A DISSERTATION ON

Impact of Experimental Exposure of Lead on Monocrotophos Intoxication in the Cultured Human Brain Cells

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
B. TECH-M. TECH DUAL DEGREE
IN BIOTECHNOLOGY

BY

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UNDER THE SUPERVISION OF

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DECLARATION

I, **Yusra Naaz Qidwai**, a student of **B.Tech.-M.Tech. Dual Degree Biotechnology** (V Year/ X Semester), Integral University have completed my six months dissertation work entitled **"Impact of Experimental Exposure of Lead on Monocrotophos Intoxication in the Cultured Human Brain Cells"** successfully from **CSIR-Indian Institute of Toxicology and Research, Lucknow** under the able guidance of **Dr AB Pant.** I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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Dr Reena Vishvakarma

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सीएसआईआर-भारतीय विषविज्ञान अनुसंधान संस्थान
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH
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CERTIFICATE

This is to certify that Ms **Yusra Naaz Qidwai** (Enrollment Number 1800100980) has carried out the research work presented in this dissertation entitled **"Impact of experimental exposure of Lead on Monocrotophos intoxication in the cultured human brain cells"** for the award of **B.Tech.-M.Tech. Dual Degree in Biotechnology** from CSIR-Indian Institute of Toxicology and Research, under my supervision. This dissertation embodies the results of original work and studies carried out by the student herself, and the contents of the dissertation do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/ Institution. The dissertation was a compulsory part of her **B.Tech.-M.Tech. Dual Degree Biotechnology**.

I wish her good luck and a bright future.

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I wish her good luck and bright future.

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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

Dr Alvina Farooqui
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Firstly, I would like to thank Almighty, for providing me this opportunity and by whose blessings I have beenable to complete my report. I'm profoundly thankful to Dr Bhaskar Narayan, Director, CSIR-Indian Institute of Technology and Research for allowing me to work in this esteemed organization and providing me with all the necessary facilities. I express my deepest sense of gratitude to my thesis supervisor Dr AB Pant, Senior Principal Scientist, CSIR-Indian Institute of Toxicology and Research, Lucknow, for providing me precious guidance, inspiring discussions, and constant supervision throughout this work. I would like to express my gratitude to Dr Alvina Farooqui, Head of the Department of Bioengineering, and my internal adviser, Dr Khwaja Osama for their constant support during this study.

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List of abbreviations

1.Ab/Am- Antibiotic/Antimycotic

2.AChE: Acetylcholinesterase

3. APS- Ammonium Persulphate

4. BCA- Bicinchoninic Acid

5. BPB- Bromophenol Blue

6.BSA-Bovine serum albumin

7.cDNA-Complementary DNA

8. DMSO- Dimethyl Sulfoxide

9. EtBr- Ethidium Bromide

10. FBS- Fetal Bovine Serum

11. MCP-Monocrotophos

12. MCT- Microcentrifuge Tube

13. MQ- Milli-Q

14. NFW- Nuclease Free Water

15. Organophosphate-OOPs

16. Pb-Lead

17. PCR- Polymerase Chain Reaction

18. P.I.- Protease Inhibitor

19. PVDF-Polyvinylidene fluoride
20. RCF- Relative Centrifugal Force
21. RIPA- Radioimmunoprecipitation assay buffer
22. RPM- Revolutions Per Minute
23. RT-PCR-Real Time Polymerase Chain Reaction
24. R.Q.- Relative Quantification
25. SDS- Sodium Dodecyl Sulphate
26. SDS-PAGE-Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
27. TEMED- Tetramethyl ethylenediamine
28. UND- Undifferentiated

TABLE OF CONTENTS

Chapter 1: Introduction

India comes 2nd in the agricultural producing country worldwide. Moreover, the agriculture sector plays a major role in India and supports 70% of the population's employability. To protect different crops, pesticides come into play for sustainable agriculture. Pesticides have subcategories based on the type of pest to be controlled. Organophosphates (OOPs) are the most widely used pesticide, which comes under the category of insecticide (Kushwaha M et al., 2016). They are biodegradable, highly effective, and have broad-spectrum of action against diverse sets of pests because of which they have been abundantly used in the agriculture sector for more than 40 years worldwide (Karpouzas and Singh 2006; Abraham and Silambarasan 2015). Roughly, they account for 22% of the global pesticide market (Kumari et al., 2018). However, only about 0.1% of pesticides applied reach their target, the rest ends up in the environment which results in reduced crop harvest, low-quality agricultural products, deteriorating quality soil, and worsened soil enzyme activity (Riah et al., 2014), contaminated water thus, giving rise to a severe risk to humans and animals (Yadav et al., 2016; Buvaneswari et al., 2017). Monocrotophos (MCP) is a type of synthetic organophosphate insecticide. It is one of the most widely used organophosphate insecticides because of Its easily available and low-cost price, making it a preference for farmers. Moreover, it is used to increase the yield of various cash crops such as cotton, wheat, rice, etc. However, various studies have indicated that exposure to MCP may result in the development of various neural disorders such as Parkinson's disease, Alzheimer's disease, etc. Lead (Pb) is one of the most environmentally reactive toxic metal and is one of the best-known neurotoxicants. It causes alterations in the function of the nervous system causing several neurophysiological as well as behavioral changes. Exposure to heavy doses of Pb may lead to permanent brain damage as it can actas a substitute of calcium ion due to which it can cross the bloodbrain barrier, disrupting various intracellular biological activities (Sanders T *et al.,* 2009). Lead is one of the most commonly used heavy metal because of its unique physical and chemical properties. It is majorly used in chemical industries along with it being used in cables, lead-based batteries, paints, ceramics, etc. due to which it has become of one

the most widely distributed environmental toxicants (Ragnarsdottir KV, 2000). According to the World Health Organization, exposure to lead gives rise to around 600,000 new cases of lead neurotoxicity per year which mainly lowers children's IQ and affects their future (Karpouzas DG and Singh BK, 2006).

To study the effect of environmental exposure of lead neurotoxicity in combination with occupational exposure of MCP-induced neurotoxicity which mainly includes farmers, SH-SY5Y is chosen as the *in vitro* model. It is widely used as an established neuronal cell model because of its human origin (Xicoy H *et.al.,* 2017). It is a thricesub-cloned cell line derived from the SK-N-SH neuroblastoma cell line. It has the property to convert into various types of functional neurons on the addition of specific compounds because of which it serves as a model for neurodegenerative disorders. It is semi-adherent naturally and has epithelial as well as neuronal morphology. The doubling time of SH-SY5Y cells during the exponential phase is 48h±5.8h.

Noncytotoxic concentrations of MCP and lead (Pb) have been already established in our lab. MCP has a safe dose of 70% cell viability at 300μm for 72hr exposure whereas lead acetate at 50μm for 72hr exposure. This study mainly focuses on analyzing the impact of environmental exposure on the toxic profile of occupational exposure of MCP in human neuroblastoma cell line, on which we used established safe dose of lead on various concentrations of MCP for 24h, 48h, and 72h. Moreover, as a control, unexposed cells, cells exposed with only MCP, and cells exposed with only Pb were used.

The objective of the proposed work:

- To determine the non-cytotoxic concentration of co-exposure of lead and MCP on the SH-SY5Y cell line using Alamar Blue assay.
- To analyze the effect of co-exposure of lead and MCP on transcriptional level via. RT-PCR using Bax, Bcl_2, Cas-3, Cas-7 and Cas-9 apoptotic markers and Nestin and NFM as neuronal markers.
- To analyze the effect of co-exposure of lead and MCP on transcriptional level via.

  GAPDH as internal control and Cas-3 and Bax as apoptotic target proteins.

Chapter 2: Review of Literature

According to WHO the Indian pesticide industry is ranked 2nd in Asia after China for the production of 79,800 metric tons of production of pesticide and has ranked 12th on global pesticide production. Moreover, the pesticide industry in India has a value of around 1500 million US dollars including the cost of exports of about 622 million US dollars. Along with that, the importance of agriculture in India can be understood from the fact that it contributes 22% of the national GDP and that 70% of the population's employability of the country depends on it.However, the pesticide management in India including the use and distribution of the pesticides is not up to the standard code of conduct set by the Food and Agriculture Organization of the United Nations (FAO). Pesticides are sub-categorized based on their action on diverse sets of pests. In Uttar Pradesh only, 11557 metric tons of pesticide is used according to a study conducted in 2020-2021.

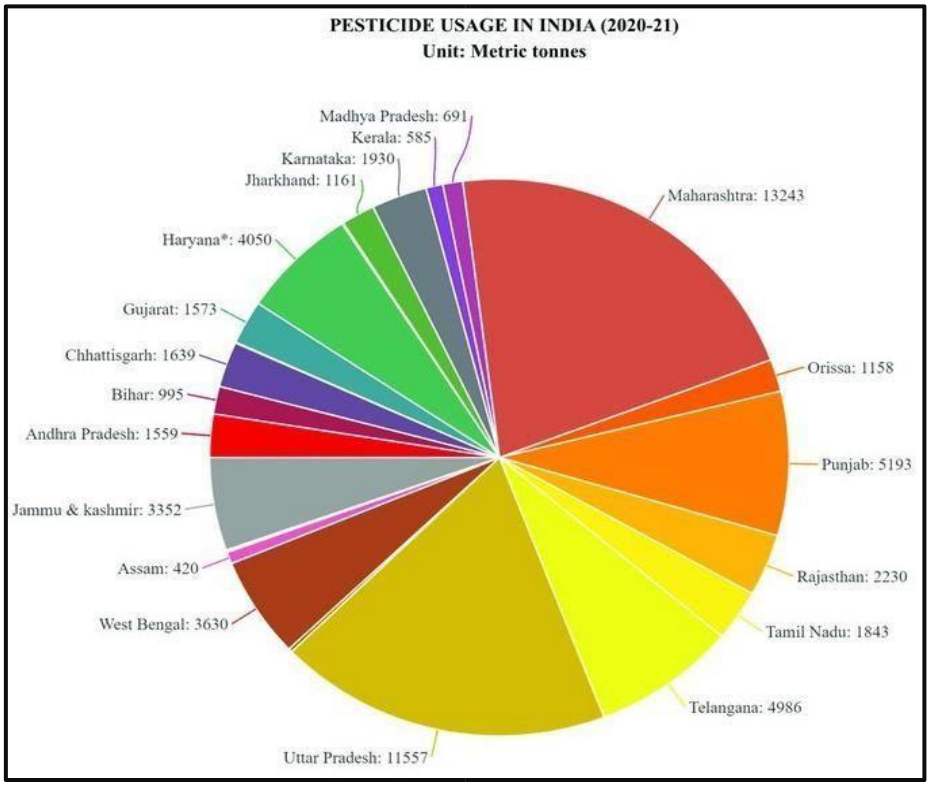


Figure 2: Pesticide usage in India in metric tons. Source: Raj A, 2021.

Organophosphates (OOPs) are one of the most widely used pesticides in the agricultural industry, which falls under the category of insecticide (Kushwaha M *et al.,* 2016). They are biodegradable so they do not accumulatein the environment. They are abundantly used in agricultural sector worldwide because they have a broad range and are highly effective in controlling pests. They have been used in the field of agriculture for protection against pests for about the past 40 years. (Karpouzas and Singh 2006; Abraham and Silambarasan 2015). Roughly, they account for 22% of the global pesticide market (Kumari *et al.,* 2018). However, only about 0.1% of pesticides applied reach their target, the rest ends up in the environment which results in reduced crop harvest, low-quality agricultural products, deteriorating quality soil, and worsened soil enzyme activity (Riah *et al.,* 2014), contaminated water thus, giving rise to a severe risk to humans and animals (Yadav *et al.,* 2016; Buvaneswari*et al.,* 2017).

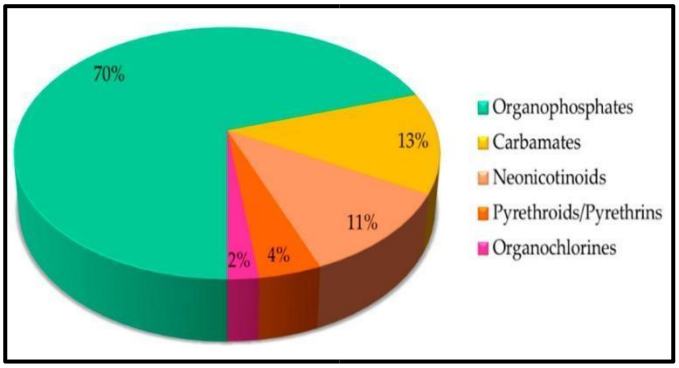


Figure 3: Percentage of various classes of insecticide present in the environment based on the optical sensors. Source: Fauzi NIM et al., 2021

Despite being biodegradable, organophosphate exposure may lead to acute as well as chronic toxicity to animals as well as humans (Gill *et al.,* 2018). Symptoms of initial organophosphate toxicity include headache, dizziness, diarrhea, weakness, vomiting, etc. While long-term exposure includes symptoms like seizures, confusion, anxiety, weakness in muscles, numbness, depression, and tingling sensation in hands and feet (Fauzi NIM *et al.,* 2021), and in the worst-case scenario it may lead to death (Kanekar *et al.,* 2004). Annually, 3 million organophosphate poisoning with 200,000 human deaths were reported in various developing countries (Ragnarsdottir 2000; Karpouzas and Singh 2006).

MCP: MCP is a type of synthetic organophosphate that is widely used among farmers even though it has a detrimental effect on human health. It is not patented and is therefore cheap because of which it has high demand.It was first registered in Australia in 1968 wherein it was highly useful in protecting various crops such as sorghum, sunflower, tomatoes, and tobacco by acting on a broad range of insects. Its IUPAC name is dimethyl [(Z)-4-(methylamino)-4-oxobut-2-en-2-yl] phosphate and has Molecular formula: C7H14NO5P. The Environmental Protection Agency classifies MCP as a class (I) highly hazardous substance (Sidhu et al., 2015). Its median fatal dose (LD50) for mammals is 18-20 mg /kg (Kaur and goyal 2019). Monocrotophos is completely soluble in water, but due to its hydrophilic nature, it is only slightly sorbed by soil particles (Subhas and Singh, 2003; Mackay *et al.,* 2006), providing a concern to underground water contamination via. leaching which may come in direct contact with drinking water resulting in dietary intake of MCP by humans.

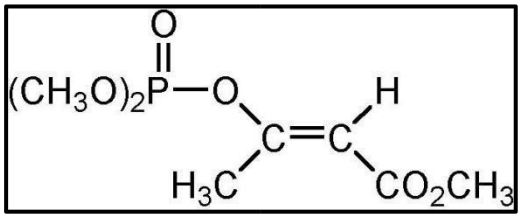


Figure 4: Structural Formula of MCP.

Characteristics of MCP: MCP is a colorless or reddish-brown, crystalline form having a mild ester odor. It is highly toxic and has a half-life of 10 hours in humans. It has a molecular weight of 223.2gm/mole. It is highly hygroscopic which is why stored in glass bottles (Jose A *et al.* 2015).

Applications of MCP: MCP is applied on various crops such as cotton, rice, castor, citrus, olives, rice, maize, sorghum, sugar cane, sugar beet, peanuts, potatoes, soybeans, cabbage, onion, pepper, tobacco, etc.

Toxicity of MCP exposure: Humans can be exposed to MCP via oral, dermal, and inhalation. In rats, the acute oral lethal dose (LD 50) of MCP is 14mg/kg whereas the uptake of 1200mg of MCP can be highly lethal to humans. According to WHO

classification of pesticides, MCP is classified as class Ib which means it is apesticide of high-risk to human as well as animal health (WHO 2009). Intake of MCP inside the body through inhalation affects the respiratory system of the body and can cause discomfort in breathing, and shortness of breath due to constriction in bronchial tubes. Dermal contact with MCP may lead to involuntary muscle contraction and sweating. Entry of MCP via any route may lead to vomiting, abdominal cramps, confusion, etc. within 12 hours of exposure. Adverse symptoms include contraction of the muscles involuntarily, loss of reflexes, and twitching which may ultimately lead to paralysis because it adversely affects the central nervous system. Recent studies on workers, mainly farmers, who were occupationally exposed to MCP indicate that workers who do not wear protective clothing in countries having hot weather conditions are more susceptible to dermal exposure to MCP (WHO 1993).

Mechanism of action of MCP: MCP mainly inhibits the enzyme called acetylcholinesterase which is important for neuronal signaling in the body. Inhibition of acetylcholinesterase (AChE) not only kills the pest by hampering their nerve signals but also causes critical neuronal defects in the exposed humans, mainly farmers who apply this pesticide for the protection of their crops. Inhibition of AChE leads to the accumulation of acetylcholine at junctions of neuromuscular regions which results in the involuntarily twitching of the muscles leading to paralysis. In a study, it was estimated that the absorption of 20mg of MCP may lead to the inhibition of the plasma AChE enzyme. Moreover, it was indicated that the major population that was affected by accidental oroccupational MCP toxicity were mainly farmers (WHO 2009).

Lead (Pb): Lead (Pb) is found naturally in earth's crust therefore it has been known and utilized by humans for day-to-day life over a long duration. It is a heavy metal and is not easily degradable and persist in nature which makes it one of the most commonly found environmental pollutant. However, even after knowing its possible toxic effect, it is still in use in day-to-day life for a variety of purposes such as in cosmetics, paints, plumbing, lead-based batteries, mining, manufacturing, etc. Lead occurs in both organic and inorganic forms. The organic form of lead is comparatively more toxic than the inorganic form. In this study, we have chosen lead acetate, an organic form of lead. It is easily absorbed via.

Dermal exposure is highly toxic to the central nervous system and brain and can lead to insomnia, cognitive deficits, tremors, hallucinations, etc. It readily dissolves in water due to which it can easily contaminate ground water.

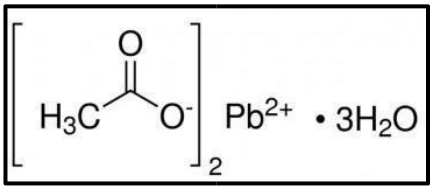


Figure 5: Structural formula of Lead acetate

The screening-guideline of Center for Disease Control and prevention (CDC) for lead was 10µg/dl. However, recent studies suggest that the screening-guideline should be reduced to 2µg/dl because it was observed that people who were exposed to Pb resulted in reduced IQ levels as well as low academic performance which hampers the future of the children. However, recent studies show that exposure to a constant low level of Pb may lead to a negative impact on neurological development especially in children. Along with that, neurophychiatric abnormalities including hyperactivity disorder are reported. Interestingly, Pb is not needed in any biological process and it cannot be metabolized into any other element inside the human body.

Lead is mainly consumed by the body via ingestion and inhalation. Lead, when enters the human body is accumulated in the bone. It may transfer from the mother to the fetus which may incorporate in the skeleton of the fetus. Exposure of lead to children may cause behavioral defects because of the onset of neurological disorder which may be only evident in the adulthood of the exposed children.

Lead mainly exists in organic as well as in organic forms. The organic form of lead is more toxic than the inorganic form. In our study, we have chosen the organic form of lead i.e., lead acetate. Lead acetate was produced by reacting lead (II) oxide with acetic acid. Because of its sweet taste, it was used as a sweetener long before its toxicity was known.

Characteristics of lead acetate: Lead acetate has a density greater than water. It is white to gray in color and appears as crystalline solid. Its molecular weight is 379.33gm/mole. Its molecular formula is (CH3COO)2Pb. It is readily soluble in water due to which it can readily contaminate ground water.

Applications of Pb: It is used in lead-based production of paint, ink, varnish, coating, hair dyes, cables, lead- based batteries, ceramics, house decoration materials, children's toys, stationery, cosmetics, batteries, automobile exhaust, renovations, repairs, etc.

Toxicity of Pb exposure: Exposure of Pb may lead to various neurological disorders such as brain damage, mental retardation, behavioral problems, nerve damage, possibly Alzheimer's disease, Parkinson's disease and schizophrenia, insomnia, cognitive deficits, tremors, hallucinations, etc. It enters the environment through varioussource and persist by being absorbed in the air or from direct discharge into the water streams. This not only hampers the human directly as well as indirectly by decreasing the growth and reproduction in plants and animalsalong with adverse neurological impact on vertebrates. Moreover, according to the World Health Organization, exposure of lead gives rise to around 600,000 new cases of lead neurotoxicity per year which affects children's IQ (Karpouzas DG and Singh BK 2006). It may enter the human body by contaminated drinking water having leached lead from corroded pipes. Various neurodegenerative disease is caused as result of exposure to lead such as Alzheimer's and Parkinson's diseases. lead exposure causes a rise in oxidative stress in brain cells resulting in cell death and neurodegenerative disorders (Martínez-Hernández M I 2023).

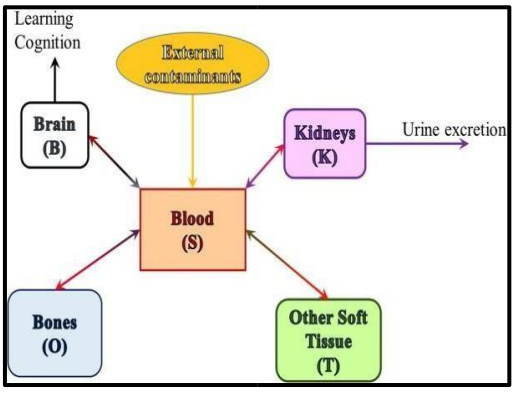


Figure 6: Environmental exposure of lead on human body. Source:(Rădulescu A and Lundgren S, 2019)

Mechanism of action of Pb: Lead when enters the body targets calcium transporters. It replaces calcium fromthe calcium-phosphate salt that contains the crystalline matrix of the bones (Rădulescu A and Lundgren S 2019). It then accumulates on the surface of the bone of the human body and is released in the bloodstream at the time ofremodelling whereby it crosses the blood-brain barrier and disrupts the various intracellular biological activities (Sanders T *et al.* 2009).

SH-SY5Y Cell Line: SH-SY5Y is selected as the model cell line. It is a thrice-subcloned cell line derived from the SK-N-SH neuroblastoma cell line. It has a property to convert into various types of functional neurons on the addition of specific compounds because of which it serves as a model for neurodegenerative disorders. It is semiadherent and has epithelial as well as neuronal morphology. The doubling time of SHSY5Y cells during the exponential phase is 48h±58h. Being semi-adherent, it has two types of cells both adherent as well as suspension cells.

Adherent Cell: These cells grow in monolayer, and attach themselves to the surface of the culture vessel. Neuronal cells require surface to grow hence they are adherent.

Suspension Cell: These cells grow in cell suspension and are not required to attach themselves to the base of the flask.

To study the effect of environmental lead neurotoxicity in combination with occupational MCP-induced neurotoxicity, SH-SY5Y is considered as the model cell line. As this is novel research, there are no articles related to the safe dose of MCP in combination with Pb. Therefore, we performed the Alamar Blue assay which is a cytotoxic assay to detect the safe dose of MCP in combination with Pb, and analyzed its molecular level expression in comparison with unexposed cells, only MCP, and only Pb exposed cells.

Chapter 3: Material and Methods

Growing cells/tissues in vitro in a suitable environment and under sterile conditions is known as animal tissue culture. These cells may be derived from previously established cell lines cell strains, or multicellular eukaryotes. To establish and maintain a cell line, suitable culture conditions are a prerequisite which mainly includes a suitable medium for cells to grow, along with that growth factors can be supplemented if needed, a controlled physio-chemical environment (pH, temperature, etc.), an incubator providing 5% CO2 and 95% air and most importantly a sterile culture environment.

1) **Materials and reagents required:** The various reagents used in in-vitro cell culture and maintenance of SH-SY5Y cells are described below:

- **EMEM alpha:** Harry Eagle developed EMEM which is one of the most widely used synthetic culture medium for mammalian culture. It contains no proteins, lipids, or growth factors hence it requires supplementation. The most commonly used supplement is 10% FBS. It uses sodium bicarbonate as a buffering system and requires 5% CO2 in a culture environment to maintain osmolarity and physiological pH.

- **Ham's F-12:** Ham's F-12 medium was mainly designed for the growth of Chinese hamster ovary (CHO) cell culture. Ham's F-12 is a modification of Ham's F-10 medium. It has a higher concentration of amino acids compared to Ham's F-10.

- **Sodium bicarbonate (NaHCO3)-** The ideal pH range for MEM-F12 medium is 7.4-7.6. To maintain this pH NaHCO3 is used as a buffer to maintain the pH of the medium. The CO2 provided to the cultured cells readily dissolves in the medium and reacts with the water content of the medium to produce carbonic acid. This carbonic acid produced lowers the pH of the medium which is not suitable for the growth and development ofthe cells. To balance it out, the bicarbonate ion produced from NaHCO3 reacts with this carbonic acid to balance the pH of the medium that is optimal for the cells.

- **Antibiotic-Antimycotic solution (Ab/Am)-** A 100X stock solution of the antibiotic-antimycotic solution is used whose working concentration is 1X. It is required to prevent the growth of contaminants such as fungi, viruses, and bacteria. The main contaminant in animal cell culture is mycoplasma, which in some cases, may persist even after adding Ab/Am.

- **Non-essential Amino Acids (NAA):** Non-essential amino acids are supplemented in the medium to enhance the growth of the cells.

- **Fetal Bovine Serum (FBS):** FBS, as the name indicates, is obtained from calf serum. It is a rich source of growth factors. Before its use, it is heat inactivated to destroy heat-liable complement proteins as well as any contaminants, if present, at a temperature of 57°C in the water bath for 30 min and is swirled at an interval of 10 min to ensure that there is uniform mixing and no protein coagulation. After heat inactivation of FBS, it is normalized at room temperature (RT). Filtration of FBS after heat inactivation is done using a syringe filter of pour size 0.2μm filters. Filtration is mainly done as a precaution to prevent any mycoplasma contamination.

- **Trypsin-EDTA complex**: Trypsin is a proteolytic enzyme that is used in combination with EDTA (Ethylenediaminetetraacetic acid) to detach adherent cells from the base of the culture vessel. Trypsin acts on C-terminal of the amino acid lysine and arginine residues by hydrolyzing the polypeptide bond. EDTA is used as a chelating agent to chelate the divalent cations mainly calcium (Ca^2+) and magnesium (Mg^2+) as these ions may hinder the enzyme activity of trypsin during trypsinization of the cells. Incubating cells with either high concentrations of trypsin or for a prolonged period may impair the cell membrane which is not desired. Phosphate buffered saline (PBS) buffered salt solution is used to dilute trypsin to the optimum concentration that is not harmful to the cells.

- **Dulbecco's Phosphate Buffered Saline (PBS):** In animal tissue culture PBS is widely used to maintain the the constant pH as well as osmolarity of the cells in a particular environment. It has a pH of around 7.4 whichis used in human cell lines as it is close to the pH of the human body. It is frequently used in animal tissue culture because it is not cytotoxic and is isotonic which minimizes the chances of cell rupture

or flaccing of cell due to changes in osmolarity. Moreover, it is also used to rinse the remaining spent medium from cell culture vessels in passaging of cells so that it may not hinder trypsinization. It also plays the role of solvent for dissolving trypsin such as in SH-SY5Y cell line, working concentration of trypsin is 0.05% which is diluted from the stock solution having concentration of 0.25% using DPBS.

- **Trypan blue:** Trypan blue dye is used to check the viability of the cell. It follows the principle that live cells excludes trypan blue dye because they have intact cell membrane whereas non-viable cells are stained blue as they have impaired cell membrane which allows entry of the trypan blue. Hence, viable cells have clear cytoplasm in contrast to non-viable cells which have blue cytoplasm.

- **Alamar blue Reagent:** Alamar blue reagent contains resazurin, which is the primary component of the reagent. It is a cell-permeable chemical that is non-toxic. It has a blue color and is non-fluorescent. When resazurin enters living cells, it is converted to resorufin, a red-colored molecule that is highly fluorescent. This is widely used in cytotoxic experiments where changes in cell viability may be easily identified at fluorescence at 560-590nm using plate reader.

- **Dimethyl sulfoxide (DMSO):** DMSO is used as a cryoprotectant in cryopreservation. It is a light-sensitive compound and is added into the freezing media to protect the cells from the formation of intracellular and extra-cellular water crystals during the freezing of the cells. It penetrates the cell membrane and replaces water molecules to prevent the formation of intracellular crystals which may cause mechanical injury to the cell and may lead to possibly cell death.

- **Disposable culture wares:** The disposable culture wares that are used for cell culture are sterile. The major culture wares used in medium preparation include micro tips, syringes, serological pipettes, parafilm, syringes, syringe filters of pour size 0.2-0.22μm or vacuum filter cup (0.22μm pour size), and falcon tubes.

2) Methodology: The methodology for the three objectives is given in step wise manner.

Objective 1: The method and various techniques for in vitro cell culture and maintenance are listed below.

a) Culture medium: The basal medium used for SH-SY5Y cell line is Eagles Minimum Essential Medium- Alpha Modification (EMEM) (1X) and Ham's F-12 Nutrient mixture (F-12) is mixed in a ratio of 1:1.

- **Requirements:** Autoclaved deionized Milli Q water, autoclaved glass bottles, EMEM alpha, Ham's F-12, Sodium bicarbonate (NaHCO3), Antibiotic-Antimycotic solutions (Ab/Am), Non-essential amino acids (NAA), Fetal Bovine Serum (FBS), micro tips, syringe, serological pipettes, parafilm, syringe- filter of pour size 0.2-0.22µm or vacuum filter cup (0.22µm pour size), falcon tubes.
- **Preparation of incomplete medium:**

1. MEM and Ham's F-12 powder sachet (MEM Gibco cat# 2533382 and F-12 Gibco cat#2209586) typically produce 1L of medium per sachet.
2. Weigh NaHCO3 beforehand for both of the mediums according to the required quantity mentioned in the specification of the individual sachets.
3. Pour autoclave Milli-Q around 800ml into the vessel and first empty the MEM sachet in the media preparation beaker.
4. Thoroughly mix the powdered medium in the autoclave Milli-Q with the help of serological pipettes.
5. Add NaHCO3 according to the specification given on the sachet.
6. Check and adjust the pH of the medium to 7.4 with the help of HCL and NaOH, as per the need.
7. When pH is adjusted, add 1% of the stock solution of 100X Ab/Am solution (10ml).
8. Volume makeup the medium solution to the final volume i.e., 1L.

9. Filter the medium in a 1L sterile autoclave glass bottle with the help of a vacuum sterilization filter cup or with the help of a syringe filter.

10. Rinse the medium preparation beaker thoroughly and repeat the same steps for F-12 preparation, adding the required amount of NaHCO3, as indicated in the sachet.

11. After filtration of both the medium, add 1ml of the freshly prepared medium in a 48-well plate and incubate it for 24hr in a CO2 incubator. This is done to check if there is any contaminant in the medium or not. The presence of any contaminant results in turbidity of the medium and the contaminant may be detected under the microscope.

12. After adding media to the check plate, fasten the medium bottle cap and warp the parafilm around the cap. Properly label the bottles and store them at 4°C.

- **Preparation of complete medium (40ml) from the incomplete medium:**

1. Before preparing complete media, normalize the MEM and F-12 media at RT for 30 minutes.

2. Place the bottles in a laminar flow hood and wipe them with 70% ethanol.

3. Take out the parafilm from medium bottles and add them in falcon tubes in a ratio of 1:1add 18ml MEM and 18ml F-12 in a 50ml falcon tube.

4. Add Non-essential amino acid (1X).

5. Add 10% FBS i.e., 4ml for 40ml medium aliquot.

6. Mix thoroughly making sure that there is no froth formation.

7. Label the tubes properly before use.

b) Revival of cells: Cells that were stored in liquid nitrogen or -80°C for cryopreservation are revived at the time of use.

- **Requirements:** Complete medium for SH-SY5Y cell line, micro tips, serological pipettes, parafilm, falcon tubes, culture vessel (T-25, T-75flask, 60mm dishes, etc.).

- **Method:**

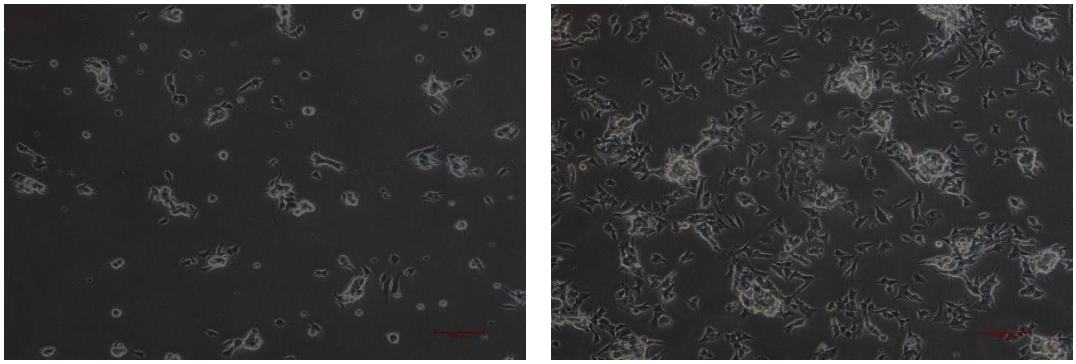
1. Set the water bath at 37°C beforehand.
2. Take your cryovials having the desired cell line from -80°C (The lower the passage number the greater the chance of revival of maximum cells).
3. Place the cryovials in the water bath for the thawing of cells until only one ice crystal remains.
4. After the vial is thawed, immediately transfer the cell suspension from the cryovial into a 15ml falcon tube containing 3ml of complete culture medium.
5. Centrifuge the cell suspension at 800rpm for 5 minutes.
6. Remove the supernatant and re-suspend the appropriate amount of cell pellet in a suitable volume of complete medium.
7. The cell suspension is then transferred to an appropriate cell culture vessel.
8. Now, Keep the cells in the incubator at 37°C so that they may take appropriate cell morphology.

c) Passage: Cells when reached to 80% confluency, are sub-cultured as the nutrient depletes, toxins and dead cells accumulate, and cells have reached their maximum cell density per surface area which leaves no room for cells to further proliferate. The process of sub-culturing of cells to obtain the large number of cells from already existing cells of the 80% confluent flask is called passage. This itself includes various techniques such as trypsinization, trypan blue assay, counting, and seeding of cells.

Trypsinization: The SH-SY5Y cell line has adherent cells as well as suspension cells. The adherent cells need to be detached from the surface of the culture vessel. To detach these adherent cells trypsin-EDTA complex is used.

Trypan Blue assay: Trypan Blue assay is mainly done to identify live and dead cells in a cell suspension. Trypan blue is a dye that is excluded by live cells as they have intact cell membranes and appear to have clear cytoplasm and is uptaken by dead

cells as their membrane integrity is lost due to which they appear to have blue color cytoplasm.



a) **b)**

Figure 7: a) SH-SY5Y cell confluency at the first day of passage
b) SH-SY5Y cells at 80% confluency

Cell counting: Cell counting is done to quantify the number of viable and dead cells in a cell suspension so that an estimate of the total number of viable cells can be obtained and cells can be seeded with a defined seeding density. The most commonly used instrument for cell counting is a hemocytometer in which we have to manually count the number of cells in a defined set of areas. For our experiment we have used an automatic cell counter in which we mix cell suspension with trypan blue dye in the ratio of 1:1 and 10µl of the reaction mixture is put on a automated countess slide which is read by the automatic counter and the total number of cells, the number of viable and dead cells per ml of cell suspension is displayed.

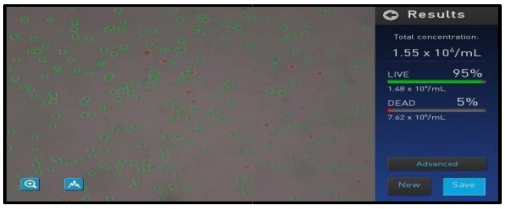


Figure 8: Automated Countess **Figure 9: Percentage of viable and dead cells**

Cell seeding: Cell is counted using a countess cell counter slide to seed the optimum amount of cell number in a culture flask or dish. As the automated cell counter gives a number of live cells per 1 ml of cell suspension, the accurate amount volume to be picked up to seed the appropriate amount of cell number according to the seeding density of the respective culture flask or dish is calculated. The list of the maximum seeding density of the respective culture vessel is mentioned below:

CULTURE WARE	SEEDING DENSITY
48-well culture plate	0.03x10^6
T-25 flask	0.7x10^6
T-75 flask	2.1x10^6

- **Requirements:** Complete medium for SH-SY5Y cell line, micro tips, serological pipettes, parafilm, falcon tubes, suitable culture vessel, trypsin-EDTA complex (0.05%), Kim wipes.

- **Method:**

1. Take the flask having 80% confluent cells and collect the spent medium having suspension cells in a sterile falcon tube.
2. Rinse the flask with 1ml of trypsin and collect it in the same falcon tube.
3. Add 1ml trypsin in a T-75 flask or 500µl of trypsin in a T-25 flask and incubate it at 37°C in an incubator for 3-5 minutes.
4. Observe the cells under the microscope to observe whether cells have detached from the surface of the vessel. The detached cells appear either floating or round in shape.
5. Add complete medium double the amount of trypsin added (e.g., 2ml for T-75 flask or 1ml for T-25 flask) to the flask to neutralize the effect of the trypsin-EDTA complex.

6. Swirl the medium in the flask gently so that all the detached cells come in the medium they can be easily collected in the form of suspension.
7. Collect the detached cells from the flask in the falcon tube.
8. Centrifuge both cell suspensions at 1000rpm for 5min.
9. Discard the supernatant having spent media, trypsin, and cell debris and dissolve the pellet containing cells in a suitable amount of complete media.
10. Does the single-celling use a serological pipette (to avoid froth formation)?
11. Take 10µl of trypan blue dye in the form of a drop and 10µl of the cell suspension to it and mix thoroughly.
12. Wipe off the countess slide and cover the slip with kim wipes wet with 70% ethanol Place 10µl of trypan blue and cell suspension mixture on the countess slide and cover it with a cover slip.
13. Place the slide in the automated cell counter and counter and note down the viable number of cells. Repeat this step 3 times to get an accurate reading.
14. Calculate the required amount of volume of cell suspension to be added according to the appropriate amount of cell number to be seeded in a culture vessel.
15. Add fresh medium to the flask /plate and seed the appropriate amount of cell suspension according to the seeding density of the culture vessel.
16. Incubate the cells at 37°C in a CO2 incubator. The cells take up their morphology after 24 hours of passage.
17. As the doubling time of the SH-SY5Y cell line is 67.3h±5.8hr, the medium is changed after every 48hr.

d) Alamar Blue Assay: Alamar blue assay is used to determine the safe dose i.e., cells having 70% cell viability, of MCP+Pb combination. As the safe dose of MCP and Pb is already established in our lab, we used the safe dose of Pb i.e., 50µm on varied

concentrations of MCP i.e., 100µm, 150µm, 300µm, 400µm, 500µm and 1000µm to assess the toxic potential of Pb on varied concentration of MCP. The duration of the exposure is of 72hr. In alamar blue, the blue color resazurin is converted into pink color resorufin in metabolically active viable cells which is highly fluorescent.

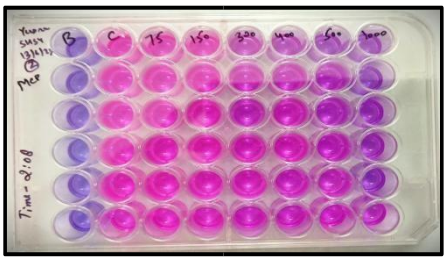


Figure 10: Alamar blue assay plate

- **Requirements:** Complete medium for SH-SY5Y cell line, micro tips, serological pipettes, parafilm, falcon tubes, suitable culture vessel, trypsin-EDTA complex (0.05%), Kim wipes, 48 well plates, multichannel pipette, aluminum foil.

- **Method:**

1) Passage the flask having 80% confluent cells and seed in three 48-well plate with a seeding density of 25,000 cells per well having blank and control in the first two rows of the plate. Make sure that there is no bubble formation while seeding the cells. Label the plates as 24hr, 48hr, and 72hr.

2) After 24 hours of seeding, prepare the stock and working concentration of MCP and Pb having a concentration of the desired concentration.

3) Add the respective amount of medium having the desired concentration of MCP and Pb in the respective rows.

4) After 20hr of first exposure, add 20µl of Alamar Blue in the 24hr labeled plate so that resazurine may be reduced. Place the plate in CO2 incubator to incubate for 4hr. 5) After the incubation is completed, take the plate out (covered with aluminum foil) and take the reading of 24hr at 560-590nm in plate reader.

6) Repeat the same step for 48 hr. incubated plate and 72hr incubated plate.

7) Calculate the percent viability and analyze the data.

e) Cryopreservation: Freezing of cells or cryopreservation is done to preserve the cells for later use. They can be stored for a long duration by cooling them at extremely low temperatures such as -80°C to -196°C to slow down their metabolism. In freezing of cells, the water content of the cell may form ice crystals which may harm the cell. To stop the formation of ice crystal inside the cell, cryoprotectants are added such as DMSO.

- **Requirements:** Complete medium for SH-SY5Y cell line, micro tips, serological pipettes, parafilm, falcon tubes, suitable culture vessel, trypsin-EDTA complex (0.05%), Kim wipes, cryovials, DMSO.

- **Method:**

1. Decant the spent medium in a sterile falcon tube and trypsinize the adherent cells.
2. Collect the total cell suspension in a sterile falcon tube and centrifuge at 1000rpm for 5 minutes.
3. Remove the spent medium and dissolve the pellet in an appropriate amount of the medium. Do the single-celling.
4. Count the cells and dissolve the cells in complete media having 5% DMSO in a way that 1ml contains10×10^5 cells/ml.
5. Label the cryovials with passage number, date, and name of the cell line and add 1ml per cryovial and cover them with parafilm. Store the cryovials at 80°C or in liquid nitrogen.

3) Objective 2: The method and various techniques used for transcriptional-level analysis are listed below.

a) RNA isolation using trizol reagent: Trizol Reagent, or Total RNA Isolation Reagent, is used to isolate total RNA from cells and tissues. During tissue lysis or homogenization, the trizol reagent breaks cells and dissolves their constituent parts while retaining RNA integrity. The addition of chloroform, followed by centrifuge,

causes the aqueous phase to be separated from the organic phase. Isopropanol is used to precipitate the extracted RNA from the aqueous phase. This RNA is redissolved in Nuclease Free Water, and the obtained RNA is quantified in terms of optical density.

- **Procedure for isolating RNA from cells using TRIzol reagent:**

1. **Cell lysis:** Decant media of the flask/dish of which the RNA lysate is to be obtained. Add 1ml TRIzol reagent per flask. Swirl it around till it covers the entire flask surface. Let it rest for 5 minutes. Collect the cells with TRIzol in an MCT.

2. **Phase separation of the cellular contents:** Add 200ul of chloroform to the RNA lysate and Vortex the sample for about 15 seconds such that the layers of TRIzol and chloroform mix properly. After that incubate the sample at room temperature for 10 minutes Centrifuge the sample at 11600 r.c.f for 15 minutes at 4ºC after the incubation period is completed.

3. **Precipitation of the RNA:** The aqueous phase of the sample after the is carefully collected in the new MCT. Now, Add 500ul of isopropyl alcohol to the aqueous phase. Mix the sample by pipetting and centrifuge it at 11600g for 10 minutes at 4ºC.

4. **Washing of the RNA pellet:** Discard the supernatant and add 1 ml of 75% ethanol to the RNA pellet. Mix the ethanol by gentle inversion. Do not vortex. After proper mixing of ethanol, centrifuge it at 10000 g for 5 minutes at 4ºC.

5. **Re-suspension of the RNA pellet:** Discard the supernatant and let the pellet air dry at room temperature till the ethanol evaporates. Resuspend the pellet in the appropriate amount of nuclease-free water (preheated at 95ºC) and mix it gently by pipetting up and down. Store the sample at -20ºC or measure the concentration of RNA in the sample.

6. **RNA quantification by bio-spectrometer:** A bio-spectrometer is used to measure the quantity and purity of RNA by measuring absorbance at wavelengths 260 and 280nm. For pure RNA samples, the A260/A280 ratios range between 1.8 to 2.0. When there is protein or phenol contamination, the A260/A280 ratio is much

lower, making it impossible to precisely detect RNA. The 260/230 ratio is a measure of nucleic acid purity check. Pure nucleic acid often has a higher 260/230 value than the corresponding 260/280 value, with expected 260/230 readings falling between 2.0 and 2.2.

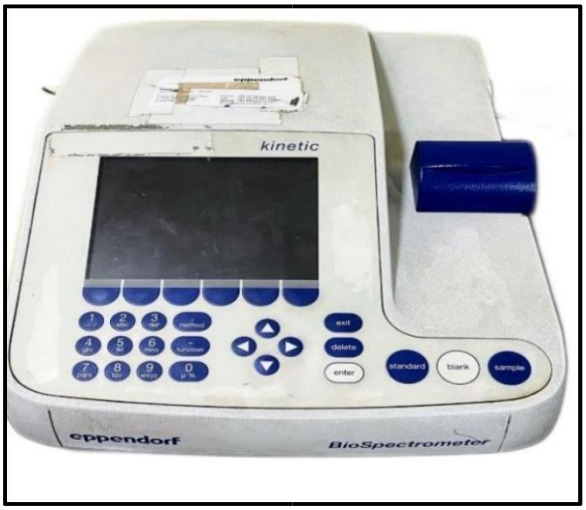


Figure 11: Bio-spectrometer used for RNA quantification.

b) Agarose gel electrophoresis: The usage of RNA-based applications that require high-quality, non-degraded RNA is a critical component in making cDNA. To analyse the integrity of the extracted RNA sample, agarose gel electrophoresis of the extracted RNA sample is run.

1. **TBE BUFFER (2X) PREPARATION:**

Component	**Amount**	**Significance**
Tris Base	21.6 g	The main role of Tris buffer is maintaining the constant pH of 8.3.
Boric Acid	11 g	The primary buffering element's main job is to keep the buffer's pH stable, usually around 8.3.

EDTA (0.5M)	8 ml	Role of EDTA is to mainly protect the RNA sample from enzymatic digestion by nuclease. EDTA chelates the divalent cations such as Ca^{+2} and Mg^{+2} which are co- factors for nuclease.
DEPC (Di-ethyl Pyrocarbonate)	1000 ml	DEPC inhibits RNase, inhibiting the RNA breakdown.

2. TBE BUFFER (1X) PREPARATION:

Components	**Volume (500ml)**
2XTBE Buffer	250ml
DEPC water	250ml

3. AGAROSE (1%) GEL PREPARATION:

Components	**Amount**	**Significance**
1X TBE Buffer	50 ml	Maintains pH of the gel
Agarose	0.5 g	Through non-covalent linking, agarose polymers create a network of bundles, and the pore widths of these bundles determine the molecular sieving capabilities of a gel.
Ethidium Bromide	3ul	EtBr binds to RNA molecule and helps visualizing the RNA bands inUV light

- **Casting of agarose gel:**

1.Use ethanol to disinfect all components used to cast and run the gel (well plate, buffer tank, well comb, tank lid).

2. Set the well comb in the gel casting tray.

3. Weigh 1% agarose powder in a foil or paper (for 50ml gel, 500mg of agarose).

4. Add agarose in the bottle having 1X TBE buffer with the exact volume of which the gel is made.

5. Heat the bottle containing agarose in a microwave without the cap of the bottle for about 30 seconds.

6. Now, add EtBr in the bottle containing semi solid gel.

7. Slowly pour the hot agarose solution into the gel tray. Avoid any bubble formation. Allow the gel to cool and solidify for about 20 minutes at room temperature.

8.Once the gel has solidified, carefully insert it in the buffer tank. Take out the well comb.

9. Add 1x TBE buffer to the tank and place the gel inside the tank after it solidifies.

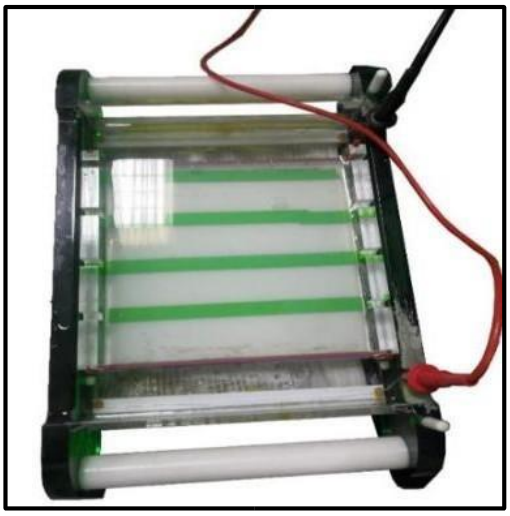


Figure 12: Gel casting chamber for agarose gel electrophoresis

- **Sample preparation and loading of the gel:**

1. Each sample loaded into the wells should have the same concentration, such as 500ng/l, and volume, such as 10µl.

2. A 1x concentration of dye (bromophenol blue) is necessary. If the total volume is 10µl, then the amount of dye required will be 5µl.
3. The amount of RNA required is determined as (300ng/RNA yield). This number, together with the 5µl dye would be added and further subtracted from the total volume. The dye would be added and then deducted from the overall volume. This determines the amount of NFW required.
4. Mix and spin the sample in the PCR tube and incubate the prepared sample for 5 minutes in a dry bath at 72°C. Remove the sample from the dry bath and place it in an ice tray.
5. Load the RNA ladder first (3µl), followed by the sample(s) in the subsequent wells (10µl).
6. Connect the electrodes to the tank and power pack in a stepwise manner.
7. Apply a constant 50-volt voltage.
8. Run the gel until the gel-loading buffer stain migrates the required distance, about 3/4 of the way down the gel.
9. Examine the gel in the imaging system with UV light. MultiScribe Reverse Transcriptase.

Figure 13: ChemiDoc Imaging System Bio-Rad

c) PCR for cDNA synthesis: Prepare the following Reverse Transcription reaction mix on ice in a PCR tube:

Component	**Amount (n=number of samples)**
Reverse Transcription Buffer	2.0 µl x n
Deoxyribonucleoside Triphosphate (dNTP)	0.8 µl x n
Random Primer	2.0 x n
Multi Scribe Reverse Transcriptase	1.0 x n
Nuclease Free Water	4.2 x n
Total Volume	(10 x n) µl

- **STEPS FOR PCR:**

1. Firstly, prepare the master mix in a PCR tube.
2. After preparing the master mix, dilute the RNA stock sample to a volume of 2000ng/l. Because the desired RNA concentration is 2000ng/l and the total volume required is 10µl per sample: If (2000/yield) =y RNA dilution, then y= RNA + (10-y) l NFW.
3. To each diluted RNA sample, add 10µl of RT master-mix.
4. Tap the tube(s) to thoroughly mix. For a few seconds, spin the samples. Configure the run method with the following parameters:

Step	**Time**	**Temperature**
1	10min	25°C
2	60min	37°C
3	60min	37°C

4	5min	85°C
5	∞	4°C

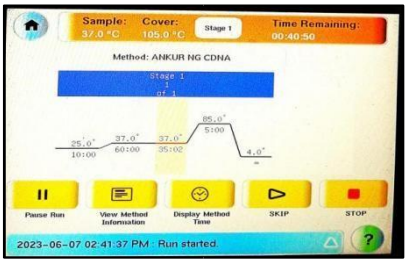


Figure 14: Thermal Cycler **Figure 15: Thermal Cycler cycles at run time**

d) REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR): The RT-PCR technique has evolved as one of the most widely used techniques for gene expression analysis due to its large dynamic range, excellent sensitivity, and exact assessment of specific gene expression. Unlike classical PCR, which uses an end-point quantification method that is not necessarily proportional to the amount of target present in the samples, RT- PCR measures the amount of PCR product after each cycle of amplification (along the reaction). PCR is an in-vitro technology for producing multiple copies of a specific DNA template from a modest starting amount through a three-step cycle procedure. For PCR amplification to occur, the target sequence is in the DNA sample, a thermostable DNA polymerase that can withstand multiple cycles of heating, dNTPs, and two synthetic oligonucleotide primers that are complementary to the regions flanking the target DNA sequence on the opposite strands are required. To begin the PCR cycle procedure, the DNA sample is thermally denatured at approximately 95 °C. In the second stage, known as renaturation or annealing, the primers in the reaction bind to their complementary DNA sequences as the temperature is gradually dropped to roughly 55 °C (depending on the primer). The third and final phase is known as "synthesis" or "extension," and it entails raising the temperature to 72 °C, which is perfect for Taq DNA polymerase's catalytic function, which extends the primer-started sequences and makes copies of the target

sequence. A fluorescent marker is widely used for quantification in RT-PCR. This marker, or reporter, emits a signal during the reaction that indicates the amount of product produced, which is subsequently recognized by a camera or detector. The signal is weak in the early cycles (there is little product), but as more product accumulates during the reaction, it becomes progressively stronger until saturation is reached. TaqMan probes and SYBR Green dye are the most often utilized detection chemicals in real-time PCR nowadays. SYBR Green dye is the most simple, versatile, and cost-effective format for detecting and quantifying PCR products in real-time PCR procedures, but it does require some fine-tuning.

- Reaction mixture for gene Preparation

Component	**Amount (n=number of wells)**
SYBR- Green	2.5µl x n
Forward Primer	0.25 µl x n
Reverse Primer	0.25 µl x n

- cDNA Dilutions:

Component	**Amount (n=number of wells)**
NFW	1.5 µl x n
cDNA	0.5 µl x n

STEPS FOR RT-PCR:

1. Take PCR tubes and add SYBR-Green dye according to the calculated volume, then add forward and reverse primer.
2. Tap the tube(s) to thoroughly mix. For a few seconds, spin the samples.
3. Now mark the 384 well plate and firstly add 3µl of gene prepared in triplicate for each sample then add2µl of c-DNA sample.
4. Seal the plate with film and cover with foil. Centrifuge the plate at 2500 r.p.m. for 2 minutes.

5. Insert the plate in the RT-PCR machine and set the parameters for the run.

4) Methodology for objective 3: The method and various techniques used for translational level analysis are listed below.

a)Protein isolation: Protein is isolated from control and exposed flask with the help of PBS (Phosphate Buffer Saline) and RIPA Buffer (Rapid immunoprecipitation assay). PBS is used to rinse the culture vessel. It is used along with P.I. so that any possible enzyme present in either medium or in M.Q. which may cleave the protein should be inhibited. Moreover, RIPA lysis buffer is used to lysis the cells for protein to separate out.

- **Requirements:** Chilled PBS, P.I., RIPA lysis buffer, MCT, cell scraper, ice packs.
- **Method:** (All work to be done on ice as protein samples are susceptible to denaturation)

1. Place the cultured flask on ice packs.
2. Discard the spent medium from the flask/dish.
3. Wash the flask/dish surface with 500-1000µl of PBS.
4. Now, add 1000µl of PBS+P.I. and scrape the adherent cells from the base of the flask/disk.
5. Collect the cells+PBS mixture in a 1ml MCT. Prior to this, label the tubes properly of control and exposed cells.
6. Centrifuge at 4000 r.p.m for 10 min.
7. Discard the supernatant and dissolve the pellet in 80-100µl of the RIPA lysis buffer+P.I. depending on the size of the pellet. Mix the pellet thoroughly by gentle vortex or by pipetting up and down.
8. Incubate it at 4°C for 1hr.
9. Sonicate the samples to properly lyse the cells.
10. Incubate the sample 1/2hr at 4°C.
11. Centrifuge at 13000 r.p.m for 25 minutes at 4°C.

12. Collect the supernatant having protein and discard the pellet containing cellular debris.

13. Quantify the protein using BCA assay.

b) Quantification of protein using Bicinchoninic acid (BCA) assay: The principal of BCA mainly involves that proteins reduce the Cu2+ ions present in BCA reagent into Cu+ ions in an alkaline solution. This results in a change in color of BCA reagent from green to purple color. This BCA reagent is light sensitive in nature as it shows absorbance at 562nm. The concentration of protein is estimated by having a standard curve of known concentration of BSA.

- **Requirements:** Ice, falcon tubes, aluminum foil, MCT, BSA, BCA reagents, 96 well plate.

- **Method:**

1. Thaw the protein samples on ice. Prepare BSA stock by adding reagent A and B in a ratio of 50:1. and cover it with a foil.

2. Prepare the BSA standard stock of 2mg/ml from which do the serial dilution of 100µl in a manner that the final concentrations are 2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml and 0.065mg/m in separate clean labeled MCT.

3. This can be done by taking 6 labeled MCT add 200µl of 2mg/ml BSA stock solution to it. In the remaining 5 tubes add 100µl of M.Q. each.

4. From the stock of 2mg/ml add 100µl of it to the 1mg/ml tube and mix well. Now, take 100µl from 1mg/ml BSA containing tube to 0.5mg/ml tube. Repeat the same step for other dilution.

5. Sample preparation: take fresh MCT according to your number of samples and add 70µl of M.Q. in each tube. Now, add 5µl of protein sample in the respective tubes and label them properly.

6. Calculate the dilution factor using the formula: total volume/volume of protein used (e.g., D.F. 70+5=75/5=15)

7. Prepare two blank solutions, one having 40µl of M.Q. for BSA and the other having 40µl of RIPA lysis buffer for protein samples.

8. In a 96 well plate, add 200µl of BSA stock solution in the required number of wells. Remember each concentration should be loaded in a triplicate.

9. Now add 20µl of blank, sample and standard in their respective wells.

10. Cover the plate with foil and incubate it at 37°C fro 30 minutes in dry bath.

11. Read the plate in plate reader at the wavelength of 560nm and plot the graph suing total optical density of the sample and BSA concentrations. Calculate the concentration of protein samples from this data.

c) Western blot analysis: [for semi-dry transfer method]

1. Lysis buffer used: for 1ml of 1X of RIPA lysis buffer following are required:

Component	**Volume**
Dithiothreitol (DTT)	(1mM) 5µL)
Protease Inhibitor	20 µL
Sodium Orthovanadate	(1mM) 15µL

2. Lamelli buffer components:

Component	**Amount**
β-Mercaptoethanol(freshly added)	0.1%
Bromophenol blue dye	0.0005%
Glycerol	10%

SDS	2%
Tris HCL (pH 6.8)	Unspecified

3. Separating Gel (Resolving Gel) (pH 8.8):

COMPONENT FOR 10% GEL	**GEL VOLUME (ml)**
Milli Q.	4
Acrylamide (30%)	3.3
Separating Buffer	2.5
10% SDS Solution	0.1
10% APS	0.1
TEMED	0.004
TOTAL VOLUME	10

4. Stacking gel (5%) (pH 6.8):

COMPONENT	**VOLUME**
M.Q.	2.7 ml
Acrylamide-Bisacrylamide	670 ml
Tris-HCl (pH6.8)	500 ml
10% SDS	40 ml
10% APS	40 ml

TEMED	0.01

5. **Gel Running Buffer (1x) (for 1 liter):**

Component	**Weight in gm**
Tris base	3.3 g
Glycine	14.3 g
SDS	1 g

6. **Transfer Buffer (1X) (for 1 liter):**

Component	**Weight in gm**
Trizma base	5.81 g
Glycine	2.93 g
SDS	37.5 g
5% Ethanol	50 ml

SDS-PAGE: SDS is the most often used method for separating proteins based on their mass. SDS, which is employed in gel preparation, is essentially an anionic detergent that denatures proteins before electrophoresis. It denatures proteins by attaching to their hydrophobic cores, resulting in a protein with an overall negative charge. It has a charge proportional to its mass. In an electric field, the negative charges on SDS break most of the complex structure of proteins and are strongly attracted to an anode
(Positively charged electrode).

This results in a denaturation of proteins which ease their separation on basis of their mass. On the SDS page, smaller proteins travel farther than larger proteins to the anode

because they have comparatively low molecular mass resulting in their rapid movement through the pores of the gel. Because the charge-to-mass ratio of denaturated polypeptides of the proteins is virtually the same, the final separation of proteins is almost entirely based on differences in relative molecular mass of polypeptides. Because this method involves protein separation based on mass, it can be used to estimate a protein's molecular weight.

Sample preparation: Lamelli buffer containing β-Mercaptoethanol and SDS was added into the samples to be run. The disulfide bridges that hold the tertiary structure together are reduced using β-Mercaptoethanol. Lamelli buffer also contains a tracking dye, commonly known as Bromophenol Blue, which allows tracking of the samples in the gel. Moreover, glycerol allows sample to settle in the wells after sample loading.

- **STEPS FOR WESTERN BLOT:**

1. SEPARATING GEL or RESOLVING GEL: When the protein-SDS complex enters the resolving gel, the molecular sieving feature of the gel causes separation of the proteins in the sample. Smaller proteins move more easily and travel further than larger proteins. Because bromophenol blue is a tiny molecule, it goes further and produces the electrophoresis front to indicate the amount of gel that has been completed. Gels are made ranging in their composition from 8-15% depending on the size and molecular weight of the protein molecule in the protein samples that we want to separate. The addition of freshly made 10% Ammonium Persulfate (APS) and N, N, N', N'-tetramethyl ethylenediamine (TEMED) initiates the polymerization process. We simply pour the solution into the space between the space and short plates in the gel casting assembly. Isopropanol must be poured after pouring the resolving gel. It has a lower density compared to the gel because it settles on top of the resolving gel. Isopropanol is used to level out the gel and break the atmospheric O2 contact from the gel because O2 hinders the polymerization of the gel.

2. STACKING GEL: Before entering the resolving gel, the protein sample is concentrated into a crisp band using stacking gel. This is accomplished by taking advantage of the ionic strength and pH differences between the electrophoretic buffer

and the stacking gel, a process known as isotachophoresis. The increased pore size of the stacking gel allows the protein samples to concentrate and move freely under the influence of the electric field. The differential in the electrophoretic mobility of glycinate ions, protein-SDS complex, and chloride ions in the loading buffer causes band sharpening [Cl^-] > [protein-SDS] > [Glycinate]. All ionic species must migrate at the same speed under the influence of the applied field in order to create a stable electric circuit.

Stacking gel of the concentration of 5% is utilization to allow for the stacking of very big proteins while retaining enough mechanical strength to form acceptable sample wells. Isopropanol should be drained off the separating gels before adding the remaining two components, which will initiate polymerization. The same proportions of APS and TEMED are employed as in the separating gel mix. We immediately whirl the mixture after adding APS and TEMED and pour it into the cassettes to the tops of the plates. We insert combs one at a time, being careful not to catch bubbles between the teeth, and adjust to make them even if necessary, scraping out extra stacking mix later.

3. **Protein separation through SDS-PAGE:** The anode (+ electrode) should be linked to the bottom chamber, and the cathode should be attached to the top chamber. Naturally, negatively charged proteins will gravitate toward the anode. Gels are typically run at a voltage that allows the tracking dye to reach the bottom of the gels as rapidly as possible without scorching the gels. Overheating might cause the acrylamide to deform or even break. We generally run our gels at 10mA for the stacking gel and 15mA once the proteins have traveled halfway through the separating gel.

4. **Western Immuno-Blotting:** Western immuno-blotting is a technique for accurately detecting and determining the presence of a certain protein. To perform a western blot, we must first run the protein samples through a denaturing SDSPAGE. SDS-PAGE separated protein samples are then placed onto a nitrocellulose/Polyvinylidene fluoride) (PVDF) membrane through capillary blotting or electroblotting, where hydrophobic interactions bind the proteins irreversibly. Using the electroblotting principle, the resolved protein bands were transferred onto a PVDF membrane. The gel was sandwiched between filter paper-gel-membrane-filter papers,

cushioned by pads, and pushed together by a support grid. The tank was then filled with transfer buffer and the supported gel sandwich was placed vertically in it. This procedure was followed at 250mA for 125mins.

Figure 16: Power blot cassette for semi-dry transfer

5. Blocking of protein on PVDF membrane: Western blotting membrane supports have a high affinity for proteins. Because the membrane was chosen for its propensity to bind protein, and because both antibodies and the target are proteins, it is critical to block the remaining surface of the membrane to prevent binding. Following steps include nonspecific binding of detecting antibodies. Various blocking buffers Milk, normal serum, and highly purified proteins have all been utilized to inhibit binding of proteins on non-specific on a membrane. As a result, when the proteins have been transferred from the gel, it is critical to inhibit the residual membrane surface to prevent nonspecific binding of detection antibodies. The blocking buffer should improve the assay's sensitivity by lowering background interference. The optimal blocking buffer will bind to all potential locations of nonspecific interaction, completely eliminating background while without changing or obscuring the epitope for antibody binding. For 1 hour, the membrane was immersed in blocking buffer. 5% of skimmed milk was dissolved in 1X TBST. Following protein blocking on the membrane, the membrane is washed three times with 1X TBST (Washing Buffer).

6. Incubation with Primary Antibody: The membrane is "probed" for the protein of interest during the detection process with a modified antibody that is coupled to a reporter

enzyme; when exposed to an appropriate substrate, this enzyme drives a colorimetric reaction and produces a color. Following the blocking, a dilute solution of primary antibody (usually between 0.5 and 5g/mL) is incubated on the membrane overnight. The antibody solution and the membrane are incubated together for 30 minutes at a time. From room temperature to 4°C overnight. The blot was stored at 4°C in 5mL of primary antibody. Gently agitate the mixture overnight.

7. **Incubation with secondary antibody:** After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to secondary antibody, which is aimed towards a species-specific region of the primary antibody. Typically, the secondary antibody is coupled to biotin or a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that a number of secondary antibodies will bind to a single primary antibody and boost the signal. A horseradish peroxidase-linked secondary is typically employed to cleave a chemiluminescent agent, and the reaction result provides light proportional to the amount of protein. After incubation with the primary antibody, the blot was washed three times with TBST buffer. The membrane is then incubated for 1 hour at room temperature with gentle agitation with 5mL of the appropriate secondary antibody.

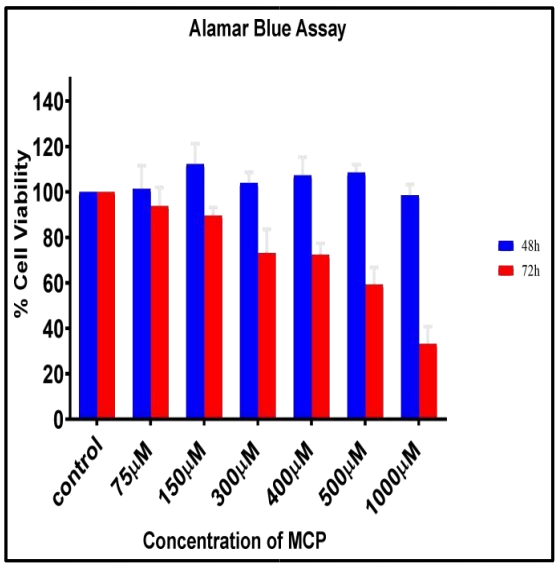
8. **Visualization of the blot:** After the unbound probes are washed away, the western blot is ready to detect the labeled and bound probes to the protein of interest. The colorimetric detection method is based on the western blot being incubated with a substrate that reacts with the reporter enzyme (such as peroxidase) coupled to the secondary antibody. Chemiluminescent detection requires 36 incubating the western blot with a substrate that will glow when exposed to the reporter antibody on the secondary antibody. CCD cameras detect the light and capture a digital image of the western blot or photographic film. Densitometry is used to measure the relative amount of protein staining and quantify the results in terms of optical density. The blot was developed using GE Healthcare LAS500 Gel Doc.

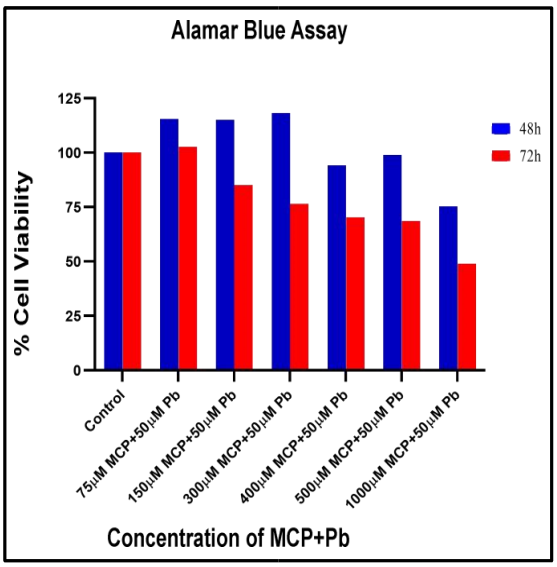
Chapter 4: Results and Discussion

A. Cytotoxic analysis:

- **Non-cytotoxic estimation of MCP exposure via. Alamar blue assay:** The noncytotoxic dose of MCP was estimated at a concentration of 75µm, 100µm, 150µm, 300µm, 400µm, 500µm and 1000µm. The exposure was given after 48 hours of seeding and for 48 hours and 72hr. From the result, it was deduced that at a concentration of 300µm of MCP, 70% of cells were viable after 72 hours of exposure. Therefore, the non- cytotoxic dose of MCP was 300µm at 72 hours of exposure.

- **Non-cytotoxic estimation of MCP+Pb co-exposure via. Alamar blue assay:** The non-cytotoxic dose of MCP+Pb co-exposure was estimated at a concentration of 75µm, 100µm, 150µm, 300µm, 400µm, 500µm and 1000µm of MCP along with the combination of 50µm of Pb. The co-exposure was given after 48 hours of seeding and for 48hr and 72hr. From the result, it was deduced that at a concentration of 300µm ofMCP+50µm of Pb, 70% of cells were viable after 72hr of exposure. Therefore, the non-cytotoxic dose of MCP+Pb co-exposure was found to be 300µm of MCP+50µm of Pb at 72hr of exposure.

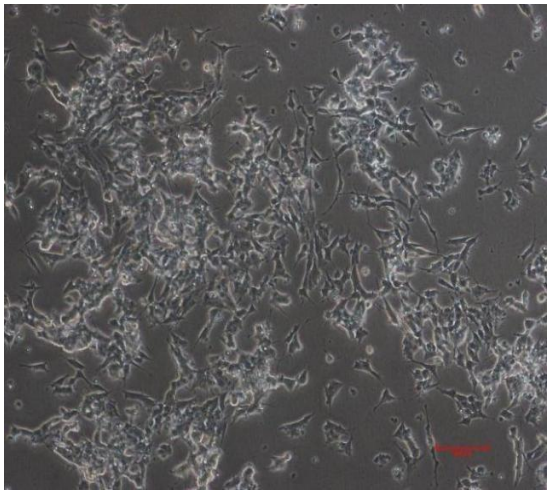




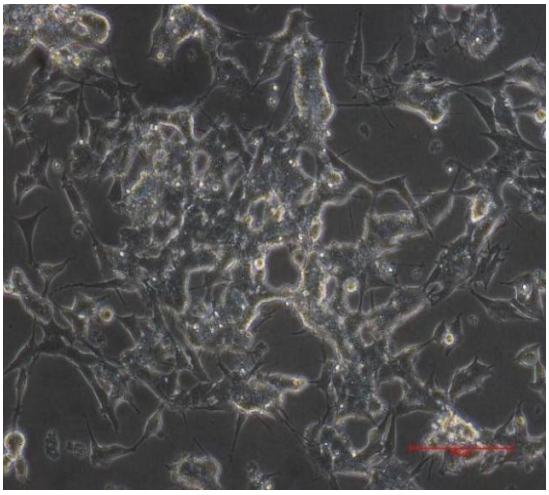
B. Morphological analysis: The morphological change on control and exposed cells was analyzed using a Nikon Eclipse Ti microscope (phase contrast microscope)

after 72 hr of exposure. Control cells showed normal cell morphology, while MCP, Pb, and MCP+Pb exposed cells appeared to be under stress which was evident by the bulges like appearance and cytoplasmic vacuolation of the cell.

- **Control flask after 72 hr**

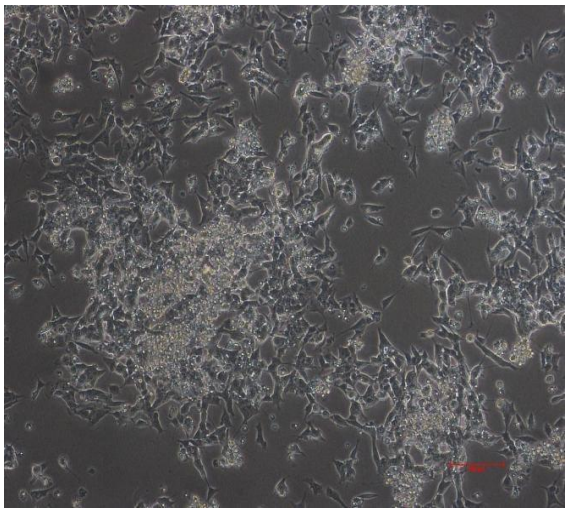


A) Image at magnification of 100X

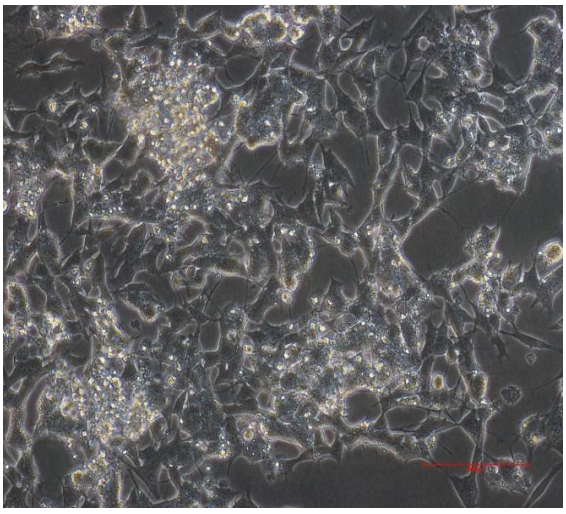


B) Image at magnification 200X

- **MCP exposed flask after 72hr of exposure**

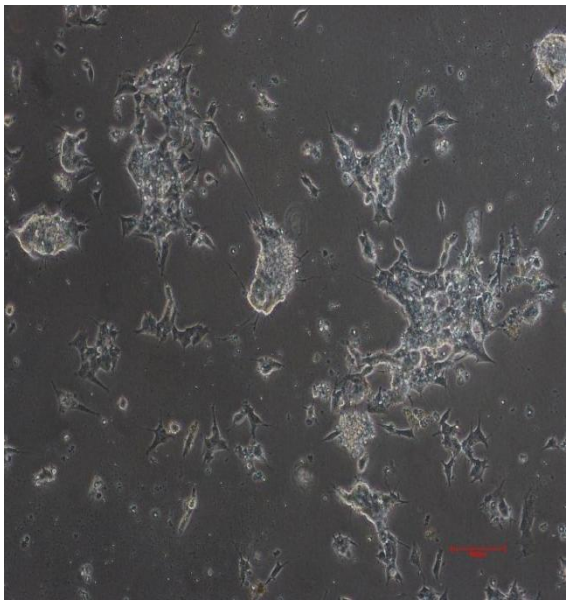


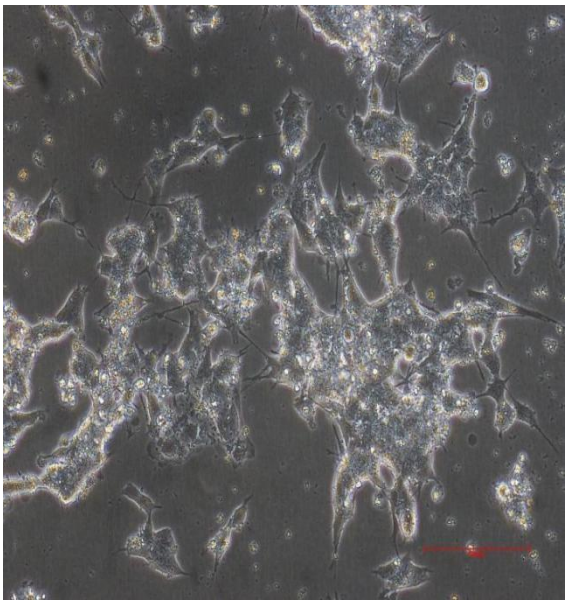
A) Image at magnification of 100X



B) Image at magnification 200X

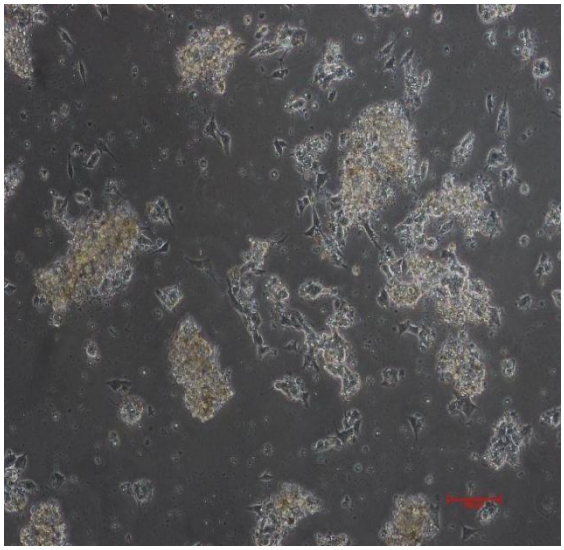
- **Pb exposed flask after 72 hr of exposure**

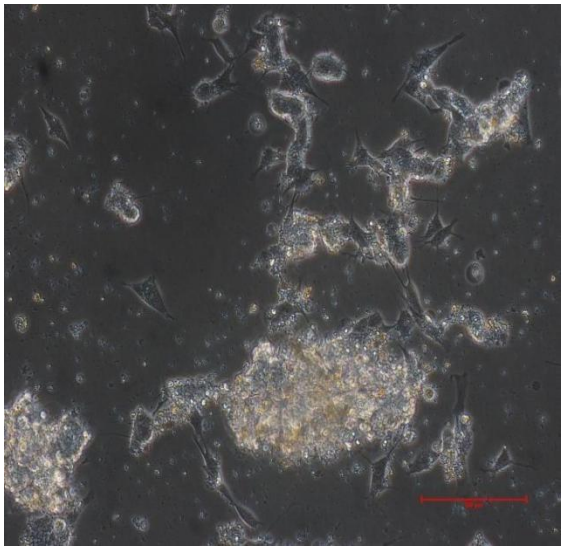




A) Image at magnification of 100X **B) Image at magnification 200X**

- **MCP and Pb Co-exposed cells after 72hr of exposure**

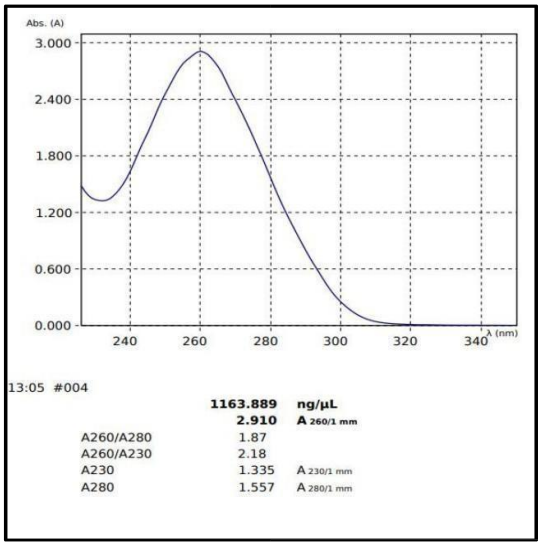




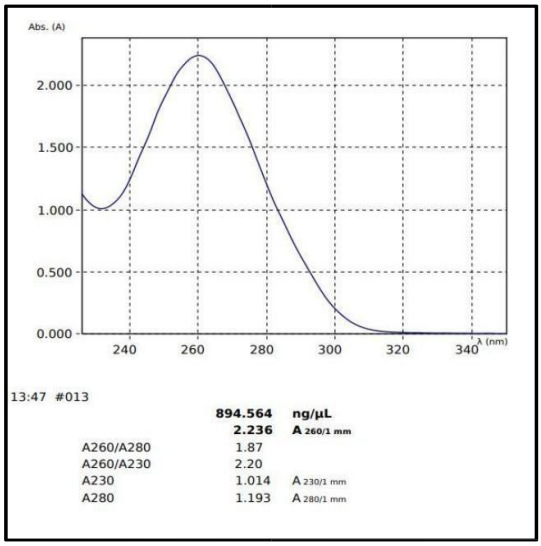
A) Image at magnification of 100X **B) Image at magnification 200X resolution**

C. Transcriptional level analysis:

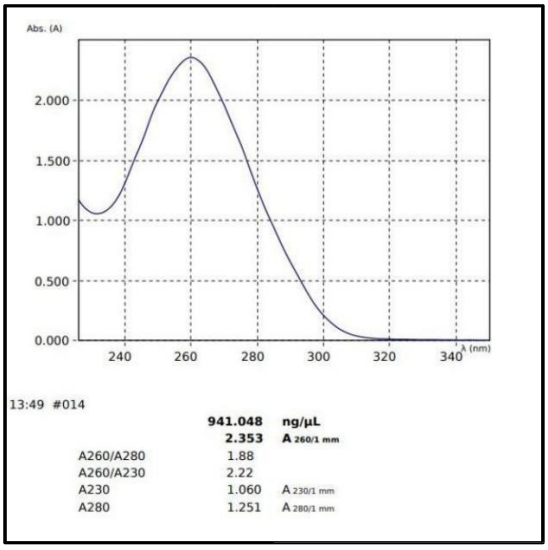
- **RNA quantification:** RNA quantification is done on bio-spectrometer. The yield of RNA samples along with their 260/ 280 and 260/230 ratio are given below.



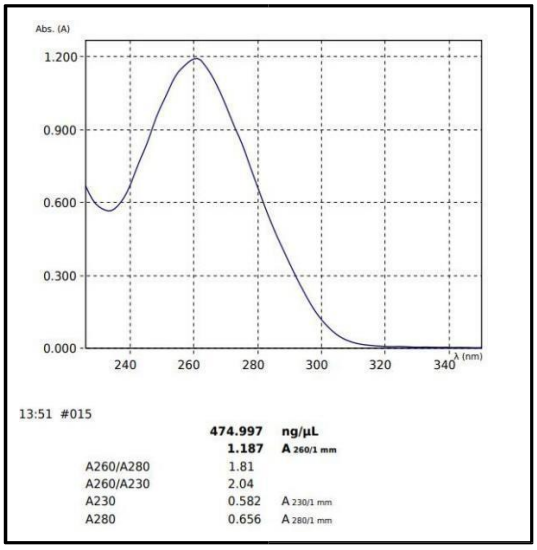
A) Yield of Control Samples



B) Yield of MCP exposed Samples



C) Yield of Pb exposed Samples



D) Yield of MCP+Pb exposed Samples.

- **RT-PCR:** RT-PCR is performed for various markers such as Bax, Bcl2, Caspase 3, Caspase 7, Caspase 9, Neurofilament medium chain (NFM), Nestin, and p53. To

analyze the up-regulation and down-regulation of these genes in the control, MCP exposed, Pb exposed and MCP+Pb exposed cells, β-actin is taken as a reference gene. The expression of these particular genes in the control and exposed cells is analyzed based on their relative quantification (R.Q.) with respect to the endogenous/reference gene.

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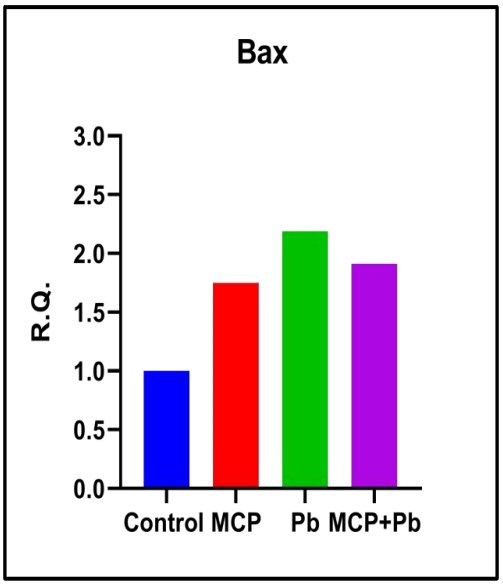
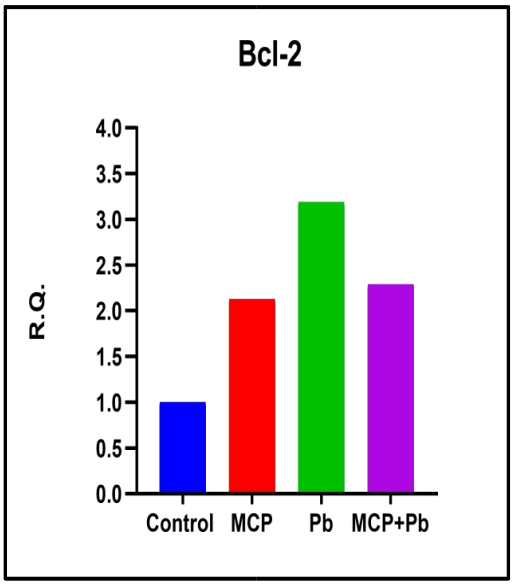
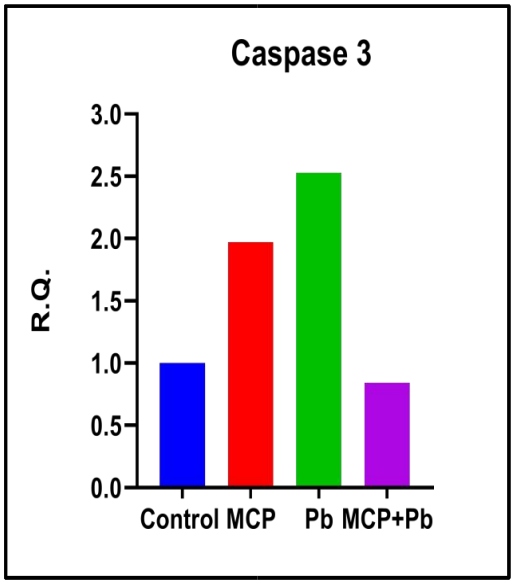
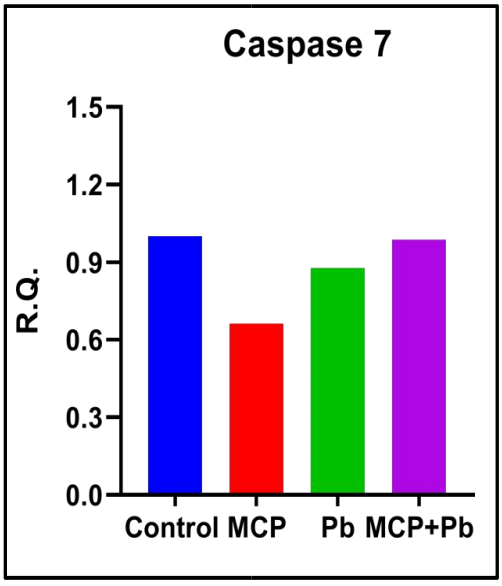
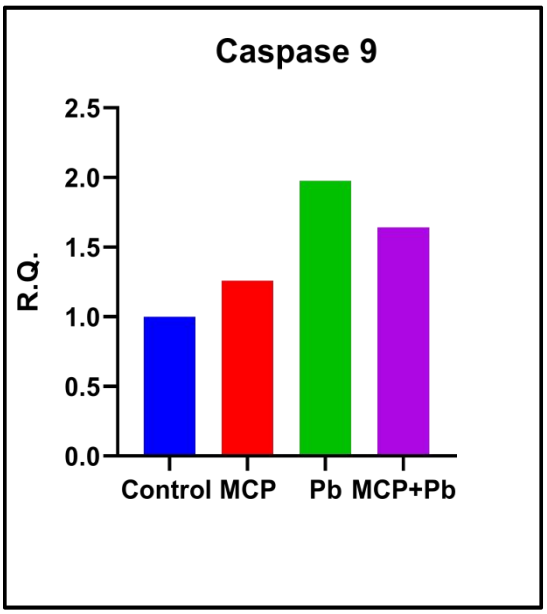
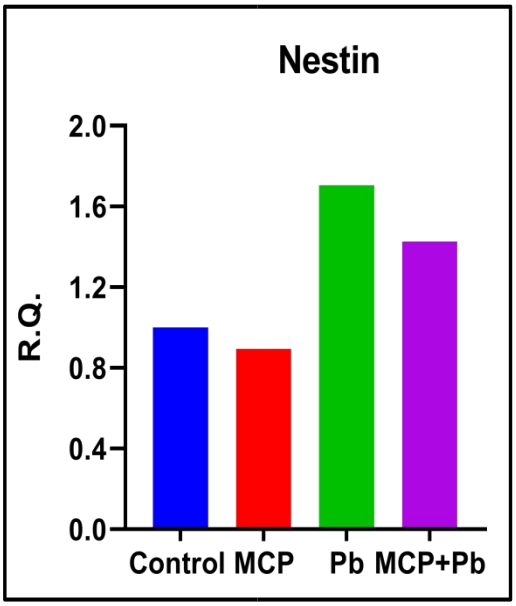


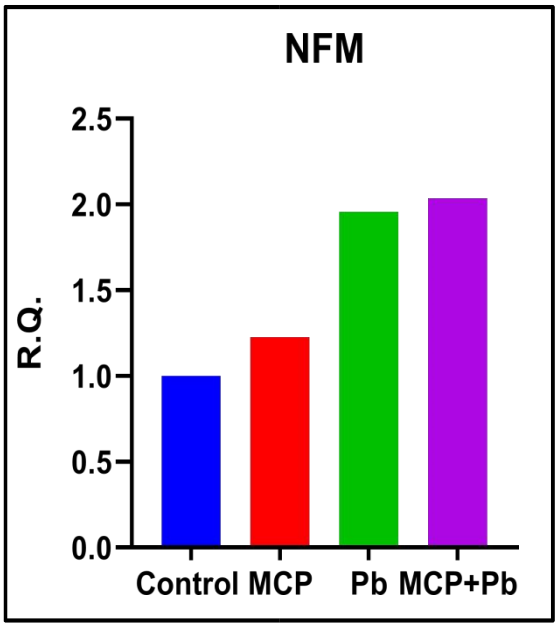


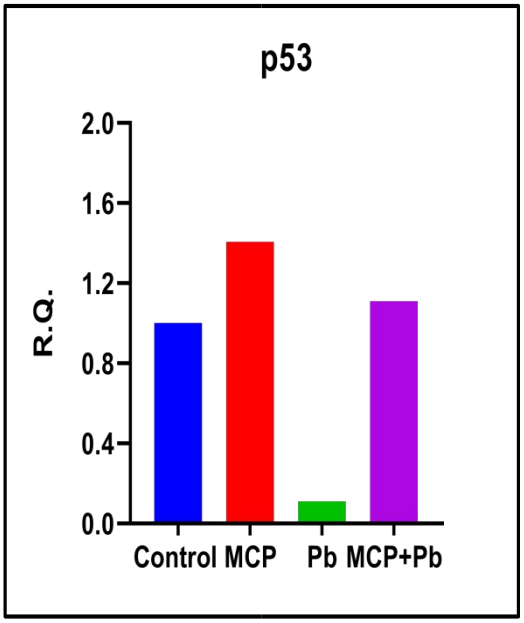





Caspase 9
R.Q.
2.5
2.0
1.5
1.0
0.5
0.0
Control MCP
Pb MCP+Pb


Nestin
R.Q.
2.0
1.6
1.2
0.8
0.4
0.0
Control MCP
Pb MCP+Pb


NFM
R.Q.
2.5
2.0
1.5
1.0
0.5
0.0
Control MCP
Pb MCP+Pb

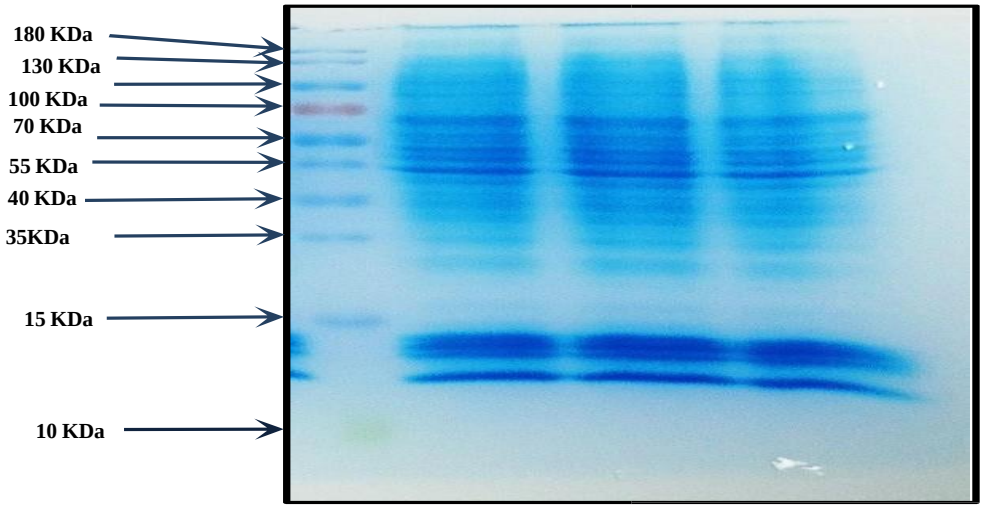

p53
R.Q.
2.0
1.6
1.2
0.8
0.4
0.0
Control MCP
Pb MCP+Pb

Relative Quantification (R.Q.)	Control	MCP	Pb	MCP+Pb
Bax	1.000	1.748	2.189	1.910
Bcl_2	1.000	2.128	3.186	2.288
Caspase 3	1.000	1.972	2.529	0.841
Caspase 7	1.000	0.662	0.878	0.987
Caspase 9	1.000	1.259	1.975	1.643
Nestin	1.000	0.894	1.705	1.425
NFM	1.000	1.225	1.957	2.036
p53	1.000	1.406	0.111	1.110

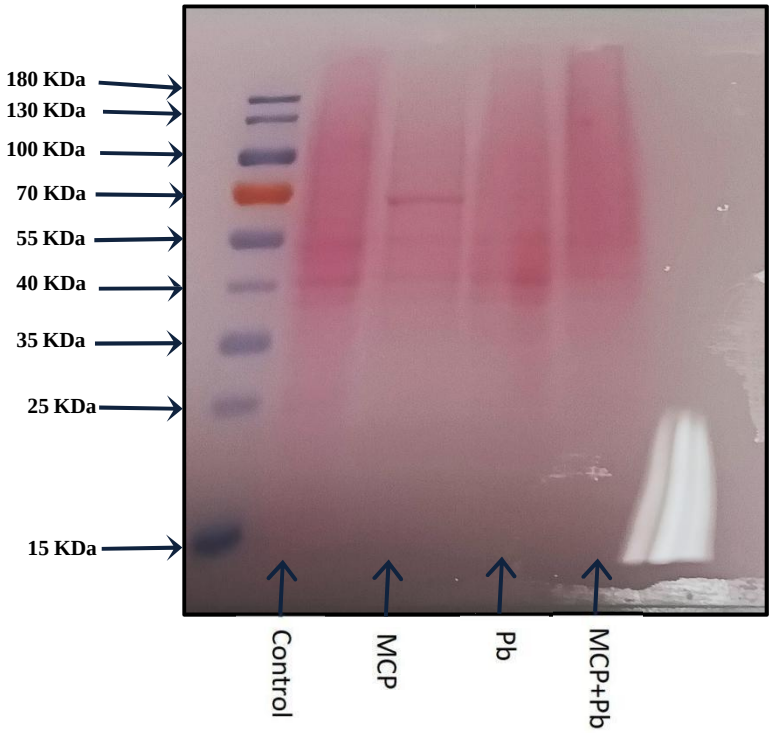
Data Sheet of R.Q value of Control, MCP, Pb and MCP+Pb exposed samples

D. Translational level analysis:

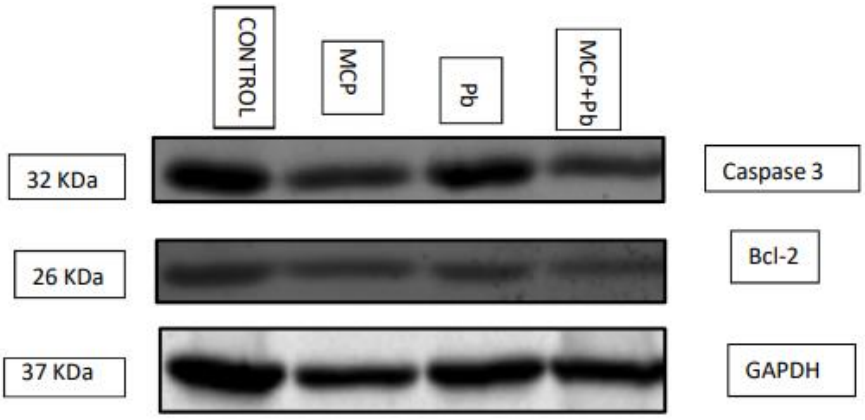
- **Coomassie brilliant blue staining:** The coomassie brilliant blue staining on the gel is done on normal SH-SY5Y cells to analyze the proper gel formation and protein separation.



- **Ponceau staining:** The ponceau staining of the control and exposed protein samples is done on the membrane after the transfer of proteins is completed to check whether the proteins have properly transferred to the membrane or not.



- **Blot development:** The blots were firstly anàlyzed for endogenous protein to check the protein samples' consistency and then primary antibody of caspase 3 and Bcl-2 were applied to check the protein expression in the control and exposed samples.



Chapter 5: Conclusion

The intricate interplay between environmental toxins and human health has garnered significant attention in recent years. Among these toxins, Monocrotophos (MCP) and lead (a heavy metal) are of particular concern due to their widespread use and potential neurotoxic effects. Monocrotophos and lead are environmental toxins with well documented neurotoxic properties. Monocrotophos, an organophosphate pesticide, and lead a heavy metal, are commonly encountered in various settings, from agricultural practices to industrial activities. The simultaneous exposure to these two neurotoxicants raises concerns about potential synergistic effects on neuronal cells. The cumulative impact of MCP and lead co-exposure on neuronal cells sheds light on the complex mechanisms that underlie their neurotoxicity. Monocrotophos, a widely employed pesticide in agricultural practices, functions by inhibiting acetylcholinesterase, an enzyme crucial for nerve impulse transmission. Chronic exposure to MCP has been associated with an increased risk of neurodevelopmental disorders, cognitive deficits, and neurodegenerative diseases. It leads to the accumulation of acetylcholine in synaptic junctions, causing overstimulation of neural pathways and subsequent cell damage. Additionally, oxidative stress induced by MCP results in mitochondrial dysfunction and DNA damage, further exacerbating the neuronal injury. Lead, a ubiquitous environmental contaminant, affects neuronal cells by disrupting calcium homeostasis, impairing synaptic transmission, and interfering with various signaling pathways. Lead exposure has been linked to cognitive impairment, learning disabilities, and behavioral disturbances, particularly in developing children. It induces neuronal apoptosis, disrupts neurotransmitter release, and alters the expression of genes critical for neuronal development and function. Furthermore, lead-induced oxidative stress and inflammation contribute to the progressive deterioration of neuronal health. Recent research has illuminated the potential synergistic neurotoxic effects of MCP and lead co exposure. The combination of these two neurotoxicants can amplify their individual impacts on neuronal cells. Their cumulative effects exacerbate oxidative stress, calcium dysregulation, and mitochondrial dysfunction, leading to more severe neuronal damage. Additionally, co-exposure may potentiate the disruption of key cellular signaling pathways, further

compromising neuronal integrity. The co- exposure of neuronal cells to MCP and lead presents a concerning scenario in terms of neurological health. Their synergistic effects contribute to a heightened risk of cognitive impairments, neurodevelopmental disorders, and neurodegenerative diseases. Understanding the intricate molecular mechanisms underlying this interaction is vital for the development of targeted interventions to mitigate the neurotoxic impact of these environmental contaminants. Regulatory measures aimed at reducing human exposure to both MCP and lead are essential to safeguard public health and ensure the well-being of future generations.

Chapter 6: Bibliography

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