzyme activity (Sedlak and Lindsay, 1968).

Glutathione reductase converts oxidized form of glutathione into reduced form of glutathione with the utilization of NADPH (Nicotinamide Adenine Dinucleotide Phosphate). This reduced form of glutathione is very important player in reducing the oxidative stress. Glutathione reductase activity can be assessed by detecting oxidation of NADPH at 340 nm in spectrophotometer.

Chemicals required: EDTA, $MgCl_2$ (Magnesium chloride), NADPH, Oxidized glutathione, Potassium phosphate dibasic **(**K_2HPO_4), Potassium phosphate monobasic (KH_2PO_4) and polyvinyl phosphate

Procedure: Prepared 0.2M Potassium Phosphate Buffer of pH-7.5 containing 1% polyvinyl phosphate, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM NADPH and 2mM oxidized glutathione. Then added 200µl potassium phosphate buffer, 100µl EDTA, 100µl $MgCl_2$, 200µl NADPH, 200µl oxidized glutathione and 200µl of sample (cell lysate) in cuvettes and taken readings at 340 nm with respect to sample containing all solution except sample.

Calculation:

Units/mg= $[(A/min)/6.22x\ 10^{-3}\ .d)]$/ mg protein

Where,

A/min = Change in Absorbance per min

d = dilution factor

$6.22x\ 10^{-3} = e^{mM}$ (extinction coefficient) for NADPH

Experiment 7: Long term cell culture with treatment

Chemicals required: Malathion, Monocrotophos, DMEM complete media.

Procedure: Two 6 well plates were seeded and incubated in incubator for 24 hours. After 24 hours treatments were given to cells. After 3 days of treatment the media in plates were changed and repeated treatment. Then again after three days repeated the process and observed cells. Then after 10 days of culture, pictures were clicked.

Chapter 4

Results

4.1: Survey of different villages

The survey was conducted to discern the pesticide usage pattern in Bathinda and Mansa Districts of Punjab. The survey conducted in different villages revealed that Malathion and Monocrotophos are mostly used pesticides. In the villages of Bathinda and Mansa district wheat, cotton, rice, vegetables (Table.4.1) are mostly grown for which Malathion and Monocrotophos (organophosphorous insecticides) are widely used. Various villages where survey performed are shown with the help of various legends in the map (Fig.4.1).

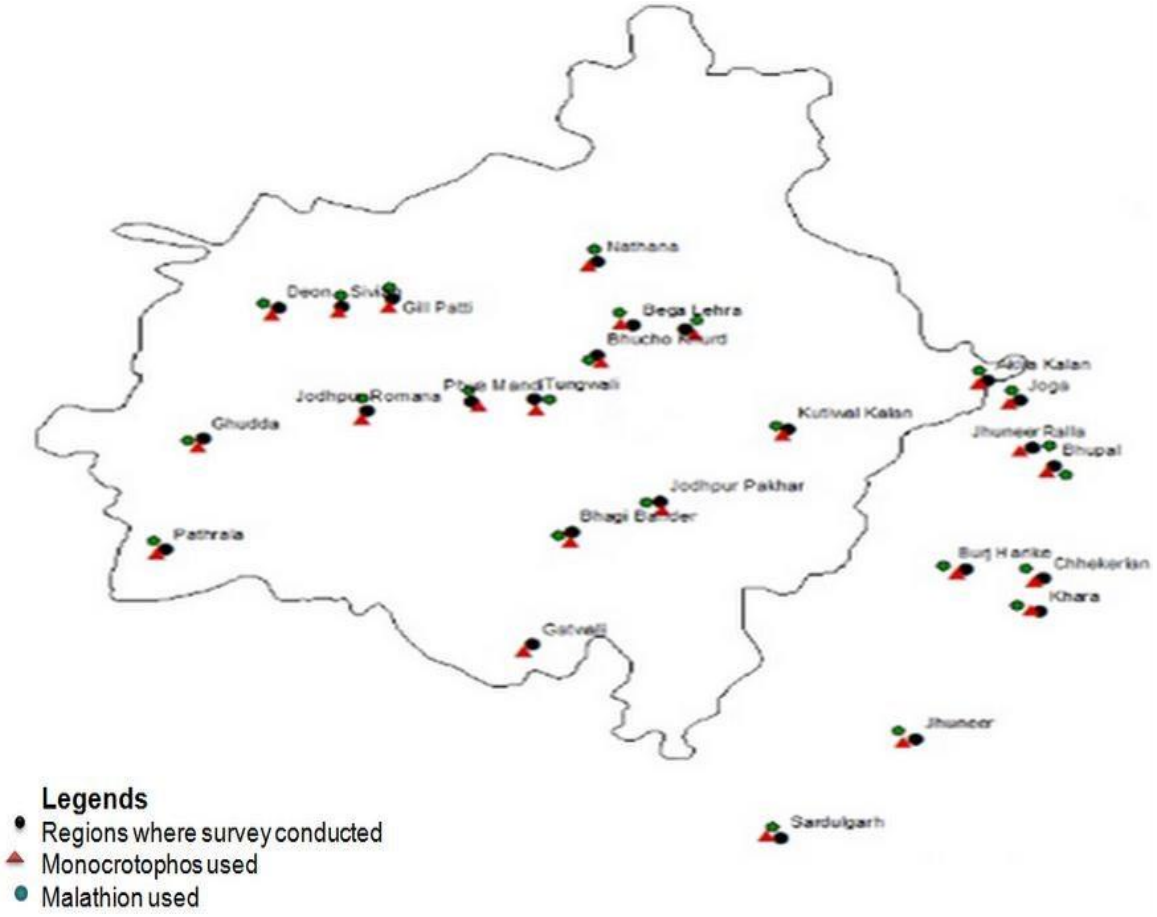


Fig.4.1 Villages depicting use of Malathion and Monocrotophos of Bathinda and Mansa district. The black boundary line denoted Bathinda dist. and outside villages are of Mansa dist.

Table.4.1 Monocrotophos and Malathion using villages of Mansa and Bathinda district

Sr. No.	Name of villages	District	Crops grown
1.	Begalehra	Bathinda	Wheat, cotton, rice, vegetables
2.	Lehramohabbat		
3.	Nathana		
4.	Gill patti		
5.	Ghudda		
6.	Akliakalan		
7.	Bhuchokhurd		
8.	Deon		
9.	Jodhpur romana		
10.	Kotshmeer		
11.	Pathrala		
12.	Phusmandi		
13.	Jodhpur pakhar		
14.	Sivian		
15.	Bhagibander		
16.	Gatwali		
17.	Korieana		
18.	Kutiwalkalan		
19.	Singo		
20.	Tungwali		
21.	Mansa khurd	Mansa	
22.	Khara		
23.	Barnala		
24.	Chkerian		
25.	Joga		
26.	Bhupal		
27.	Ralla		
28.	Burjharike		
29.	Burj jhabran		
30.	Jhuneer		
31.	Sardulgarh		

4.2: Evaluation of Malathion in ground water

According to some studies it was found that residues of pesticides seep into water which is then consumed by human beings and other animals (Younes and Galal-Gorchev, 2000).Various studies highlighted the presence of various pesticides in the water (Sankararamakrishnan *et al*., 2005; Jayashree and Vasudevan, 2007). Survey highlighted that Malathion and Monocrotophos were widely used, so presence of Malathion in water was also evaluated. Evaluation for presence of Malathion in water, with NBS assay, confirmed presence of Malathion in underground water.

To achieve results of objectives of this study, treatments were given to cells with different concentration of pesticides individually or in combination. The abbreviated forms to represent pesticides and their respective concentrations are as, M denoted for Monocrotophos, S for Malathion and C for control (Table.4.2**)**.

Table.4.2 Concentrations and abbreviated forms of pesticides, used during following sections.

Name	**Abbreviation**	**Concentration of pesticide**
Control	C	Without pesticide treatment
Monocrotophos (M)	M1	1µM
	M2	10µM
	M3	100 µM
	M4	250 µM
Malathion (S)	S1	10 µM
	S2	25 µM
	S3	50 µM
	S4	100 µM
Monocrotophos (M) and Malathion (S) Combination	M1S1	1µM of (M) + 10 µM of (S)
	M2S2	10µM of (M) + 25 µM of (S)
	M3S3	100µM of (M) + 50 µM of (S)
	M1S4	1µM of (M) + 100 µM of (S)
	M4S1	250µM of (M) + 10 µM of (S)

4.3: Evaluation of cell viability by MTT assay

4.3.1: Evaluation of cell viability in pesticides treated cells

In order to determine the extent of toxicity of the pesticides individually and in combination, MTT assay for cell viability was carried out.

To achieve results, cells were treated with different concentrations of Monocrotophos, Malathion and a combination of Monocrotophos-Malathion for duration of 48 hours, followed by MTT Assay.

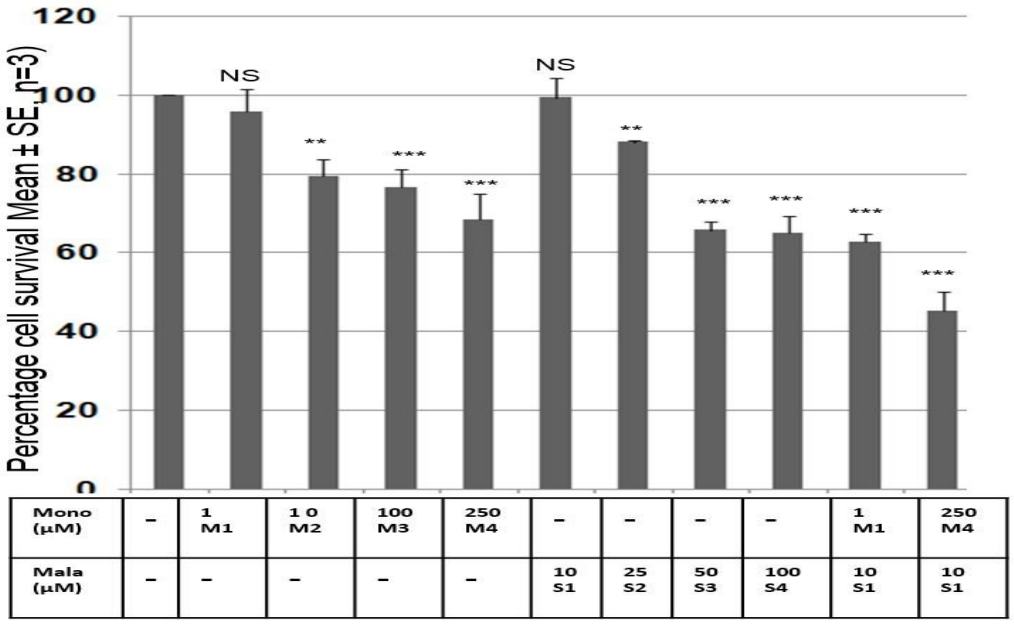


Fig.4.2 (A) Percent cell viability of HCT116 WT in response to different concentrations and combinations of pesticides. Here, Mono (M) is denoted for Monocrotophos and Mala (S) is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3) and has been analyzed by student t-Test and compared with respect to control. Statistically significant results were indicated by - "*" and nonsignificant denoted by NS.

The results demonstrated that cell viability decreased (after 48 hours of treatment) from 5%-32% with an increase in concentration from 1µM- 250 µM of MCP (Fig.4.2 (A), Bar no. 2-5), highlighting dose dependent toxicity of the MCP pesticide. Cell viability decreased from 1%-35% with the increase in concentration from 10µM-50µM in Malathion, highlighting dose dependent toxicity of the Malathion also (Bar no. 6- 8). The

cell viability significantly reduced from 38%-55% with increase in concentration of Monocrotophos in case of combination (Bar no. 10-11). This decrease in cell viability was considerable compared to control as well as individual pesticide, thus, representing that combination of pesticides are more cytotoxic in nature.

Numerous genotoxic stress responses have been observed to be mediated through p53 (Appella and Anderson, 2001; Schwartz and Rotter, 1998). So in order to determine the role played by p53 that is mostly implicated in numerous genotoxic stress responses, HCT116 p53 mutant cells were used.

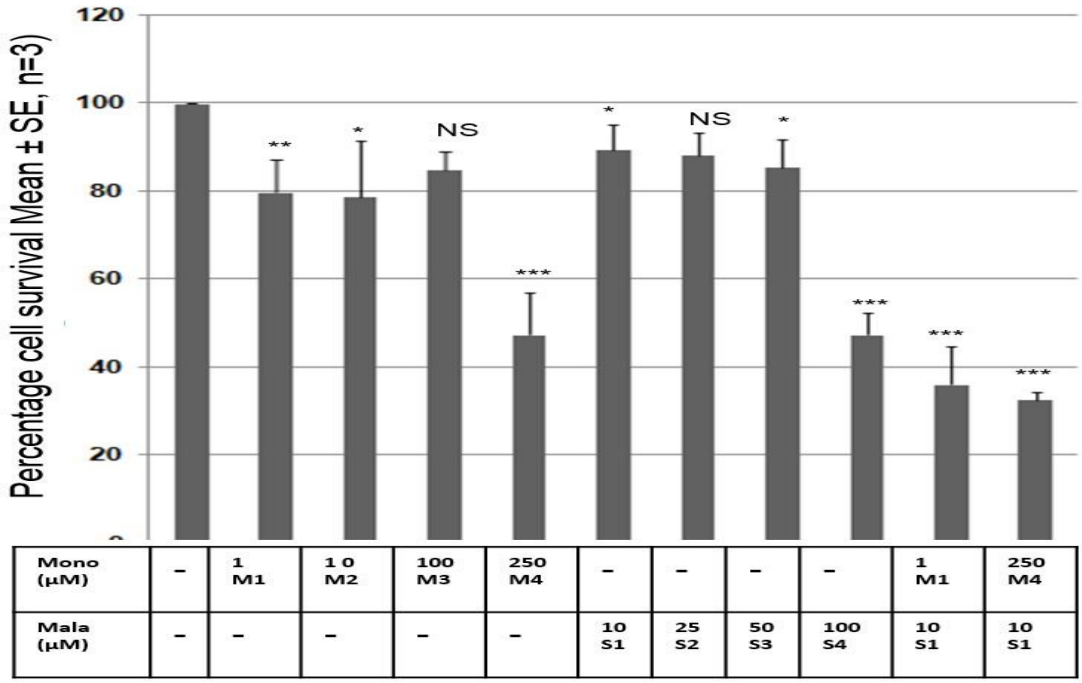


Fig.4.2 (B) Percent cell viability of HCT116 p53 mutant in response to different concentrations and combinations of pesticides. Here, Mono (M) is denoted for Monocrotophos and Mala (S) is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3) and has been analyzed by student t-Test and compared with respect to control. Statistically significant results were indicated by "*****" and nonsignificant denoted by NS.

There was decreased cell viability in all pesticide treated cells. Monocrotophos showed almost 20 % decrease in cell viability at low doses (Fig.4.2 (B), Bar no.2-4), but abrupt decrease of almost 52% at 250 µM (Bar no. 5). This indicated that, in these cells, high dose is highly cytotoxic but low doses didn't show increased cytotoxicity as with increase in dose. There was a slight decrease of almost 10% cell viability at 10µM to 50µM dose (Bar no.6-8) and then abrupt decrease of almost 52% at 100µM concentration (Bar no. 9) of Malathion. Similarly, the higher dose of Malathion was found to be extremely cytotoxic, but low doses didn't show significant increase in cytotoxicity with increased dose. Cell viability decreased significantly with the increase in concentration of Monocrotophos in case of combination i.e. S1M1 (Bar no. 10) and S1M4 (Bar no. 11). This difference was significant as compared to individuals and control. Both of the combinations results in immense decreased cell viability indicating highly cytotoxic nature.

Both of the pesticides used in the study observed cytotoxic in nature. There was gradual decrease in cell viability with respect to increase in concentration in case of HCT WT. But there is slight or negligible change in cell viability in lower dose and abrupt decrease at higher dose in case of HCT p53 mutant.

4.3.2: Evaluation of cell viability in pesticides treated cells having pesticide pretreatment.

To determine whether pretreatment of low dose of pesticides prior to usual treatment of pesticides show activation of defence mechanism to already exposed cells or not, evaluation of the cell survival in pretreated cells has been done.

To achieve this target, the cells were pretreated with low dose of individual and combined pesticides, followed by usual treatment of various doses individually and in combination. This is followed by MTT assay.

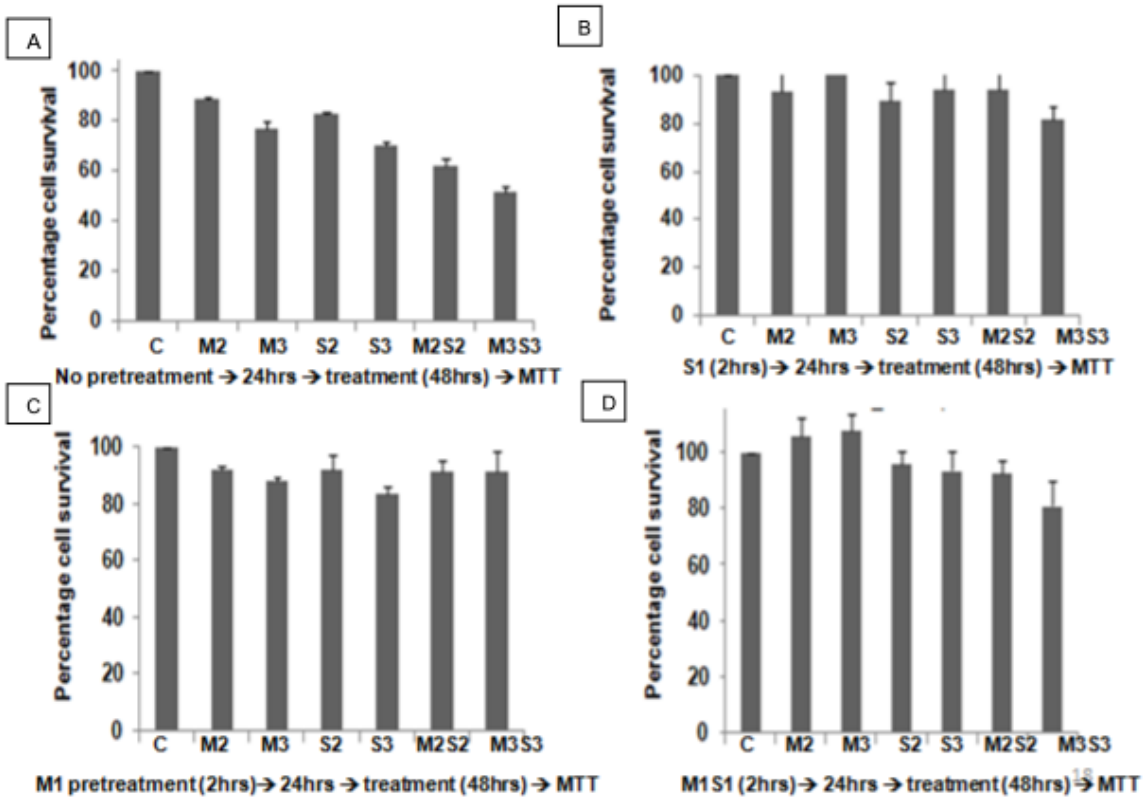


Fig.4.3 Percent cell viability of HCT116 WT in response to different concentrations and combinations of pesticides, pretreated with low dose of pesticides individually and in combination. Here, M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

It was observed that Malathion low dose (S1) pretreated cells showed more cell survival compared to without pretreated cells (Fig.4.3 (B)). Cells were almost 5% more in case of S2 and almost 27% more in S3 compared to without pretreated cells. Monocrotophos resulted in almost 5% and 25% more cell survival in M2 and M3 respectively compared to control. There was almost 30 % and 28% more cell survival compared to control in case of M2S2 and M3S3 combination respectively.

Cell survival also increased as compared to control in M1 pretreated cells (Fig.4.3 (C)). There was almost 4% and 11% increase in cell survival in M2 and M3 treated cells respectively, as compared to control. Cells were almost 13% and 16% more in S2 and S3 treated cells respectively as compared to control. Treatment of combination resulted

almost 32% and 42% increase in cell survival in M2S2 and M3S3 respectively as compared to control.

As far as pretreatment of combination is concerned (Fig.4.3 (D)), the cells showed that S2 and S3 resulted in almost 15% and 33% increase in cell survival respectively, compared to control. M2 and M3 resulted in almost 13% and 27% increase in cell survival respectively. There was almost 30% increase in M2S2 and M3S3 with respect to cell survival of control.

Experiment.4.4: Determination of reactive oxygen species and their expected effect

4.4.1: ROS determination in pesticide treated cells

Evaluation of oxidative stress is a very crucial parameter for the present study. Various studies suggested there is increase in production of reactive oxygen species during pesticides exposure. Thus we evaluated the content of ROS production during individual and combined pesticides exposure in both of cell lines (HCT116 WT and p53 mutant). As the H2DCFDA is oxidized within the cell by ROS into Di-Chlorodihydro fluorescein (DCF) which is fluorescent in nature and this is measured under spectrophotometer, so higher absorbance illustrate high level of ROS.

To evaluate oxidative stress cells were treated with pesticides for 24 hours and measured ROS extent with the help of H2DCFDA dye.

ROS content increased (as shown in Fig.4.4 (A)) with the increase in concentration of Monocrotophos in HCT-116 WT cells. Low doses were observed to be less toxic and produced negligible increase in ROS content (Bar no. 2 and 3). ROS content increases almost 10 times and 18 times at 100µM and 250µM dose of MCP respectively (Bar no.4 and 5).

Similar trend observed in case of Malathion, there was negligible increase in ROS content from 10 µM – 50 µM (Bar no. 6-8), but at high dose of 100 µM (Bar no. 9) almost 11 times increase observed as compared to control.

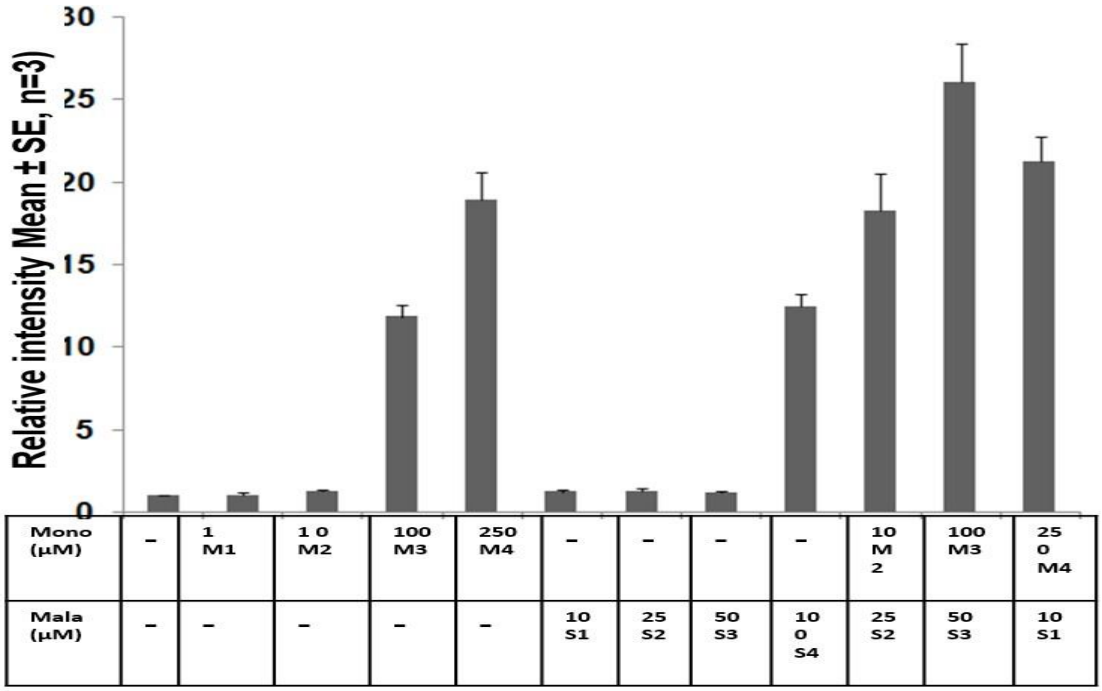


Fig.4.4 (A) ROS relative intensity in HCT116 WT cells in response to different concentration and combination of pesticides. Here, M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

There was significant increase in the ROS production in combination of pesticides, almost 17, 27 and 21 fold increase in M2S2, M3S3 and M4S1 respectively in HCT-116 WT cells (Bar no.10 -12).

Monocrotophos resulted in negligible difference in ROS production at low concentration of 1-10 µM but higher at doses of 100 µM and 250 µM resulting in almost 13 and 14 fold significant increase in ROS production respectively in HCT-116 p53 mutant (Fig.4.4 (B)). There was negligible effect at low dose, but resulted in rapid increase of almost 13 times than control at high dose of 100 µM of Malathion (Bar no. 6-9). Combination of both pesticides resulted in almost 14, 21 and 17 fold increase in ROS in M2S2, M3S3 and M4S1, that means combination even at low dose is more toxic.

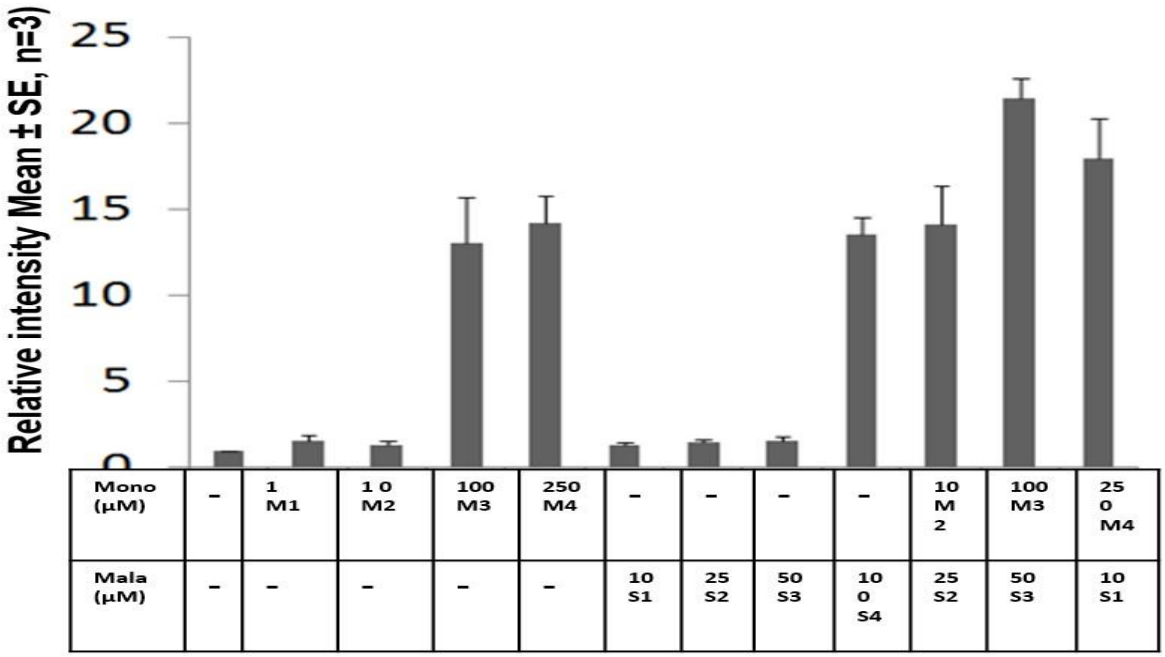


Fig.4.4 (B) ROS relative intensity in HCT116 p53 mutant cells in response to different concentration and combination of pesticides. Here M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

4.4.2: DNA Damage assay

DNA Damage is a very important parameter of present study. As DNA is vital part of cell, it is also vulnerable to various types of stress. In present study stress is imposed by pesticides individually and in combination. To find out effect of imposed stress experiment was performed with treatment of low concentrations of pesticides to cells.

Since numerous studies evidenced the DNA damage caused by individual pesticides. So in this study evaluated the combined effect of pesticides on DNA damage. That further leads to apoptosis (a phenomenon of programmed cell death).

Cells were seeded and after 24 hrs treatment of low dose pesticides individually and in combination was given for duration of 48 hrs. Then cells were taken and extracted DNA from cells with Zhou*et al*., 2004 method with little modifications, followed by subsequent run on agarose gel electrophoresis.

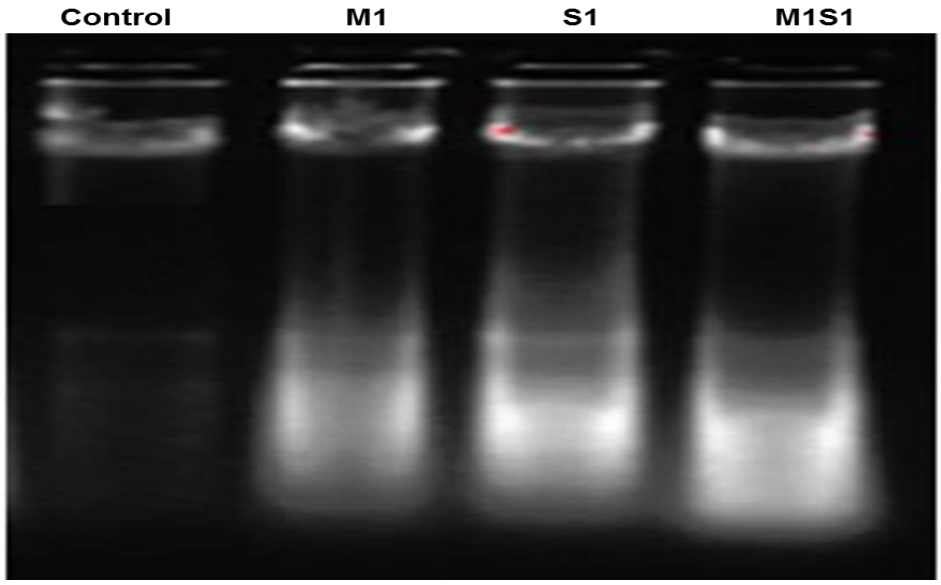


Fig.4.5 DNA damage in HCT116 WT in response to lower concentrations and combinations of low dose of both pesticides. Here first band is of control of wild type, M is denoted for Monocrotophos and S is denoted for Malathion.

In the control of wild type there was no DNA damage, however exposure of pesticides low dose individually and in combination resulted in DNA damage.

4.4.3: Lipid Peroxidation

Lipid peroxidation is an important parameter to determine the impact of stress on cells. Some studies explored that the increase in oxidative stress results in increased lipid peroxidation (Kashyap *et al*., 2011; Prakasam *et al.,* 2001). In the present study, the lipid peroxidation has been evaluated after imposing stress of pesticides. This is based on the principle that when cells are exposed to stress, they may result increase in milondialdehyde (MDA) as a result of increased lipid peroxidation. These increased MDA reacts with TBA and form adduct of pink colour that can be measured at 538 nm under spectrophotometer.

For this experiment, cells were treated, and lysate were prepared followed by estimation of lipid peroxidation using spectrophotometer.

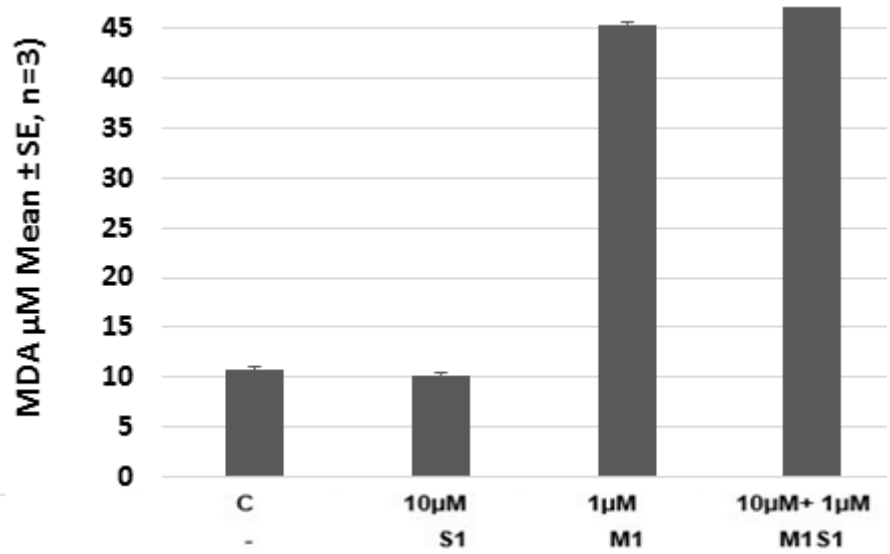


Fig.4.6 Lipid peroxidation in response to individual and combined pesticide exposure. Here M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

It was observed that Malathion didn't show any effect on lipid peroxidation at low dose (S1), but there was almost 4.5 fold increase with low dose of Monocrotophos (M1) (Fig.4.6). Combination of low dose of both (M1S1) resulted in significant increase of almost 4.8 fold compared to control, but slight change with respect to low dose of Monocrotophos. So in combination increased lipid peroxidation is due to Monocrotophos.

4.4.4: Mitochondrial membrane integrity evaluations

The results demonstrated, there was increase in depolarization with respect to control in all pesticide treated cells (Fig.4.7). There was abrupt increase of almost 36% and 27% at M1 and M2 respectively, in mitochondrial depolarization with respect to control. Mitochondrial depolarization increases almost 38% in both S1 and S2 compared to control. In case of combination of pesticides, there was almost 37% and 27% increase in mitochondrial depolarization at M1S1 and M2S2 respectively.

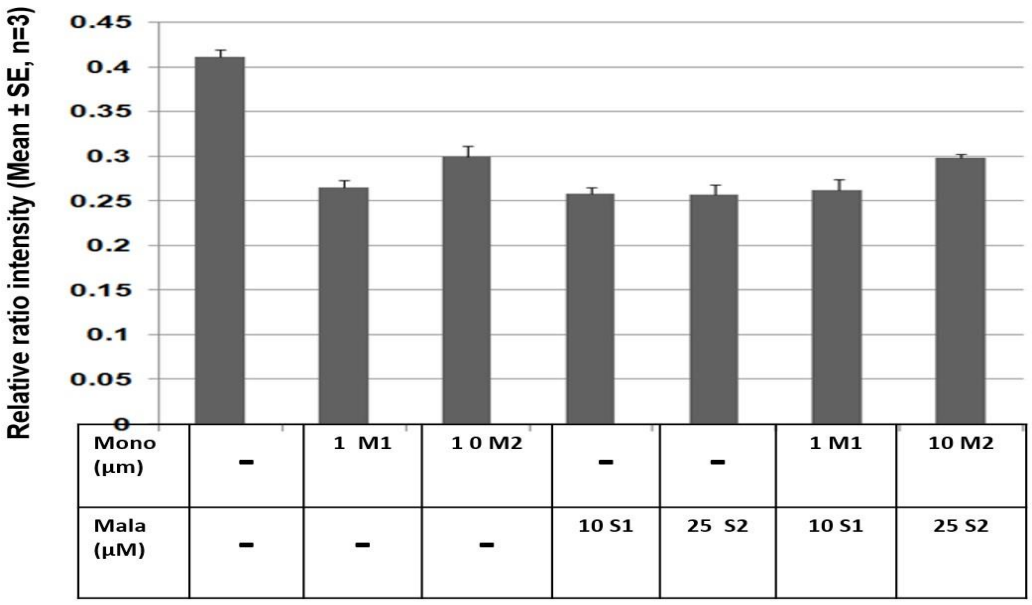


Fig.4.7 Mitochondrial membrane potential in HCT116 WT in response to different concentrations and combinations of pesticides. Here M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

Experiment 4.5: Assessment of antioxidant enzymes activity

4.5.1: SOD assay

Superoxide dismutase is the antioxidant enzyme which converts superoxide anion ($O_2^{\cdot-}$) into H_2O_2. To evaluate the effect of pesticides on the antioxidant system, the activity of superoxide dismutase has been evaluated.

Results showed that activity of superoxide dismutase decreased in all treated cells with respect to control. There was almost 70% and 42% decrease in SOD activity compared to control in S1 and S2 respectively (Fig 4.8, Bar no. 2 and 3). SOD activity decreased almost 38% and 46% in M1 and M2 respectively, compared to control (Bar no. 4 and 5). There was decrease of almost 42% and 51% decrease in M1S1 and M2S2 respectively, compared to control (Bar no. 6 and 7).

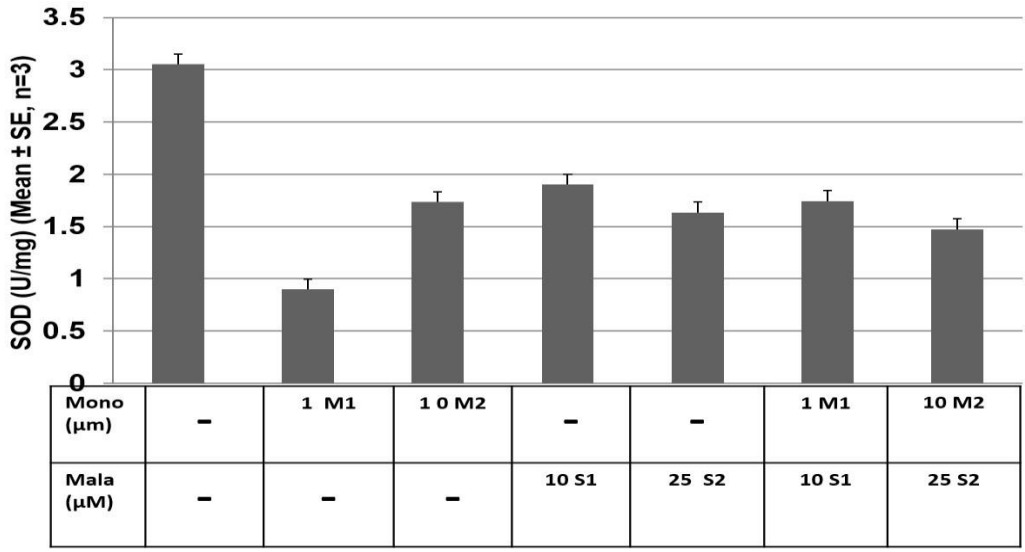


Fig 4.8 Superoxide dismutase activity in HCT116 WT cells in response to different concentration and combination of pesticides. Here M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

4.5.2: Catalase Activity Evaluation

As far as the antioxidant system is concerned, the catalase enzyme plays a significant role in scavenging the free radicals and in maintaining balance, as it breaks hydrogen peroxide into water. According to various studies pesticides affect the activity of antioxidant enzymes. Thus to evaluate effect of individual and combined pesticides on antioxidant enzymes, catalase activity was evaluated. This assay is on the basis of disappearance of H_2O_2 which act as substrate for catalase and absorbs at 240 nm.

Activity of catalase decreased almost 25% and 77% with increased concentration i.e. M1 and M2 respectively (Fig.4.9, Bar No.2 and 3).There was almost 15% increase and 50% decrease in activity of catalase at S1 and S2 respectively (Bar No. 4 and 5). There was significant decrease of almost 93% and 20% in catalase activity in M1S1 and M2S2 respectively (Bar No. 6 and 7), compared to control.

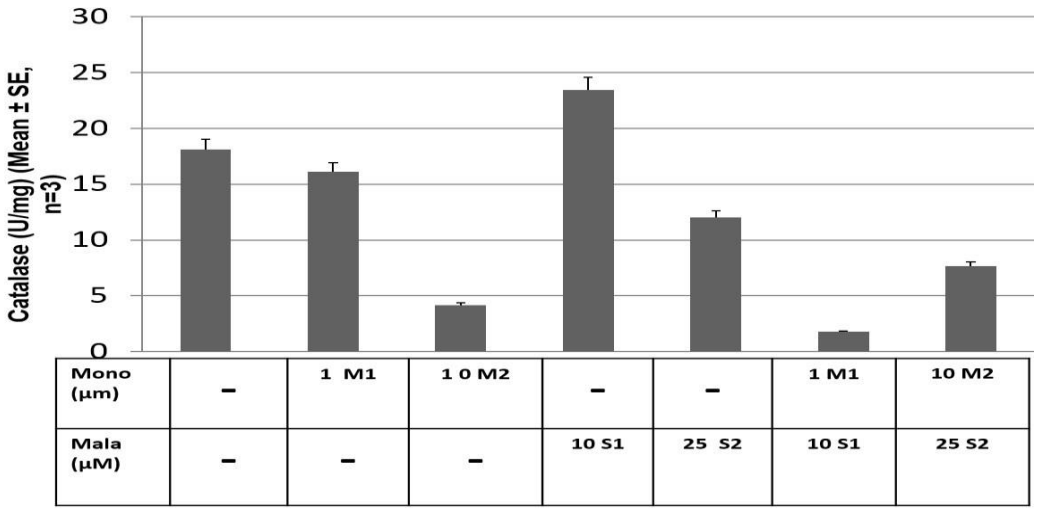


Fig.4.9 Activity of catalase in response to different types and concentration of pesticides. Here M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

4.5.3: Glutathione reductase (GR) activity evaluation

Glutathione reductase is a vital enzyme for the restoration of reduced form of glutathione from oxidized form of glutathione, therefore its activity is necessary for replenishing the reduced glutathione in cells. So glutathione reductase is an important parameter to evaluate the effect of individual and mixture of pesticides on cells. This experiment was performed with the lysate of various cells treated with different concentrations of pesticides individually or in combination.

Glutathione reductase activity increased in all treated cells with respect to control i.e. without any pesticide treatment (Fig.4.10). The activity was increased by almost 0.5 and 2 fold at 1µM and 10µM dose of Monocrotophos (Bar No. 2 and 3). Enzyme activity increased by 3 and 2 fold at 10µM and 25µM concentration of Malathion respectively (Bar No. 4 and 5). There was almost 3 and 4 fold increase in GR activity in M1S1 and M2S2 respectively (Bar No. 6 and 7). This increase in Glutathione reductase activity may be due to increase in oxidative stress during pesticide treatment.

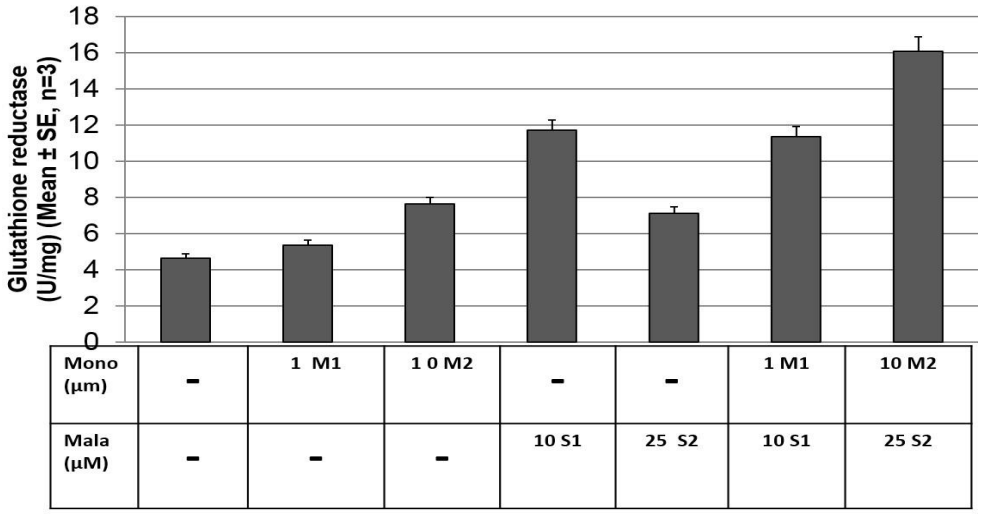


Fig.4.10 Glutathione reductase activity in HCT116 WT in response to different concentrations and combinations of pesticides. Here C denotes for control, M is denoted for Monocrotophos and S is denoted for Malathion. Data is expressed as mean values ± S.E. (n=3).

4.6 Long term culture

All of the parameters evaluated were during short term treatment of pesticides, so to evaluate long term effect; seeded cells were treated with different concentrations of Malathion and Monocrotophos and their combination. Media was changed after each and every three days and repeated treatment. Then, the effect of long term exposure was evaluated after ten days under fluorescence microscope.

There was increase in cell debris (as floating mass) and also cell morphology changed after 10 days (As shown in fig 4.11 (A) and (B)). Even the low dose of Monocrotophos (M1) resulted in a remarkable amount of debris. Slightly higher dose of Monocrotophos (M2) resulted in more damaging effect compared to M1, resulted in more debris and less cell number. Low dose of Malathion (S1) is less damaging and forms less floating bodies, but high dose of Malathion (S2) resulted in lots of debris as indicated in the image (black arrows). Combination of low dose and slight high doses of both pesticides resulted in numerous floating bodies shown with the help of arrow. At the end of 10 days, negligible cells were observed to be attached to the surface thus no MTT result could be deduced for any of the samples.

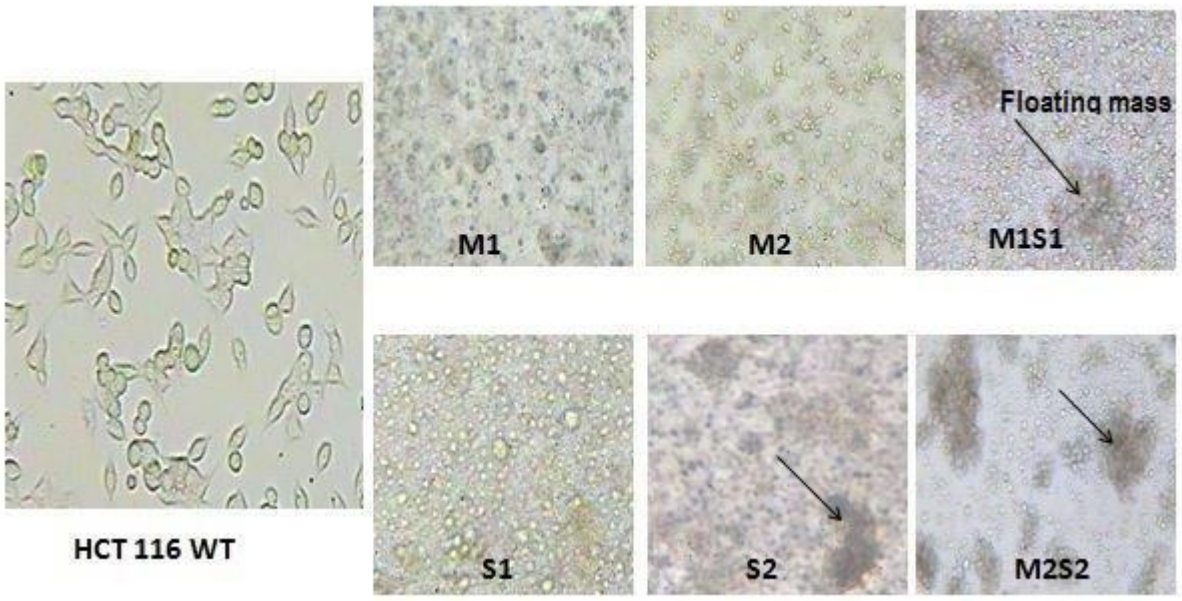


Fig 4.11 (A) Long term culture results with the treatment of individual and combined pesticides in HCT-116 WT cells. Here arrows show the cell debris or dead mass as floating bodies.

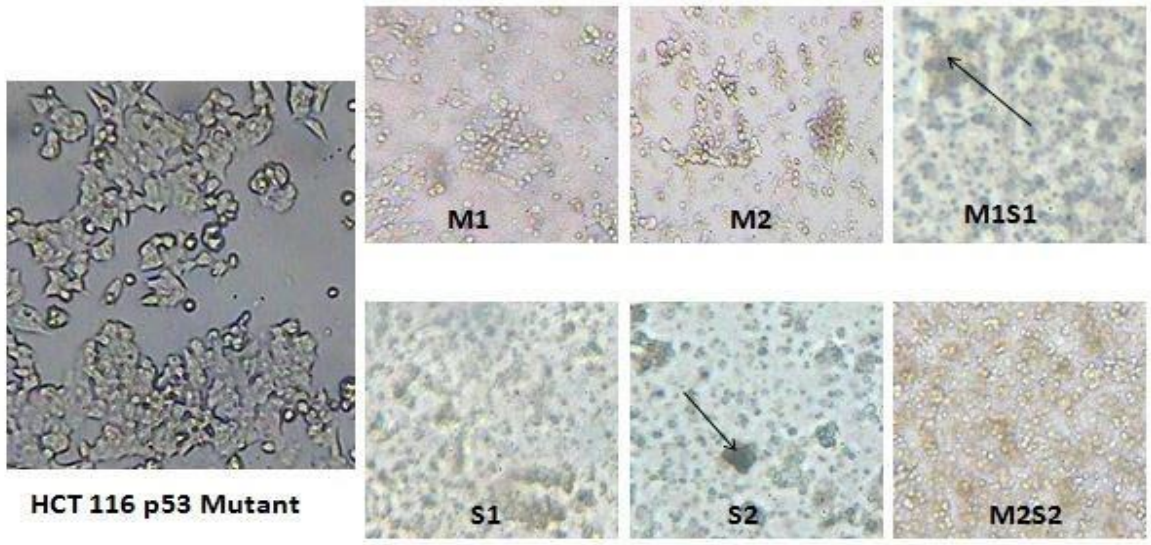


Fig 4.11 (B) Long term culture results with the treatment of individual and combined pesticides in HCT-116 mutant cells. Here arrows show the cell debris or dead mass as floating bodies.

Chapter 5

Discussion

Uses of pesticides such as of organophosphorous insecticides, is increasing day by day. Pesticides are also present in environment, air, food, water etc. they may present together and cause serious health problems to people. So it is very important to evaluate combined effect of pesticide on human cells. In this study, it was aimed to evaluate the combined effect of two commonly used pesticides Malathion and Monocrotophos, both which are reported to be present in the blood sample of people of Punjab (Mathur *et al*., 2005).

In the present study firstly different villages of Mansa and Bathinda district were surveyed for evaluation of commonly used pesticides. As a result of this survey it was found that Malathion and Monocrotophos were mostly used. Since various studies stated that the pesticides are present in water, in this study presence of Malathion in water was evaluated, resulting in the conclusion that Malathion is present in water. Presence of Malathion in water is consistent with the study by Thakur *et al.,* (2008). Then to evaluate the cytotoxic effects of Malathion and Monocrotophos, individually and in combination cell lines were cultured and maintained.

To determine the extent of cytotoxicity, cell survival assay was performed. According to some studies, it is shown that exposure of Monocrotophos or Malathion results in decreased cell survival (Kashyap *et al*., 2011 and Moore *et al*., 2010), but these studies had evaluated the individual impact of pesticides. In the present study, results showed that both pesticides, considered under study, are cytotoxic in nature. They resulted in decrease in cell viability with increase in concentration, in case of Malathion and Monocrotophos, and the combinations of M1S1 (1µM of Monocrotophos+ 10 µM of malathion) and M4S1 (250µM of Monocrotophos + 10 µM of malathion) was more toxic and show significant decrease compared to their individual respectively, so both M1S1 and M4S1 seems to be combinational in nature in both types of cell lines i.e. HCT-116 and HCT-116 p53 mutant. It is found that p53 mutant cells are resistant to low dose of

pesticide but result in abrupt increase in response to high dose that may be due to mitotic catastrophe at high dose.

In mice and rat it has been proved that the survival of recipient can be improved, if they are exposed to low dose of similar or different cytotoxic agent prior to treatment. The various pathways involved in response to toxicity may be initiated with prior exposure (Rose *et al*., 1975; Millar and McElwain, 1978). Keeping this in view, in the present study, cell survival was evaluated, and results of present study are consistent with the literature and showed increase in cell survival with pretreatment of pesticides.

To determine the plausible mechanism of cell death, various parameters were evaluated such as oxidative stress during pesticide exposure. Free radicals are produced in cells during normal metabolic processes such as mitochondrial electron transport of aerobic respiration, processes of biosynthesis and biodegradation, but various stress conditions results in increase of ROS production. Various studies suggested that exposure to both pesticides resulted in increase in ROS production (Kashyap *et al*., 2011; Yaduvanshi *et al.,* 2010; Saleha Banu *et al.,* 2001). In these studies ROS production was explored after exposure of individual pesticides, but in present along with exploring ROS production during single pesticide treatment also the effect of pesticide combination was also explored. It is found that high doses of pesticides impose more stress on cells compared to low dose which resulted in elevated ROS production at high dose. Combination of pesticides resulted in significant increase in ROS production in all combinations of M2S2 (10µM of Monocrotophos + 25 µM of Malathion), M3S3 (100µM of Monocrotophos + 50 µM of Malathion) and M4S1 (250µM of Monocrotophos + 10 µM of Malathion) compared to control. Which illustrate combination is more toxic in nature.

Severe increase in ROS production may lead to DNA damage, lipid peroxidation and mitochondrial instability, therefore evaluated these parameters in response to individual and combined pesticide exposure.

Various studies have explored DNA damage occurring in response to the exposure of pesticides results in DNA damage and there is evidence that both pesticides taken in

this study cause DNA damage individually at various doses (Yaduvanshi *et al.,* 2010; Saleha Banu *et al.,* 2001; Moore *et al*., 2010; Ojha and Srivastava, 2014). According to the results of present study exposure of pesticides results in DNA damage even at low dose. This DNA damage is indication of apoptosis.

One effect of increased ROS may be on lipid peroxidation. Some studies shows that stress conditions may result in increase in ROS production which further increases lipid Peroxidation (Kashyap *et al*., 2011; Prakasam *et al.,* 2001). Results of present study are in accordance with literature that states there was significant increase in lipid Peroxidation with Monocrotophos exposure individually and when present with Malathion. Malathion low dose did not show any lipid Peroxidation.

Another effect of increased ROS was found to be on mitochondrial stability. Mitochondria is primary target for most of the organophosphate pesticides and causes inhibition in enzymatic activity and ATP generation also results in structural alterations of the matrix (Carlson *et al*., 1999; Tos-Luty *et al*., 2003). Some studies found that exposure of pesticides results in production of free radicals and in inactivation of factors of the mitochondrial respiratory chain (Barja *et al*., 1998; Barja *et al.,* 1999; Massicotte *et al.,* 2005). The results of present study also present the effects of pesticide exposure on mitochondrial stability, that decreases during the exposure of pesticides individually and in combination.

Increase in ROS of production or direct pesticides may interfere in antioxidant defence mechanism so evaluated various antioxidant enzymes such as SOD with changes superoxide into hydrogen peroxide. Hydrogen peroxide is converted into water and oxygen with the help of Catalase. This made it important to evaluate Catalase enzyme activity. Other enzyme evaluated was glutathione reductase, which is important to restore reduced form of glutathione.

In case of SOD results demonstrated that there is decrease in SOD in all pesticide treated cells, whether, individually or in combination. Some studies such as of Lopez *et al.,* 2007 are in consistent with results of present study, according to this study pesticides results in decrease in SOD activity.

In case of Catalase evaluation, pesticide treatment, whether given individually or in combination, resulted in decrease in catalase activity except in case of low dose of Monocrotophos. Various studies are also in accordance of our observation that the exposure of pesticides results in decreased level of catalase (Zhou *et al.,* 2004; Lopez *et al.,* 2007). The prime reason for the inhibition of catalase activity may be accredited to the increased superoxide production following pesticide treatment and this may also be the reason for decreased catalase (Lukaszewicz-Hussain *et al.,* 2001). According to another study reason for inhibition of catalase activity may be increased free radicals that may result in inhibition of thiol group of catalase enzyme (Kono and Fridovich, 1982). Decrease in catalase activity results in high levels of free radicals which may further leads to cellular and tissue injury.

In case of glutathione reductase it showed increased glutathione reductase activity in all treated cells. This is consistent with the Lukaszewicz-Hussain (2008) study which shows increase in glutathione reductase activity with the exposure of organophosphate pesticides.

Followed by all parameters evaluated long term effect of pesticides and found that long term exposure results in increase in cell debris(increased number of dead cells) and changed cell shape after ten days, this is due to stress imposed by pesticides.

Conclusion and future perspectives

Both of pesticides showed genotoxicity individually as well as in combination. To evaluate the response of Malathion and Monocrotophos in combination with respect to cell survival cell, the deviation between the observed and expected (for dose addition) were evaluated. According to various studies, if the observed response of combination, deviate from expected for dose addition, thus implying the active role of interaction. If response observed more toxic than dose addition then it is synergism (Hertzberg and MacDonell, 2002), if low toxicity then it is antagonism.

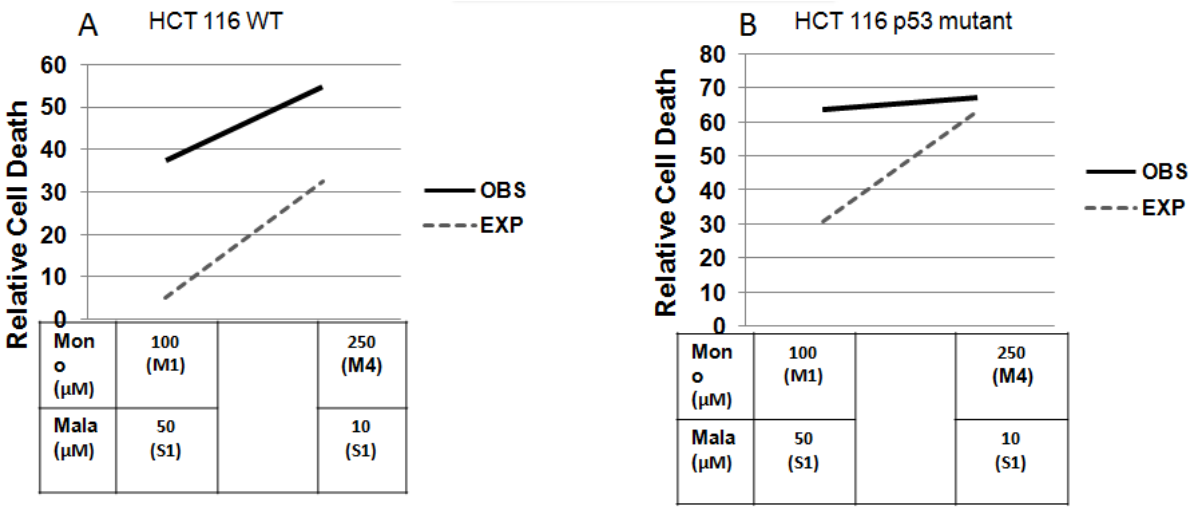


Fig 4.12 Deviation between observed and expected values for dose addition in (A) HCT 116 WT cells (B) HCT 116 mutant cells in response to combination of Malathion and Monocrotophos.

Result show that there is significant deviation between the observed and expected values. So it is concluded that there may be some interaction in Monocrotophos and Malathion.

Because this deviation is towards more cell death (more toxicity) so it is concluded that this interaction may be synergistic in nature.

Combination results in more severe toxicity by following plausible mechanism, such as severe increase in oxidative stress, that leads to DNA damage, unstable membranes, imbalance in antioxidant enzymes activity, all together further leads to cell death.

For further studies there should be evaluation of toxicokinetics and toxicodynamics with respect to combination of pesticides. This study can further be carried out with other pesticides as well used in various areas. The effect of combination of pesticides can be evaluated on various cell signalling pathways for further studies. On a large scale this can be employed *in vivo* to look for various other effects as well as to devise methods to prevent them. By considering this type of interaction studies, one may be able to find out possible reasons for various diseases.

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