

EXPLORING THE ROLE OF ROTENONE INDUCED DEVELOPMENTAL TOXICITY AND THE REVERSIBLE EFFECT OF AROMATIC TURMERONE IN ZEBRAFISH EMBRYOS

*Submitted in partial fulfilment of the requirements for the award of Master of Science
in Medical Biotechnology and Clinical Research*

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Mr. RUBESH C (42101011)** who carried out Project entitled **"Exploring the role of rotenone induced developmental toxicity and the reversible effect of aromatic turmerone in zebrafish embryos"** under the supervision of **Dr. Y. BERYL VEDHA, Assistant Professor (Research)** from September 2023 to April 2024.

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DECLARATION

I, **Mr. RUBESH C (42101011)** hereby declare that the Project Report entitled **"Exploring the role of rotenone induced developmental toxicity and the reversible effect of aromatic turmerone in zebrafish embryos"** done by me under the guidance of **Dr. Y. BERYL VEDHA, Assistant Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

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ABSTRACT

Rotenone is an isoflavone compound that naturally occurs in the *jicama* vine plant as well as many *Fabaceae* plants. It has a broad spectrum insecticide and pesticide activity and is also found to be toxic in aquatic environment. The ability of rotenone to inhibit microtubule assembly by binding to tubulin, resulting in the mitotic arrest and inhibition of cell proliferation leads to disease condition. In this study, the toxic effects of rotenone on the development of zebrafish embryos have been analysed taking into considerations the morphological changes and mortality caused by the drug. Rotenone was used on three different concentration groups and the lethal points were noted. Apoptotic activity of rotenone was studied in zebrafish larvae. On the other hand, aromatic turmerone a biologically active compound found in turmeric has been explored, to study its beneficial properties on the rotenone treated zebrafish embryos. The reversible effects of aromatic turmerone was observed by co-treating it with rotenone. Apoptosis assay and blood stasis reversibility was performed for the co-treated zebrafish embryos.

Keywords : Rotenone, aromatic turmerone, apoptosis, blood stasis, zebrafish, proliferation.

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ABBREVIATIONS

AO	Acridine orange
APS	Ammonium per sulphate
Ar-turmerone	Aromatic turmerone
ATP	Adenosine triphosphate
Bax	Bcl-2 associated protein
Bcl-2	B-cell leukemia/ lymphoma 2 protein
BV 2	Microglia cell
Ca ²⁺	Calcium ions
CAT	Catalase
COX2	Cyclooxygenase2
DMSO	Dimethyl Sulfoxide
EB	Ethidium bromide
g	Gram
GST	Glutathione S-transferase
H&E Staining	Hematoxylin and Eosin Staining
hr	Hour
IL- β	Interkeukin 12 beta
IL-6	Interleukin 6
IPA	Isopropyl alcohol
LPO	Lipid peroxidation
ml	millilitre
NF κ B	Nuclear Factor kappa Beta
NO	Nitric oxide
NSCs	Neuronal stem cells
OCT Compound	Optimal Cutting Temperature Compound

OECD	Organisation of Economic Co-operation and Development
PAGE	Polyacrylamide gel electrophoresis
PAX-2	Paired Box-2
PC12	Pheochromocytoma of the rat adrenal medulla
ppb	Parts Per Billion
Pqq	Pryoquinoline quinone
RO	Reverse Osmosis
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SOD	Superoxide Dismutase
TEMED	N,N,N',N' – Tetra methyl ethylene diamine
UV	Ultra Violet
µg	microgram
µl	microlitre

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CHAPTER – 1

INTRODUCTION

1.1 ROTENONE

Rotenone is a naturally occurring compound used worldwide as a herbicide and insecticide. It is extracted from plants such as *Lonchocarpus*, *Millettia pachycarpa*, and *Mundulea suberosa*. Rotenone was first used as a fish poison until its potential for insect control was noticed (Wen *et al.*, 2020). Rotenone in the late 1800s, rotenone has been widely used as a piscicide to reduce invasive fish in lakes and reservoirs and as a pesticide to control insects, ticks, and lice (Khaled Radad, *et al.*, 2013). Fisheries managers have using rotenone as a management technique for almost fifty years. A few initiatives in recent years have sparked public controversy, and the use of rotenone has been restricted or even outlawed in many states. Using Federal Aid Administrative Funds, the American Fisheries Society's Task Force on Fishery Chemicals created and executed the Rotenone Stewardship Programme for fisheries management.

The most recent method for simulating Parkinson's disease in rats uses rotenone, a mitochondrial toxin whose significance to the aetiology of the disease is uncertain. (Sherer *et al.*, 2003). When Lewis rats are given rotenone (Rot) continuously, several symptoms of Parkinson's disease are mimicked. Using Alzet minipumps, male Lewis rats received subcutaneous injections of Rot (3 mg/kg/day) for a duration of 6 days. Rotenone attached to the mitochondria was totally eliminated during the isolation process when 0.1% bovine serum albumin was present. As a result, every functional alteration was a result of rotenone poisoning *in vivo*. State 3 and State 3U respiration in Rot rat brain mitochondria (Rot-RBM) was 30–40% inhibited when succinate and Complex I (Co-I) substrates were present. The State 4 $\Delta\psi$ of RBM and rat liver mitochondria (RLM) were unaffected by rot. On the opposite hand, Rot-RBM needed twice as much Ca²⁺ to start the permeability transition (mPT) (Alezandar panov, *et al.*, 2005). Rotenone regularly causes *in vivo* loss of substantia nigra dopaminergic cells, which is a characteristic of Parkinson's disease neuropathology, according to laboratory research. Furthermore, rotenone causes α -synuclein to aggregate *in vivo*, which is a hallmark of Parkinson's disease neuropathology and the main component of

Lewy bodies and Lewy neurites observed in the brains of PD patients. Certain peripheral symptoms of Parkinson's disease (PD), like decreased intestinal motility and peripheral α -synuclein aggregation, are also replicated in several in vivo rotenone models. These indicators are believed to come before traditional PD symptoms in humans, like bradykinesia, cogwheel rigidity, and resting tremor (Innos and Hickey 2021). Although it was previously used as a pesticide and piscicide, it is important to note that rotenone strongly inhibits complex I of the mitochondrial respiratory chain (MRC).

The mechanism of action of rotenone involves inhibiting the transfer of electrons from the iron-sulfur centers in complex I to ubiquinone, which leads to a blockade of oxidative phosphorylation and limited ATP synthesis. Furthermore, incomplete electron transfer to oxygen can cause the formation of reactive oxygen species (ROS), which are believed to damage mitochondrial components, including mitochondrial DNA, and can ultimately lead to apoptosis. Furthermore, incomplete electron transfer to oxygen can cause the formation of reactive oxygen species (ROS), which are believed to damage mitochondrial components, including mitochondrial DNA, and can ultimately lead to apoptosis (Ce line Perier *et al.*, 2003). Rotenone is a promising candidate for Parkinson's disease research due to its ability to inhibit microtubule assembly by binding to tubulin, resulting in mitotic arrest and inhibition of cell proliferation. These mechanisms of action showcase the potential of rotenone in Parkinson's disease research. Furthermore, its high lipophilicity allows it to easily cross biological membranes, including the blood-brain barrier. Some patients with PD demonstrate defective mitochondrial function, including decreased complex I activity and increased oxidative stress. Microtubule dysfunction may also contribute to PD progression due to their role in neuronal polarity, axonal transport, and synaptic plasticity. Furthermore, exposure to rotenone has been linked to several PD-like symptoms in humans. Chronic administration of rotenone causes selective degeneration of nigral dopaminergic neurons with histopathological hallmarks of Parkinson's disease (PD) and PD-like locomotor symptoms in animal models. Therefore, rotenone-based PD models have been developed, which are widely used in research to study the pathogenesis of PD (Sherer *et al.*, 2003).

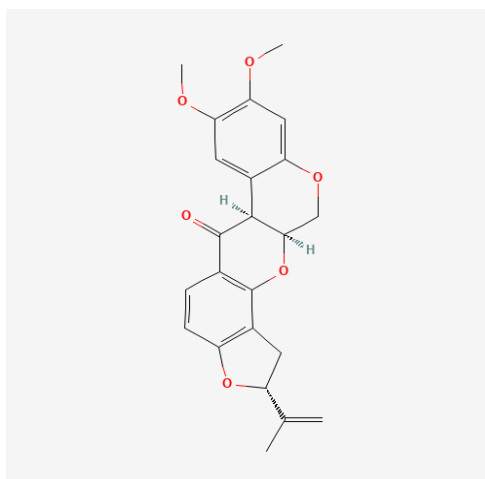


Fig 1. Rotenone

1.2 AROMATIC TURMERONE

Aromatic turmerone is a bioactive compound derived from *Curcuma longa*, a spice commonly used in culinary, cosmetic, and medicinal practices across various cultures, particularly in Asian countries. Ar-turmerone has been found to inhibit microglia activation and may have potential in treating neurodegenerative diseases. Ar-turmerone is a member of the turmerone compound class and has potential anti-inflammatory, antioxidant, and neuroprotective properties. (Kunnumakkara *et al.*, 2023).

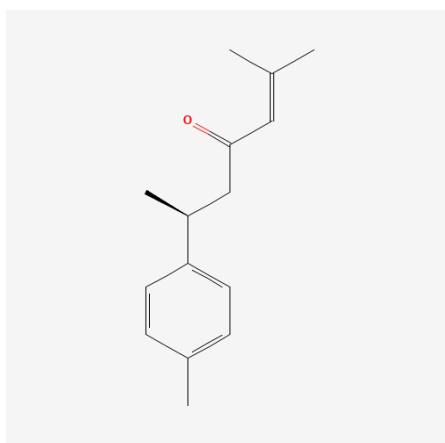


Fig 1.2 Ar-turmerone

Ar-turmerone is a sesquiterpenoid compound found in turmeric. Its chemical structure is characterized by a bicyclic ring system with a substituted cyclohexene ring, consisting of three isoprene units forming a 10-carbon backbone. The compound is mainly composed of two fused six-membered rings and one five-membered ring. (Gupta *et al.*, 2013).

1.2.1 Properties of Ar-turmerone: Ar-turmerone exhibits various biological properties including anti-cancer, anti-inflammatory, and antioxidant activities.

1.2.1.1 Anti-inflammatory: Ar-turmerone has shown significant anti-inflammatory activity inhibiting the production of inflammatory cytokines and enzymes such as COX2. It can alleviate inflammation-related conditions.

1.2.1.2 Antioxidant: Ar-turmerone possesses antioxidant properties that enable it to scavenge free radicals and reduce oxidative stress. This property was helpful in contributing to the protective effects against cellular damage.

1.2.1.3 Neuroprotective: Ar-turmerone has demonstrated antimicrobial activity against various pathogens including bacteria, fungi, viruses, and fungi. It may be effective in combating infections and supports immune health.

1.2.1.4 Anti-cancer: Ar-turmerone exhibits anti-cancer properties by inhibiting the growth of cancer cells, inducing apoptosis, and suppressing the tumor progression (Gupta *et al.*, 2013).

1.2.1.5 Proliferative property of Ar-turmerone: The study of (Hucklenbroich *et al.*, 2014) represents a cornerstone in the research on aromatic turmerone effects on neural stem cells. Their investigations revealed that aromatic turmerone significantly enhances the proliferation of NSCs in vitro and in vivo. Notably in vivo administration of ar-turmerone led to an increased number of newly generated neural cells in the subventricular zone of adult rats, highlighting its potential to stimulate neurogenesis.

1.3 ZEBRAFISH

Zebrafish (*Danio rerio*) is a commonly utilized model organism in scientific research, especially in the fields of genetics, developmental biology, and neuroscience. They are small freshwater fish native to the Ganges region in South Asia and belong to the minnow family (Cyprinidae). Zebrafish have become increasingly popular as a

research model due to their transparent embryos, which allow for clear observation of developmental processes. Zebrafish embryos offer excellent opportunities to examine internal development due to their transparency which is significant in studying early organ development and embryogenesis. Zebrafish are ideal for laboratory study due to their small size, which allows for easy housing and cost-effective maintenance of large populations for experiments. They have been utilized as animal models to investigate a vast array of human diseases, making them an invaluable asset in biomedical research. Zebrafish are a common freshwater aquarium fish that, over the last 40 years, has become a popular and powerful model organism for research into vertebrate genetics, development, regeneration, and toxicology (Meyers, 2018).

1.4 ZEBRAFISH USED AS A MODEL ORGANISM

Zebrafish are small easy to house, and cost effective to maintain in large numbers compared to other vertebrate models like mice or rats. This makes Zebrafish an attractive model for laboratories with space and resources. In recent years, zebrafish have been widely used as a model for studying the developmental processes in vertebrates. Due to its rapid embryonic development and the transparency of its embryos making them an ideal model for understanding the genes that regulate embryonic development as well as the physiology to the adult organism. Zebrafish can be able to genetically manipulated easily, allowing easy to study the effects of specific genes on development, disease, and behavior (Briggs, 2002). *Danio rerio*, widely recognized as the zebrafish, is a small tropical freshwater fish that has become a staple in biomedical research due to its genetic, developmental, and physiological similarities to humans. Originating from the Ganges region in South Asia, including India, Nepal, Bangladesh, and Myanmar, the zebrafish has adapted to a variety of aquatic environments, from slow-moving streams to rice paddies (Engeszer *et al.*, 2007).

1.4.1 Taxonomy

Kingdom : Animalia
Phylum : Chordata
Class : Actinopterygii (ray-finned fishes)

Order : Cypriniformes
Family : Cyprinidae
Genus : *Danio*
Species : *rerio*

This classification highlights the zebrafish's position within the vertebrate lineage, sharing a common ancestry with other fish in the Cyprinidae family, as well as with more distant terrestrial vertebrates.

1.4.2 Nomenclature: The scientific name "*Danio rerio*" was officially given by Francis Hamilton (formerly Buchanan) in 1822, based on specimens collected from India. The genus name 'Danio' is derived from the Bengali word 'Dhani,' which means 'of the rice field.' This reflects the natural habitat of these fish in the rice paddies of South Asia. The species name 'rerio' is believed to have originated from a local indigenous name for the fish. *Danio rerio* has undergone several taxonomic revisions and was initially described under the genus *Cyprinus*. Later, the fish was moved to the genus *Brachydanio*, a name that was widely used until the late 20th century. Taxonomic studies based on more detailed morphological and molecular analyses reclassified it back to the genus *Danio* (Fang, 2003).

CHAPTER – 2

LITERATURE SURVEY

2.1 ANTI-OXIDANT

This study shows the locomotor activity decreased in the rotenone exposed zebrafish. LPO increased in both brain and intestines whereas NO increased only in the brain. Decreased GST and CAT activities were found in both tissues whereas SOD activity decreased only in the intestines. As a conclusion, the results of this study supported the connection between the gut and brain axis in rotenone exposed zebrafish by means of oxidative stress and NO for the first time in the literature (Cansız *et al.*, 2021).

This study illustrates investigated the antioxidant and neuroprotective effect of kaempferol against rotenone-induced Parkinson's disease (PD). Kaempferol inhibited apomorphine-induced rotational behavior and inhibited lipid peroxidation and interleukin (IL)-6 and tumor necrosis factor (TNF)- α , while antioxidant markers were increased in a PD rat model and SH-S5Y5 cells. that kaempferol acted as an antioxidant, anti-inflammatory, and neuroprotective agent against the rotenone-induced PD model of rats and SH-S5Y5 cells by preventing the loss of tyrosine hydroxylase (Pan *et al.*, 2020).

2.2 MITOCHONDRIAL DYSFUNCTION

This study demonstrates that Co-I is the major site of reactive oxygen species generation with glutamate or succinate. Furthermore, Co-II generates substantial amounts of reactive oxygen species that increase 2-fold in the Rot-RBM. The primary mechanism of the Rot toxic effect on RBM involves a significant increase in O₂ generation that causes damage to Co-I and Co-II, presumably at the level of 4Fe-4S clusters. This leads to a decreased respiratory activity, which reduces the resistance of RBM to Ca²⁺, increasing the likelihood of mPT and apoptotic cell death. The study clearly demonstrates that the damage caused by Co-I and Co-II shifts O₂ generation from the CoQ10 sites to more proximal sites, such as flavines, making it independent of the RBM functional state. (Panov *et al.*, 2005).

Asiatic acid (AA) has been reported to possess antioxidant and anti-inflammatory properties, as well as provide neuroprotection against glutamate toxicity. This study examines the impact of (AA), a triterpene derived from *Centella asiatica*, on a rotenone-induced in vitro model of Parkinson's disease. After exposing SH-SY5Y cells to rotenone, there was a significant increase in ROS production, mitochondrial dysfunction (indicated by decreased mitochondrial membrane potential), and apoptosis. Additionally, AA was found to have protective effects against rotenone-induced apoptosis in SH-SY5Y cells by reducing oxidative stress, maintaining mitochondrial membrane potential, and regulating the expression of Bcl-2, Bax, and caspases. Hence, AA has been suggested as a possible treatment for neurodegenerative disorders, including PD, either as a standalone therapy or in conjunction with other anti-parkinsonism medications. (Nataraj *et al.*, 2017).

The study shows that protocatechuic acid (PCA), a phenolic compound found in *Alpinia (A.) oxyphylla* kernels, has significant neuroprotective effects against hydrogen peroxide (H₂O₂) or MPP⁺-induced apoptosis in cultured PC12 cells. The observed rotenone-induced PC12 cell apoptosis was accompanied by mitochondrial membrane potential loss, reactive oxygen species (ROS) formation, total glutathione depletion, caspase-3 activation, and Bcl-2 down-regulation. PCA significantly reduces the mitochondrial dysfunction caused by rotenone in a dose-dependent manner. Treating PC12 cells with PCA can prevent rotenone-induced apoptosis by improving mitochondrial function (Mao YuRong *et al.*, 2007).

The study showed that rotenone had significant effects on mitochondrial function, resulting in decreased complex I activity and mitochondrial membrane potential. Additionally, the study found changes in antioxidant status, including glutathione depletion, enzymatic disorders (glutathione peroxidase and superoxide dismutase), and increased lipid peroxidation. This was observed through an increase in the activities of complex I, superoxide dismutase, and glutathione peroxidase, as well as a reduction in lipid peroxidation and loss of mitochondrial membrane potential in mice treated with rotenone. The study found that catalpol may have a potential role in ameliorating the oxidative stress associated with Parkinson's disease and the cellular dysfunction found in PD (Liu *et al.*, 2008).

2.3 ANTI INFLAMMATORY PROPERTIES

Mitochondrial and autophagic dysfunction, as well as neuroinflammation, were associated with the pathophysiology of Parkinson's disease (PD). Rotenone, an inhibitor of mitochondrial complex I, had been associated as an environmental neurotoxin related to PD. Our previous studies reported that rifampicin inhibited microglia activation and production of proinflammatory mediators induced by rotenone, but the precise mechanism had not been completely elucidated. BV2 cells were pretreated for 2 hr with rifampicin followed by 0.1 mM rotenone, alone or in combination with chloroquine. Here, we demonstrated that rifampicin pretreatment alleviated rotenone-induced release of IL-1b and IL-6, and its effects were suppressed when autophagy was inhibited by chloroquine. Moreover, preconditioning with 50mM rifampicin significantly increased viability of SH-SY5Y cells cocultured with rotenone-treated BV2 cells in the trans well coculture system. Chloroquine partially abolished the neuroprotective effects of rifampicin pretreatment. Rifampicin pretreatment significantly reversed rotenone-induced mitochondrial membrane potential reduction and reactive oxygen species accumulation. We suggested that the mechanism for rifampicin-mediated anti-inflammatory and antioxidant effects was the enhancement of autophagy. Indeed, the ratio of LC3-II/LC3-I in rifampicin-pretreated BV2 cells was significantly higher than that in cells without pretreatment. Fluorescence and electron microscopy analyses indicated an increase of lysosomes colocalized with mitochondria in cells pretreated with rifampicin, which confirmed that the damaged mitochondria were cleared through autophagy (mitophagy). Taken together, the data provided further evidence that rifampicin exerted neuroprotection against rotenone-induced microglia inflammation, partially through the autophagy pathway. Modulation of autophagy by rifampicin was a novel therapeutic strategy for PD (Liang & Zhou, *et al.*, 2017).

Neuroinflammation was a feature common to neurodegenerative diseases, such as Parkinson's disease (PD), which might be responsive to therapeutic intervention. Rotenone had been widely used to establish PD models by inducing mitochondrial dysfunction and inflammation. Previous studies had reported that pyrroloquinoline quinone (PQQ), a naturally occurring redox cofactor, could prevent mitochondrial dysfunction in rotenone-induced PD models by regulating

mitochondrial functions. In the present study, the effect of PQQ on neuroinflammation and the mechanism involved was investigated. BV2 microglia cells were pre-treated with PQQ followed by rotenone incubation. The data showed that PQQ did not affect the cell viability of BV2 cells treated with rotenone, while the conditioned medium (CM) of BV2 cells pre-treated with PQQ significantly increased cell viability of SH-SY5Y cells. In rotenone-treated BV2 cells, PQQ dose-dependently decreased lactate dehydrogenase (LDH) release and suppressed the up-regulation of pro-inflammation factors, such as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) in the cultured media, as well as nitric oxide (NO) release induced by rotenone. PQQ pretreatment also increased the ratio of LC3-II/LC3-I and expression of Atg5 in BV2 cells stimulated with rotenone. Additionally, the autophagosome observed by transmission electron microscopy (TEM) and co-localization of mitochondria with lysosomes indicated that mitophagy was induced by PQQ in rotenone-injured BV2 cells, and the PINK1/parkin mediated mitophagy pathway was regulated by PQQ. Furthermore, the autophagy inhibitor, 3-methyladenine (3-MA), partially abolished the neuroprotective effect of PQQ and attenuated the inhibition of inflammation with PQQ pretreatment. Taken together, the data extended our understanding of the neuroprotective effect of PQQ against rotenone-induced injury and provided evidence that autophagy enhancement might be a novel therapeutic strategy for PD (Zhang, *et al.*, 2020).

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM To determine the developmental toxicity of Rotenone in zebrafish embryos and evaluating the reversible effect of Ar-turmerone co-treated with rotenone.

3.2 OBJECTIVES

- To analyse the toxicological parameters of rotenone in zebrafish embryos.
- To explore the developmental delay of rotenone induced zebrafish embryos using gene expression studies.
- To examine the apoptosis and histopathological parameters of rotenone induced zebrafish embryos.
- To evaluate the reversible effects of aromatic turmerone co-treated with rotenone in zebrafish embryos.

CHAPTER 4

MATERIALS AND METHODS

4.1 ZEBRAFISH OBTAINING

Zebrafish are an excellent choice for scientific research due to their affordability and wide availability at pet shops and fish stores. AB, TL, Tubingen, and WIK strains are particularly valuable due to their unique genetic and behavioral traits, as demonstrated in studies by (Gorissen *et al.*, 2015). Zebrafish wild-type *Danio rerio* from a nearby pet store and entrusted their care to the Regeneration and Stem Cell Biology team at the esteemed International Research Centre of Sathyabama Institute of Science and Technology. The fish were expertly housed in a glass tank filled with RO water.

4.2 ZEBRAFISH HOUSING CONDITION

The zebrafish were bred in a well-maintained glass tank with either dechlorinated or filtered water. The water temperature was maintained between 26-28°C, and the pH level was kept at 7.0-7.5 to ensure a suitable environment for the fish. Male and female fish were kept in separate tanks to prevent overcrowding and maintain water quality. The fish tank is cleaned every two days and the water is refilled to maintain the health and safety of the fish. During cleaning, the fish are temporarily transferred to a holding tank with water from the main tank. After cleaning and refilling the main tank, the fish are returned to their original tank. An air pump motor is installed in the tank to ensure that the fish have enough oxygen. The machine aerated the water with air via a tube, providing ample oxygen for the zebrafish to breathe and ensuring a healthy and safe environment for their growth.

4.3 SOLUBILITY OF ROTENONE

Rotenone ($C_{23}H_{22}O_6$) is a natural substance derived from certain plants, particularly the roots of the species of the genera *Lonchocarpus* and *Derris*. 100mg of Rotenone were taken and dissolved in DMSO (stock). From prepared stock 1mg were taken and diluted in 1ml of DH_2O (working). In that working concentration we had immersed different concentrations in zebrafish embryos.

4.4 ROTENONE DOSAGE STANDARDIZATION

To study the toxicity effect of the rotenone were given to the zebrafish embryos. During 16 cell stage the embryos were collected and then treated with different dosage of rotenone in embryos, Rotenone has immersed at different concentrations 2 µg, 4 µg, 6 µg , 8 µg, and 10 µg.

4.5 EMBRYO TOXICITY ASSAY

Zebrafish embryos are widely used in research for various purposes due to their unique characteristics and suitability as model organisms. Zebrafish embryos were extensively used to study vertebrate embryonic development and external fertilization.

4.5.1 Principle of the test

Newly fertilized eggs were exposed to the test chemical for a period of 96 hrs. Every 24 hrs upto four apical observations were recorded as indicators of lethality, (coagulation of fertilized eggs, lack of somite formation, lack of tail detachment of the tail bud from the yolk sac, and lack of heartbeat). At the end of the exposure period, acute toxicity was determined based on positive outcomes in any of the four apical observation recorded, and the LC₅₀ was calculated.

4.5.2 Conditions of exposure

Twenty embryos per concentration (one embryo per well) were exposed to test chemicals. Normally five concentrations of the test chemical should be tested. Respective negative control, internal control, and solvent control should be maintained. If more than 1 dead embryo is observed in the internal plate control, the plate is rejected. The test is initiated as soon as possible after fertilization of the eggs and terminated after 96 hrs of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences or at the latest, by the 16 cell stage.

4.5.3 Distribution of the eggs over 24 well plates

Eggs are distributed to well plates in the following numbers;

- 20 eggs on one plate for each test concentration

- 20 eggs as solvent control on one plate (if necessary)
- 20 eggs as positive control on one plate
- 4 eggs in dilution water as internal plate control on each of the above plates
- 24 eggs in dilution water as the negative control on one plate.

4.5.4 Preparation of E3 Media (60x stock)

60x stock for 1 litre

Sodium chloride (NaCl) – 17.4 g

Potassium chloride (KCl) – 0.8 g

Calcium chloride ($\text{CaCl}_2, 2\text{H}_2\text{O}$) – 2.9 g

Magnesium chloride ($\text{MgCl}_2, 6\text{H}_2\text{O}$) – 4.88 g

Weighing the amount of each salt as mentioned and the salts were added in 800ml of DH_2O in a beaker. The beaker were stirred until they completely dissolved. Once they dissolved, the volume of the solution were adjusted into 1 litre by adding DH_2O . Check the pH for 7.2 and autoclaved it and kept it under refrigerator.

4.5.5 Preparation of 1x E3 Media

16.5 ml of 60X E3 media was added to the autoclaved water and it was make up in to 1litre. To this 2 to 3 μl of methylene blue was added to prevent from fungal contamination and stored it under refrigerator

4.5.6 Procedure

- The fertilized embryos were collected within 1-2hrs after mating and the embryos were removed from the tank gently using Pasteur pipette.
- The embryos were rinsed with RO water with 2 times and then it was transferred to E3 medium washed with 3 times to remove debris.
- The embryos were distributed in 24 well plate in such a way that, each well contain single EMBRYO
- Ensure that the volume of the compound in each well is sufficient to cover the embryos adequately.
- The embryos were incubated under standard conditions, typically at controlled temperature 28-30°C.

- After 24hrs, the embryos were observed under microscope to analyze the morphological changes such as developmental abnormalities, growth retardation, or mortality, using a standardized scoring system

This toxicity assessment were strictly followed by OECD guidelines, for analysis the embryotoxicity three different concentration group of rotenone was used.

Group I – In microgram level

Group II – In ppm level

Group III – In ppb level

4.6 Protein sample preparation

Beta-mercaptoethanol (β -ME-reducing agent) is used for breaking disulfide bonds of protein. 2X sample buffers were brought to room temperature before use. After that, 2X sample buffer was added with 5% of Beta-Mercaptoethanol (reducing agent) (stock 1). Samples were transferred into the fresh 1.5ml vial which has an equal amount of sample buffer at 4°C. A homogenizer was used for homogenization which was done manually. After that samples were transferred into the dry bath at 95°C for 5 minutes and then stored at -20°C.

4.7 SDS-PAGE

SDS PAGE is an analytical technique that is used to separate the proteins based on their molecular weight. Electrophoresis is the study of the movement of charge molecules in an electric field. Their rate of movement depends on the strength of the field, net charge, size and shape of the molecules, and also the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. Polyacrylamide gels are widely used for separating larger molecules like proteins. Based on the resistance of the gel matrix proteins are separated viz., smaller proteins generally have less resistance as well as larger proteins have more resistance.

The molecular weight of the protein was estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a

reducing agent β Mercaptoethanol, it can bind with the protein backbone and denaturing effect of SDS is very strong and that disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds (both inter-disulfide and intra-disulfide bonds).

Polyacrylamide creates a mesh-like matrix that helps in the separation of proteins. During PAGE electrophoresis linear or primary structures of the proteins were stacked or arranged properly, once reached the separating gel, proteins started to separate based on their size.

4.7.1 Materials

SDS glass plates, comb, Gel tank, Eppendorf's; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.7.2 Chemical preparation of SDS-PAGE

30% Acrylamide and 0.3% Bis acrylamide

Acrylamide – 30 g

N, N' Methylene Bis acrylamide - 0.3 g

Makeup into 100 ml with distilled water

4.7.3 4X Separating Buffer

Tris HCL - 18.2 g

Distilled water – 60 ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Makeup into 100 ml with distilled water.

4.7.4 4X Stacking Buffer

Tris Hcl - 6.05 g

Distilled water – 40 ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Makeup into 100 ml with distilled water.

4.7.5 2X Sample Buffer

Tris stacking buffer (pH 6.8) – 25 ml

Sodium Dodecyl Sulphate (SDS) – 4 g

20% Glycerol

20 ml makeup into 100 ml with distilled water. During protein sample preparation, 5% β -Mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

4.7.6 10% Ammonium Per Sulphate (APS)

APS – 100 mg

Makeup into 1 ml with distilled water.

4.7.7 1X Running Buffer

Tris HCl - 3.94 g

Glycine - 15.014 g

SDS -1 g

Makeup in 1 litre with distilled water.

4.7.8 Staining Solution

Commassie brilliant blue R-250 – 200 mg

Methanol – 50 ml

Glacial Acetic acid – 7 ml

Makeup into 100 ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

4.7.9 Destaining Solution

Methanol – 30 ml

Glacial Acetic acid – 7 ml

Makeup into 100 ml with distilled water.

Table. 4.1 Separating gel concentration

Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0
4X Tris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
10% APS (μl)	200	200	200	200	200	200	200	200	200	200
TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table.4.2 Stacking gel concentration

Sl.no	Stock Solution	5 ml	10 ml	15 ml	20 ml
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	0.65	2.5	3.75	5.0
2.	4X Tris Stacking Buffer (ml) pH 6.8	1.25	2.5	3.75	5.0
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	10% APS (μ l)	100	100	100	100
5.	TEMED (μ l)	10	10	10	10

4.7.10. Preparation of gel

The glass plates were cleaned by using ethanol and manually assembled. Glass plates were fixed in the proper equalized manner with each corner covered by using spacers so that they are used as a primary baseline on gel which helps in the process of agarose sealing or second level baseline system. 1% agarose was prepared and boiled until liquid then corners were sealed in both surface and bottom area of the spacer. Separating gel was manually prepared and loaded on the gel carefully. The gel was overlaid with isopropyl alcohol so that it excludes the air. Then IPA was removed and washed with distilled water, after the drain stacking gel was prepared and added, instantly comb was fixed on the stacking gel. The gel was allowed a few minutes to solidify, then the comb was carefully removed and wells manifestly washed with water to eliminate the bubbles the bottom of the spacer was carefully removed and the setup was fixed in the bottom chamber which carried 1X running buffer as well as an upper chamber.

4.7.11 Sample loading and electrophoresis

A protein sample of 80 µg and the appropriate molecular markers were loaded into the wells. The apparatus was connected to the power pack with proper polarity and 60 v was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulled away from the gel.

4.7.12 Staining and Destaining the Gel

The gel was carefully taken out from the glass plate and the stacking gel was removed by using a cleaver. The separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the Coomassie brilliant blue R250 (stainer solution). The gel should be fully submerged in the staining solution for 60 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to a destaining solution until protein bands are visualized clearly. Commonly, the first destainer wash is about 30 minutes, then the second wash is about overnight.

4.8 IMMUNOBLOTTING

4.8.1 Buffer preparation

10X Transfer Buffer (Stock)

Glycine - 72.1 g

Tris-base - 15.2 g

Distiller water- 500 ml

The 10X transfer buffer was stored at room temperature, and during blotting the 1X transfer buffer was prepared from the 10X in the ratio of preparation viz.,

For a Total Volume of 1 litre

100 ml - 10X Transfer Buffer

200 ml – Methanol

700 ml of Distilled water

4.8.2 Running the gel

Prepare 12% SDS gel and place it in SDS-PAGE setup. Pour 1X running buffer (100 ml of 10X running buffer + 900 ml of distilled water) into the setup. A protein ladder of 3 μ l is loaded at the first lane of the gel for molecular weight determination. In other wells, the prepared protein samples were added (80 μ g/well) concentration. After loading all the samples, the setup started to run at a 60 v unit. The samples were left to run till it reaches the bottom line.

4.8.3 Membrane transfer

After the protein runs fully till the end, take off the glass slides from the setup and cut off the stacking gel. Then gently take the gel and keep it in the transfer buffer for 5 minutes and simultaneously, cut the PVDF membrane by the same gel size. Soak the PVDF membrane in the methanol for 1-2 minutes for activation of the membrane. Then, keep the membrane in 1X transfer buffer until preparing the sandwich for blotting.

Prepare blotting apparatus, sponge, and filter paper to arrange the sandwich setup and soak it in 1X transfer buffer before using.

4.8.4 Blocking

Blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% of Bovine serum albumin (BSA) with of 1X PBST buffer was used as blocking buffer. The membrane was incubated in blocking buffer for 1 hr in RT.

4.8.5 Primary antibody

The primary antibody was diluted in 5% BSA in the following dilutions and incubated overnight in cold condition. Beta-actin (produced from rabbit polyclonal Ab clonal AC026) – (1:5000 in 5% BSA), Pax-2 (produced from rabbit polyclonal, Abcam ab 229318) – (1:2000 in 5% BSA) and Nf-kb (produced from rabbit polyclonal, Abcam ab 16502) – (1:2000 in 5% BSA). After undergoing the incubation period overnight, keep the membrane at room temperature for 10 mins and start the washing process.

4.8.6 Secondary antibody

Secondary antibody (Anti Rabbit IgG produced from goat was added in the 5% Bovine serum albumin (BSA) at 0.5: 5,000 dilutions and incubated for 1 hour at room temperature (NOTE: starting from adding the secondary antibody, every step should be performed in dark condition because these antibodies are light sensitive). After undergoing a dark incubation period, start the washing process.

4.8.7 Gel imaging

Gel imaging was carried out using the sophisticated Bio-Rad ChemiDoc™ XRS+ imaging system for faster and more accurate western blot quantification. After the incubation with secondary antibody the membrane is washed with 1X PBST every 5 minutes for 5 times. The blot is visualized in the system using ECL as a substrate by introducing solution A and B in equal volume. The software used for imaging is Bio-Rad Image Lab 6.1 software.

4.9 APOPTOSIS ASSAY

Apoptosis assay was carried out using acridine orange (AO) and Ethidium bromide (EB) assay. 1 mg/ml stock of AO/EB was prepared and stored in the dark at -20°C. To perform the analysis, live zebrafish embryos/ larvae were treated with different concentrations of any chemicals. Embryos without any treatment were used as the control group. After treatment, the embryos/larvae were washed with E3 media (Dechoriation using pronase must be performed, particularly for embryos, and washed with E3 medium thrice to remove the traces of the pronase enzyme). Further, embryos/larvae were incubated in acridine orange and ethidium bromide solution for 10 mins in the dark and observed under the fluorescence microscope to document the apoptotic cells.

4.10. HISTOLOGY

4.10.1 Cryo microtome

A Cryostat is a sophisticated and high-grade device used to generate low temperatures for cutting tissue sections.

4.10.2 Components of the Cryostat-Microtome

4.10.2.1 Waste container

It holds water generated by the continuous frost-defrost cycles inside the cryostat. This water can be a source of sample contamination; thus, it is important to dispose of it in the waste container.

4.10.2.2 Freezing chamber

The chamber is designed to house the microtome and maintain a low temperature during the sectioning process.

4.10.2.3. Specimen disc

This device secures the tissue specimen in place while it is being sectioned with the knife.

4.10.2.4 Microtome blade and blade holder

To examine tissue under microscopes, it must be cut into slices of a suitable thickness, often a few microns wide. Precision cutting necessitates the use of extremely sharp blades. The blades are secured by the blade holder.

4.10.2.5. Glass slide door

This is the cover for the cryostat.

4.2.10.6. Anti-roll plate

The anti-roll plate prevents curling of the tissue sections after they have been sliced by the blade.

4.10.2.7. U.V source

Ultraviolet light effectively sterilizes surfaces and is utilized for sterilizing the cryostat chamber. Usually 35 minutes UV irradiation is typically adequate for sterilizing.

4.10.2.8. Source of freezing temperature

The cryostat maintains freezing temperatures using condensers. In certain machines, liquid nitrogen or helium is also used.

4.10.2.9. Peltier stage

This is a thermoelectric device that removes heat from the cryostat chamber by enhancing diffusion towards a cold stage.

4.10. 2.10 Control panel

The control panel, as the name suggests is the hardware interface for the user to operate the Cryostat.

4.10.2. 11. Specimen chuck and chuck holder

Chuck is the adapter on which the tissue sample is mounted. It has a crossing grid pattern which provides better gripping. These chucks are made of stainless steel, which makes them more durable.

4.10.3 HISTOLOGY

Histology is the technique used to study the complete architecture of the tissue patterns employing examination and analysis of cell/tissue physiology and morphology at the microscopic level. The principle behind the histology for cryo-sections involves preserving tissue structure through rapid freezing, cutting thin sections for microscopic examination, staining to enhance contrast, and analysing tissue morphology to gain insights into normal and pathological processes.

4.10.3.1. *Reagents used for processing cryo-tissue sections*

OCT Compound

95% Ethanol

100 % Ethanol

10 % Formalin in Phosphate Buffer

Hematoxylin

Eosin

Xylene

DPX Mount

4.10.3.2. *Processing and staining the cryo-sections*

1. Coat the slides with 0.1 % Gelatin. Air dry.
2. Place the cryosection on the coated slide and allow it to stay in RT for 5-10 minutes.
3. Staining Procedure-
 - (i) 95 % Ethanol (10 dips)
 - (ii) 10 % Formalin in Phosphate Buffer (10 dips)
 - (iii) Distilled water (10 dips)
 - (iv) Hematoxylin (30 seconds)
 - (v) Distilled water (10 dips)
 - (vi) Distilled water (10 dips) - optional
 - (vii) 95 % Ethanol (10 dips)
 - (viii) Eosin (15 seconds)
 - (ix) 95 % Ethanol (10 dips)

- (x) 95 % Ethanol (10 dips) - optional
- (xi) 100 % Ethanol (10 dips)
- (xii) 100 % Ethanol (10 dips) - optional
- (xiii) Xylene (10 dips)
- (xiv) Xylene (10 dips) - optional

Mount with DPX mount. Observe under microscope.

4.11 STATISTICAL ANALYSIS

All statistical analysis was performed using the software GraphPad Prism v9.5.1. Ordinary one way ANOVA with Brown-Forsythe test was used to analyse the western blotting data $p < 0.05$ was considered as significant.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 TOXICITY ANALYSIS OF ROTENONE IN ZEBRAFISH EMBRYOS USING GROUP I CONCENTRATION (IN MICROGRAM)

Fertilized embryos were treated to study the rotenone toxicity and their lethality. During the zygote stage, the embryos were treated with rotenone at 1µg, 2µg, 4µg, 6µg, 8µg, and 10µg concentrations, control embryos were not treated with any chemicals, and dimethyl sulfoxide (DMSO) was used as solvent control. After 24 hours of treatment, we could able to notice the developmental delay in the embryos. The major morphological feature of 24 hrs embryos is detachment of tail from the yolk sack (Kimmel *et al.*, 1995; Yesudhason *et al.*, 2020). But the embryos treated with rotenone remained in the zygote stage without progressing to development, whereas the control and DMSO-treated embryos developed normally and it was presented in Fig. 5.1 and 5.2.

After 48 hours rotenone treated embryos were completely coagulated in all concentrations (Fig. 5.3). The reason behind this developmental delay in rotenone treated embryos is it blocks the mitochondrial electron transport chain through the inhibition of complex I (Talpage et al 2000) and hence the production of ATP was disrupted result in various physiological consequences, including impaired organ function and potentially cell death. The similar consequence was observed in our result..

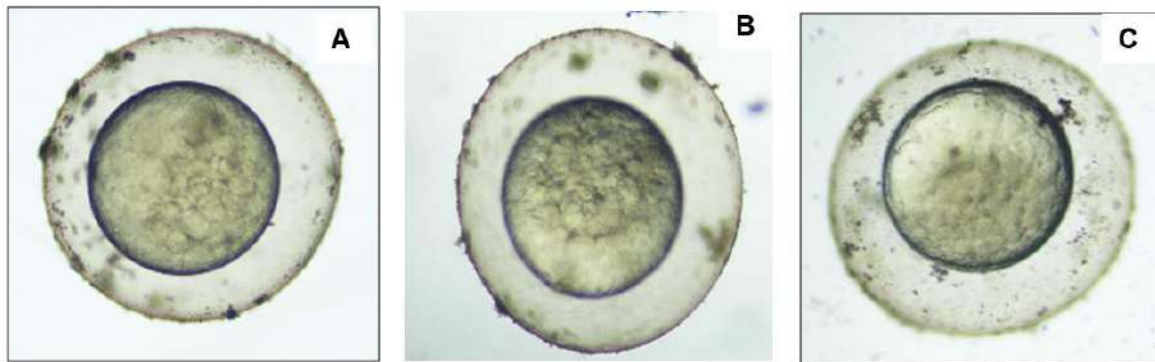


Fig: 5.1 : Toxicity assessment of Rotenone in zebrafish embryos. A) control embryos, B) Rotenone treated embryos, C) DMSO treated embryos.

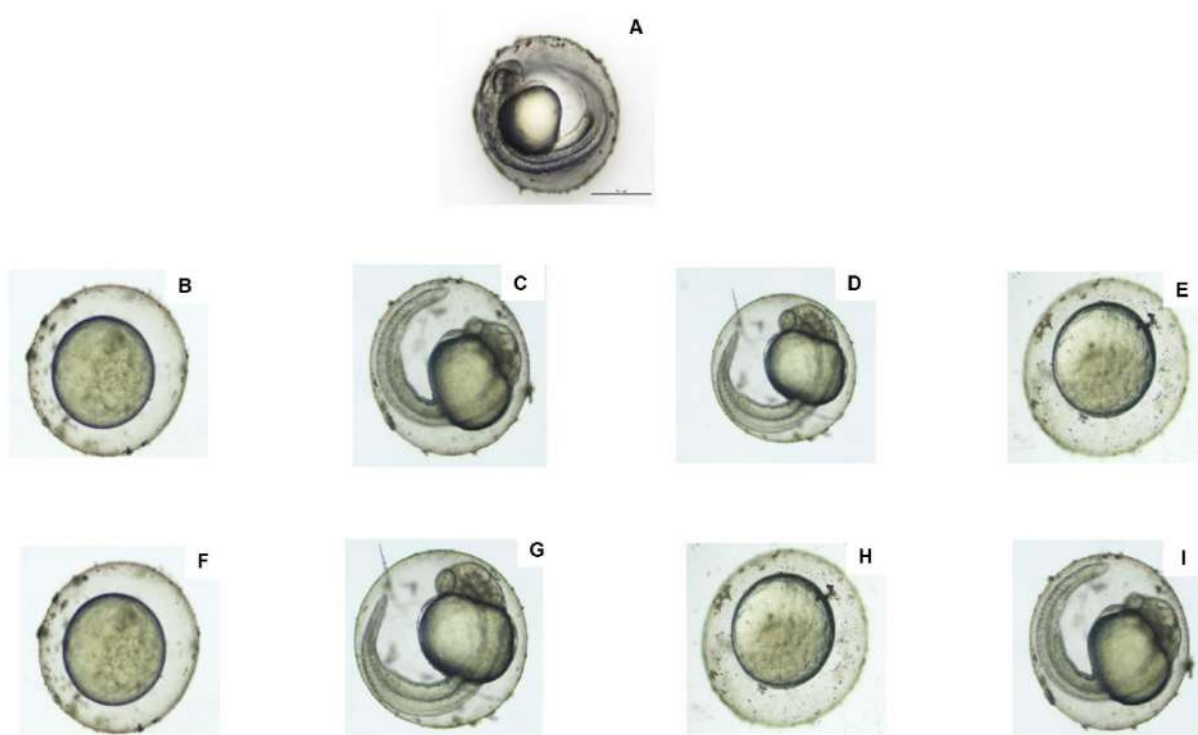


Fig: 5.2 : Rotenone treated with different concentrations in zebrafish embryos 24 Hrs. A) Control embryos, B) Rotenone 1 µg/ml treated embryos, C) DMSO 1 µl/ml treated embryos, D) DMSO 2 µl/ml treated embryos, E) Rotenone 2 µl/ml treated embryos, F) Rotenone 6 µg/ml treated embryos, G) DMSO 6 µl/ml treated embryos, H) Rotenone 10 µg/ml treated embryos I) DMSO 10 µl/ml treated embryos

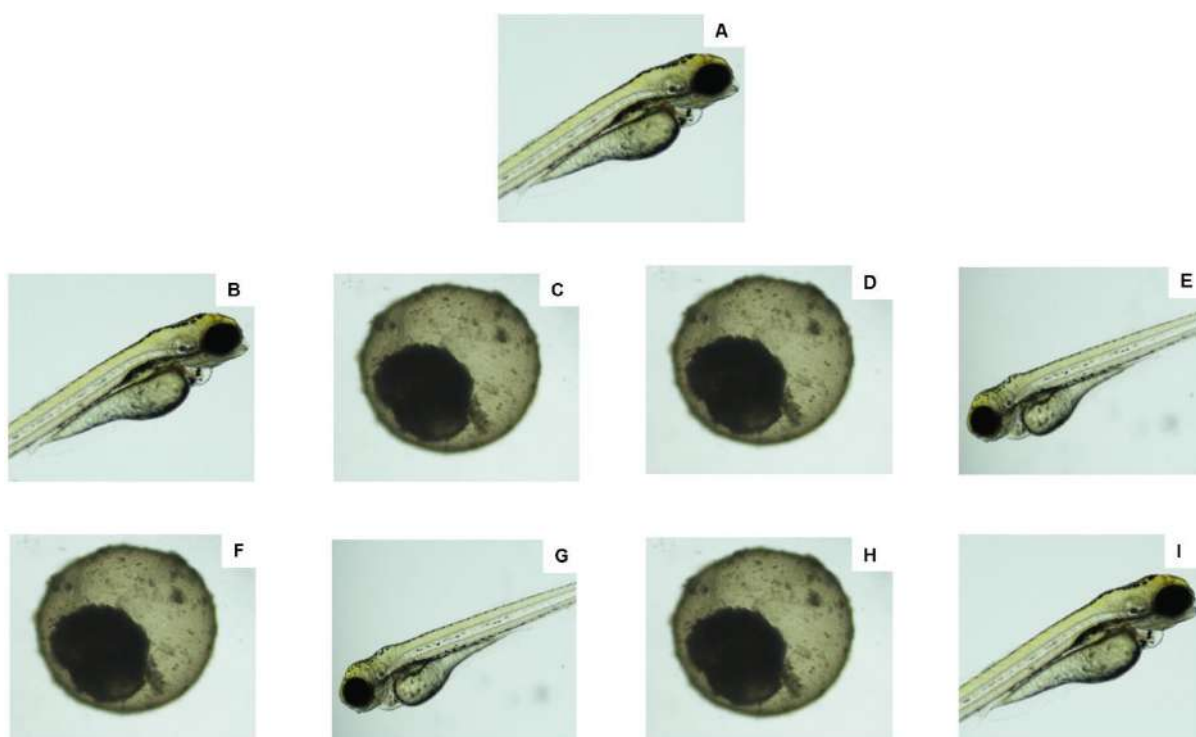


Fig: 5.3 : Rotenone treated with different concentrations in zebrafish embryos 48Hrs. A) Control embryos, B) DMSO 1 μ l/ml treated embryos, C) Rotenone 1 μ g/ml treated embryos, D) Rotenone 2 μ g/ml treated embryos, E) DMSO 2 μ l/ml treated embryos, F) Rotenone 6 μ g/ml treated embryos, G) DMSO 6 μ l/ml treated embryos, H) Rotenone 10 μ g/ml treated embryos, I) DMSO 10 μ l/ml treated embryos,

Table 5.1. Observation of lethal endpoints at 24 to 48 hours

Sl.no	Lethal endpoints	Control	DMSO	Rotenone
1.	Coagulation	Nil	Nil	Noticed at 1 μ g to 10 μ g concentrations at 48 hrs
2.	Somite formation	Detected in all healthy	Detected in all healthy	Embryos were not developed

		embryos	embryos	upto 24 hrs and all embryos were coagulated at 48 hrs
3.	Detachment of tail	Detected in all healthy embryos	Detected in all healthy embryos	Tail not detached
4.	Heart beat	29 ± 2/10s	29 ± 2/10s	Cardiac tissue was not formed in all concentrations.
5.	Pigmentation	Detected in all healthy embryos	Detected in all healthy embryos	Pigments were not formed.
6.	Developmental delay	Nil	Nil	Noticed at all concentrations.

5.2 TOXICITY ANALYSIS OF ROTENONE IN ZEBRAFISH EMBRYOS USING GROUP II CONCENTRATION (IN PPM)

The data obtained from the previous experiment shows high lethality in zebrafish embryos and hence parts per million (ppm) concentration of rotenone-induced to zebrafish embryos. For this treatment, 2.5 hpf embryos (Fig. 5.5) were used and treated with different concentration (2ppm, 4ppm, 6ppm, 8ppm, 10ppm, 20ppm, 25ppm) and it was observed for 48 hours. From this experiment we observed that 2ppm treated embryos develop normally similar to that of control and depicted in Fig.5.6 B. Further to the previous experiment 4ppm, 6ppm, 8ppm, 10ppm, 20ppm and 25ppm showed very delayed development (Fig. 5.6 C, D E).

After 48 hours all the embryos get coagulated except 2ppm treated one (5.7 C, D, E). Even though it was alive it showed delay in hatching (Fig. 5.7 B) when compared with the control (Fig. 5.7 A). The effects of rotenone persist indefinitely in the adult zebrafish when induced with rotenone which in turn it acts as the model

animal for Parkinson's disease (Wang et al., 2017). This finding is consistent with our findings, which revealed that the effect of rotenone lasts forever in the embryos.

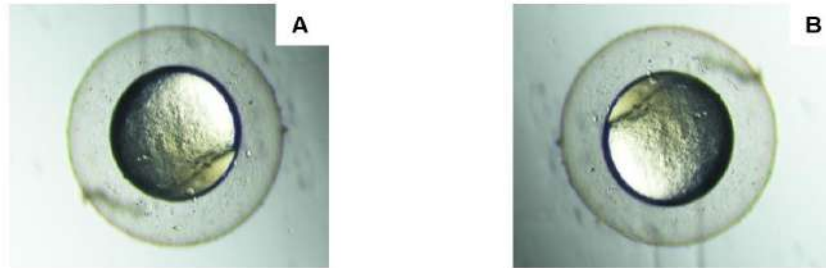


Fig: 5.4 : Zebrafish embryo 2.5 hr post fertilization A) Control embryos, B) Rotenone treated embryos

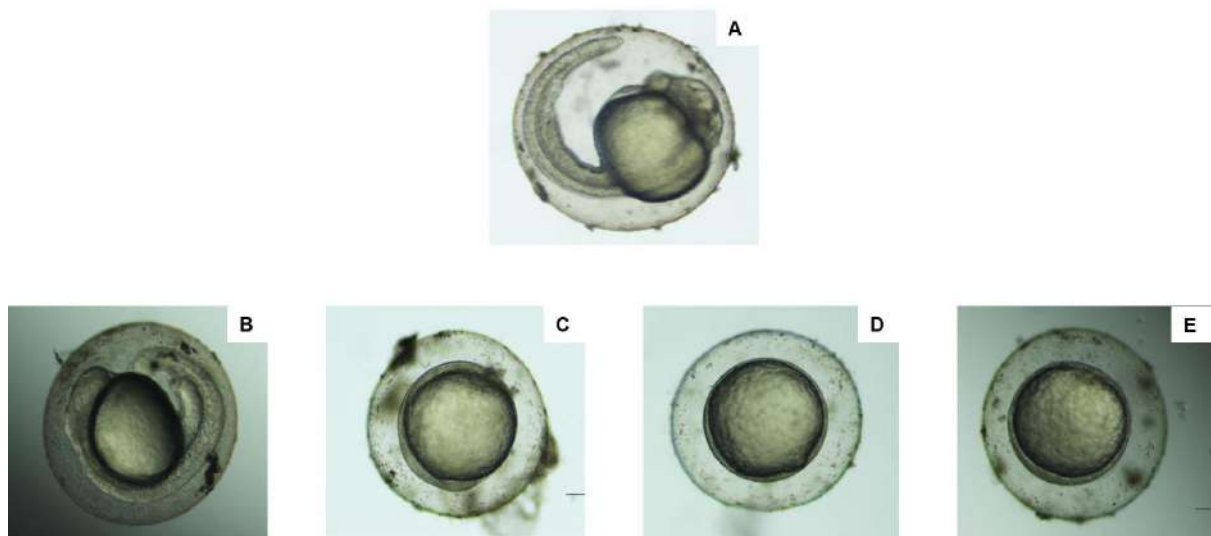


Fig: 5.5 : Rotenone treated with different concentrations in zebrafish embryos 24 Hrs. A) Control embryos, B) Rotenone 2 ppm treated embryos, C) Rotenone 6 ppm treated embryos, D) Rotenone 10 ppm treated embryos E) Rotenone 25 ppm treated embryos.

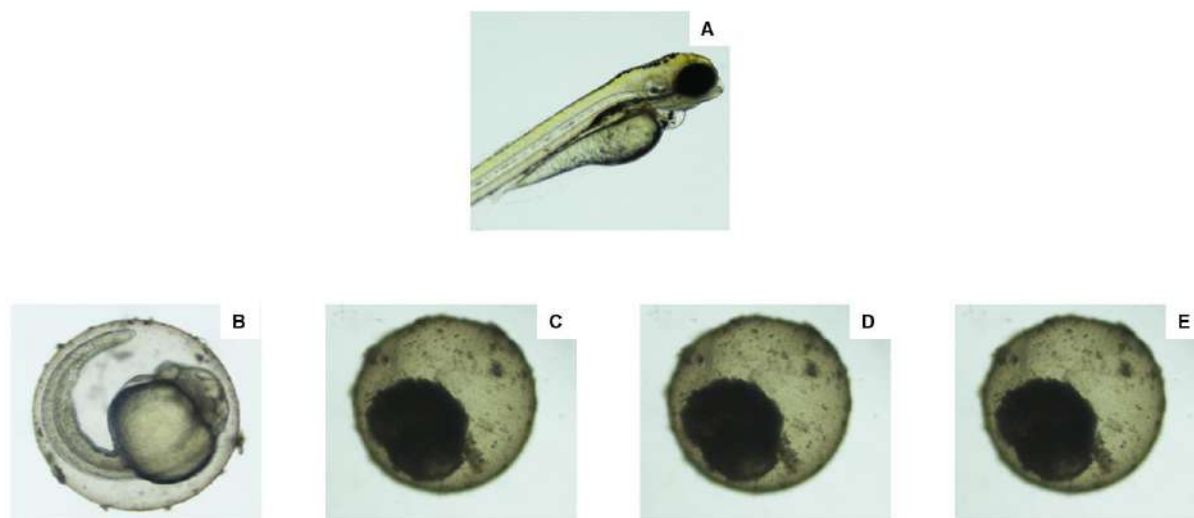


Fig: 5.6 : Rotenone treated with different concentrations in zebrafish embryos 48 Hrs A) Control embryos, B) Rotenone 2 ppm treated embryos, C) Rotenone 6 ppm treated embryos, D) Rotenone 10 ppm treated embryos, E) Rotenone 25 ppm treated embryos

Table 5.2 Observation of lethal endpoints upto 24 to 48 Hrs

Sl.no	Lethal endpoints	Control	Rotenone
1.	coagulation	Nil	Noticed at 4 ppm, 6 ppm, 8 ppm, 10 ppm and 25 ppm
2.	Somite formation	Detected in all healthy embryos	Embryos were not well developed in 4 ppm, 6 ppm, 8 ppm, 10 ppm and 25 ppm, coagulated at 48 hrs
3.	Detachment of tail	Detected in all healthy embryos	Tail was not formed in all concentration after 24 hrs except 2

			ppm. Tail detachment was found after 48 hrs in 2 ppm. After 48 hrs all embryos were dead except in 2 ppm.
4.	Heart beat	29 ± 2/10s	Cardiac tissue were not formed in all concentration after 24 hrs except 2 ppm.
5.	Pigmentation	Detected in all healthy embryos	Pigments were not formed in all concentration after 24 hrs except 2ppm.
6.	Developmental delay	Nil	Noticed at all concentrations.

5.3 EMBRYO TOXICITY – SURVIVAL GRAPH

Fertilized embryos were used to study the rotenone toxicity and their lethality. During the blastula stage, the embryos were treated with rotenone at 2 ppb, 4 ppb, 6 ppb, 8 ppb, 10 ppb and 25 ppb concentrations, control embryos were not treated with any chemicals. After 24 hrs developmental delay was observed in all the rotenone treated concentrations, the developmental delay was directly proportional to the increasing concentration of rotenone, indicating rotenone plays a negative effect in the growth of zebrafish embryos. Death count was noted after 24 hrs for each concentration, no dead embryos were found in control group. After 48 hrs of treatment all the embryos were found dead in 6 ppb, 8 ppb, 10 ppb, 25 ppb rotenone treated group. 2 ppb showed delayed growth but no death was observed.

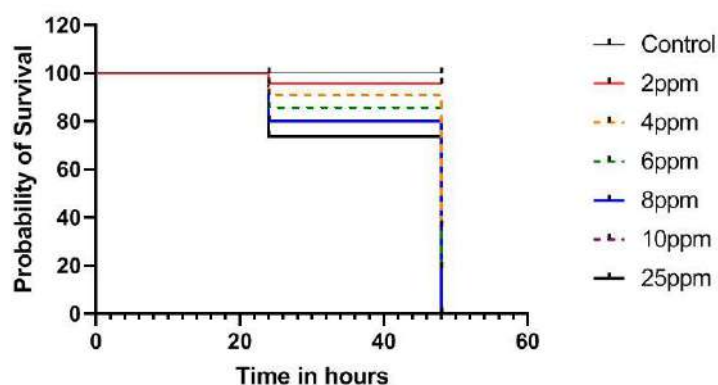


Fig: 5.7 : Survival graph of zebrafish embryos after exposure to rotenone

5.4 TOXICITY ANALYSIS OF ROTENONE IN ZEBRAFISH EMBRYOS USING GROUP III CONCENTRATION (IN PPB)

With the result obtained from ppm concentration, again another set of embryos were exposed to rotenone with minimal concentration (parts per billion). The blastula stage, the embryos were treated 0.5ppb, 2ppb, 3ppb, and 4ppb of rotenone. After 24hrs developmental delay was observed in 3ppb and 4ppb concentration (Fig. 5.8 D, E) whereas the 0.5 and 2ppm concentration developed normally and it was shown in fig. 5.8 B, C. The developmental delay was directly proportional to the increasing concentration of rotenone, indicating rotenone plays a negative effect in the growth of zebrafish embryos. Death count was noted after 24hr for each concentration, no dead embryos were found in control group. After 48 hr of treatment almost all embryos were found dead in 3ppb and 4ppb (Fig. 5.8 D, E) 0.5 ppb and 2ppb showed delayed growth but no death was observed (Fig. 5.8 B, C).

Similar types of experiments were carried out in different vertebrate animals like adult zebrafish, guinea pigs, and chick embryos that showed developmental toxicity, hatching delay, and other malformations respectively (Melo *et al.*, 2015). Apart from this exposure to Rotenone also reduces oxidative stress enzymes like CAT, and GST. Siddiqui *et al.* (2013) found that rotenone generated a dose-dependent cytotoxic response in HepG2 cells while also decreasing the activity of oxidative stress indicators like glutathione, CAT, and superoxide dismutase. Changing the

normal oxidative state in zebrafish embryos can cause cellular damage, leading to mortality or morphological changes. The developmental delay that we noticed in our finding also might be related to the inhibition of oxidative stress enzymes

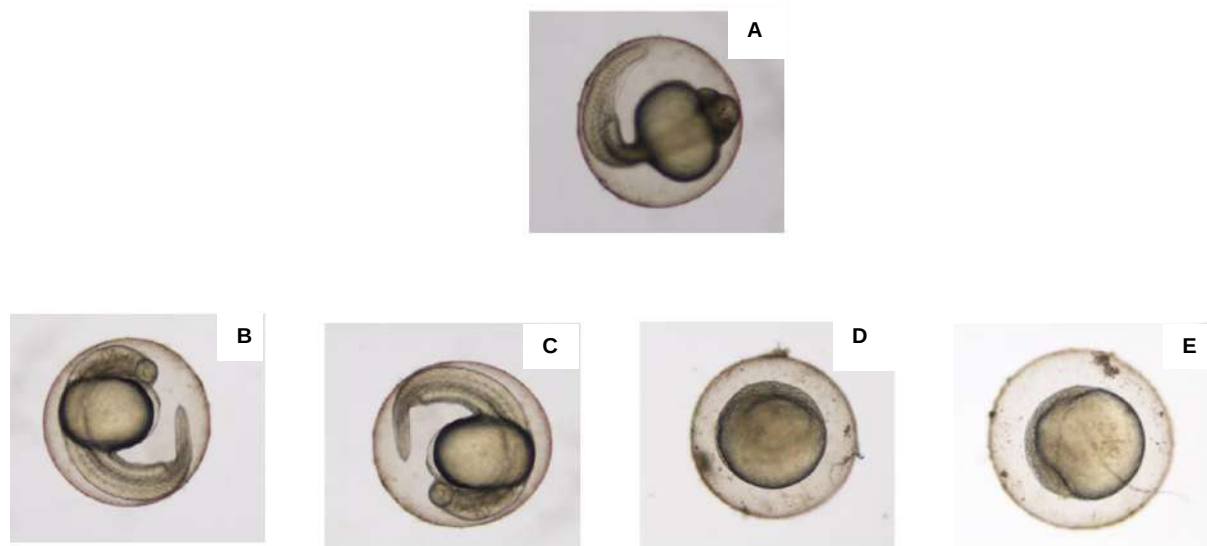


Fig: 5.8 : Rotenone treated with different concentrations in zebrafish embryos. A) Control embryos, B) Rotenone 0.5 ppb treated embryos, C) Rotenone 2 ppb treated embryos, D) Rotenone 3 ppb treated embryos, E) Rotenone 4 ppb treated embryos.

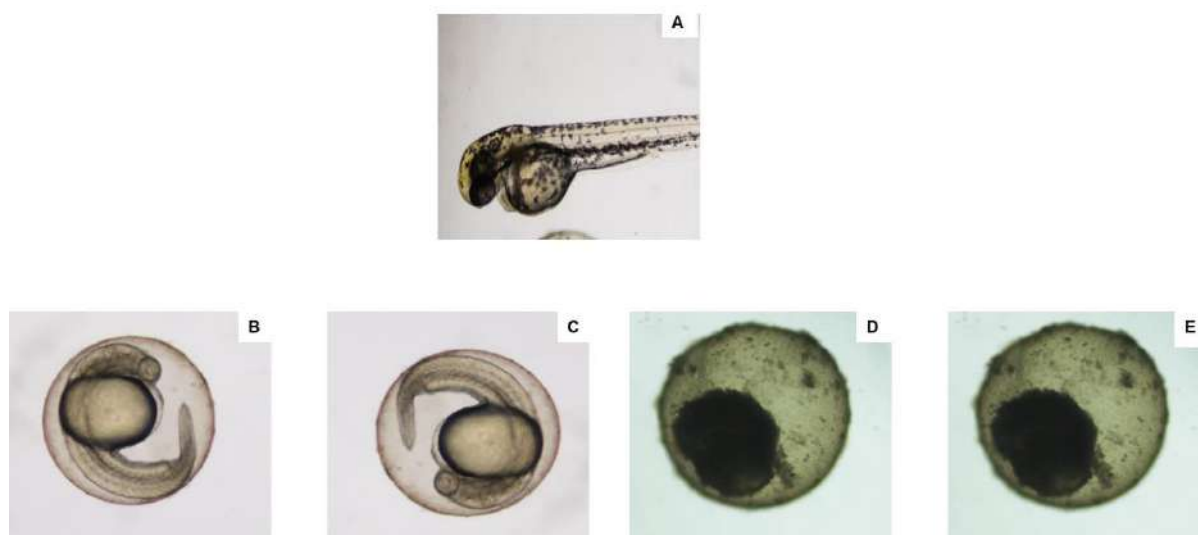


Fig: 5.9 : Rotenone treated with different concentrations in zebrafish embryos. A) Control embryos, B) Rotenone 0.5 ppb treated embryos, C) Rotenone 2 ppb treated embryos, D) Rotenone 3 ppb treated embryos, E) Rotenone 4 ppb treated embryos

Zebrafish embryos were treated with rotenone to study the developmental toxicity of the compound. 3 different groups were analysed using different concentration ranges – in microgram, ppm and ppb. All the experiments were conducted upto 48 hrs of exposure to the compound. Higher concentrations indicated death of almost all of the treated embryos. Morphological changes and developmental delay of the zebrafish embryos were observed in rotenone as compared with control groups. Rotenone leads to compensatory glycolysis induction and bioenergetic capacity loss which in turn activates apoptotic cascade and cell death. Head malformation, spinal curvature and dorsal curvature were observed in rotenone exposed embryos at 48 and 72 hrs exposure of 10 µg/L of rotenone (Üstündağ, *et al.*, 2022)

Table 5.3 Observation of lethal endpoints upto 24 to 48hrs

Sl.no	Lethal endpoints	Control	Rotenone
1.	coagulation	Nil	Noticed at 3 ppb and 4 ppb

2.	Somite formation	Detected in all healthy embryos	Embryos were not well developed in 3 ppb and 4 ppb, coagulated at 48 hrs
3.	Detachment of tail	Detected in all healthy embryos	Tail were not detached in 3 ppb and 4 ppb concentration
4.	Heart beat	29 ± 2/10s	Cardiac tissue were not formed in 3 ppb and 4 ppb concentrations
5.	Pigmentation	Detected in all healthy embryos	Pigments were not formed in and 3 ppb and 4 ppb concentrations.
6.	Developmental delay	Nil	Noticed at all concentrations.

5.5 ROTENONE TREATED WITH DIFFERENT CONCENTRATIONS IN 3RD DAY LARVAE

Larvae (3dpf) were used to analyze the rotenone toxicity and their lethality. Rotenone concentrations 2 ppb, 10 ppb, 25 ppb, 50 ppb and 100 ppb. Control larvae groups were not treated with any chemicals. After 2 hr of incubation rotenone treated larvae showed a decrease in heartbeat. Rotenone acts on the cardiac tissue by inhibiting the blood circulation to the heart causing a condition called blood stasis and it leads to the formation of edema. Larvae treated with 25 ppb, 50 ppb and 100 ppb showed blood stasis after 2 hr of treatment. Aside from rotenone, some research showed that zebrafish embryos exposed to Methylparaben (MeP) causes sublethal modifications such as pericardial edema,

yolk edema, blood stasis, decreased blood circulation, decreased heartbeat, and notochord curvature (Merola, 2020).



Fig: 5.10: Toxicity assessment of Rotenone in zebrafish larvae. A) Control larvae. B) Rotenone 2 ppb treated larvae, C) Rotenone 10 ppb treated larvae, D) Rotenone 25 ppb treated larvae, E) Rotenone 50 ppb treated larvae, F) Rotenone 100 ppb treated larvae.

5.6 APOPTOSIS ASSAY FOR ROTENONE TREATED LARVAE

2dpf larvae were used to analyse the apoptotic assay by incubating it with rotenone for 2 hr. Larvae were treated with rotenone 25 ppb concentration and control group were not treated with any chemicals. Rotenone treated larvae showed more apoptotic cells compared with control. Due to the rotenone activity more cells will get damaged compared to control group. Rotenone induces programmed cell death and the possible mechanism behind this is might be that rotenone inhibits the mitochondrial respiratory chain at the complex I site, decreasing the cellular ATP level. Few research findings showed that rotenone

causes apoptosis or necrosis in cell lines (Siddiqui, et al., 2013). Increase on ROS accumulation also consider as crucial mediator of cell death (Bergamini, 2004). Dornetshuber et al. 2007 proposed that there is a diminished preservation of mitochondrial integrity. This could be attributed to the generation of oxygen radicals during mitochondrial respiration, implying a potential link between oxidative stress and mitochondrial function (Mronga, et al 2004), ultimately resulting in apoptosis (Sharma et al 2012).

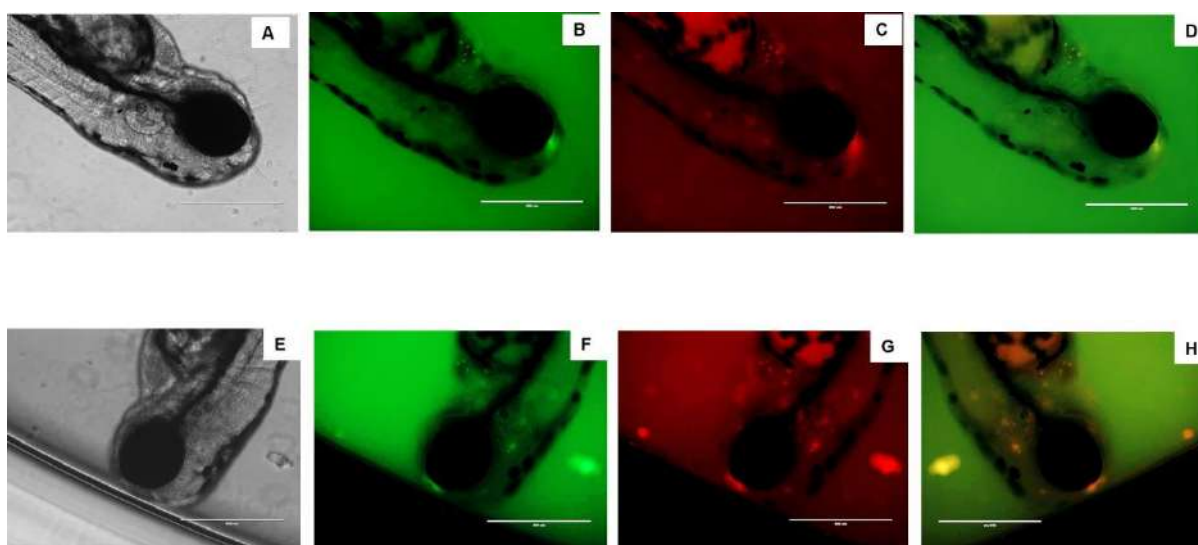


Fig: 5.11 : Apoptosis of Rotenone treated larvae. A) Control larvae, B) Control larvae with AO, C) Control larvae with EB, D) Control larvae with AO/EB, E) Rotenone treated larvae, F) Rotenone 25 ppb treated larvae with AO , G) Rotenone 25 ppb treated larvae with EB, H) Rotenone 25 ppb treated larvae merge(AO-EB).

5.7 HISTOLOGICAL ANALYSIS OF ROTENONE INDUCE ZEBRAFISH LARVAE

Rotenone exposed larvae were subjected to histological analysis to find out the pathological changes that occur in the brain of zebrafish larvae. The reason behind selecting the brain part is rotenone can cross the blood brain barrier also it affects the dopaminergic neurons which results in parkinson's disease. Moreover, our results showed developmental delay zebrafish embryos were exposed to rotenone. To find out the reason behind this histopathological analysis was carried

out. The larvae were sectioned with the help of cryomicrotome and then stained using Hematoxylin Eosin. staining.

The results were displayed in Fig. 5.12. In the control larva (5.12 A) the development of brain was normal and shows fibrillar like process where as in the case of rotenone treated samples (Fig. 5.12 B) clumps of cells were accumulated at the brain region and the fibrillar process were absent also the disintegration cells also noticed. Similarly the structure of the eye was completely disturbed with the absence of lens and tissue stratification often associated with an undetermined cellular mass, and a large empty space was noticed in the eyes. In the control larva, the structure of eye was found to be normal with a clear tissue stratification, inner nuclear layer, ganglion cell layer, and mass of fibre cells also observed. This results also coincides with the finding of Van Woudenberg et al., 2013. They found that when embryos were exposed to certain neurotoxic compounds they could observe the disrupted of brain cells and the formation of proteinaceous blobs in the brain region.

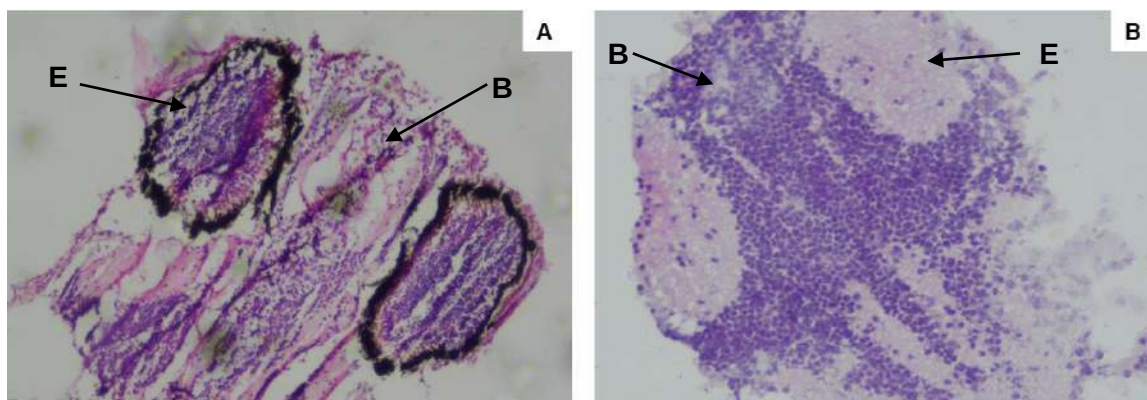


Fig 5.12 : Histopathological analysis of Rotenone induce zebrafish larva
A) Control larvae B) Rotenone treated larvae, B – Brain, E – Eye.

5.8 GENE EXPRESSION ANALYSIS

The protein lysate was prepared from the samples viz, control and rotenone-treated groups. The protein lysate was resolved in SDS-PAGE and western blotting was performed with the following antibodies used, Nfkb and Pax-2. Results showed that (Fig 5.4.2 and 5.4.3) Nfkb and Pax-2 expressed normally in the control sample, however, the expression were reduced in rotenone-treated samples. The paired box (pax) gene family is a group of transcription factors that expressed during embryonic development. Pax genes are developmental control genes that encode nuclear transcription factors (Mansouri and Gruss 2001). Since rotenone cause the developmental toxicity in the embryos it reflected in the expression pattern of PAX gene. Moreover, The role of NF-KB is it promotes cell survival (Luo et al., 2005). The expression of Nfkb in control cell was normal were as in rotenone treated due to the presence of apoptotic cells, the expression was reduced and it was implicated in the result which was shown in Fig. 5.13

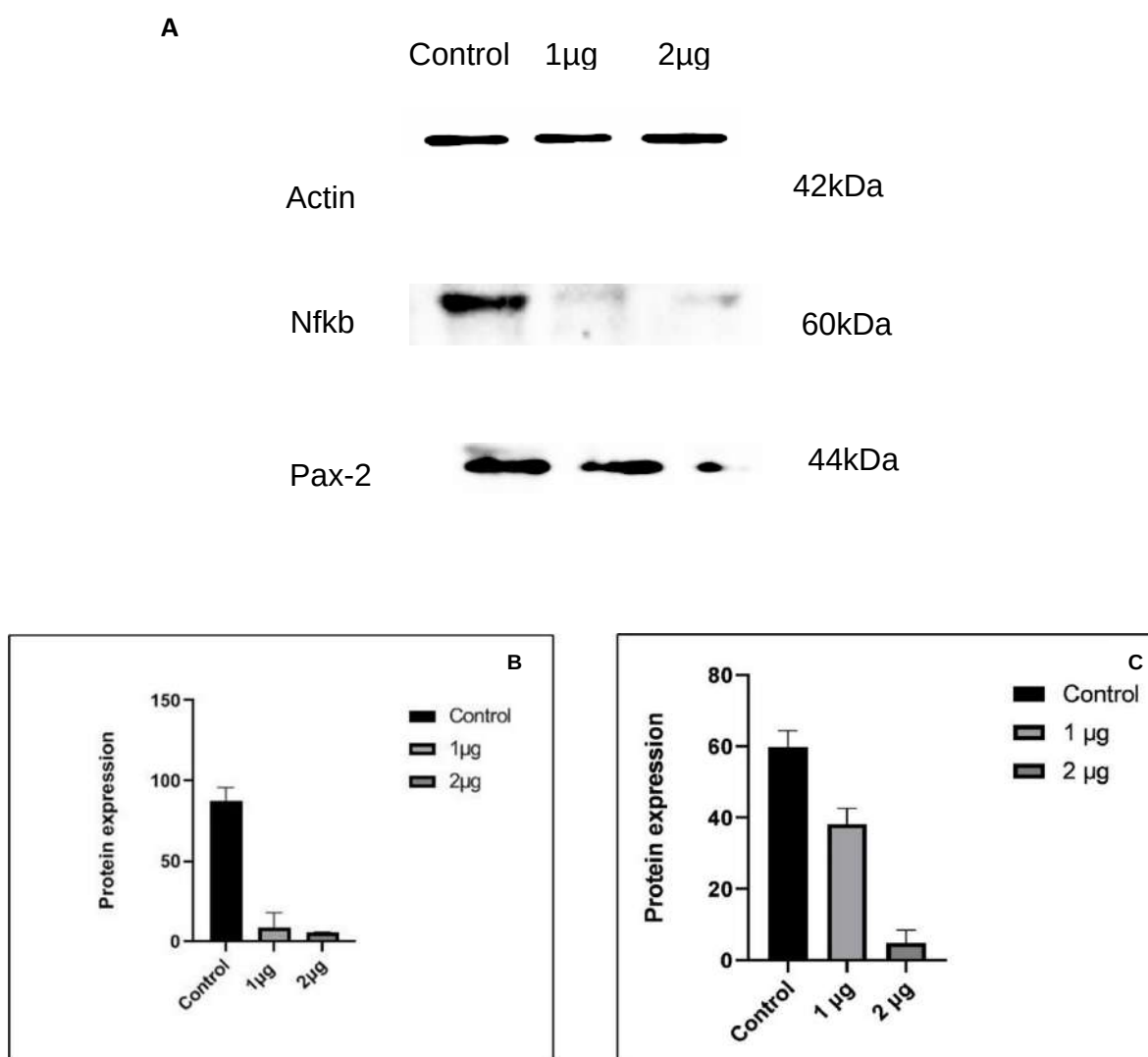


Fig. 5.13: Gene expression analysis of *Nfkb* and *Pax-2* exposure to rotenone on zebrafish embryos. A) Western blot images of Actin, *Nfkb* and *Pax-2*. B) Protein expression graph of *Nfkb*. C) Protein expression of *Pax-2*.

5.9 ROTENONE AND AR-TURMERONE CO-TREATED

Ar-turmerone is a wonderful phytochemical isolated from the *Curcuma longa*. It possess lot of medicinal property and it play a role in the proliferation of neural stem cells by blocking the ability of activated microglial cells (Hucklenbroich et al,2014). In order to find the reversible effect of ar-turmerone, the embryos were co treated with rotenone and ar-turmerone. Rotenone alone treated sample showed delayed development whereas the co treated sample the ar-turmerone promote the developmental process and it was depicted in fig. 5.14. This study concludes that, ar-turmerone helps in the development of the zebrafish embryo even though treated with rotenone a negative regulator of growth development, suggesting ar-turmerone may inhibit the activity of rotenone directly or indirectly.

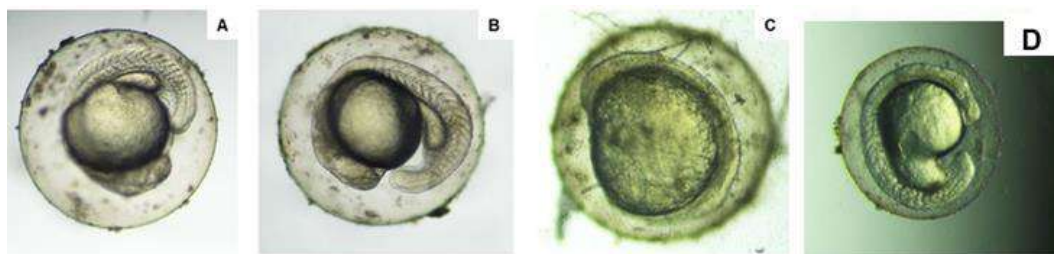


Fig: 5.14 : Rotenone and Aromatic turmerone co-treated with different concentrations in zebrafish embryos 24 Hrs. A)Control embryos, B) Aromatic turmerone treated embryos, C) Rotenone treated embryos, D)Rotenone and Aromatic turmerone co- treated embryos.

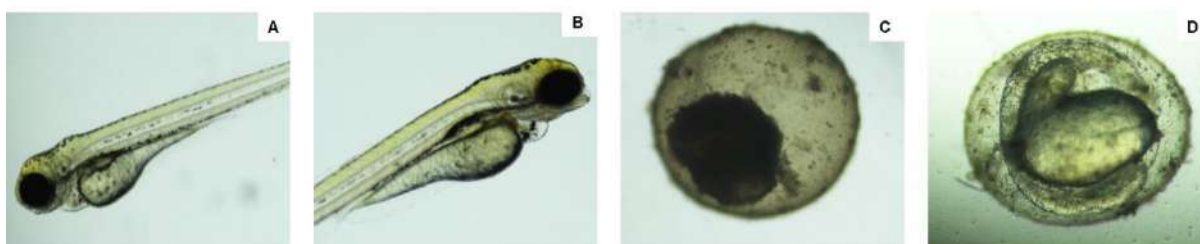


Fig: 5.15 : Rotenone and Aromatic turmerone co-treated with different concentrations in zebrafish embryos 48Hrs. A) Control embryos ,B)Aromatic turmerone treated embryos, C) Rotenone treated embryos, D)Rotenone and Aromatic turmerone co -treated embryos.

Table 5.4 Observation of lethal endpoints upto 24 to 48 Hrs

Sl. no	Lethal endpoints	Control	Aromatic turmerone	Rotenone	Rotenone + Aromatic turmerone
1.	Coagulation	Nil	Nil	Yes	Nil
2.	Somite formation	Detected in all healthy embryos	Detected in all healthy embryos	Embryos were found dead.	Delayed development was found.
3.	Detachment of tail	Detected in all healthy embryos	Detected in all healthy embryos	Embryos were found dead.	Detected
4.	Heart beat	29 $\pm$ 2/10s	29 $\pm$ 2/10s	Embryos were found dead.	Cardiac tissue had delayed development.
5.	Pigmentation	Detected in all healthy embryos	Detected in all healthy embryos	Embryos were found dead.	No pigments formed
6.	Developmental delay	Nil	Nil	Embryos were found dead.	Detected

5.10 APOPTOSIS ASSAY FOR ROTENONE AND AR-TURMERONE CO-TREATED LARVAE

The Larvae were co treated with rotenone and ar- turmerone to find whether ar- turmerone has the ability to reduce the apoptosis. Rotenone exhibited a higher number of apoptotic cells compared to the control group and ar-turmerone co-treated group. Rotenone triggers programmed cell death, possibly by inhibiting the mitochondrial respiratory chain at complex I, leading to a decrease in cellular ATP levels. Some studies have demonstrated that rotenone induces apoptosis or necrosis in cell lines (Siddiqui et al., 2013). The accumulation of reactive oxygen species (ROS) is also considered a critical mediator of cell death (Bergamini, 2004). Dornetshuber et al. (2007) suggested a decline in mitochondrial integrity, possibly due to the generation of oxygen radicals during mitochondrial respiration, indicating a potential connection between oxidative stress and mitochondrial function (Mronga et al., 2004), ultimately resulting in apoptosis (Sharma et al., 2012). In the ar-turmerone and co-treated larva also apoptotic cells were noticed but it was same as that of the control (Fig. 5.16). The result indicates that due to the property of inducing neural stem cells (NSCs) ar-turmerone has reduced apoptotic ability. Ar-turmerone rescues the cells from apoptosis caused by rotenone. From this analysis it has been found out that, ar-turmerone has played a role in healing the damaged cells.

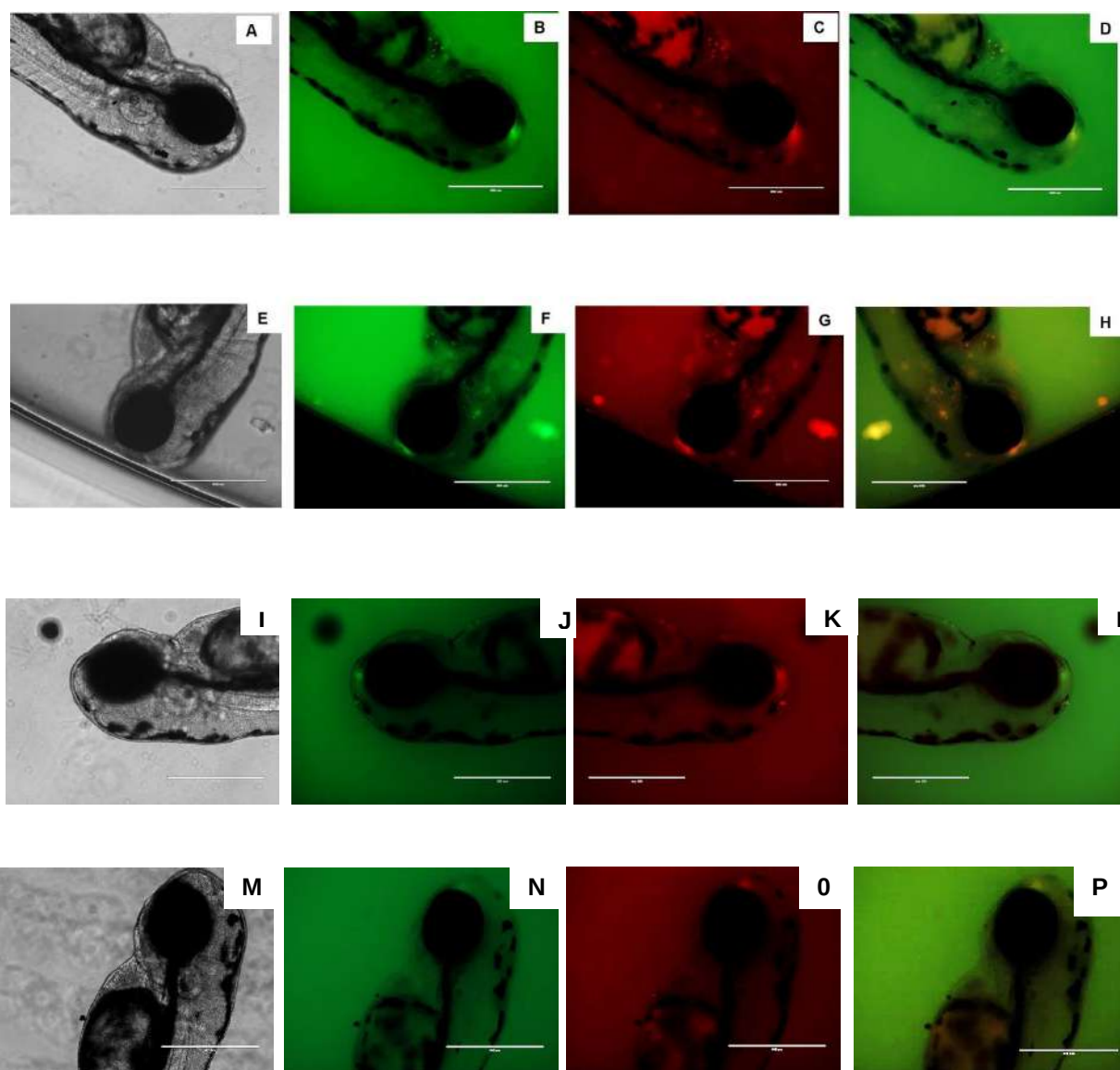


Fig: 5.16 : Apoptosis of Rotenone and Ar-turmerone co-treated larvae. A) Control larvae, B) Control larvae with AO, C) Control larvae with EB, D) Control larvae with AO/EB, E) Rotenone treated larvae, F) Rotenone 25 ppb treated larvae with AO stained, G) Rotenone 25 ppb treated larvae with EB stained H) Rotenone 25 ppb treated larvae merge(AO-EB), I) Ar-turmerone treated larvae, J) Ar-turmerone treated larvae with AO, K) Ar-turmerone treated with EB stain, L) Ar-turmerone merge (AO-EB), M) Cotreated larvae, N) Cotreated larvae with AO, O) Cotreated larvae with Eb stain, P) Cotreated larvae merge (AO-EB).

5.11 BLOOD STASIS

From the studies, it is evident that rotenone showed developmental delay, malformation in larva, delay in heart beat respectively. The delay in heart beat is due to the formation of blood stasis. Blood stasis in zebrafish larvae refers to a condition where there is a slowdown or cessation of blood flow within the circulatory system of zebrafish larvae. This can occur due to various factors such as vascular abnormalities, blood clotting disorders, or impaired heart function. Blood stasis can lead to insufficient oxygen and nutrient delivery to tissues, compromising their health and function. It can also disrupt normal physiological processes and contribute to developmental abnormalities in zebrafish larvae. To check whether ar-turmerone has the ability to reduce the formation of blood stasis the larva was co-treated with rotenone and Ar-turmerone. Ar-turmerone significantly reduce the formation of blood stasis and increased the heart beat similar to that of control and it was illustrated in Fig. 5.17. Certain studies showed that when larve exposed to Methylparaben that result in the formation of blood stasis that could be related to the slow the blood flow (Merola, 2020) and this correlates with our result exposed to rotenone. But ar-turmerone reversed the effect by reducing the formation of blood stasis. This indicates that ar-turmerone may possess properties that help to maintain or improve normal blood circulation within the larvae. This finding could imply potential therapeutic applications for ar-turmerone in conditions where blood stasis is a concern, potentially offering a natural remedy to mitigate circulatory issues. Further research would be necessary to fully understand the mechanisms behind this effect and to explore the potential clinical implications.

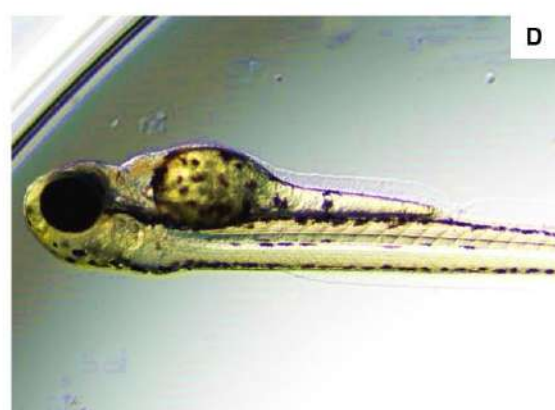


Fig: 5.17 : Blood stasis. A) Control, B) Ar-turmerone, C) Rotenone, D) Co-treated

CHAPTER 6

SUMMARY AND CONCLUSION

In this study we aimed to determine the developmental toxicity of Rotenone in zebrafish embryos and evaluating the reversible effect of ar-turmerone co-treated with rotenone. To analyse the toxicological parameters of rotenone in zebrafish embryos, different range of concentration was used. The lethal endpoints for all the treated samples was noted. Rotenone showed greater mortality in zebrafish embryos with its increasing concentrations. Developmental delay was observed in all the tested concentrations with dead embryos in maximum treated concentrations, suggesting rotenone does act on the cell proliferation pathway. It was also confirmed with apoptotic and gene expression studies. To evaluate the reversible effect of aromatic turmerone on the rotenone treated embryos, co-treatment was performed. Aromatic turmerone demonstrated promising results, as the development of the embryos was seen slowly moving into the next phase compared to the sole rotenone treated groups where the development delay was observed. Aromatic turmerone also reduced a condition termed blood stasis in zebrafish embryos where the circulatory system receive a blockage due to the inhibitory effect of rotenone, suggesting aromatic turmerone may have a solid reversible effect on the activity of rotenone.

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