# Evaluating the Toxicity of Manganese Dioxide ($MnO_2$) Nanostructures on the Liver Cell Culture of *Oreochromis mossambicus*


Submitted by

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# Evaluating the Toxicity of Manganese Dioxide ($MnO_2$) Nanostructures on the Liver Cell Culture of *Oreochromis mossambicus*

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Examined by: Seal of the School

## DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, "Evaluating the Toxicity of Manganese Dioxide ($MnO_2$) Nanostructures on the Liver Cell Culture of *Oreochromis mossambicus*" is based on the results of investigations carried out by me in the Zoology discipline at the School of Biological Science and Biotechnology, Goa University under the Supervision/Mentorship of Dr. Avelyno H. D'Costa and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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# CERTIFICATE

This is to certify that the dissertation / internship report "Evaluating the Toxicity of Manganese Dioxide ($MnO_2$) Nanostructures on the Liver Cell Culture of *Oreochromis mossambicus*" is a Bonafide work carried out by **Miss. Antara Poi Raiturker** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Zoology Discipline at the School of Biological Science and Biotechnology, Goa University.

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# INDEX

# LIST OF GRAPHS

# LIST OF PLATES

# CHAPTER 1:

# INTRODUCTION

# 1. INTRODUCTION

The term heavy metal refers to a chemical metallic element that has a relatively high density and is toxic to organisms at certain concentrations. They naturally occur in Earth's crust. They cannot be degraded or destroyed and hence they tend to bioaccumulate. As trace elements, some heavy metals elements like copper and zinc are essential to maintain the metabolism in the body. However, at higher concentrations they can create poisoning, that could result, from intake of polluted water, air or via the food chain, that can cause bioaccumulation of that metal in the body. Bioaccumulation refers to an increase in the concentration of a substance in an organism over time, compared to its concentration in the environment. The substances that are taken up, are stored faster than they are metabolized or excreted.

Heavy metals enter water bodies causing heavy metal pollution in aquatic ecosystem which can pose a serious threat to aquatic life. Due to toxicity and persistency, it bioaccumulates and biomagnifies in aquatic organisms and in turn, has harmful effects on environment. Sources of heavy metals may be natural or anthropogenic, with the latter having serious implications on health of the ecosystem. Natural source comprises of weathering of bedrocks, volcanic eruptions, etc. while the anthropogenic source of heavy metal pollution is mining, industrial production and use of fertilizers and pesticides, sewage discharge etc. (F.W.Ntengwe, 2006). Activities like mining and processing for extraction of mineral resources and their application in various sectors has led to increase in concentrations of heavy metals in environment (Zhou, et al., 2020).

The most common heavy metal pollutants found in aquatic ecosystems are arsenic, chromium, cadmium, nickel, lead, copper, mercury, manganese, etc. These can be sourced from point sources or non-point sources. Point sources are identifiable sources, such as industries, while non-point sources are dispersed sources, wherein, pollution in waterbody results from multiple

small sources. Metals are known to react with acids, hence acids are used in mine drainage systems, to release heavy metals from their ores. After drainage process, the acid containing high levels of metals, seeps in and get dispersed in groundwater. When pH of water falls, the solubility of metal increases, thus causing heavy metals from the corroded pipe to become more soluble and bioavailable (Saalidong, Aram, Otu, & Lartey, 2022).

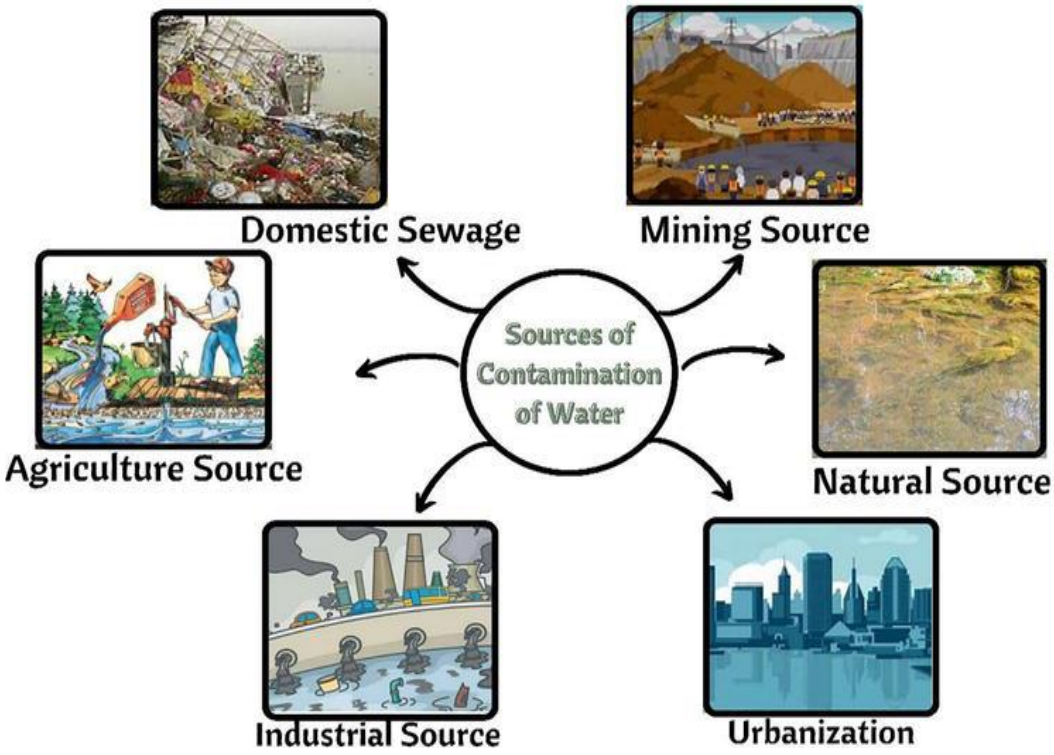



Fig 1.1: Sources of Heavy metal in water

Source: Heavy Metal Contamination of Water and Their Toxic Effect on Living Organisms | IntechOpen

Goa is a state, that is rich in iron and manganese ores. There are iron and manganese ore deposits found in Bicholim, Sanguem and Sattari taluka (Indian Bureau of Mines, 2014). Moreover, there are many mineral-based fertilizer, pellets, iron and alloy processing organized industries present in Goa, which are present near the waterbodies. Hence, there are high chances that these heavy metals leaching into the water and harming aquatic organisms.

Toxicity of heavy metal occurs via various route of exposures, such as oral, dermal, respiratory, etc. In fishes and aquatic organisms, the potential route of entry of these contaminants is via gills, digestive tract and body wall. The affinity of the metals to bind to gills is higher than that of the body wall (Tekin-Özan & Aktan, 2012). These further alter the physiological and biochemical functioning of the body. Some compounds can cause cancers in the fish, cause changes in the hematological parameters. Chronic exposures of metals may have a nephrotoxic, hepatotoxic or neurotoxic effect on the organism. Certain heavy metals like zinc, cadmium, copper get accumulated in the gonads of the fish, reducing the fertility of fish and also causing defects in the embryonic development.

In humans, the route of exposure and causes might slightly differ. In addition to this, the toxic dose may also be higher as compared to other organisms. The main organ systems that are usually seen to be affected by heavy metal toxicity are respiratory system, excretory system, central nervous system etc. with lungs, brain, kidney and liver being the target organ for bioaccumulation (Singh, et al., 2022).

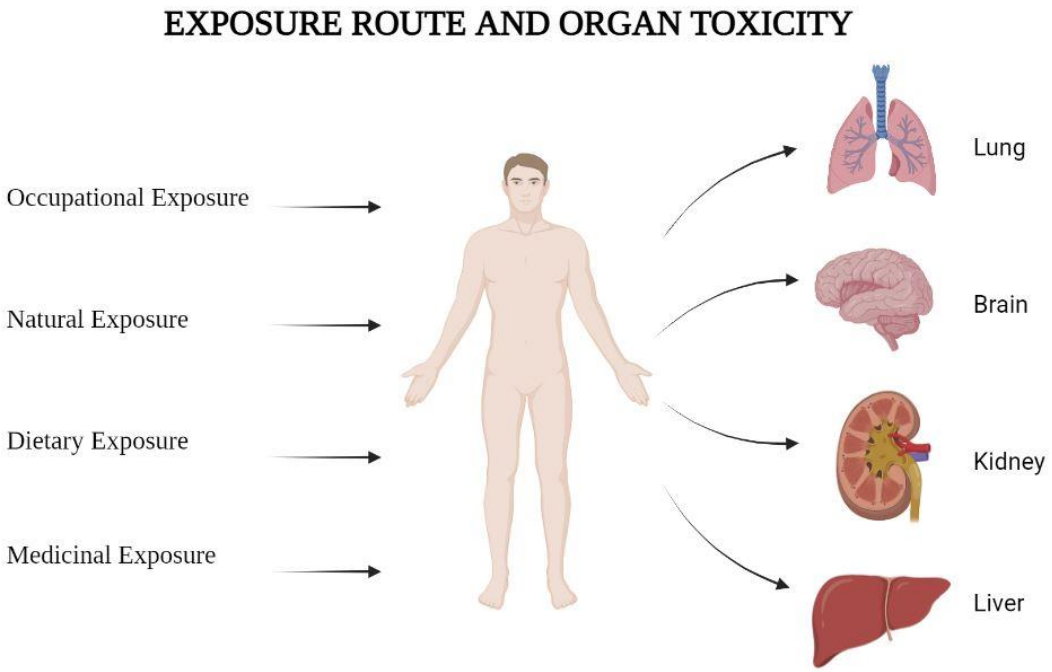



Fig 1.2: Exposure route and Organ Toxicity

## 1.1 *In vitro* Liver Cell Culture

*In -vitro* models have gained importance over the years in the field of research and medicine. The sole purpose of these models is to reduce experimental variables and effectively isolate components of an organ to be studied under controlled conditions. The most widely used *in vitro* models for liver toxicity studies involves the usage of immortalized cell lines and primary isolated liver cells. In recent times, bioartificial livers, and 3D culture systems have also been developed to study *in vitro* hepatotoxicity. *In vitro* results can be used to interpret cellular-response and cell communication pathways, in presence of a drug or a toxicant. It can be used to determine the modes and mechanisms of action of a substance, boost dose-response relationships, evaluate the effect of varying concentrations than *in vivo* results. Certain concentrations relative to human exposure, can also be tested in *in vitro* models, in order to provide information for generation of pharmaco-kinetic models, that lead to molecular investigations of toxicity pathways (Soldatow, et al. 2013).

The largest organ, Liver, has heterogenous cellular composition consisting of hepatocytes, Kupffer cells, fibroblasts, immune cells, liver sinusoidal endothelial cells (LSECs), biliary epithelial cells, and adult stem cells (Collins, et al., 2019). Hepatocytes are polygonal in shape and primary functional cells of the Liver. They are known to carry out a number of metabolic, endocrine and secretory functions of the body. These have a distinct nucleus, with 2 or more nucleoli. Majority of cells contain a single nucleus, however, binucleate hepatocytes are quite common. They are exceptionally active in biosynthesis of lipids and proteins, which also substantiates the presence of abundant quantities of rough and smooth endoplasmic reticulum. These cells also contain several stacks of Golgi membranes, that mostly assist in transport of bile.

Studies have shown that, in addition to metabolism, hepatocytes also play a role in activating the innate immunity by secreting innate immunity proteins. These proteins are either

bactericidal proteins that attack the bacteria or opsonins that cause phagocytosis of foreign substances. They also secrete several transcriptional factors that regulate the expressions of various innate immunity proteins (Zhou, Xu, & Gao, 2015).

Primary hepatocytes have been commonly cultured for *in vitro* testing as they can maintain functional activities for up to 24–72 hours, and can be used for studies concerning enzyme induction and inhibition, moderate screening of compounds, and are ideal for examining interspecific and inter-individual differences in metabolism of a substance. Primary hepatocytes cultures are often used as a predictive model with the anticipation that a substance will affect or be affected by an isolated cell in the same manner that would occur in the whole organ. In traditional primary hepatocyte cell culture, cells are plated on a rigid substratum in which they proceed to establish a monolayer across the bottom of the culture plate. During this process, primary hepatocytes undergo a process referred to as de-differentiation, where in there are changes in its morphology, structure, polarity, and liver-specific functions. This de-differentiation is an important drawback to *in vitro* system.

As the isolated cells have lost their normal hepatic microenvironment, there are alterations in cell-to-cell communications and cell membrane structures that triggers the response to differ than that occurring *in vivo*. Another limitation of the use of primary hepatocytes for toxicological research is the drastic decrease in cell viability and functionality over time. Studies have shown that liver specific functions such as production of albumin and expression of cytochrome P450 decline over the first 24–48 hours of the culture set up as the cells begin to lose their differentiated status (Soldatow, et al. 2013).

## 1.2 Manganese Dioxide ($MnO_2$) and Its Toxicokinetic

Manganese dioxide naturally occurs as a main ore of manganese called as pyrolusite. Manganese in nature, never occurs in its pure form. It is always found in combination with oxygen, chlorine and sulphur. Human bodies require manganese ions in trace amounts as it acts as a cofactor that enhances activity of various enzymes.

Artificially, manganese dioxide can be synthesized by hydrothermal processing of Potassium permanganate ($KMnO_4$). The synthesized nanostructure may have α, β or δ- $MnO_2$ crystal phase depending upon the concentration of $KMnO_4$ and treatment procedure. Differences in the crystal phases, further confer different morphology, structure and electrochemical activity to the synthesized nanostructures (Duan, et al., 2012).

Manganese dioxide is used in manufacture of dry cell batteries, in cleaning and safety equipments, in production of steel, in synthesis of paints, textile industries, and glass manufacturing. In recent times, manganese dioxide nanoparticles have gained importance in the field of medicine and in drug delivery system. They are also used in biosensors. Scientists have developed hollow nanoparticles of δ-$MnO_2$, in order to deliver certain chemical agents within the body (Greene et al, 2021). It has also been used in cancer therapies and as scaffold for stem cell transplantation. Some hybrid scaffolds that are biodegradable and biocompatible are also developed to deliver therapeutics. A study has also reported that manganese dioxide nanoparticles have antimicrobial activity towards gram-negative and gram-positive bacteria (Cherian et al, 2016).

Manganese in body is absorbed by 2 means, that is via ingestion and via inhalation. It is seen that the nanomaterials of manganese enter the hepatic system via blood circulation. These cross the air-blood barrier and gastrointestinal tract barrier. The liver is known to capture about 33-90% of the nanomaterials based on their origin and route of exposure (Zhang et al, 2016).

Manganese is known to have a cytotoxic effect that can impair a cell's transport systems, enzyme activities, and receptor functions. Studies have shown that it primarily targets the central nervous system, predominantly the globus pallidus of the basal ganglia. It is believed that the manganese ion ($Mn^{2+}$) enhances the autoxidation process, leading to increased production of free radicals, reactive oxygen species (ROS), and other cytotoxic metabolites, impairing the cellular antioxidant defence mechanisms. This it leads to oxidative damage and selective destruction of dopaminergic neurons. In addition to dopamine, manganese is known to trigger other neurotransmitters, such as GABA and glutamate (Crooks et al, 2007). The neurotoxicity of $Mn^{2+}$ has also been associated with its ability to substitute for $Ca^{2+}$ under physiological conditions. It enters mitochondria by means of the calcium uniporter and inhibit mitochondrial oxidative phosphorylation and may further inhibit the efflux of $Ca^{2+}$, which can result in a loss of mitochondrial membrane integrity. $Mn^{2+}$ has been shown to inhibit mitochondrial aconitase activity to a significant level, altering cellular iron homeostasis (Kwik-Uribe & Smith, 2006) and amino acid metabolism.

Manganese binds to alpha-2-macroglobulin, transferrin or albumin, in the plasma and is circulated to the brain and all other mammalian tissues. Nevertheless, it tends to accumulate excessively in the liver, pancreas, and kidney. Manganese is capable of existing in various oxidation states, however, is believed to undergo changes in oxidation state within the body such that there is its influence on the tissue toxicokinetic behaviour, giving rise to probable toxicity (Crooks et al, 2007). A transmembrane metal-ion transporter, SLC39A14, is a that can mediate the cellular uptake of manganese, in addition to iron and zinc, in liver and pancreas. Thus, maintaining Mn homeostasis. A recent study in mice reported that knockout of SLC39A14 led to elevated levels of Mn in blood, bones and brain, due to impaired uptake by liver and pancreas and reduced gastrointestinal excretion (Jenkitkasemwong, et al., 2018).

## 1.3 OBJECTIVES

### Use of $MnO_2$ as Potential Larvicide

A recent work by Naik, et al. (2021) reported that α-$MnO_2$ nanostructures had a larvicidal activity against 3 species of mosquito larvae namely, *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi*. There was 100% mortality observed at the concentration of 20, 30 and 200 ppm for *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* respectively. Similarly, $LC_{50}$ values were 5.39ppm against *Culex quinquefasciatus*, 8.83 ppm against *Aedes aegypti* and 168.324 pp against *Anopheles stephensi*. Histopathological studies of larvae treated with $MnO_2$ showed damaged cells and tissues in the mid-gut region. The damaged tissues suffered rupture and disintegration of the epithelial layer, cellular vacuolization, and accumulation of the nanoparticles in the thorax and abdomen.

Hence this study proved that small size, chemical neutrality and good physical activity of $MnO_2$ nanostructures makes it an effective mosquito larvicide, which will help to control the mosquito population, which is a renowned vector for many vector-borne diseases like malaria, filariasis, chikungunya, yellow fever, Japanese encephalitis, Zika, and dengue etc.

However, before administering the larvicide in the form of $MnO_2$ nanostructures, its toxicity has to be determined and the doses have to be strictly monitored.

Since it is evident from the literature that heavy metal compounds cause hepatoxicity, and usually liver is the primary organ to be affected due to its detoxification action on the body, the objective of the present study is to:

**Evaluate the Toxicity of Manganese Dioxide ($MnO_2$) nanostructures on the liver cell culture of Tilapia fish, *Oreochromis mossambicus*.**

# CHAPTER 2:

# REVIEW

# OF

# LITERATURE

# 2. REVIEW OF LITERATURE

Heavy metals are known to cause various problems in the bodies of living organisms. Various studies have indicated that heavy metals and their compounds cause severe damage to tissues at a cellular and genomic levels. Webera, et al., 2020 conducted a study to check the effect of metal contamination on liver of 2 species of fish. It was observed that the site having higher contamination of heavy metal showed a higher degree of hepatic damage such as cytoplasmic vacuolization, hepatic tissue necrosis, flat nucleus at cell periphery etc. The immunological assay of Metallothionein (MT) & Cytochrome P450 1A (CYP1A) indicated positive result in the site having higher contamination, thus indicating higher oxidative stress to the tissue. Rajeshkumar, et. al. (2013) monitored the effect of heavy metals contamination on expression of HSP70 protein in *Chanos chanos* and concluded that heavy metal increased the expression of HSP70, thereby decreasing the activity of antioxidant enzymes; and also lead to formation of large lipid droplets in the hepatocytes which in turn changed the morphology of the organelles. Padmini & Rani (2009) evaluated the oxidative stress faced by hepatocytes of *Mugil cephalus* that were obtained from polluted estuary, wherein they observed extensive vacuolization, enlarged size and increased cell necrosis and membrane disruption in the hepatocytes. The heavy metal pollution had also impacted the antioxidant enzyme activity and lowered it to a drastic level.

Agrawal & Srivastava's (1980) study on a teleost, *Colisa fasciatus* revealed that manganese poisoning can lead to erythropenia, wherein there is significant decrease in total erythrocyte count, in addition to leukocytosis & increase in the number of small lymphocytes upon acute exposure to manganese sulphate. Moreover, giving rise to the respiratory system failure. Rodrigues, et al. (2020) revealed that $MnCl_2$ causes behavioral aberrations, and at cellular

levels, it caused vacuolization of hepatocytes after a prolonged *in-vivo* exposure, in addition to formation of micronucleus in erythrocytes upon acute and chronic exposures.

Herrera & Catap (1991) exposed *Tilapia niloticus* to manganese chloride, and exhibited that it triggered hyperplasia of gill lamellar epithelium, vacuolations in liver and small intestines and renal lesions in kidney. The liver tissue showed severe necrosis as a result of hepatocyte hypertrophy, nuclear pyknosis and mitochondrial damage. Moreover, ovarian tissue showed a significant decrease in number of oocytes. Browning, et. al. (2021) synthesized $MnO_2$ nanosheets and administered them to RTG cells at sub-toxic levels, which induced severe mitochondrial dysfunction and impaired mitochondrial membrane potential. It was conclusive that, $MnO_2$ nanosheets decrease the responsiveness of the gill cells to the energy demands, inhibiting the ATP production drastically.

Compounds containing heavy metals and other substances can cause severe oxidative stress to cells and organelles. In turn, they can cause an alteration in the activity of anti-oxidant enzymes that is used by the cells to cope up with the oxidative stress. Vieira, et.al. (2012) determined effect of acute toxicity of manganese in goldfishes and revealed that manganese induced changes in functioning of antioxidant enzymes, wherein it provoked increase in superoxide dismutase activity in renal, hepatic and gill tissue and caused inhibition of catalase activity in liver and kidney, thus generating an oxidative stress in hepatocytes and other organs of the fish. An experiment conducted by Hoseini, et. al. (2014) on *Rutilus rutilus*, exposed to waterborne manganese showed that at higher concentrations, manganese caused an increase in plasma cholesterol, glucose and triglyceride levels. Similarly, a significantly higher activity of Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was noted in the exposed fish. Higher thyroid hormone (T4 and T3) levels were observed as a result of Mn toxicity.

Nath & Kumar (1987) enumerated the impacts of manganese on carbohydrates metabolism of *Colisa fasciatus*, that showed a decrease in liver glycogen over the 48h exposure set up, thereby accounting for a significant hypoglycemic response. However, the blood glucose showed a significant elevation, reaching its peak at 96h exposure period. This study depicted that manganese induced stress produced a hyperglycemic effect on the freshwater fish. Falfushynska et. al. (2011) depicted in their study that, *Carassius auratus* samples collected from polluted site, had high concentrations of manganese and copper, showed genotoxic effects and decrease in total glutathione activity in the liver. There was a significant oxidative stress and impaired responsivity to metallothionein in the samples obtained from polluted site.

Heavy metals and their compounds hold a significant potential of getting bioaccumulated in the tissues and cells. There have been several studies carried out to get an idea about the bioaccumulation potential of heavy metal compounds in tissues and cells. Abdullah et. al. (2007) exposed *Labeo rohita* to heavy metals like iron, manganese, nickel, zinc and lead, and concluded through their results that the fish was highly sensitive to lead and showed high tolerance to manganese. However, it showed highest ammonia excretion under the manganese toxicity. Further analysis had also suggested that manganese had acute toxic effects on the fish. Another study carried out by Oliveira et. al. (2008) on *Prochilodus lineatus,* revealed that manganese showed highest degree of bioaccumulation in bile, gills and liver tissue. Accumulations were also noted in kidney tissues, post 96 h treatment. It also caused oxidative damage in liver and DNA damage in blood cells. Baden & Eriksson (2006) suggested that in crustaceans, manganese accumulations prominently occur in gills, haemolymph, midgut gland and muscles. The highest concentrations of manganese, however, are seen in exoskeleton of decapod crustaceans.

Dunier's (1996) study conducted on freshwater fish exposed to agricultural fertilizers and pesticides and industrial pollutants revealed that, manganese was heavily accumulated in lymphoid organs such as kidney and spleen. It also suggested that Mn *in vitro* stimulated the spleen to induce phagocytosis of cells. Mn can be thus considered to have certain an immunotoxic effect, as it inhibited lymphocyte proliferation. Nair et. al. (2006) analysed 17 species of fish collected from a polluted waterbody and concluded that highest accumulation of heavy metals occurs in liver, followed by gills and muscles. It was observed that of commercially important fishes like *Sardinella longiceps* (Sardines) and *Rastrelliger kanagurta* (Mackerel) were found out to hold maximum concentrations of manganese in their liver and gill tissue respectively. Coppo, et al. (2015) study suggested that in *Oreochromis niloticus*, bioaccumulation of manganese is highest in liver than gills and lowest in muscle. In addition to this, the results indicated that, exposed individuals showed presence of micronuclei in erythrocytes and increase in glutathione S-transferase and catalase enzyme activity. Kaur et. al. (2018) analysed *Labeo rohita* samples exposed to heavy metals and revealed that it had induced histopathological alterations in liver, muscle and kidney causing hepatic lesions, severe necrosis, cytoplasmic and nuclear degeneration, pyknosis and infiltration of leukocytes in the liver tissue. The accumulations of manganese were also observed in muscle tissue histology, which led to shortening and elongation of muscle bundles. Renal alterations like edema, degeneration and atrophy of renal tubules were also noted.

# CHAPTER 3:

# METHODOLOGY

# 3. MATERIALS AND METHODOLOGY

## 3.1 $MnO_2$ synthesis and Characterisation (Naik et. al, 2021)

$MnO_2$ nanostructures were synthesized in the lab of Dr. Shrikanth Naik, Assistant Professor, School of Chemical Sciences, Goa University. The nanostructures were prepared using a wet chemical redox reaction between $KMnO_4$ and $MnSO_4$. The reaction led to formation of brownish-black precipitate that was confirmed to be hydrated $MnO_2$. The precipitate was purified by washing several times to remove the ionic by-products. The characterisation of the nanostructures was carried out in Central Sophisticated Instrumentation Facility (CSIF), BITS Pilani, Goa campus. The morphology of the nanostructure was determined using Quanta FEG 250 Field Emission Scanning Electron Microscope. The crystallinity, crystal structure and phase identification were determined using Bruker D8 Advance X-ray diffractor. The zeta potential was also analysed to get an idea of the charge that the synthesized nanoparticle carries.

## 3.2 Sterility Maintenance

The glassware and dissection kit used for the experiments was washed and cleaned using detergents. It was rewashed using distilled water, packed and autoclaved. This was further placed in hot air oven in order to dry. The falcon tubes, magnetic beads and micropipette tips were subjected to double autoclave in order to remove any impurity. The nanoparticles were double autoclaved, prior to suspending in media and eventually exposing it to the cells. The working surface inside the laminar air flow hood, was thoroughly wiped with 70% ethanol, prepared in distilled water. The UV was switched on for 30 mins so as to ensure sterile environment prior to starting the experiment. The incubator was wiped with 70% ethanol prior to using it for culture maintenance. Only autoclaved MilliQ water was used for preparation of

1x PBS from 10x stock. Additionally, antibiotics obtained from Hi-Media were used in formulating complete working media, to avoid bacterial and fungal contamination in the culture, during the study period. All experiments were carried out wearing a lab coat, face mask and sterile pair of latex gloves.

## 3.3 *In-vitro* liver cell culture

Apparatus required

Dissection scissors, blunt and fine forceps, scalpel, glass petri plates, beakers, autoclave pouches, 15 ml falcon tubes, 50 ml falcon tubes, adjustable micropipettes (100-1000µl, 20-200µl, 2-20µl), micropipette tips, 70µm nylon mesh, magnetic bead, 3.5mm Cell Culture treated Petri dish, etc.

Chemicals Preparation

- Complete/Working L-15 media

  45 ml L-15 media was supplemented with 5 ml FBS and 200µl antibiotic solution. This was prewarmed in water bath at 37ºC, prior to subjecting it to the cells. This was used for maintenance of the cell culture

- 1x PBS

  5 ml 10 x PBS stock solution was diluted with 45 ml sterile autoclaved MilliQ water, to prepare 50 ml of 1x PBS. This was used to wash the cell pellet.

- 70% ethanol

  70 ml alcohol was diluted with 30 ml distilled water to prepare 70% ethanol. This was used in maintaining sterility in the experiment.

Procedure

The primary liver cell culture was set up using enzymatic disaggregation method as specified by (Figueiredo et al, 2021), with modifications. Warm trypsinization method was used to isolate the cells from the liver tissue. The fish was euthanized by placing in cold water for 30 mins. The specimen was sprayed with 70% ethanol and taken inside the Laminar Air Flow hood. The dissection procedure was carried out in Laminar Air Flow hood in order to avoid contamination. The liver tissue was washed 3 times using Hank's Balanced Salt Solution (HBSS), containing HEPES Buffer. The liver was then cut into smaller pieces and placed in the beaker contained pre-warmed trypsin (37ºC) and placed on a magnetic stirrer with a magnetic bead for about 15-20 mins. Once the liver tissue was completely disaggregated, it was filtered through a nylon mesh, having pore size of 70µm, into a 15 ml centrifuge tube. Equal part of Foetal bovine serum (FBS) was added to the same, in order to denature trypsin and further stop the enzymatic digestion. The cells were pelleted using slow centrifugation (1000 rpm for 5 mins) and the supernatant was discarded. The pellet was washed twice with 1x Phosphate Buffered Saline (PBS). The pellet also subjected to 1-time plain media wash using Leibovitz, L-15 media. The cells were resuspended in complete L-15 media, supplemented with 10% FBS and 2% Antibiotic-Antimycotic solution.

The cells yield was counted using haemocytometer and viability was assessed using Trypan Blue exclusion assay, prior to seeding in 48-welled plate. The culture was maintained for 24-, 48- and 72-hours post exposure in a humidified BOD incubator at 33 ºC.

## 3.4 Trypan Blue Exclusion Assay

Apparatus required

Haemocytometer, Coverslip, Eppendorf tube, 0.4% trypan blue stain prepared in PBS, 20-200µl micropipette.

Chemicals Preparation

- 0.4% Trypan blue stain

  0.04 g or 40 mg of research grade trypan blue powder was mixed in 10 ML of 1x PBS. The solution was then filtered through Whatman filter paper and used for counting.

Procedure

The haemocytometer was cleaned thoroughly with 70% ethanol. 10 µl of cell suspension was taken in a sterile Eppendorf tube and 10 µl of Trypan Blue stain was added to it. The contents of the Eppendorf tube ere thoroughly mixed and added to the Neubar chamber or the haemocytometer. The cells were counted under 10x magnification of the objective. The cell density was determined using the formula:

Cell density (cells/ml) = Average no. of viable cells x 2 x $10^4$

The percentage of viable cells was determined using the formula:

% of viable cells = (No. of viable cells/ Total no. of cells) x 100

## 3.5 Toxicity tests

### 3.5.1 MTT Test (Mosmann, 1983)

Apparatus required

48-welled cell culture plate, Eppendorf tubes, weighing balance, autoclave, water bath Ultra-sonicator, incubator, micropipettes, micropipette tips, 96-welled plate, ELISA plate reader with 595nm wavelenth filter.

Chemicals Preparation

- $MnO_2$ stock (5 mg/ml)

  5 mg $MnO_2$ nanostructures were weighed in a 2ml eppendorf tube. This was further autoclaved to reduce chances of contamination. The nanostructures were suspended in 1 ml of complete media, making the concentration of stock to 5 mg/ml.

- MTT Reagent

  30 mg of MTT powder was added to 10 ml 1x PBS in sterile condition. The falcon tube was wrapped in a foil as MTT is light sensitive. The regent was freshly prepared every time, prior to the assay.

Procedure

Isolated cells, showing >90% viability, were seeded in 48-welled plate ($10^5$ cells/well) and incubated. Post the incubation, the remnant culture media was removed and replaced with media containing 5 different concentrations of $MnO_2$, namely 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml. The plated were incubated for the period of 24hr, 48 hr and 72 hrs. Upon the period of every exposure, the media was aspirated out from the wells and replaced with 300 µl of freshly prepared MTT reagent. The plate was incubated in the incubator

for 3-4 hrs. upon the incubation period, excess of the reagent was aspirated out, keeping the formed formazan crystals undisturbed. The formed formazan crystals were dissolved in DMSO and the absorbance was read at 595nm.

### 3.5.2 Exposure Set Up

For post-MTT toxicity analysis, the cells were exposed to 3 concentrations of 100 µg/ml, 150 µg/ml and 200 µg/ml. A stock solution of 5 mg/ml was prepared by suspending $MnO_2$ nanostructures in complete media and sonicating the mixture in water bath ultra-sonicator. The stock was further diluted with complete media to obtain the required concentrations. The cells were cultured in 3.5mm petri-dish prior to the exposure. The cells were than exposed to prepared concentrations and further analysis was made.

### 3.5.3 Protein estimation (Lowry et. al., 1951)

Apparatus required

Test tubes, test tube stand, glass cuvettes, nanodrop, spectrophotometer, reagent bottles, micropipettes.

Chemical Preparation

- Lowry's Reagent

Solution A (100 ml): 0.57g NaOH + 2.86g $Na_2CO_3$ dissolved in 100 ml distilled water

Solution B (50 ml): 0.7g $CuSO_4.5H_2O$ dissolved in 50 ml distilled water.

Solution C (50ml): 1.4g Sodium tartrate dissolved in 50 ml distilled water.

Solution A, B and C were mixed in the ratio 100:1:1 respectively to prepare Lowry's reagent.

❖ BSA stock

10 mg BSA was dissolved in 40 ml NaOH to make BSA stock solution.

❖ Folin-Ciocalteau Reagent

10 ml Folin-Ciocalteau reagent was mixed with 20 ml distilled water to make the reagent.

Procedure

The standards were prepared by adding 5ml Lowry's reagent to series of concentration of BSA and incubating it for 10 mins at room temperature. Post incubation, 0.5 ml Folin's reagent was added to obtain the colour. The blank solution was prepared adding distilled water. For the samples, the cultured cells were dissociated from the plate and centrifuged at 1200rpm for 7 mins, in order to obtain the pellet. The supernatant was discarded and the cells were suspended in 1 ml cold phosphate buffered saline, and centrifuged for 3000 rpm for 10 mins. The supernatant was used estimate protein. The absorbance was read against the blank at 660nm.

### 3.5.4 **Catalase test** (Sinha, 1972)

Apparatus required

Test tubes, test tube stand, glass cuvettes, reagent bottles, micropipettes, homogeniser.

Chemical Preparation

❖ 0.2 M $H_2O_2$

2.06 ml $H_2O_2$ was diluted with 98ml distilled water, making the final volume of the solution, up to 100ml.

❖ 5% Potassium Dichromate Reagent

5% potassium dichromate solution was prepared by dissolving 0.5g potassium dichromate crystals in 10 ml distilled water. The reagent was prepared by mixing 30 ml glacial acetic acid and 10 ml potassium dichromate solution making the final volume up to 40 ml.

❖ Phosphate buffer

6.8 g $KH_2PO_4$ and 8.9g $Na_2HPO_4$ was weighed and dissolved separately in 100 ml distilled water to make 0.5 M concentration of both the solutions. 31.3 ml 0.5 M $KH_2PO_4$ and 22.8 ml of 0.5 M $Na_2HPO_4$ was mixed and diluted with distilled water to make the final volume up to 500 ml. The pH was adjusted by adding 1N HCl and monitored using a pH meter.

Procedure

In the test tube, 1.5ml phosphate buffer and 0.4ml 0.2 M $H_2O_2$ was added and incubated for 5 min at 37ºC in a water bath. In one set, 0.1 ml Distilled water was added to prepare blank, followed by preparation of standard series having different concentrations of $H_2O_2$. The cultured cells were homogenized and 0.1 ml of the control and each experimental cell homogenate were also added in different test tubes to prepare the reaction mixture. These were further incubated for 15 min at 37ºC in a water bath. The reaction was stopped using 2 ml of potassium dichromate reagent. The test tubes were heated in boiling water bath for 10 mins. the solution was mixed well and the absorbance was read against the blank at 620nm. The concentration of $H_2O_2$ in samples was determined using the formula;

Amount of $H_2O_2 = (A_{test}/A_{std})$ x Conc. of std

Where A → Absorbance

The catalase estimation was carried out using the formula;

Activity= 1/t log Conc. of std/Conc. of test

Where t → time of incubation

### 3.5.5 Micronucleus Test (Nagpure, et al., 2007)

Apparatus required

Slides, coupling jars, micropipettes, beakers, light microscope, immersion oil

Chemical Preparation

❖ Giemsa stain

0.5g Giemsa powder was dissolved in 33 ml glycerol. This solution was kept for stirring at 60ºC for 2 hr. It was cooled to room temperature and 33 ml methanol was added to the mixture. The solution was filtered through the Whatman filter paper. The stock was stored in tinted glass bottle. For the working solution, 5 ml of Giemsa stock was mixed with 45 ml Phosphate Buffer and used for staining.

Procedure

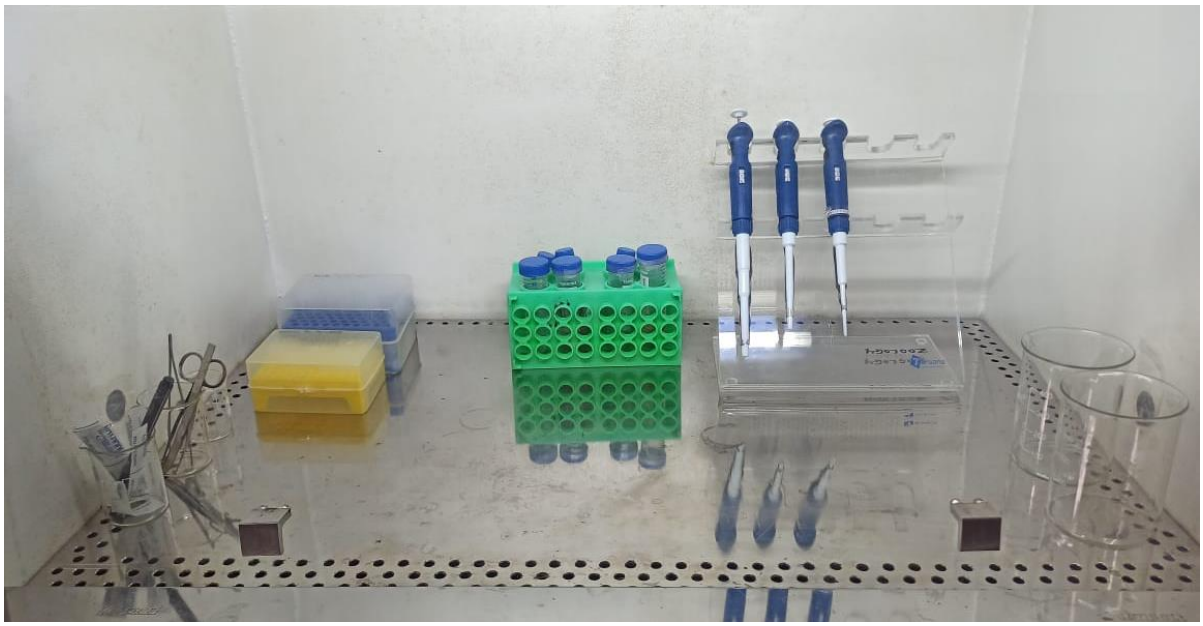
The glass slides were thoroughly cleaned and dried. A drop of cultured cell suspension was added on each slide and smeared. The slides were air dried and dipped in methanol for fixation. Slides were again air dried, followed by staining with Giemsa stain for 30 mins. The slides were washed in phosphate buffer and were allowed to air dry. These were mounted on a light-microscopes and were viewed using immersion oil under the 100x power of the objective.

About 2000 cells were counted and the cells were checked for formation of micronuclei. The cells containing micronuclei were also recorded.

## 3.6 Statistical Analysis

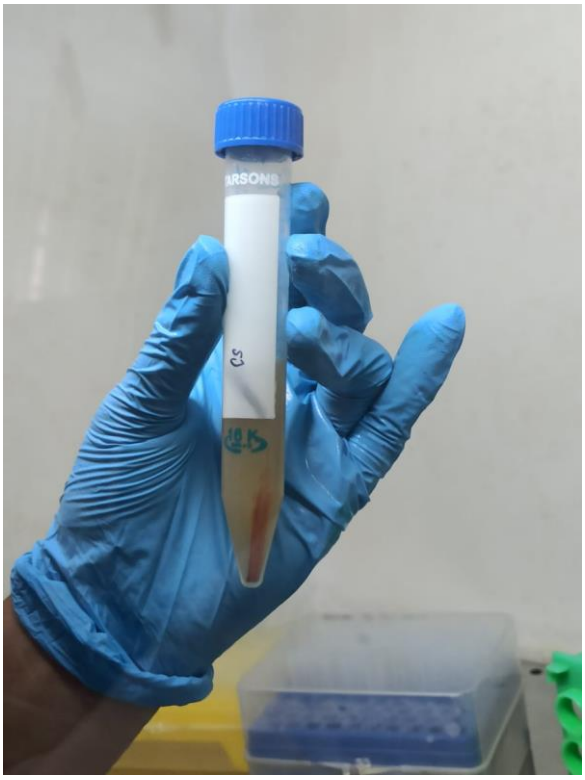
The data sets were given a mean value along with standard deviation. Statistical analysis was carried out using GraphPad Prism 8.01 for windows. The different parameters were analysed using one-way ANOVA. Post hoc tests such as Tukey's test were also used for analysis to obtain a significant result. Data was considered significant at $p<0.05$, $p<0.01$, $p<0.001$.

Plate 1: Setting up of culture



(a) Set up in Laminar air flow

(b) Dissection of Fish



(c)Cell pellet post-tissue disaggregation

# CHAPTER 4:

# RESULTS

# 4. RESULTS

## 4.1 Characterisation of $MnO_2$ Nanostructures

The synthesized $MnO_2$ nanostructures were subjected to X-ray diffraction studies, SEM analysis and Zeta Potential. The XRD pattern showed that the $MnO_2$ has nanosized dimensions. The broad peaks are indicative of its fine nature and poor crystallinity. The XRD data also confirms the phase of the nanostructure to be α-$MnO_2$. The SEM images reveal a sea-urchin like morphology of the nanostructure, with spikes seen consistently throughout the sample. The size of the same varies between 200-400 nm. The Zeta Potential analysis shows that the charge on the sample is -4.81mV, which falls in between -10 mV to +10mV and is considered to be neutral (Clogston & Patri, 2011).

## 4.2 *In vitro* culture of liver cells

The culture of liver cells in *in vitro* set up was successfully established. Upon isolation, the liver cells appreared as single cells, showing spherical, rounded morphology, as seen in the microscopic analysis (Plate.2-a). It was seen that the viable cells were attached to the substratum (cell culture treated petri plate) post 24-36 hours of the incubation at 33ºC. 24 hours post incubation, some cells appeared to form smaller clusters, and there was loss of spherical morphology seen in the cells (Plate.2-b and Plate.2-c). The cell viability was seen to reduce greatly post 72 hour incubation, as seen through the microscopic analysis (Plate.2-d)

Fungal comtamination was a major problem faced during the maintenance as seen in (Fig 4.2e) The trouble-shooting of the same was carried out by maintaining strict sterile condition at every step of the culture set up. The dose of the antibiotic in culture media had to be increased from 1% to 2% in order to avoid the grown of unwanted microorganism.

## 4.3 Trypan Blue Exclusion Assay

The viability of the cells as tested via trypan blue exclusion assay was approximately 90-95%, post enzymatic disaggregation of the tissue (Plate. 3). The cells were counted prior to seeding in 48-welled plate. The seeding density for each well was approximately kept around $3x10^4$ cells in order to maintain a good sample size and obtain accurate results.

## 4.4 Toxicity Tests

### 4.4.1 MTT Assay

The results of the MTT are shown in graph no. 4.2.1, 4.2.2 and 4.2.3 for 24 hr, 48 hr and 72 hr respectively. The MTT assay showed the lowest viability of cells at highest concentration of nanostructures, that is 500µg/ml. When compared to the control values, the exposed values of all 3 exposure set up have shown decreasing viability of cells with increasing concentration, as seen in graph no. 4.2.4.

Significance was tested using one-way ANOVA analysis for the MTT assay, and the results obtained for 24 hr, 48 hr and 72 hr were $p<0.001$***, Hence, relationship between the exposure concentration and cell viability was found to be very significant for the time frame of 24 hr, 48 hr and 72 hr. Tukey's test was performed as a part Post hoc test to further validate the results, the values of which are presented in the graph no. 4.2.1, 4.2.2 and 4.2.3 for 24 hr, 48 hr and 72 hr respectively.

### 4.4.2 Protein Estimation

The results of protein estimation of the control and treated cells are depicted in graph 4.2.5. It was seen that the protein content of the cells initially showed an increase, however, at the highest concentration of $MnO_2$, the protein concentration shows a decline. Significance tested

using one-way ANOVA showed the p-value to be $p<0.01^{**}$. Post hoc analysis (Tukey's test) showed significance between control and highest concentration (200 µg/ml), but no significance between the control and lower exposure concentrations ($p>0.05$).

### 4.4.3 Catalase Test

The catalase enzyme activity is depicted in graph 4.2.6. A decrease in enzyme activity was observed as the exposure concentrations were increased. Simultaneously, graph 4.2.7 indicating the $H_2O_2$ concentrations, showed an increasing trend. The enzyme activity was seen to be at its lowest at the highest concentration of the exposure set up. Whereas, the $H_2O_2$ concentrations found in the sample treated with highest concentration of $MnO_2$ was found to be highest.

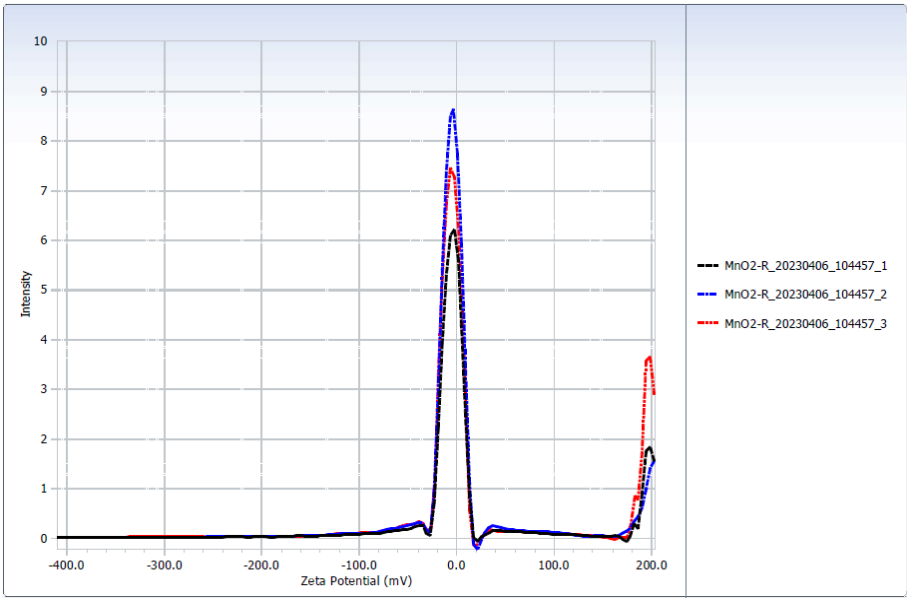
One-way ANOVA results showed the p value to be $p<0.001^{***}$ for catalase activity. The Tukey's test for the same had significant outcome as depicted in the graph

### 4.4.4 Micronucleus Test

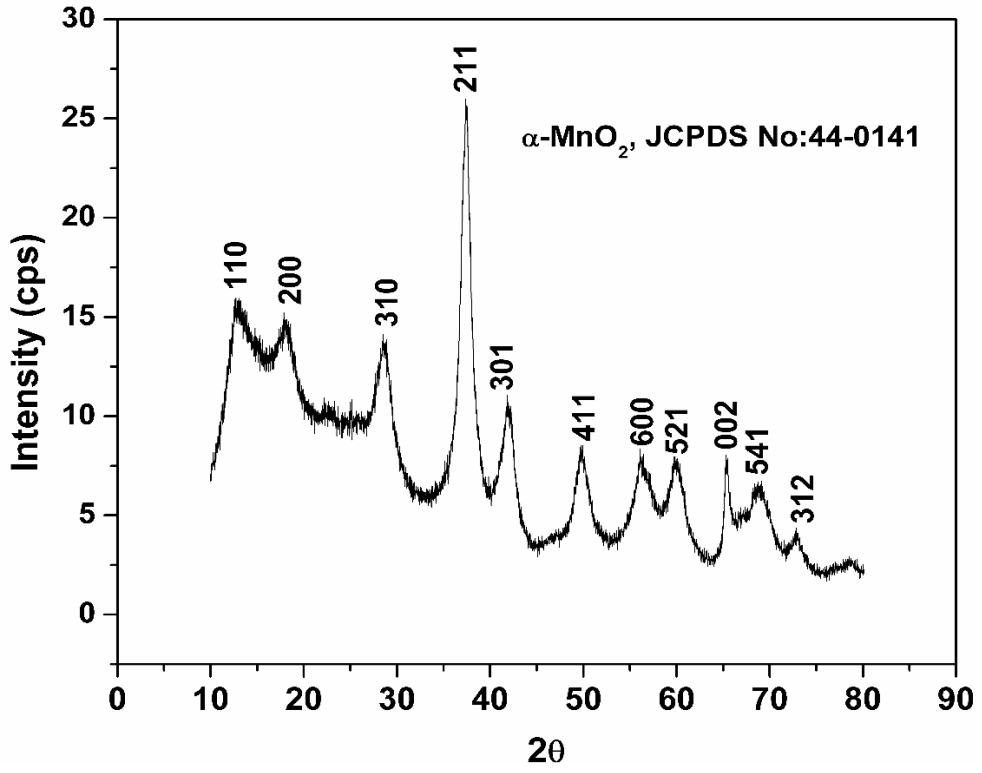
The results of the micronucleus test are depicted in the graph 4.2.6. The exposed cells have shown the presence of a prominent micronuclei (Plate 4). The results confirm that, percentage of micronuclei found in exposed cells were higher than the control. The highest number of micronuclei found in the cells exposed to 150 µg/ml concentration.

One way ANOVA results showed significant variation in the micronuclei found in cells exposed to different concentrations of $MnO_2$, since p-value was $p<0.05^{*}$.

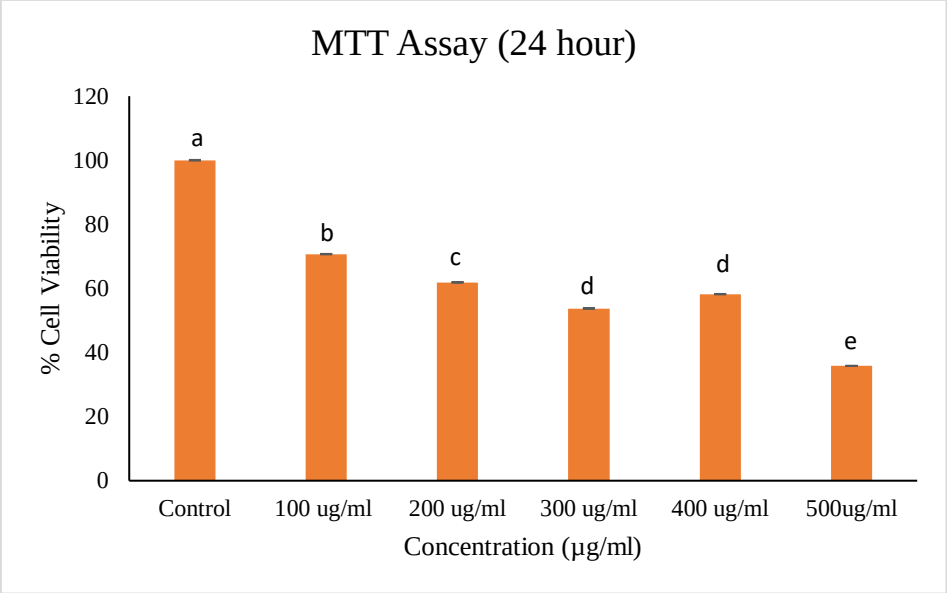
**Graph 4.1.1: Zeta Potential curve for $MnO_2$ nanostructures**




**Graph 4.1.2: X-ray Diffraction of $MnO_2$ nanostructures**



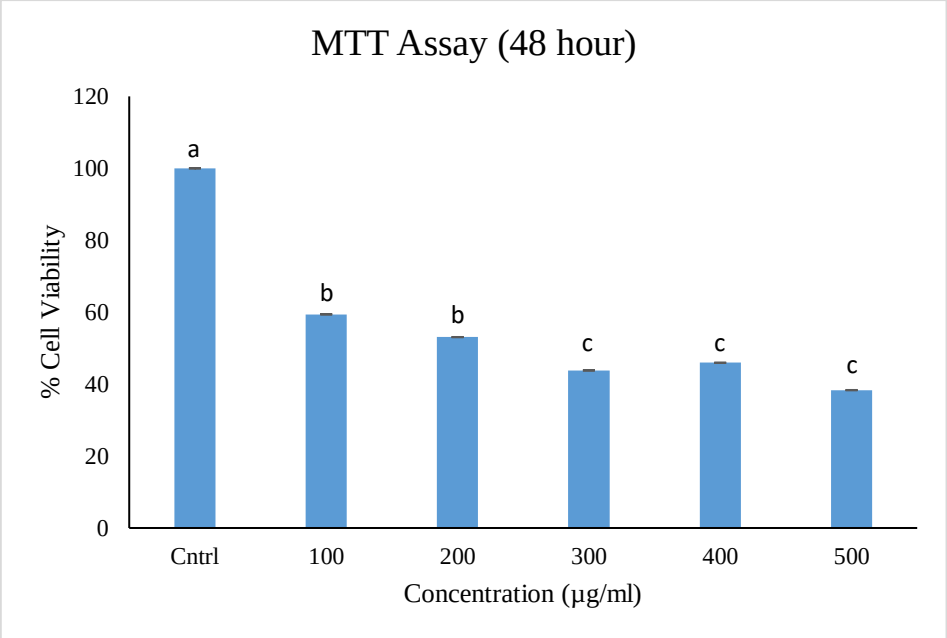
## Graph 4.2.1: Percent Cell Viability post 24 hr exposure




*p<0.001****

*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

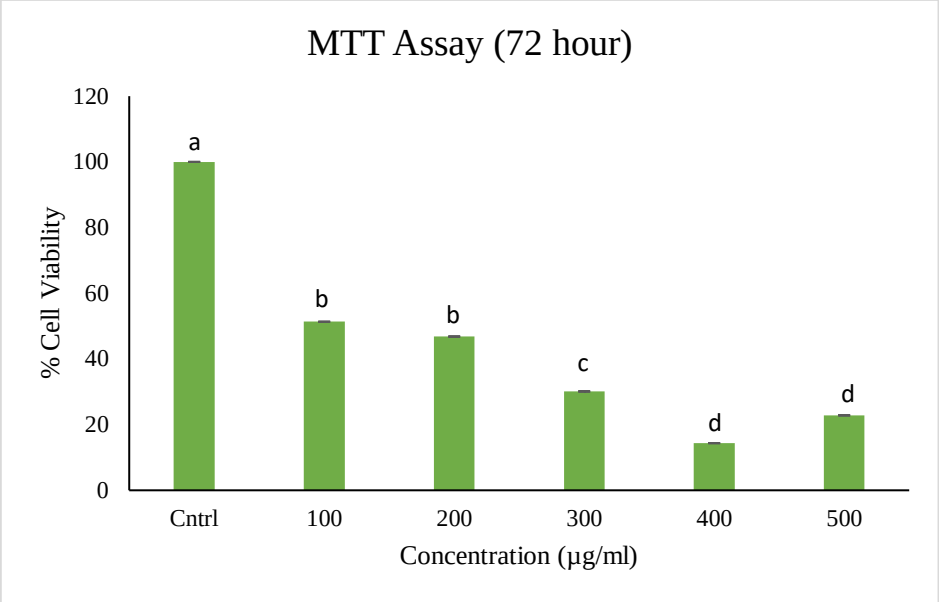
## Graph 4.2.2: Percent Cell Viability post 48 hr exposure




*p<0.001****

*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

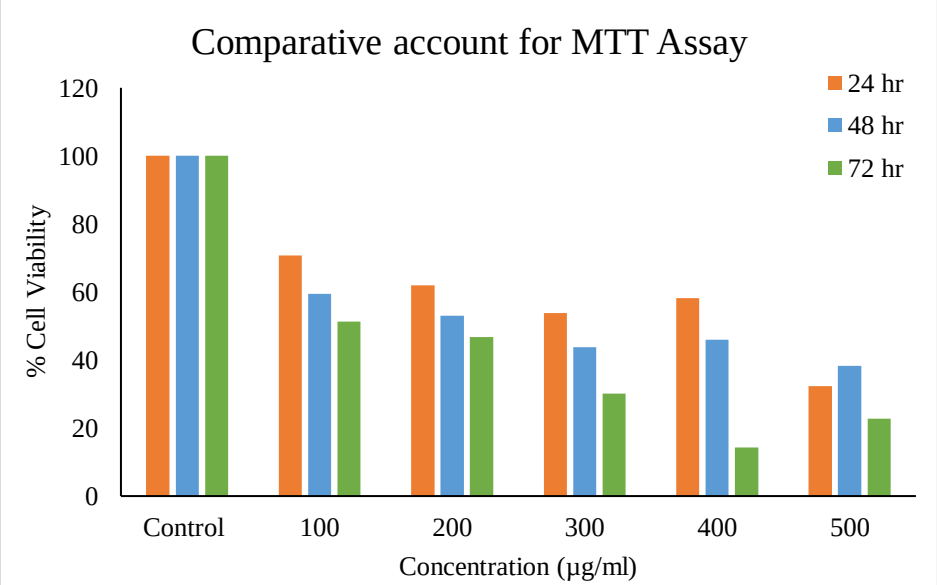
## Graph 4.2.3: Percent Cell Viability post 72 hr exposure



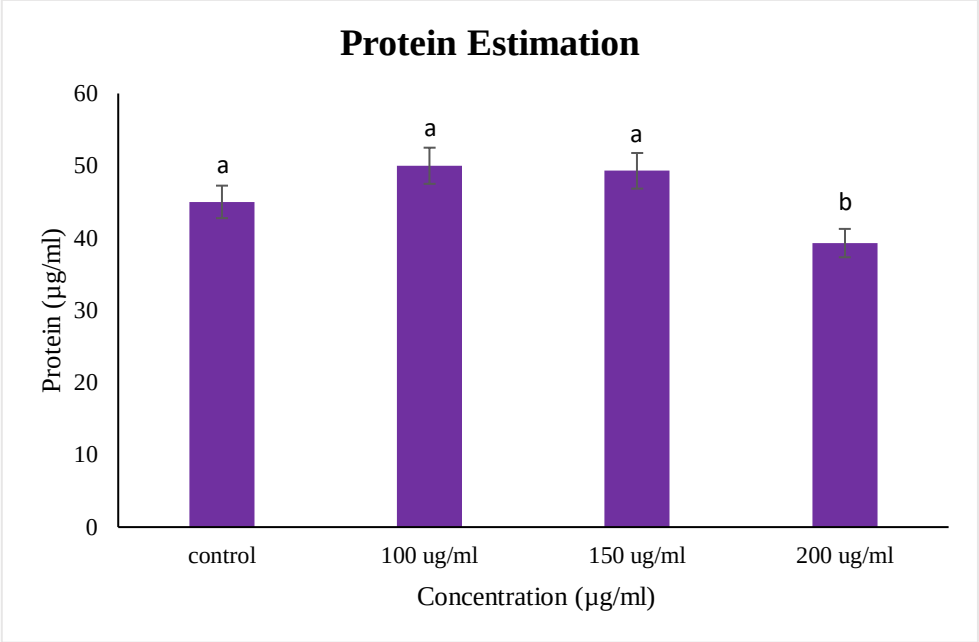

*p<0.001****

*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

## Graph 4.2.4: Variation in Percent Cell Viability



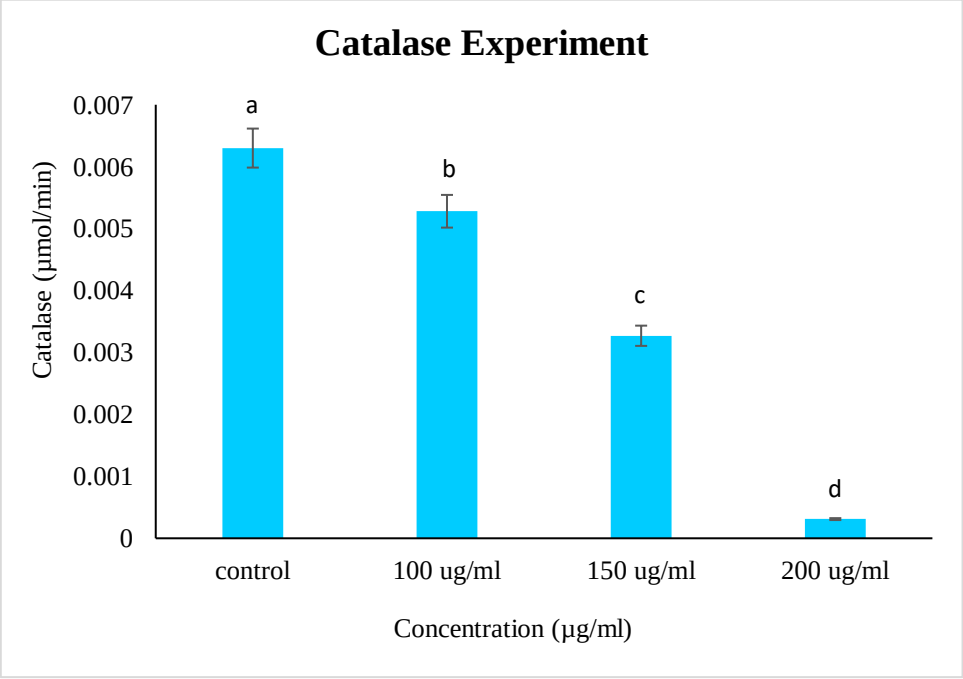
## Graph 4.2.5: Protein Estimation of Cultured Cells




*p<0.01***

*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

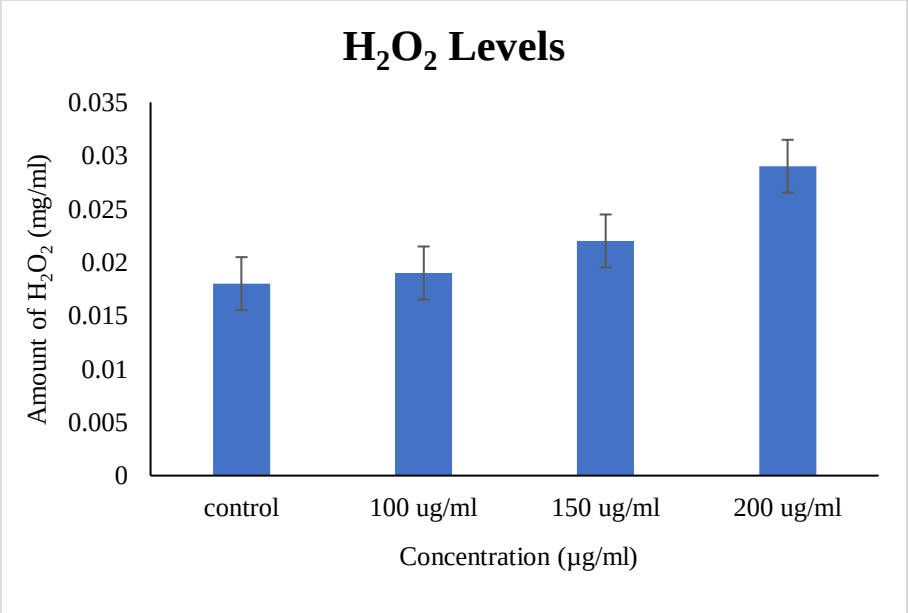
## Graph 4.2.6: Catalase Estimation of Cells



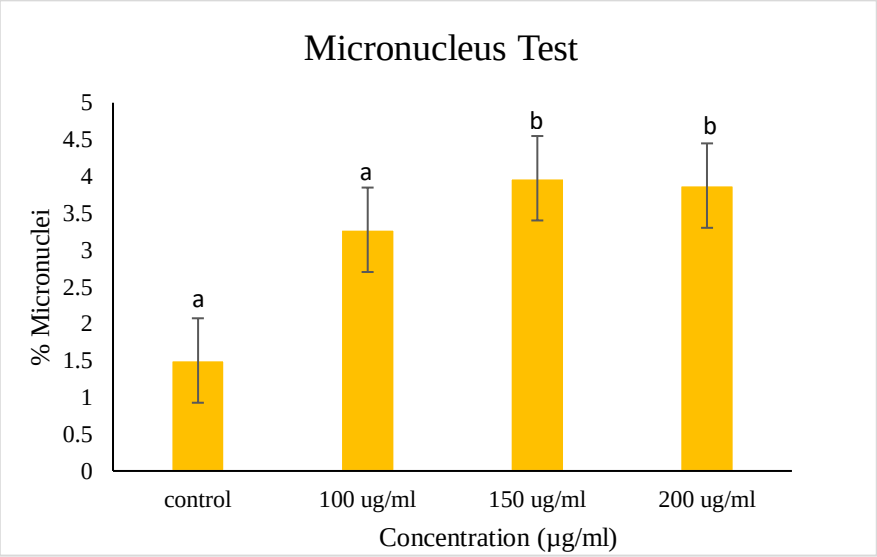

*P<0.001****

*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

## Graph 4.2.7: Levels of $H_2O_2$ in Cells



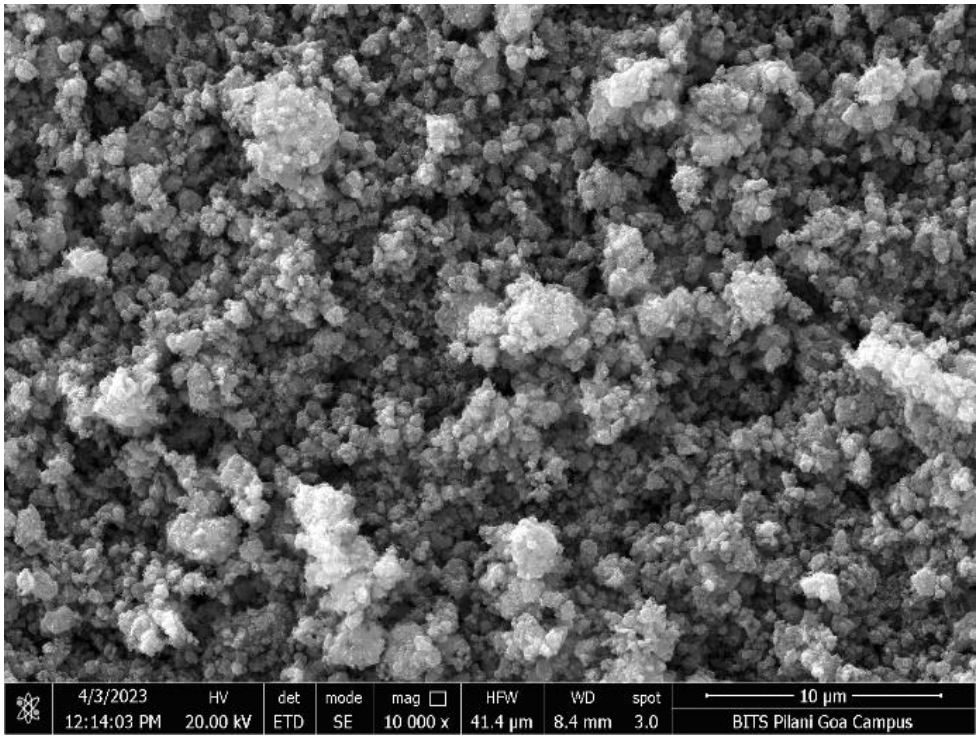

## Graph 4.2.8: Micronucleus Test of Exposed Cells

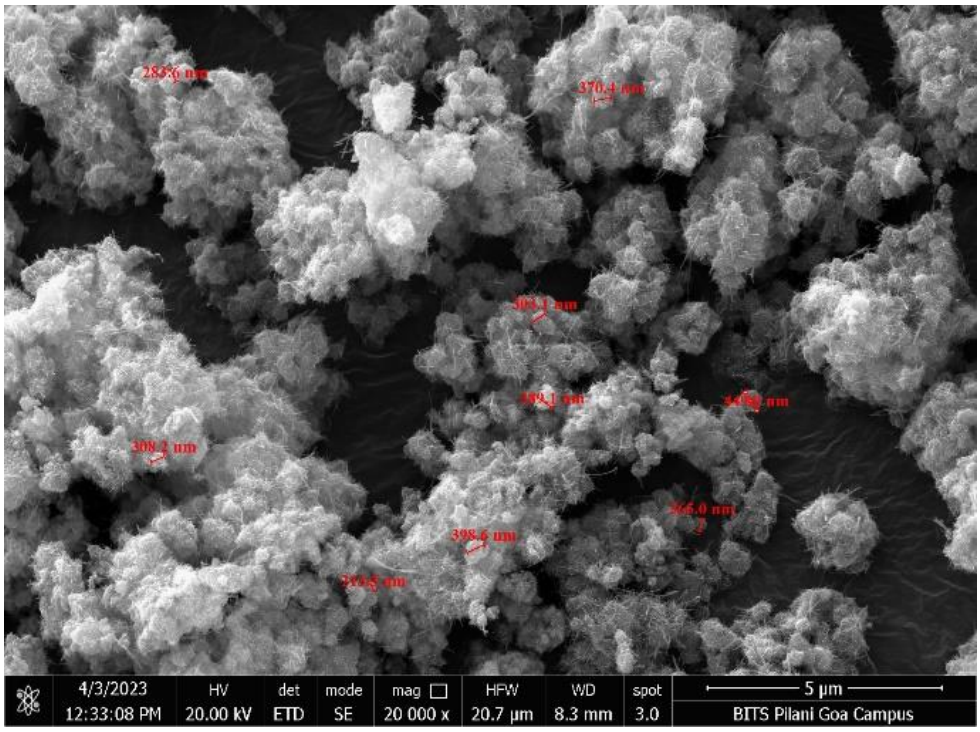



*p<0.05**

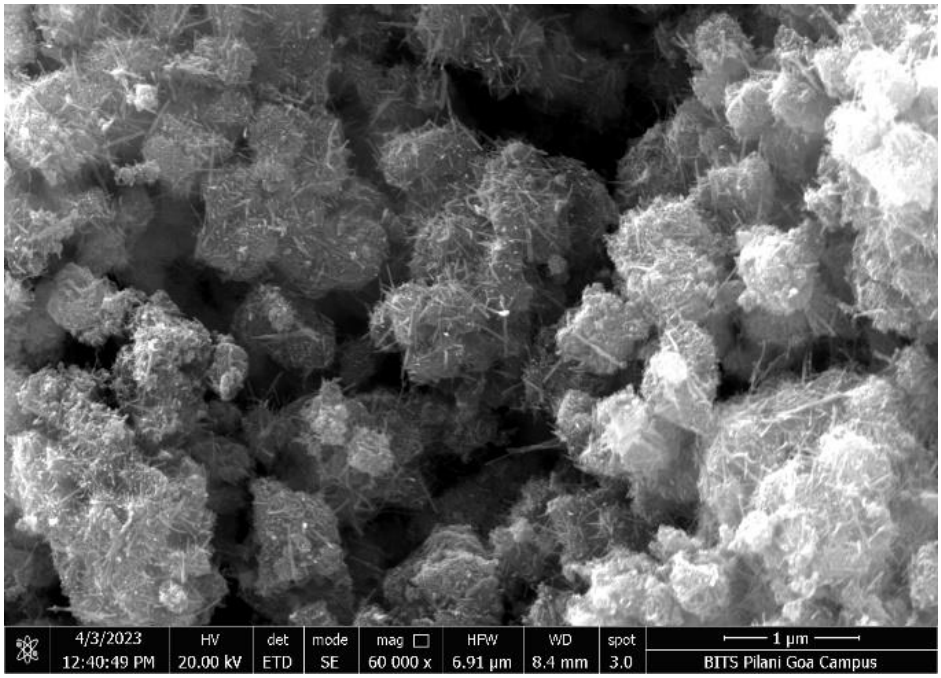
*Different letters indicate significant difference between groups, same letters indicate no significant difference between groups as per Tukey's analysis*

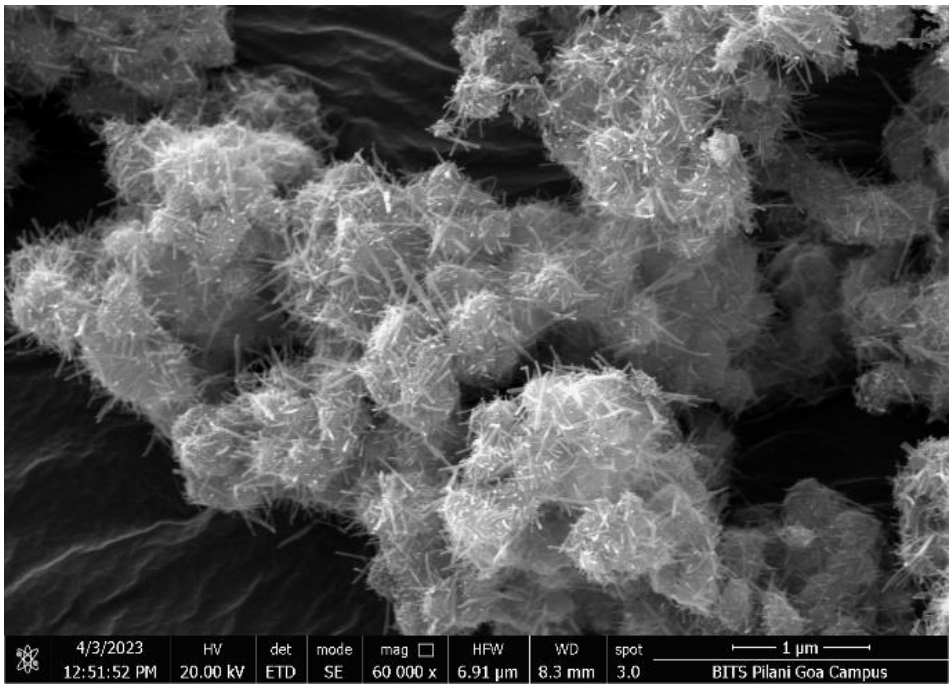
## Plate 2: SEM Analysis of $MnO_2$ nanostructures



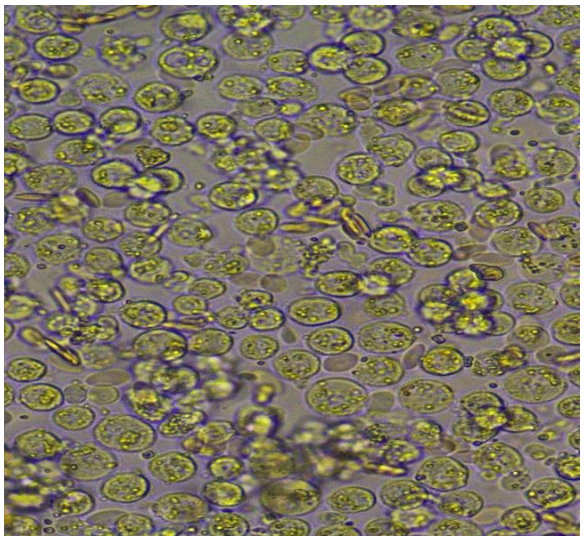



## Plate 2: SEM Analysis of $MnO_2$ nanostructures

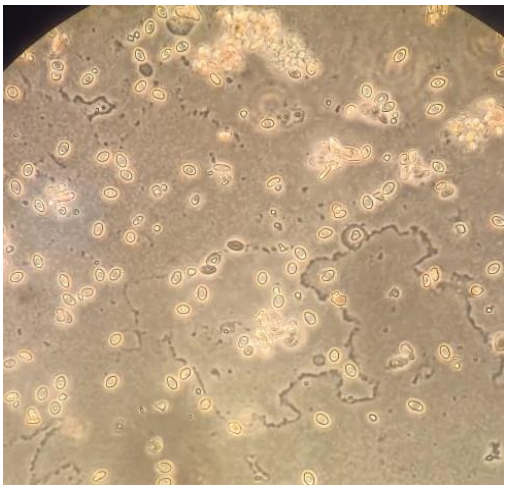


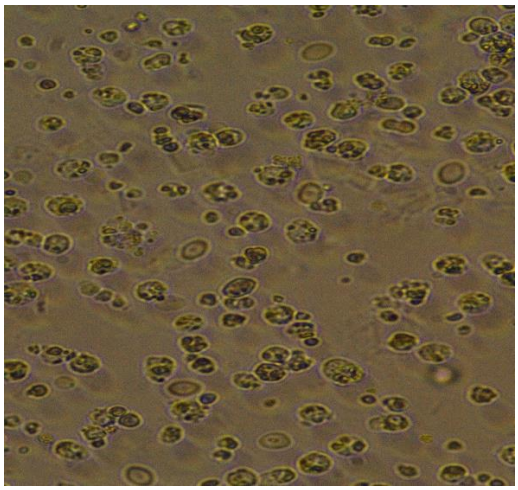
## Plate 3: *In vitro* LIVER CELL CULTURE



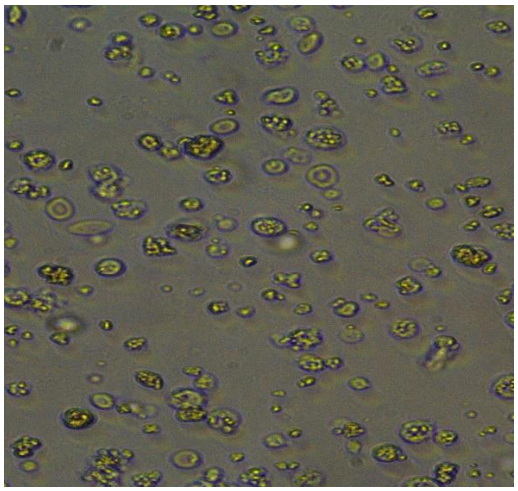
(a)



(b)



(c)

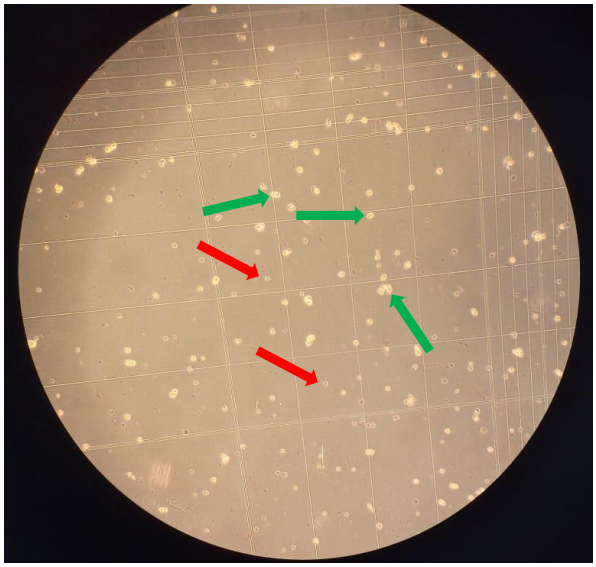


(d)

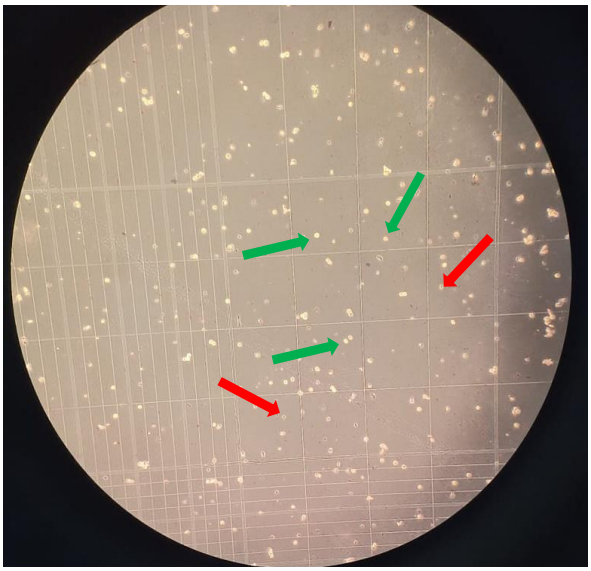
(e)

Maintained Cultures as seen on 45x of the objective (a) day 0; (b) and (c) 24 hr; (d) 72 hrs (e) fungal contamination

## Plate 4: TRYPAN BLUE EXCLUSION TEST



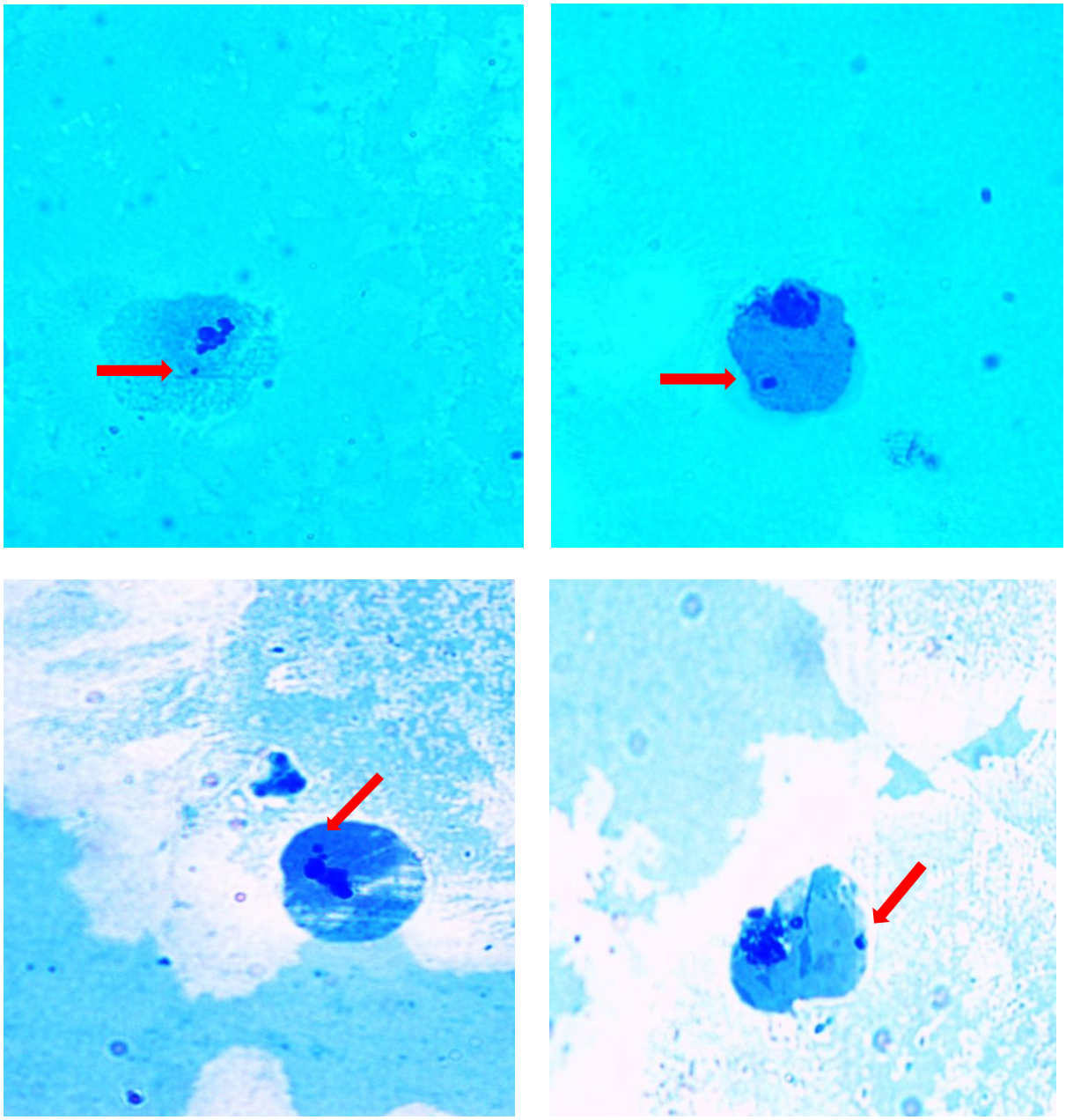
(a)



(b)

(a) and (b) The green arrows indicate viable cells and red arrows indicate non-viable cells as seen on the haemocytometer

## Plate 5: MICRONUCLUES TEST



Red arrows indicate the micronucleus within the cells.

# CHAPTER 5:

# DISCUSSION

# 5. DISCUSSION

The present study aimed to analyse the *in vitro* toxic effects of a synthesized manganese dioxide nanostructure. The dimensions of the synthesized particle were found to be nano-sized based on XRD data. The obtained peaks were compared to a standardised graph, in order to check the purity and crystallinity of the $MnO_2$. The data was conclusive of the phase of nanostructure to be α-$MnO_2$. The zeta potential analysis revealed that the charge on nano-structure was neutral, since it is falling between -10 mV to +10mV (Clogston & Patri, 2011).

The set up of the primary liver cell culture yielded 90-95% viable cells as proved by trypan blue exclusion assay, that has spherical and rounded morphology. Post-incubation, the cells attained nearly oval morphology and few also adhered in small clusters on the culture vessel. The viability of the cells was seen to decrease over time, as the non-viable cells were seen floating in the media during microscopic analysis. The attached cells were not seen to attain complete confluency during the study period. (Bols, et al. (1994) in their study suggested that primary cells showed difficulty in adhering to the surface and also proliferated very slowly, in order to attain confluency.

The MTT test was carried out post exposure to the toxicant. The lowest viability of cells was seen at highest concentration of the toxicant. This is because, the concentrations of manganese dioxide significantly affected the viability of cells in the culture. Findings of the studies conducted by Tillman (2018), also suggested that excessive concentrations of manganese were unable to preserve cell viability of the hepatocytes. This study was also suggestive of the fcat that manganese toxiciy was time and dose-dependent. Another study conducted by Zhang, et al. (2012) suggested that oxides of had caused cell death due to disruption of lysosomal membrane. A study by Rovetta, et al. (2007) also implied that treatment with $MnCl_2$ caused a

rapid decline in the cell viability after 2 days of treatment and the cells were seen floating in the media. A morphological change in the liver cell lines was also reported in the study wherein, the cells had lost their ability to proliferate in the *in-vitro* environment.

From the MTT analysis it was evident that 24 hr $LC_{50}$ concentrations lied between 250 µg/ml to 300 µg/ml. hence for further toxicity analysis, the exposure concentrations lesser than $LC_{50}$ were selected, that is 200 µg/ml, 150 µg/ml and 100 µg/ml.

The protein content of the cells exposed to lower concentrations showed an initial increase in the total protein content. However, at the highest concentrations, total the protein content of the cells were seen to drastically reduced. A study by Damelin et al. (2000) also had results that showed increase in protein content and especially HSP-70 protein, in response to cell stress induced by low concentratiosn of heavy metal toxicant. However, the decrease in the same was seen at higher concentrations in response to higher concentration, as cells were considered to seize its functions.

The catalase test showed an a significant decline in the activity of the enzyme as the exposure concentrations were icreased in the cells. Similarly, the test also showed a significant increase in $H_2O_2$ levels with the increase in exposure concentrations. These results suggest that the higher concentrations of the toxicant produced higher $H_2O_2$ in response to oxidative stress and had an inhibitory effect on the activity of catalase enzyme. An investigation by Singh & Sivalingam (1982) suggested that at high concentration of a heavy metal toxicant, the activity of catalase is inhibited. The study also indicated that heavy metal affected enzyme activity by binding by either binding to the active site or allosteric site. An analysis by Gabriel et al., (2013) showed a significant decrease in the activity of catalse on exposure to $Mn^{2+}$ for a longer duration.

The micronucleus test was performed to assess the genotoxic effect of manganese dioxide nanostructures on the cells. The results in the present study depicted that higher percentage of micronuclei were observed in the moderate concentrations of 150 µg/ml. However, not much of a difference in the percentage of micronuclei was observed in 200 µg/ml concentration, as compared to 150 µg/ml. As compared to control, the higher concentrations show an increase in the percentage of micronucleus. Similar results were also observed in the study conducted by Francisco, et al., (2021) wherein, an increase in the number of micronuclei were seen at higher concentrations of Manganese. Dutta et. al, (2006) in their study showed that manganese dioxide induced DNA strand breakage, micronucleus formation and chromosomal aberration and concluded to be an established genotoxicant.

# CHAPTER 6:

# CONCLUSION

# 6. CONCLUSION

Heavy metals, in any form hold a potential to get accumulated in the living organisms, wherein there is a faster uptake and storage of them in the tissues than their metabolism and excretion. Bioaccumulation occurs where there is an increase in the level of a toxicant in a living being over time, which has further detrimental effects on the body of the organism (Pandey & Madhuri, 2014). Manganese is required in trace quantities for metabolism. It acts as a cofactor or activator in different electron transport reaction, phosphorylation etc. Hence the accumulation of manganese is mainly seen to occur in organelles like mitochondria, Golgi apparatus and vesicles, and there are very low concentrations of the same found in the cytoplasm (Baden & Eriksson, 2006).

The present study successfully showed the acute effects of one such heavy metal compound, manganese dioxide ($MnO_2$) on the liver cells of tilapia. At higher concentrations of the $MnO_2$ nanostructures, detrimental effects on cells were observed *in vitro*. However, at lower concentrations, below $LC_{50}$, cytotoxicity and genotoxicity show a decline with decreasing concentrations. At the concentrations above 300 µg/ml, cytotoxic effects were visible. The concentrations below 150 µg/ml, as compared to control, did not show much variation in toxicity. Hence, it is safe to conclude that concentrations below 100 µg/ml, may not have much toxic effects on the hepatic cells as compared to higher concentrations.

The larvicidal activity of synthesized manganese dioxide ($MnO_2$) nanostructures reported that, here was 100% mortality observed at the concentrations 20ppm, 30ppm and 200 ppm (20 µg/ml, 30 µg/ml and 200 µg/ml, respectively) for *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* respectively. Hence to effectively use $MnO_2$ as a potential larvicide,

without causing any hepatotoxicity in the aquatic vertebrates, 20ppm and 30ppm concentrations against *Culex quinquefasciatus* and *Aedes aegypti* can be used.

Further analysis is required to test the chronic effects of $MnO_2$ on the liver and other organs of the aquatic organisms, in order to fully understand the toxicokinetic, physiological response, defence mechanisms in them upon long term exposure of $MnO_2$.

# CHAPTER 7:

# REEFERENCES

# 7. REFERENCES


- Abdullah, S., Javed, M., & Javid, A. (2007). Studies on Acute Toxicity of Metals to the Fish (Labeo rohita). International Journal of Agriculture & Biology, 9(2), 333-337.
- Agrawal, S. J., & Srivastava, A. (1980). Haematological Responses in freshwater fish to experimental manganese poisoning. Toxicology, 97-100.
- Baden, S. P., & Eriksson, S. P. (2006). Role, Routes and Effects of Manganese in Crustaceans. Oceanography and Marine Biology: An Annual Review, 44, 61-83. doi:10.1201/9781420006391.ch2
- Browning, C. L., Green, A., Gray, E. P., Hurt, R., & Kane, A. B. (2021). Manganese dioxide nanosheets induce mitochondrial toxicity in fish gill epithelial cells. Nanotoxicology, 15(3), 400-417. doi:10.1080/17435390.2021.1874562
- Bols, N., Barlian, A., Chirino-Trejo, M., Caldwell, S. J., Goegan, P., & Lee, L. E. (1994). Development of a cell line from primary cultures of rainbow trout, Oncorhynchus mykiss (Walbaum), gills. Journal of Fish Diseases, 17(6), 601-611. doi:10.1111/j.1365-2761.1994.tb00258.x
- Cherian, E., Rajan, A., & Baskar, G. (2016). Synthesis of manganese dioxide nanoparticles using co-precipitation method and its antimicrobial activity. International Journal of Modern Science and Technology , 17-22.
- Clogston, J., & Patri, A. K. (2011). Zeta Potential Measurement. Methods in molecular biology, 63-70. doi:10.1007/978-1-60327-198-1_6
- Collins, S. D., Yuen, G., Tu, T., Budzinska, M. A., Spring, K., Bryant, K., & Shackel, N. A. (2019). Hepatocellular Carcinoma. Brisbane (AU): Codon Publications. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK549191/ doi: 10.15586/hepatocellularcarcinoma.2019.ch3

- Coppo, G. C., Passos, L. S., Lopes, T. O., Pereira, T. M., Merçon, J., Cabral, D. S., . . . Chippari-Gomes, A. R. (2015). Genotoxic, biochemical and bioconcentration effects of manganese on Oreochromis niloticus (Cichlidae). Ecotoxicology. doi:10.1007/s10646-018-1970-0
- Crooks, D. R., Ghosh, M. C., Braun-Sommargren, M., Rouault, T. A., & Smith, D. R. (2007). Manganese targets m-aconitase and activates iron regulatory protein 2 in AF5 GABAergic cells. Journal of Neuroscience Research, 85(2), 1797–1809. doi:10.1002/jnr.21321
- Damelin, L., Vokes, S., Whitcutt, J., Damelin, S., & Alexander, J. (2000). Hormesis: a stress response in cells exposed to low levels of heavy metals. Human & Experimental Toxicology, 19, 420-430.
- Duan, X., Yang, J., Gao, H., Ma, J., Jiaoa, L., & Zheng, W. (2012). Controllable hydrothermal synthesis of manganese dioxide nanostructures: shape evolution, growth mechanism and electrochemical properties. CrystEngComm, 14, 4196–4204. doi: 10.1039/c2ce06587h
- Dunier, M. (1996). Water pollution and immunosuppression of freshwater fish. Italian Journal of Zoology, 63, 303-309. doi:10.1080/11250009609356150
- Dutta, D., Devi, S., Krishnamurthi, K., & Chakrabarti, T. (2006). Anticlastogenic Effect of Redistilled Cow's Urine Distillate in Human Peripheral Lymphocytes Challenged With Manganese Dioxide and Hexavalent Chromium. Biomedical and Environmental Sciences, 487-494. Retrieved from https://www.besjournal.com/fileSWYXYHJKX/journal/article/swyxyhjkx/2006/6/PDF/bes200606014.pdf

- F.W.Ntengwe. (2006). Pollutant loads and water quality in streams of heavily populated and industrialised towns. Physics and Chemistry of the Earth, Parts A/B/C, 832-839. Retrieved from https://doi.org/10.1016/j.pce.2006.08.025
- Falfushynska, H. I., Gnatyshyna, L. L., Stoliar, O. B., & Nam, Y. K. (2011). Various responses to copper and manganese exposure of Carassius auratus gibelio from two populations. Comparative Biochemistry and Physiology, Part C, 242-253.
- Figueiredo, N., Matos, B., Diniz, M., Branco, V., & Martins, M. (2021). Marine Fish Primary Hepatocyte Isolation and Culture: New Insights to Enzymatic Dissociation Pancreatin Digestion. *International Journal of Environmental Research and Public Health, 18*. doi:10.3390/ijerph18041380
- Francisco, L. F., Baldivia, D. d., Klafke, S. M., Castilho, P. F., Crispim, L. F., Santos, E. L., . . . Barufatti, A. (2021). Acute Toxic and Genotoxic Effects of Aluminum and Manganese Using In Vitro Models. Toxics. doi:10.3390/toxics9070153
- Gabriel, D., Riffel, A. P., Finamor, I. A., Saccol, E. M., Ourique, G. M., Goulart, L. O., . . . Baldisserotto, B. (2013). Effects of Subchronic Manganese Chloride Exposure on Tambaqui (Colossoma macropomum) Tissues: Oxidative Stress and Antioxidant Defenses. Archives of Environmental Contamination and Toxicology. doi:10.1007/s00244-012-9854-4
- Greene, A., Hashemi, J., & Kang, Y. (2021). Development of MnO2 hollow nanoparticles for potential drug delivery applications. Nanotechnology, 32(2). doi:10.1088/1361-6528/abb626
- Herrera, A. A., & Catap, E. S. (1991). Histopathological Effects of Manganese Intoxication. Transactions of the National Academy of Science and Technology, 13, 465-483.

- Hoseini, S. M., Hedayati, A., & Ghelichpour, M. (2014). Plasma metabolites, ions and thyroid hormones levels, and hepatic enzymes' activity in Caspian roach (Rutilus rutilus caspicus) exposed to waterborne manganese. Ecotoxicology and Environmental Safety, 84-86.
- Indian Bureau of Mines. (2014). Indian Minerals Yearbook 2012 (51 ed.). Nagpur.
- Jenkitkasemwong, S., Akinyode, A., Paulus, E., Weiskirchen, R., Hojyo, S., Fukada, T., . . . Knutson, M. D. (2018). SLC39A14 deficiency alters manganese homeostasis and excretion resulting in brain manganese accumulation and motor deficits in mice. Proceedings of the National Academy of Sciences, 115(8), 1769–1778. doi:10.1073/pnas.1720739115
- Kaur, S., Khera, K. S., & Kondal, J. K. (2018). Heavy metal induced histopathological alterations in liver, muscle and kidney of freshwater cyprinid, Labeo rohita (Hamilton). Journal of Entomology and Zoology Studies, 6(2), 2137-2144.
- Kwik-Uribe, C., & Smith, D. R. (2006). Temporal responses in the disruption of iron regulation by manganese. Journal of Neuroscience Research, 83(3), 1601–1610. doi:10.1002/jnr.20836
- Lowry, O. H., Rosebrough, N. J., & A. Lewis Farr, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry*, *193*, 265-275.
- Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of lmmunological Methods*, 55-63.
- Nagpure, N. S., Kumar, R., Kushwaha, B., Singh, P., Srivastava, S., & Lakra, W. S. (2007). *Genotoxicity Assessment in Fishes: A Practical Approach.* . Lucknow: NBFGR-ICAR.

- Naik, S., Javeer, D., Gawde, C., Palni, S., Koli, S., Jalmi, S., . . . Velho-Pereira, S. (2021). Larvicidal activity of pristine α-MnO2 nanostructures: An environmentally benign approach for combating mosquito (Diptera: Culicidae) menace. Materials Today Communications. doi:10.1016/j.mtcomm.2021.102184
- Nair, M., Jayalakshmy, K. V., Balachandran, K. K., & Joseph, T. (2006). Bioaccumulation of Toxic Metals by Fish in a Semi-Enclosed Tropical Ecosystem. Environmental Forensics, 7, 197-206. doi:10.1080/15275920600840438
- Nath, K., & Kumar, N. (1987). Toxicity of manganese and its effect on some carbohydrate metabolism of fresh water teleost, Colisa fasciatus. The Science of the Total Environment, 67, 257-262. doi:10.1016/0048-9697(87)90216-6
- Oliveira, L. F., Santos, C., Risso, W. E., & Martineza, C. B. (2008). Triple-Mixture of Zn, Mn, and Fe Increases Bioaccumulation and Causes Oxidative Stress in Freshwater Neotropical Fish. Environmental Toxicology and Chemistry, 1-8. doi:10.1002/etc.4133
- Padmini, E., & Rani, M. U. (2009). Evaluation of oxidative stress biomarkers in hepatocytes of grey mullet inhabiting natural and polluted estuaries. Science of the Total Environment, 4533–4541. doi:10.1016/j.scitotenv.2009.04.005
- Pandey, G., & Madhuri, S. (2014). Heavy metals causing toxicity in animals and fishes. Research Journal of Animal, Veterinary and Fishery Sciences, 2(2), 17-23. Retrieved from https://www.researchgate.net/publication/270169412
- Rajeshkumar, S., Mini, J., & Munuswamy, N. (2013). Effects of heavy metals on antioxidants and expression of HSP70 in different tissues of Milk fish (Chanos chanos) of Kaattuppalli Island, Chennai, India. Ecotoxicology and Environmental Safety, 98, 8-18. doi:10.1016/j.ecoenv.2013.07.029
- Rodrigues, G. Z., Staudt, L. B., Moreira, M. G., Santos, T. G., Souza, M. S., Lúcio, C. J., . . . Simoes, L. A. (2020). Histopathological, genotoxic, and behavioral damages induced

by manganese (II) in adult zebrafish. Chemosphere. Retrieved from https://doi.org/10.1016/j.chemosphere.2019.125550

- Rovetta, F., Catalani, S., Steimberg, N., Boniotti, J., Gilberti, M., Mariggiò, M., & Mazzoleni, G. (2007). Organ-specific manganese toxicity: a comparative in vitro study on five cellular models exposed to MnCl2. Toxicology In Vitro, 284-292. doi:10.1016/j.tiv.2006.08.010
- Saalidong, B. M., Aram, S. A., Otu, S., & Lartey, P. O. (2022). Examining the dynamics of the relationship between water pH and other water quality parameters in ground and surface water systems. PloS one, 17(1). doi:https://doi.org/10.1371%2Fjournal.pone.0262117
- Singh, A., Sharma, A., Verma, R. K., Chopade, R. L., Pandit, P. P., Nagar, V., . . . Sankhla, M. S. (2022). The Toxicity of Environmental Pollutants. doi:10.5772/intechopen.98127
- Singh, S., & Sivalingam, P. M. (1982). In vitro study on the interactive effects of heavy metals on catalase activity of Sarotherodon mossambicus (Peters). Journal of Fishery Biology, 683-688.
- Sinha, A. K. (1972). Calorimetric Assay of Catalase. *Analytical Biochemistry*, 389-394.
- Soldatow, V. Y., LeCluyse, E. L., Griffith, L. G., & Rusyn, I. (2013). In vitro models for liver toxicity testing. Toxicology Research, 2(1), 23-39. doi:10.1039/c2tx20051a
- Tekin-Özan, S., & Aktan, N. (2012). Relationship of Heavy Metals in Water, Sediment and Tissues with Total Length, Weight and Seasons of Cyprinus carpio L., 1758 From Işikli Lake (Turkey). Pakistan Journal of Zoology, 44(5), 1405-1416. Retrieved from http://zsp.com.pk/pdf44/1405.pdf

- Tillman, L. (2018). Manganese toxicity and effects on polarized hepatocytes. Bioscience Horizons. doi:10.1093/biohorizons/hzy012
- Vieira, M. C., Torronteras, R., Cordoba, F., & Canalejo, A. (2012). Acute toxicity of manganese in goldfish Carassius auratus is associated with oxidative stress and organ specific antioxidant responses. Ecotoxicology and Environmental Safety, 212–217. doi:10.1016/j.ecoenv.2011.11.015
- Webera, A. A., Salesa, C. F., Fariaa, F. d., Meloa, R. M., Bazzolib, N., & Rizzoa, E. (2020). Effects of metal contamination on liver in two fish species from a highly impacted neotropical river: A case study of the Fundão dam, Brazil. Ecotoxicology and Environmental Safety. doi:10.1016/j.ecoenv.2020.110165
- Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., . . . Jeffrey. (2012). Use of Metal Oxide Nanoparticle Band Gap To Develop a Predictive Paradigm for Oxidative Stress and Acute Pulmonary Inflammation. ACS Nano, 4349–4368. doi:10.1021/nn3010087
- Zhang, Y.-N., Poon, W., Tavares, A. J., McGilvray, I. D., & Chan, W. C. (2016). Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination. Journal of Controlled Release. doi:10.1016/j.jconrel.2016.01.020
- Zhou, Q., Yang, N., Li, Y., Ren, B., Ding, X., Bian, H., & Yao, X. (2020). Total concentrations and sources of heavy metal pollution in global river and lake water bodies from 1972 to 2017. Global Ecology and Conservation. doi:10.1016/j.gecco.2020.e00925
- Zhou, Z., Xu, M.-J., & Gao, B. (2015). Hepatocytes: a key cell type for innate immunity. Cellular & Molecular Immunology, 301–315. Retrieved from https://www.nature.com/articles/cmi201597