

BIOCHEMICAL VARIATION IN LIVER OF DANIO RERIO EXPOSED TO SODIUM ARSENATE HEPTAHYDRATE

(PROJECT – REPORT)

Submitted in partial fulfilment of the requirements for the award of
master of science degree in medical biotechnology and clinical research

by

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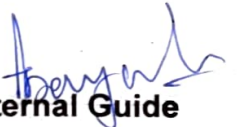
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. KAMALAYAZHINI A (41101004)** and who carried out the project entitled "BIOCHEMICAL VARIATION IN LIVER OF DANIO RERIO EXPOSED TO SODIUM ARSENATE HEPTAHYDRATE" under our supervision from December 2022 to May 2023.


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DECLARATION

I **Ms. KAMALAYAZHINI A (41101004)** hereby declare that the project report entitled ***"BIOCHEMICAL VARIATION IN LIVER OF DANIO RERIO EXPOSED TO SODIUM ARSENATE HEPTAHYDRATE"*** done by me under the guidance of **Dr. A. DAYANANDAN** at **Sathyabama institute of Science and Technology, Chennai** is submitted in partial fulfilment of the requirements for the award of Master of science degree in Medical Biotechnology and clinical research.

DATE: 05/05/2023

PLACE: CHENNAI

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ABSTRACT

Zebrafish is a widely used animal model in biomedical sciences and toxicology. Although evidence for the presence of phases I and II xenobiotic defense mechanisms in zebrafish exists on the transcriptional and enzyme activity level. Liver is the most important organ concerned with the biochemical activities in the human body. It has great capacity to detoxicate toxic substances and synthesis useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. A number of pharmacological and chemical agents act as hepatotoxin and produce variety of liver ailments. Almost all the metals are toxic at higher concentrations and some are lethal even at a very low concentration. The priority list of pollutants compiled by the Environmental Protection Agency (EPA) of the United States contains eight widespread heavy metals, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc. Among these, arsenic is more significant from environmental and toxicological point of view. In the present study was to assess the histopathology, agarose gel electrophoresis in DNA, protein estimation and antioxidant enzymes in sodium arsenate heptahydrate exposed in Zebrafish (*Danio rerio*). In the present experiment study, the sodium arsenate heptahydrate toxicity causes the physical aspects. It was observed that there was decrease in body and organ weight (liver) of sodium arsenate heptahydrate treated zebra fish due to the arsenate stress. A reduction in the protein content in the present investigation in zebrafish suggests that the tissue protein might have undergone proteolysis, during the stressful situation in the sodium arsenate heptahydrate intoxicated fishes. The DNA pattern has been slightly disturbed in 20 μ L treated animal. The entire variable tested i.e., SOD, CAT, GST, reduced glutathione and vitamin E recorded a decrease on sodium arsenate heptahydrate intoxicated fishes indicates that the protective mechanism in response to ROS generated by arsenate. In histopathology it is used to find any changes in liver, to study the sign of the damage using microscopic examination of a biopsy. Overall, it can be concluded that sodium arsenate heptahydrate induced the liver toxicity in dose dependent manner via oxidative stress confirmed by biochemical, antioxidants and histological studies.

Keywords: Zebrafish, Sodium arsenate heptahydrate, Liver, Antioxidants

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LIST OF ABBREVIATIONS

µg	:	Microgram
µL	:	Microliter
As	:	Arsenate
CAT	:	Catalase
EDTA	:	Ethylene diamine tetra-acetic acid
EPA	:	Environmental Protection Agency
gm	:	Grams
GPx	:	Glutathione peroxidase
GSH	:	Glutathione
GST	:	Glutathione-S-transferase
HE	:	Hematoxylin-Eosin
L	:	Liter
mg	:	Milligrams
ml	:	Milliliter
mM	:	Millimole
Na ₂ HAsO ₄ ·7H ₂ O	:	Sodium arsenate heptahydrate
ROS	:	Reactive Oxygen Species
SOD	:	Superoxide dismutase

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CHAPTER – 1

1. INTRODUCTION

Liver, the largest gland is a vital organ. It is the metabolic “engine-room of the body”. Almost all the drugs, foods and water constituents are metabolized and detoxified in the liver, and as such it is often exposed to maladies resulting in a number of clinical syndromes. Many chemicals, foods, drugs and infections (parasitic, bacterial, viral or fungal) can cause variety of liver diseases such as hepatitis, jaundice, cirrhosis, liver cancer, etc. Because of variations in liver dysfunctions and difficulties encountered in reaching to a proper diagnosis, a physician is rarely able to provide specific treatment. At the most, supportive and symptomatic treatments are given but the multiplicity of deranged functions renders the treatment still more complicated. Besides this, modern (allopathic) drugs exhibit severe toxicity, thus there is a definite need to search alternate drugs having maximum therapeutic value with no or least toxicity” (Pandey Govind, 1980, 1990).

Liver has a pivotal role in regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Further, detoxification of a variety of drugs and xenobiotics occurs in the liver itself. The bile secreted by the liver has, among other things, an important role in digestion. Liver diseases are among the most serious disorders. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatose (non-inflammatory diseases) and cirrhosis (degenerative disorder resulting in fibrosis of the liver). The liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidase oil, aflatoxin, carbon tetrachloride, acetaminophen, chlorinated hydrocarbons, etc.), excess consumption of alcohol, infections and autoimmune disorders” (Kumar *et al.*, 2011; Pandey Govind, 1980, 1990). Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis. It has been estimated that about 90% of the acute hepatitis is due to viruses. The major viral agents involved are Hepatitis B, A, C, D (delta agents), E and G. Of these, Hepatitis B infection often results in chronic liver diseases and cirrhosis of liver. Primary liver cancer has also been shown to be

produced by these viruses. It has been reported that about 14-16 million people are infected with Hepatitis B virus in South East Asia region and approximately 6% of the total population in the region are carriers of this virus. A vaccine has become available for immunization against Hepatitis B virus. Hepatitis C and Hepatitis E infections are also common in countries of South East Asia region (Kumar *et al.*, 2011).

1.1 Functions of the liver

The livers probably perform more separate tasks than any other organ in the body. Its chief functions are to help the body digest and use food and to help purify the blood of wastes and poisons (Hand Sharma, 1990). The liver also regulates the amount of certain nutrients that the cells of the body receive. digestive enzymes in the intestine break down proteins into amino acids, and carbohydrates into amino acids, and carbohydrates into simple sugars primarily glucose. The blood carries these nutrients as well as vitamins, minerals and fatty acids and glycerol to the liver. The liver also plays an essential role in the storage of certain vitamins. The liver stores vitamin A, as well as vitamins D, E and K and those of the B complex group. It also stores iron and other minerals (Mitratur, 1997). Liver cells filter harmful substances from the blood. Such substances include insecticides, drugs food additives, and industrial chemicals. Enzymes in the liver cells connect some of these substance into products that dissolve in water other functions. The liver manufacture various blood protein including albumin globulin and fibrinogen. Albumin helps to prevent plasma (the liquid portion of the blood) from seeping through the walls of the blood vessels. Globulin help the body fight infections fibrinogen enables the blood to seal broken blood vessels the blood to seal broken blood vessels by forming a clot (Robbins, 2004).

1.2 Toxicity

Toxicity of a substance could be defined as capacity to cause internal or external injury to living organisms. A highly toxic substances will damage an organism if administered in a very small concentrations, a substance of low toxicity cannot be defined without reference to the quantity of a substance administered or absorbed (dose) the way in which this quantity is administered (e. g. inhalation, ingestion, injection) and distributed in time (e.g. single dose, repeated doses) the type and severity of injury and the time needed to produce that injury Mohammad

Reza (2012).

Toxicity is relative property of a chemical which refers to its potential to have a harmful effect on a living organism. It is a function of the concentration of the chemical and duration of exposure, if toxicity and related data are commonly used in comparing chemical (Selvanathan 2011). Toxicants may found either natural in origin or artificial or synthetic ones. Naturally occurring toxicants may originate from animal, plant or mineral sources (Qixiao *et al.*, 2015).

Toxic effect of the chemicals may be physiological, biochemical and pathological in nature (Stephenson, 1987). The changes produced by toxic chemical may be complex, damage one or different organs, tissues or cells. There are additional quantities of effluents of industrial waste illegally discharged in to canals showed by researches (Alabaster and Lloyd, 1982). In agriculture land the excess use of herbicide and pesticide are the main source of water pollution in the modern age. They are related to the mismanagement of industrial wastes (Prabha and Selvapathy, 1997, Khurshid *et al.*, 1998).

The pollution of water resources soils, sediments and aquatic biota by heavy metals is of major concern especially in many industrialized countries due to their toxicity and bio- accumulative nature. (Gautam and Gupta, 2008). Acute and chronic are types of toxicity study. The acute toxicity determined toxicant effect within few hours or a few days while in the chronic type toxicity, study for long turn basis is carried out including few days to many months.

The exposure time for acute toxicity is generally 24, 48, 72 and 96 hours. The acute toxicity which is used to determine the concentration of toxic metals that produces adverse effects on the test organisms. The lethality is used on the basis of habit of organism. The lethal concentration (LC) for aquatic animals while the (LD) for terrestrial animals lethal dose represented as (LD) is expressed in the term of μg or Mg /kg body weight of animal and lethal concentration represented as (LC) in the form of milligram per litre (mg/l) or part per million (ppm) or part per billion (ppb).

In acute toxicity studies on aquatic organism the time period is generally extended up to 96 hours. The evaluation of toxic impact of the different toxicant on the aquatic habitat fresh water fish selected for the toxicological study. The study on the toxic effects of organism has evolved the determination of lethal dose for

particular time periods. LC₅₀ value is the concentration at which 50 % kill of the exposed animals within a given time periods .The LC₅₀ values were determined by graphical probit analysis method for 24, 48, 72 and 96 hours' time periods.

In aquatic toxicology the traditional LC₅₀ test are used to measure potential risk of a pollutant. Krishna and Govil (2004) studied heavy metal contamination of soil around Pali industrial area. The LC₅₀ value are determined by many workers using different methods includes-statistical method, probit analysis method (Finney 1971), Graphical method (Fisher 1964).

In aquatic organisms' changes in behavioral activities than normal activities showed due to the presence of water toxicants and signs of stress physiology normally at small concentration of a toxicant. The aquatic animals try to adapt itself to the changing environment but at high concentration of toxicant the physiological activities slow down and death is the result. At low concentration the animals becomes weak such toxicant content animal may put into the human life into danger. The presences of toxicant into aquatic environment give the effect on physiological and behavioral activities of the aquatic organisms. (Mohammad Reza and Vahide Alipoor, 2012., Agarwal S. 1991) The toxicity of heavy metals which is influenced by fresh-water fish (Eisler 1973, Holden 1972, Bhaskar Reddy, *et al*, 1999, Banerjee, *et al.*, 1978). Pesticides, herbicides and other pollutants acts as a source of toxicity that ultimately burden on the living organism (Siddiqui *et al.*, 2009).

The acute and chronic toxicity of heavy metals to fresh-water fish has been widely summarized. Chronic exposure and accumulation of these chemical by aquatic living system can result in tissue burdens that produce adverse effects not only in the exposed aquatic animals but it produces its adverse effect including human beings also. The fishes are the main supply of cheap and healthy protein to a large percentage of the world's population. In some area of the India it is the main food for human beings and is main protein of diet. They are particularly valuable for supply proteins of high comparable with meat, eggs or milk and also a good source of fatty acids and vitamin A for human beings. Industrial effluents contain highly toxic chemicals like heavy metals that cause pollution of aquatic environment including rivers, lakes and ponds, ditches etc. The accumulations and persistence of metal and pesticides on the aquatic environment constitute a threat

to living life as witnessed by the chronic and acute poisoning of fish and other aquatic ecosystem. (Guedenon *et al.*, 2012 ; Janardhan Reddy, 1999).

The science of toxicology has progressively made from being an activity based on the ability to observe and classify the harmful effects of chemicals in the animal body, relying principally on the tools of classical pathology to achieve these ends, to a discipline explain the effects of toxic compounds. This change has resulted from the wide spread application of techniques and concepts from a wide range of basic sciences, triggered by the realization of the potential impact of exposure to a wide range of chemicals on human health. These changes were initially a reaction to the harmful effects of thalidomide and the key molecules in the development of our understanding of toxic mechanisms.

1.3 Mechanisms of Toxicity

The heavy metal toxic compounds are chemically stable and produce their characteristic effects by interference with that a full understanding of the pharmacology of toxic compounds is essential. (Sambasivarao *et al.*, 1988) Even if the compound under consideration has no specific actions appropriate for therapeutic application, it is still critical to have a proper knowledge of the compounds biochemical and molecular sites of action. Cytotoxicity causing damage and death of the cell is often the consequences of exposure to a harmful chemical, but the number of cells which must be killed before the function of a tissue is noticeably impaired is highly variable. Some cell types, notably the epithelia including the liver, have the ability to regenerate in response to insult while others, most notably neurons cannot. Some histological organs such as the liver, lung and kidney, have a substantial reserve capacity in excess of normal requirements and normal body function can be maintained in the presence of marked impairment.

The degradation of aquatic system is a worldwide phenomenon developed from the intense population and from the corresponding increase in agriculture practices, urbanization, industrial development and domestic activities studied by Rish and Rish (2011). Effects of the pesticides in agricultural fields on different non-target flora and fauna (Ural and Simsek, 2006,).

Industrial chemicals and agricultural waste mix and pass with the stream of water and ultimately reach to water body cause adverse effects on the aquatic organisms. Heavy metal compounds accumulated in aquatic organisms and

damage to different body tissue. (Tucker 1997, Khan *et al.* 2004, Jain and Salman 1995, APHA. 1998). The pesticides, herbicides and heavy metals are non-biodegradable cause pollution potential and as a result it is danger to the aquatic organisms like fishes (Lloyd R, 1965).

Heavy metals are the metallic elements which are toxic and have highly density and atomic weight according to (IUPAC) International Union of Pure and Applied Chemistry. Heavy metals are toxic metals they are derived from agriculture and industrial waste dissolves into the freshwater of lake, ponds, rivers, reservoirs and other water bodies. It produces adverse effects on the aquatic biota as well as on the human beings and other animals.

They are observed by some researchers (Johansson and Ramussen 1977, Vinodhini and Narayanan 2008, Karbassi *et al.*, 2006). The essence of toxicology is that it is a discipline concerned with studying and potential of chemicals or mixture of them to produce harmful effects in living organisms and determining the implications of these effects overview definition covering the various facets of toxicology (Panigrahi and Mishra 1978).

Toxicology is the interaction study between chemical substances and biological systems in order to quantitatively determine the potential for chemicals to produce injury to the living organisms. It gives results in adverse effects and to investigate the nature, incidence, mechanisms of production, factors influencing their developments and reversibly of such adverse effects on animals. Chemicals or their conversion products, require coming into close structural and functional contact with tissue or organs of the body and causing injury to the affected organisms shown by (Adami *et al.*, 2002).

The fish are the good indicator of water quality because they are aquatic animals and highly sensitive to water toxicants and they will react increasingly unfavorable as dissolved oxygen content of water is decreased due to pollution (Prosser Ladd 1973; Janardhan and Ramamurthy 1987). Fishes have been widely used as test organisms to evaluate the toxicity of water pollutants. The reason for the selection of fishes due to their sensitivity to toxic substances of water and their easily adaptability to the laboratory condition. (Natarajan and Sreenivasan 1962, Jana and Bandopadhyaya 1987).

The heavy metals released by industrial are the sources to increases the

contamination of aquatic ecosystem (Mali, 2002). Pesticides, herbicides and heavy metals are mostly and daily misused chemicals in the world. Heavy metals and pesticides are finally mixed in water bodies and they produce their adverse effect to the aquatic animals. (Krishna and Govil, 2004) showed the aquatic contamination of heavy metals cause acute and chronic poisoning of fish and other aquatic animals. (John Prentera *et al.*, 2004, Srinivasa Gowd and Pradip Govil, 2007).

Metals are substances with high electrical conductivity, malleability and lustre, voluntarily lose their electrons to form cations. Metals are found naturally in the earth's crust and their compositions vary among different localities, resulting in spatial variations of background concentrations (Zhang *et al.*, 2016). The metal distribution in the atmosphere is correlated with the properties of the given metal and by various environmental factors. Anthropogenic uses have led to global dispersion of metals in the environment. Man, animals and plants are exposed to a variety of metals through environment, food, water and soil. Metals being non-biodegradable persist in the environment for a long period and cause serious ecotoxicological problems (Khelifi & Hamza-Chaffai, 2010).

Toxic wastes like acids, alkalis, heavy metals, oil, grease, dyes, pesticides and radioactive materials are released into the water bodies by many industrial units. Some other important pollutants include polychlorinated biphenyl (PCBs) compounds, lubricants and hot water discharged by power plants. The pollutants discharged into the water bodies usually dissolve or remain suspended in water. Sometimes they also accumulate on the bottom of the water bodies. Several pollutants are also carcinogenic in nature. Some pollutants like sodium can cause cardiovascular diseases, mercury and lead cause nervous disorders while DDT can cause chromosomal changes.

1.4 Free radicals

Free radicals are playing adverse role in etiology of wide continuum of disease from cancers to neurodegenerative diseases. They induce alterations in forms and function of various biological molecules which persuade to diseased conditions. Oxidative stress and apoptosis have prominent role in pathogenesis of several cardiac and neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) etc. Reactive oxygen species (ROS) produced

enormously due to endogenous and exogenous stimuli leads to induction of signals that result in mitochondrial dysfunction and cell death. Inflammation is one of the evident processes of oxidative stress and the formation of inflammatory mediators like interleukins, adhesion molecules which are having significant role in diseases like diabetes, neurodegenerative diseases, arthritis, and cardiovascular diseases therefore inhibition of inflammation is important in treating such diseases.

1.6 Oxidative stress

Oxidative stress is described generally as a condition, under which increased production of free radicals, ROS and oxidant-related reactions result in damage to cells (Chung *et al.*, 2007). The extent of oxidative stress in a cell is determined by the amounts of superoxide, hydrogen peroxide and hydroxyl radicals that are produced by different cellular mechanisms in response to endogenous and environmental cues (Cho *et al.*, 2009). Oxidative stress initiated by free radicals, such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite, play a vital role in damaging various cellular macromolecules. These include DNA molecules, proteins and lipids. This damage may result in many diseases, including diabetes mellitus, atherosclerosis, myocardial infarction, arthritis, anemia, asthma, inflammation, neurodegenerative diseases and carcinogenesis (Oyedemi *et al.*, 2010).

1.7 Antioxidant

The continuous interaction of the animal physiological systems with free radicals, generated either indigenously or inhaled/ingested from exogenous sources leads to excess load of free radicals and causes cumulative damage of protein, lipid, DNA, carbohydrates and membrane, resulting in oxidative stress. Therefore, living creatures have evolved a highly complicated defense system with antioxidants, composed of enzymes and vitamins, against oxidative stress. These defence systems are mainly classified as

(i) suppression of generation of ROS, (ii) scavenging of ROS, (iii) clearance, repairing and reconstitution of damage, and (iv) induction of antioxidant proteins and enzymes (Veena *et al.*, 2007).

Antioxidants are substances that either directly or indirectly protect cells against the adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions (Janani *et al.*, 2010). An antioxidant is a molecule capable of slowing or

preventing the oxidation of other molecules. In a biological system, they protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants terminate the chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. They are believed to play a role in preventing the development of chronic diseases like cancer, heart disease, stroke, AD, RA and cataracts (Chakraborty *et al.*, 2009).

1.7.1 Superoxide dismutase (SOD)

SOD is a metallo protein and is the first enzyme involved in the antioxidant defence. SOD plays an important role in protecting cells against ROS by lowering the steady state level of $O_2^{\bullet-}$. In general, there are three types of SOD, containing Mn, Fe or Cu and Zn as prosthetic metals (Corpas *et al.*, 2006). SOD converts superoxide radicals to H_2O_2 and molecular oxygen, which, in turn, can be counteracted by catalase or GPx reactions, thereby reducing the level of cellular damage (Subash and Subramanian, 2009). SOD is widely distributed to protect such cells against the toxic effects of superoxide anion (Rao *et al.*, 2009). The enzyme SOD is considered the first-line defense because it catalyses the first reaction in the system harvesting oxygen-free radicals. Therefore, SOD prevents the oxidation of biological molecules, performed by the same radicals or their derivatives (Sanchez-Venegas *et al.*, 2009).

1.7.2 Catalase (CAT)

Catalase is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red blood cells and in the liver. CAT decomposes H_2O_2 and protects the tissue from highly reactive $OH^{\bullet}$. Therefore, the reduction in the activity of these enzymes may result in an accumulation of $O_2^{\bullet-}$ radicals and H_2O_2 (Deepak *et al.*, 2007). Hence, catalase has been considered an important regulator of oxidative stress (Souza *et al.*, 2008). Catalase, localized in peroxisomes, has two enzymatic activities, depending on the concentration of H_2O_2 . If the concentration of H_2O_2 is high, catalase acts catalytically, that is, it removes H_2O_2 by forming H_2O and O_2 (catalytic reaction). However, at a low concentration of H_2O_2 and in the presence of a suitable hydrogen donor, eg., ethanol, methanol, phenol and others, catalase acts peroxidically, removing H_2O_2 , but oxidizing its substrate (Turkseven *et al.*, 2005).

1.7.3 Glutathione S-transferase (GST)

GST comprises a multigene family of proteins involved in the metabolism of many disease-causing electrophilic substrates that protects the cells against oxidative stress, and is also useful in monitoring of cellular induction (Sarkar *et al.*, 2010). It consists of a large family of GSH-utilizing enzymes that play an important role in the detoxification of xenobiotics in mammalian systems (Sohini and Rana, 2007; Tabrez and Ahmad, 2009). GSTs are suspected to have an important protective function (Polit *et al.*, 2009). The GSTs are a supergene family of dimeric enzymes that catalyze the conjugation of GSH to a variety of electrophiles, including arene oxides, unsaturated carbonyls, organic halides and other substrates (eg., by-products of ROS activity). These enzymes are ubiquitously present in living organisms. A number of chemical agents, including some antioxidants, have cancer chemopreventive properties that can induce one or more GST isozymes (Bickers and Athar, 2006).

1.7.4 Vitamin E

Tocopherols, collectively known as vitamin E, are a class of lipid-soluble antioxidants synthesized exclusively by photosynthetic organisms. Tocopherols are essential components of the human diet because they perform numerous crucial functions, including quenching and scavenging of GSH and ascorbate, which accumulate in response to oxidative stress (Mani *et al.*, 2008). α -tocopherol, found in the green parts of plants, scavenges lipid peroxy radicals through the concerted action of other antioxidants (Kiffin *et al.*, 2006). α -tocopherols interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various reactive oxygen species (ROS) and lipid soluble by-products of oxidative stress (Shao *et al.*, 2008), thus acting as a powerful terminator of lipid peroxidation (Arulmozhi *et al.*, 2010).

Tocopherols are essential components of biological membranes. They play both antioxidant and non-antioxidant functions (Bosch, 2005). Because of its fat-soluble nature, it safeguards cell membranes from damage by free radicals. Vitamin E has been proposed for the prevention against colon, prostate and breast cancers, some cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders (Huy *et al.*, 2008; Janani *et al.*, 2010).

1.7.5 Reduced glutathione

Glutathione (GSH), a tripeptide, is the most abundant intracellular free thiol and an important antioxidant (Liu and Pravia, 2010). It is believed to scavenge ROS directly or act as a substrate for other antioxidative or repair enzymes (Gao *et al.*, 2009). This most abundant, non-protein antioxidant in the cells, plays a pivotal role in the defense against oxidative stress-induced cell injury and mitochondrial damage (Lakshmidevi and Anuradha, 2010). GSH also preserves the cellular levels of active forms of vitamin C and vitamin E (Janani *et al.*, 2010). It is an important defense mechanism against potentially toxic hydrogen peroxide by glutathione peroxidase, which reduces hydrogen peroxide to water, and along with that, the oxidation of GSH (Shieh *et al.*, 2010). Glutathione in mammalian cells maintains the intracellular thiol redox status, and detoxifies exogenous and endogenous reactive molecules. Depletion of intracellular GSH predisposes cells to proapoptotic stimuli and can also activate apoptosis in the absence of such stimuli (Valko *et al.*, 2007).

1.8 Sodium arsenate heptahydrate

Sodium arsenate heptahydrate is mainly used in the production of pesticides and herbicides, as well as in the manufacturing of glass, ceramics, and other chemicals. Sodium arsenate heptahydrate is a chemical compound with the formula $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$. It is a white crystalline substance and it's a water soluble. Molar mass was 312.01 g/mol (heptahydrate). Sodium arsenate heptahydrate is a toxic compound that can have significant negative impacts on both human health and the environment. Ingestion of sodium arsenate heptahydrate can lead to acute poisoning symptoms such as abdominal pain, nausea, vomiting, and diarrhea. Long-term exposure to arsenic compounds like sodium arsenate heptahydrate has been linked to several chronic health effects, including skin lesions, peripheral neuropathy, cardiovascular disease, and various types of cancer. When released into the environment, sodium arsenate heptahydrate can contaminate soil and water, potentially harming plants and wildlife. Therefore, it is essential to handle and dispose of sodium arsenate heptahydrate carefully and to avoid any unnecessary exposure to this toxic compound. The crystal structure of disodium hydrogen orthoarsenate heptahydrate, or sodium arsenate heptahydrate, $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, has been determined by single-crystal X-ray analysis by Ferraris & Chiari (1970a) and,

together with its phosphorus isomorph, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ independently by Baur & Khan (1970).

1.9 Experimental fish of *Danio rerio* (Zebrafish)

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Actinopterygii
Order	:	Cypriniformes
Family	:	Cyprinidae
Subfamily	:	Danioninae
Genus	:	<i>Danio</i>
Species	:	<i>D. rerio</i>



Figure 1.1: Experimental fish of *Danio rerio* (Zebrafish)

Danio rerio the zebrafish, is increasingly being used as a model for studying genetic effects in vertebrate development. Zebrafish are native to the slow freshwater streams and rice paddies of East India and Burma, including the Ganges River. They are small, hardy fish, about an inch long that have been bred for many years by fish hobbyists in home aquariums where they may survive for about 5 years. The standard “wild-type” is clear-colored with black stripes that run lengthwise down its body.

CHAPTER – 2

2. LITERATURE SURVEY

Chemical contamination of aquatic ecosystem is unquestionably a global environmental problem. With the increasing anthropogenic activities, toxic residues are continuously mobilized from different sources in to the aquatic systems. In addition to the existing substances new chemicals are produced for industrial, domestic and agricultural applications and most of them are non-biodegradable. Heavy metals are group of elements that are redistributed in various environment at different times (Morais *et al.*, 2012).

Hasty growth of industry and agriculture in the past few decades have resulted in increased heavy metals pollution significantly affects invertebrates, fish, and human beings (Uluturhan and Kucuksezgin 2007). Heavy metals in aquatic environment are currently major polluting chemicals in both developed and developing nations (Lloyd, 2008) due to their toxicity and threat to plants and animals. As a result of heavy metal contamination, consequent devastating effects are potentially ensuing in the receiving environment, thus upsetting the ecological balance (Dimari *et al.*, 2008; Farombi *et al.*, 2007). The overt problem associated with heavy metals in the environment is their accumulation through food chain and persistence in nature (Dimari *et al.*, 2008).

2.1 Heavy metals

The term heavy metal refers to any metallic chemical element that has a relatively high density and is toxic at low concentration. Examples of heavy metals include mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), and lead (Pb). Heavy metals are natural components of the earth's crust. They cannot be degraded or destroyed. Heavy metals are dangerous because they tend to bioaccumulate in organisms (Babu, 1995). Bioaccumulation means an increase in the concentration of a chemical in an organism over time, compared to the chemical's concentration in the environment. Compounds of heavy metals accumulate in organisms any time they are taken up and stored faster than they are metabolized or excreted. Heavy metals enter an aquatic system from industrial and consumer waste, or even from acidic rain, breaking down soils and releasing heavy metals into streams, lakes, rivers and groundwater. Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft

tissues.

After the world war in Japan, the break out of diseases, Itai-itai among the inhabitants along the lower stream of the Jinzu river by cadmium found in the effluents of a mine (Kobayashi, 1970) and Minamata (Irukayama *et al.*, 1961) among the inhabitants living in the vicinity of Minamata bay by mercury attract the attention of toxicologist to decipher the efficacies of various heavy metals on biota. In spite of the fact that some metals are essential at low concentrations for living organisms such as micronutrients (Cu, Zn, Fe, Mn, Co, Mo, Cr, and Se) and macronutrients (Ca, Mg, Na, P, and S) at higher concentrations, they could induce toxic effects disturbing organisms' growth, metabolism and reproduction that consequently affect the entire trophic chain, including human beings (Dimari *et al.*, 2008). In addition to that non-essential metal such as Pb, Cd, Ni, **As**, and Hg elicit toxic effect to organisms even at very low concentrations.

2.2 Causes of metal pollution in aquatic ecosystems

Metals are constantly released in aquatic systems from natural and anthropogenic sources such as industrial activities, domestic sewage discharges, mining, farming, electronic waste, accidents, navigation traffic as well as climatic change event like floods (Babu *et al.*, 1990; Sandu *et al.*, 2008; Zhang *et al.*, 2016).

Metal pollution can arise most commonly from the mining, smelting and nuclear fuel production. Electroplating activity is the primary source of chromium and cadmium contamination in aquatic systems. Through precipitation of their compounds or by ion exchange in soils, heavy metal pollutants can localize and lay dormant. Unlike organic pollutants, heavy metals do not decay and thus pose a different kind of challenge for remediation.

Plants and microorganisms are tentatively used to remove some heavy metals from the contaminated site. Plants which exhibit hyper accumulation can be used to remove heavy metals from soils by concentrating them in their biomass. Some plants are used in the treatment of mining tailings where the vegetation is eventually incinerated to recover the heavy metals. Heavy metals like lead, mercury, iron, cadmium, aluminium and magnesium are present in water bodies reached sediment and enter the food chain through plants and other aquatic organisms cause heavy metal poisoning in case their level in the water is high.

Smelting is a form of extractive metallurgy to produce a metal from its ore. This includes iron, copper and other base metals extraction from their ores. Smelting uses heat and a chemical reducing agent, commonly a fuel that is a source of carbon such as coke, or in earlier time's charcoal, to change the oxidation state of the metal ore. The carbon or carbon monoxide derived from it removes oxygen from the ore to leave the metal. The carbon is thus oxidized, producing carbon dioxide and carbon monoxide. As most ores are impure, it is often necessary to use flux, such as limestone, to remove the accompanying rock gangue as slag. Plants for the electrolytic reduction of aluminium, while not using carbon, are also generally referred as smelters.

Electroplating is a plating process that uses electrical current to reduce cations of a desired material from a solution and coat a conductive object with a thin layer of the material, such as a metal. Electroplating is primarily used for depositing a layer of material to a surface that otherwise lacks some properties such as abrasion and wear resistance, corrosion protection, lubricity and aesthetic qualities. Another application of electroplating is to build up thickness on undersized parts. Water pollution is caused by discharge of domestic or urban sewage, agricultural waste and industrial wastes into water bodies that makes the water unsuitable for drinking and other purposes. This renders the water useless for humans and also endangers aquatic life.

2.3 Metal uptake by aquatic organisms

Metal uptake by aquatic organisms is a two-phased process involving initial rapid adsorption or binding to the surface, followed by slower transport into the cell interior (Crist *et al.*, 1988). In epithelial tissues the last step is the rate-limiting factor in transepithelial movement of metals (Foulkes, 1988). Metal transport inside the cell is carried out either by diffusion of the metal ion across the cell membrane or by carrier protein driven active transport (Brezonik *et al.*, 1991).

2.3.1 Metal uptake by the liver

Liver is the main organ for metal regulation in mammals and other organisms (Heath, 1987). Exposure of organisms to metals results in induction of metallothionein and subsequent binding of metals to the protein (Hogstrand and Haux, 1991). According to Brown and Parsons (1978) formation of metallothionein is a *de novo* process because metallothionein always exists in a metal-saturated

form.

According to Pickering (1993) the stress response can be regarded as a mechanism that enables fish to avoid or overcome potentially threatening or harmful situations. Even though the response may vary according to different circumstances, there appears to be a common element in response to all forms of environmental stress. This involves the activation of the sympathetic chromaffin system to release catecholamines and the hypothalamic-pituitary-interrenal axis for secretion of corticosteroid hormones into the circulatory system. Catecholamines' secretion is particularly rapid in response to respiratory stress and promotes the fish survival by increasing the gill blood supply, blood oxygen carrying capacity and increasing intermediate metabolism to provide a readily accessible energy supply (Pickering, 1993).

Catecholamines induce glycogenolysis (Klee *et al.*, 1990), gluconeogenesis (Morata *et al.*, 1982) and lipid mobilization (Sheridan, 1987) in fish liver. In addition to other hormones catecholamine are responsible for inducing other adaptive changes in liver tissue. Internal metal fluxes may have occurred in reaction to stress-induced hormone secretions, thereby resulting in the release of the naturally bound metals in the liver. These metals are depurated through biliary and renal excretions.

Bioaccumulation is the accumulation of pollutants by organisms in concentrations that are orders of magnitude higher than the ambient concentration. Bioaccumulation is the sum of two processes viz. bioconcentration and biomagnification. Bioconcentration is the direct uptake of a substance by a living organism from the medium via skin, gills or lungs, whereas biomagnification takes place through dietary uptake. Fish that actively filter large amounts of water through their gills are subjected to high proportion of bioconcentration type of bioaccumulation, whereas biomagnification process takes place in predatory organisms. The heavy metals found in the prey are transferred to the predator through food chain.

2.4 Effects of metal contamination

Several heavy metals at appropriate concentrations are essential and at

high concentration they are toxic to organisms. Chronic low exposure of organisms to heavy metals can have serious health effects in the long run. By and large, heavy metal pollution threats to human health are attributed to lead, arsenic, cadmium and mercury poisoning. International legislative bodies focused their attention on these elements for their environmental toxicity.

Aldavood *et al.*, (2020) reported that effect of cadmium and nickel exposure on early development in Zebrafish (*Danio rerio*). The toxicity of nickel and cadmium in zebrafish (*Danio rerio*) embryos exposed to environmentally relevant concentrations of each metal alone or in combination from 4 h through to 72 h postfertilization. These results imply that nickel and cadmium are toxic to developing embryos, even at very low exposure concentrations, and that these metals act via different mechanisms.

Vasanthi *et al.*, (2019) have investigated the toxic effect of mercury on the freshwater fish *Oreochromis mossambicus* and the LC₅₀ values for 24, 48, 72 and 96 hours were 0.73, 0.69, 0.63 and 0.58 ppm respectively.

Elaiyaraja *et al.*, (2018) have studied the effects of zinc sulphate on the morphometrical and biochemical changes in the fish *Cyprinus carpio* sampled from the fish farm of Tamil Nadu Fisheries Development Corporation Limited, Mettur, Tamil Nadu, India. Gradual accumulation of zinc sulphate in the muscle and gill tissues was observed. The severity of zinc toxicity was depicted by the gradual reduction of glucose level in the gill and muscle region of the fish. Zinc toxicity was increased related to duration of the exposure, concentration of zinc, age and sex of the fish.

LeFauve and Connaughton, (2017) have assessed the exposure to heavy metals alters visually-guided behaviors in zebrafish. These results suggest that initial developmental exposure to cadmium and nickel significantly impacts visually-guided larval behavior. The absence of significant differences in gross morphology suggests that the effects of these metals are subtle and may occur at the cellular level. By using this ecologically relevant model and pollutant type, this study has broad application and implications with regard to safe levels of contaminant in drinking water and freshwater ecosystems.

Zaki *et al.* (2011) have observed a significant increase in cholesterol, alkaline phosphatase, alanine aminotransferase (ALT), aspartate

aminotransferase (AST) and cortisol levels and a significant decrease in haemoglobin (Hb), haematocrite (Ht), mean total protein values of serum of *Clarias gariepinus* fish fed with a diet containing 15 ppm mercuric oxide for 4 weeks.

Firat and Kargin (2010) have found that Cr (VI) exposure caused significant elevation in transaminases (serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase), acid phosphatase (ACP), and alkaline phosphatase (ALP) levels in the fish Nile tilapia. Decreased erythrocyte count and changes in other hematological indices were also observed.

Hameed and Muthu (2006) have studied two sublethal concentrations (10% and 30% of LC 50) of Cd for a period of 96 hrs on the fish *Oreochromis mossambicus*. Carbohydrate, protein and the lipid content of fish decreased with the increase in Cd concentrations.

Kothari and Soni (2004) have found that sub-lethal exposure of 150mg/L of ZnSO₄ on fresh water cat fish *H. fossilis* for 30 days caused significant alteration in gill function due to elevated levels of alkaline phosphates over control. Simultaneous feeding of polyunsaturated fatty acids to fish, maintained alkaline phosphate value near normal.

Nevalenyyi and Bednyakov (2004) have assessed the influence of Cd ions on the alpha-amylase, maltase and alkaline phosphatase activities of carp, *Cyprinus carpio*. Alkaline phosphatase activity remained unchanged until 10 days then gradually increased and reached a peak on 30th day.

Bhatkar *et al.* (2004) have tested sub-lethal effects of CrCl₂, NiCl₂ and ZnCl₂ on the biochemical parameters of the fish *L. rohita* for 30 days. After 10 days of exposure to CrCl₂, NiCl₂ and ZnCl₂, blood glucose level increased by 48.11%, 71.71% and 68.35% respectively. However, after 20 and 30 days of treatment, significant reduction in glucose level was observed. Protein and glycogen content of liver and muscle were depleted in all the experimental fish.

Martinez *et al.* (2004) have studied the gill morphology, haematocrite, blood sodium, glucose, lipid, protein and cholesterol levels of fish *Prochilodus lineatus* exposed to sub-lethal concentrations of lead for 96h found histopathological alterations of the gills.

Sesha Srinivas and Rao (1999), and Sharma (2003) have noticed the effect of hexavalent chromium on the gills of *L. rohita* and revealed damage, fusion,

bulging at the distal parts, necrosis of the secondary gill lamellae, atrophy of the central axis, ruptured tips of villi and wide intercellular spaces between villi.

Virk and Sharma (2003) have assessed the effects of acute toxicity of nickel and chromium on fingerlings of the *C. mrigala*. After 45 days of exposure, significant decline in the protein and carbohydrate content of gills was observed.

Earlier studies disclosed that metallothioneins can be induced in fish by exposure to bivalent metal ions of zinc, copper, lead, iron, cobalt and mercury (Carginale *et al.*, 1998; Riggioa *et al.*, 2003).

Rani (2000) has observed congestion of primary rachis, bulging of primary and secondary gill lamellae, hypertrophy and hyperplasia of interlamellar cells, atrophy of several of secondary gill lamellae and necrosis in the gills of *Oreochromis mossambicus* exposed to cadmium. Hypertrophy and thinning of epithelial cells in gill structure are due to adaptive response to enhance oxygen diffusion during stress and secretion of mucous to prevent the entry of toxicant through gill surface.

James *et al.* (1991) have reported the toxic effects of Cu, Zn and Cd in *Oreochromis mossambicus* and the toxicity was in the order of Cu > Zn > Cd. Combination of Zn + Cd was less toxic than the combination of Cu + Cd and Cu + Zn. A significant reduction in protein, carbohydrate and lipid were observed in muscles, liver, gills and whole body of fish *O. mossambicus* exposed to aforementioned metals individually and combinedly but for copper alone.

Gill *et al.*, (1990) have exposed rosy barb (*Puntius conchoni*) to 181 µg/L of mercuric chloride for 48 hours and found increased activities of acid and alkaline phosphatases, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase and acetylcholinesterase in several organs.

Hilmy *et al.* (1987) have examined the changes in total protein of liver, heart, gill and serum enzymes after exposing fish *Mugil cephalus* to cadmium. An increase in the total protein content of the liver, gill and serum was reported with no change in heart protein. The sensitivity of the assayed enzymes to cadmium varied in different tissue.

Abel and Papoutsoglou (1986) have explored the sub-lethal toxicity and accumulation of Cd in *Tilapia aurea*. The Cd appeared to have no effect on the growth or on the moisture, fat protein or ash content or hemoglobin concentration

of fish. However, hematocrit values of fish examined after ten weeks exposure showed a general decline with increasing Cd concentration.

Studies on cadmium induced metallothionein expression in a variety of fish such as turbot *Scophthalmus maximus* (George *et al.*, 1996), carp *Cyprinus carpio* (Smet *et al.*, 2001), goldfish *Carassius auratus* (Choi *et al.*, 2001), sole *Solea solea* (Rovira, 2005), zebra fish *Danio rerio* (Chen *et al.*, 2007), rainbow trout *Oncorhynchus mykiss* (Lange *et al.*, 2007) and tilapia *Oreochromis mossambicus* (Wu *et al.*, 1999) showed increase in the concentration of metallothioneins with the increase in the concentration of Cadmium.

Kling and Olsson (1993) have characterized four metallothionein genes and demonstrated that these genes share common promoter regions and carry several metal responsive elements, which effectively bind metal transcription factors to enhance large synthesis of MTs in target organs. Studies on regulation of MT gene expression provided evidence that there is an induction of MT gene expression with respect to increase in intercellular heavy metal concentration that suggests increase in metallothionein level in cells is correlated to heavy metal contamination in cells (Thiele *et al.*, 1992).

Heavy metals have become a major environmental pollutant today. In higher concentrations they may be lethal to aquatic organisms (Schroder, 1973; Rubin, 1974). The effect of heavy metals on behaviour, reproduction, blood chemistry, survival, growth of fish and other aquatic organisms have been reviewed by Katz (1975). A number of authors have reported the effect of heavy metals on aquatic organisms particularly fishes (Geetha *et al.*, 1996; Olsson *et al.*, 1998; Van den Belt *et al.*, 2000).

Number of physiological changes in response of lead stress have been reported in fish such as disturbances in hormone secretion, ion regulatory processes, oxygen uptake rate and biochemical parameters (Tewari *et al.*, 1987; Thomas, 1990; Ay *et al.*, 1999; Spencer *et al.*, 2000; Baker and Bird, 2002). The extent of such effects depends on the inherent toxicity of the metal, its concentration, its chemical form, the species affected, the animal's condition and the characteristics (Ghazaly, 1992).

Virk and Kaur *et al.* (1998) have pointed out that indiscriminate discharge of heavy metals by the industries have resulted in serious water

pollution problems due to the toxic nature of these metals and their adverse effect on water quality; these may contaminate the aquatic environment, which in turn is likely to affect the aquatic organisms including fish. The seriousness and longevity of the presence of metal in water are compounded by the facts that generally they are water soluble, non-degradable, vigorous oxidizing agents and are strongly bounded to many biochemicals, especially polypeptides and proteins, but also to other materials which possess electron rich function groups such as sulfhydryl, amino and imidazole (Christensen, 1971).

CHAPTER – 3

3. AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM

The aim of the study was to investigate the histological, agarose gel electrophoresis in DNA, protein estimation and antioxidant enzymes in control and sodium arsenate heptahydrate treated Zebrafish (*Danio rerio*).

3.2 OBJECTIVES

- To assess the protein content, the body weight and organ (Liver) weight of *Danio rerio* in control and sodium arsenate heptahydrate treated fish.
- To examine the fragmentation of DNA by using agarose gel electrophoresis in control and sodium arsenate heptahydrate treated fish.
- To observe the histopathology changes in liver of the *Danio rerio* using staining and haematoxylin-eosin (HE) in control and sodium arsenate heptahydrate treated fish.
- To determine the enzymatic antioxidant activities in the liver of control and sodium arsenate heptahydrate treated fish.
- To estimate the non-enzymatic antioxidant activities in the liver of control and sodium arsenate heptahydrate treated fish.

CHAPTER - 4

4. MATERIAL AND METHODS

4.1 EXPERIMENTAL DESIGN

4.1.1 MAINTENANCE AND CARE OF ZEBRA FISH

This protocol describes regular care and maintenance of a zebrafish laboratory. Zebrafish are now gaining popularity in genetics, pharmacological and behavioural research. As a vertebrate, zebrafish share considerable genetic sequence similarity with humans and are being used as an animal model for various human disease conditions. The advantages of zebrafish in comparison to other common vertebrate models include high fecundity, low maintenance cost, transparent embryos, and rapid development. Wild-type zebrafish were obtained from a Local laboratory and were acclimatized to laboratory conditions for 25 days in reverse osmosis water (RO) before the experiments. And they were fed daily with normal fish feed. The room or water temperature is usually maintained between 26-28.5°C. This allows for adequate growth and breeding. The pH level of the water is maintained at 7.0 – 8.0 and is tested regularly.

4.1.2 WORKFLOW

Three tanks were placed in 28°C of room temperature. Taken about 12 *Danio rerio* and separated into 4 representatives in 3 tanks.

4.2 PREPARATION OF TOXICITY SOLUTION

Stock solution (1):

2000µg of the chemical of sodium arsenate heptahydrate were taken and dissolved in 1ml distilled water.

Stock solution (2):

From stock solution 1 0.5ml is taken and dissolved in 0.5ml of distilled water.

4.2.1 WORKFLOW AFTER PREPARING TOXICITY

Each tank covered with 1liter of reverse osmosis water (RO). Take the stock solution 2 from that add the different concentration of each three tanks

Tank 1 – Take 5µL of preparing toxicity solution and add into the tank 1 with the help of pipette.

Tank 2 - Take 10µL of preparing toxicity solution and add into the tank 2 with the help of pipette.

Tank 3 - Take 20µL of preparing toxicity solution and add into the tank 3

with the help of pipette.

Subsequently, adding a toxic solution (SODIUM ARSENATE HEPTAHYDRATE) in different concentration they will be taken for observations and viewing after 96hrs.

4.3 PROTOCOL OF PROTEIN PREPARATION IN TISSUE

2X – SAMPLE BUFFER – 950 + β -mercaptoethanol - 50 μ L} – 1ml of sample buffer. Cover it with the aluminium foil sheet. Take fresh 1.5ml eppendorf tube. Add tissue into that. Add 200 μ L of sample buffer. Homogenize it. Dry bath / water bath at 100°C for 5 minutes. Centrifuged it for 30sec to 1min. Transfer the supernatant in a fresh tube. Store it in -20°C from start to end. Observe the results with the help of nanodrop.

4.4 AGAROSE GEL ELECTROPHORESIS (EXTRACTION OF DNA)

Collect Tissue of danio rerio (zebra fish), 200mg of tissue in 2ml of homogenesis buffer. Add 300 μ L of supernatant into another 2ml eppendorf tube. Add 30 μ L of SDS + 300 μ L phenol + 300 μ L chloroform – isoamyl alcohol mixture. Mix thoroughly shake vigorously and centrifuged in 11,000 rpm for 5 minutes. Collect the aqueous layer. Add 9 μ L of 4M NaCl and 2 volume of 100% ethanol (approximately 500 μ L). Again centrifuged at 12,000rpm for 5 minutes and wash it in 60% ethanol twice. Air dry and dissolve in (100 μ L) or (50 μ L) with milli-Q water. After that note the value by using nanodrop.

Figure 4.4: Dissection of tissue



4.4.1. Preparation of the Gel

Weigh out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask. Add running buffer to the agarose-

containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA). Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved. Add ethidium bromide (EtBr) to a control – 3.65 μ L and concentration of 2.09 μ L. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 2 μ L EtBr for 15-30 min, followed by destaining in running buffer for an equal length of time.

Note: EtBr is a suspected carcinogen and must be properly disposed of per institution regulations. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however EtBr remains the most popular one due to its sensitivity and cost.

Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath. Failure to do so will warp the gel tray. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use

4.4.2. Setting up of Gel Apparatus and Separation of DNA Fragments

Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel. Program the power supply to desired voltage (1-5V/cm between electrodes). Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working. Remove the lid. Slowly and carefully load the DNA sample(s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples. Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are

plugged into the correct slots in the power supply. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

4.4.3. Observing Separated DNA fragments

When electrophoresis has completed, turn off the power supply and remove the lid of the gel box. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer. Remove the gel from the gel tray and expose the gel to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Properly dispose of the gel and running buffer per institution regulations.

4.5 HISTOLOGY

4.5.1 PROTOCOL

Histology is the branch of anatomy that focuses on the study of tissues of animals and plants. The term tissue refers typically to a collection of cells. In humans, organs comprise two or more tissue types, including epithelial, connective tissue, nervous, and muscular. The word "histology" stems from the Greek word "histos," meaning web or tissue, and "logia," meaning branch of learning. In brief, histological processing involves obtaining fresh tissue, preserving it (i.e., fixing it) in order to allow it to remain in as life-like a state as possible, cutting it into very thin sections (3–8 microns), mounting it on glass microscopic slides, and then staining the sections so that they can be observed under a microscope to identify different histological components within the tissue.

Types of Histological preparation:

The histological specimen can be prepared as

1. Whole mount,
2. Sections and
3. Smears.

I did histology in section method

Sections- Most of the preparations in histology are sections. The tissue is cut in about 3-5 mm thick pieces processed and 5 microns thick sections are cut on a microtome. These are then stained and permanently mounted. Microtomes are special instruments which have automatic mechanism for cutting very thin sections. To cut the sections on the microtome; the tissue must be made hard

enough to not get crushed. There are 2 methods of hardening the tissues. One is by freezing them and the other is by embedding them in a hard material such as paraffin wax or gelatin.

4.6 Estimation of Antioxidant activity

4.6.1 Assay of Catalase:

The activity of catalase was assayed by the method of Beers and Sizemore (1952).

Reagents:

- Phosphate buffer : 50 mM/L, pH: 7.0
- Hydrogen peroxide : 0.059 M/L in phosphate buffer pH: 7.0.

Procedure:

1.9 ml of distilled water and 1ml of the hydrogen peroxide reagent, as substrate was added and incubated for 4-5 minutes. Added 0.1 ml of serum sample and recorded the decrease in absorbance for 2-3 minutes at 240nm. Enzyme activity was expressed as $\mu\text{g}/\text{mg}$.

4.6.2 Assay of superoxide dismutase (SOD)

Copper-zinc superoxide dismutase activity was determined by the procedure of Kakkar *et al.* (1984) in plasma.

Reagents

1. Sodium pyrophosphate buffer: 0.025 mol; pH 8.3
2. Phenazine methosulphate: 186 μmol
3. Nitroblue tetrazolium: 300 μmol
4. NADH: 780 μmol
5. Glacial acetic acid
6. n-Butanol
7. Chloroform
8. Ethanol

Procedure

0.5ml of plasma was diluted to 1ml with water. Then 2.5ml of ethanol and 1.5ml chloroform (all reagents chilled) were added. This mixture was shaken for one minute at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.01ml

of phenazine methosulphate, 0.3ml of nitroblue tetrazolium, 0.2ml of NADH, appropriately diluted enzyme preparation and water in a total volume of 3ml. Reaction was initiated by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml of n-butanol. The intensity of the chromogen in the n-butanol layer was measured at 560nm against butanol blank. A system devoid of enzyme served as control. Enzyme activity was expressed as μ /mg.

4.6.3 Determination of Reduced glutathione (GSH):

Reduced glutathione was estimated by method of Moron *et al* (1979).

Reagents:

- 10% TCA solution
- 0.6 mM DTNB in 0.2M sodium phosphate.
- 0.2M phosphate buffer (pH 8.0)

Procedure:

0.5ml of serum sample was precipitated with 1ml of 10% TCA and the precipitate was removed by centrifugation. To 0.5ml of the supernatant 1ml of DTNB was added and the total volume was made upto 3ml with phosphate buffer. The absorbance was read at 412nm. The level of glutathione was expressed as μ /mg.

4.6.4 Estimation of α -tocopherol (Vitamin E):

α -tocopherol was estimated by the method of Baker *et al* (1980).

Reagents:

- Ethanol
- Double distilled water
- 2,2'-dipyridyl solution : 0.2%in ethanol
- Ferric chloride solution : 0.5% in ethanol

Procedure:

To 0.5ml of serum sample, 1.5ml of ethanol was added, mixed and centrifuged. The supernatant was dried at 80°C, to this 0.2ml of 2, 2'-dipyridyl and 0.2ml of ferric chloride solution were added, mixed well and 4.0ml of butanol was added. The developed red colour was read at 520nm. The level of α -tocopherol was expressed as mg/dl in serum. μ g/ mg.

4.6.5 Assay of glutathione-s-transferase (GST):

GST was assayed by the method of Habig *et al* (1974).

Reagents:

1. Phosphate buffer : 0.3M, pH 6.5
2. Reduced glutathione (GSH) : 30mM
3. 1-chloro-2, 4-dinitrobenzene (CDNB) : 30mM

Procedure:

The reaction mixture contained 1.0 ml of phosphate buffer, 0.01ml of CDNB, 0.1 ml of sample and 0.7 ml of distilled water. This mixture was **preincubated** at 37°C for 5 minutes, and then the reaction was started by the addition of 0.1 ml of GSH. The change in the absorbance was read at 340 nm for 5minutes and the reaction mixture without the enzyme was used as the blank. The enzyme activity was expressed as $\mu\text{moles CDNB conjugated/ min/ } \mu\text{ /mg}$.

CHAPTER 5

5. RESULTS AND DISCUSSION

Fish as a taxonomic group are the only primarily aquatic vertebrate class and have, thus, traditionally been regarded as an indispensable component of integrated toxicity testing strategies. In fact, fish may differ not only from vertebrates, but also from most invertebrates in terms of their metabolic capacities, example, in biotransformation competence for certain chemical classes. Moreover, given the occasionally high pollution levels and frequencies of chemical spills, fish have frequently been the targets of overt chemical pollution. From both political and emotional points of view, fish kills provided a straightforward tool to communicate the need for reduction of chemical discharge via direct spills and by continuous release from sewage treatment plants. Last, but not least, from anthropogenic perspective, fish have traditionally been used as sentinels for the quality of waters that serve as sources for human drinking water. Given the importance of fish in aquatic pollution monitoring, both at the national and international levels, fish have thus intensively been implemented in aquatic toxicity testing regulations. At the OECD level, a whole set of test guidelines using fish as test organisms has been established for the testing of acute toxicity (OECD 2003). The zebrafish, a small teleost species, has rapidly become a worldwide fundamental animal model in many scientific branches such as development analysis, molecular biology, toxicity, genetics, immunity and for the study of several diseases. The zebrafish is considered one of the most versatile experimental animal models. The transparency of the embryos, the small size, the rapid development and the homology with higher vertebrates have made the zebrafish a valuable model also for drug screening. Its use is closely related for the determination of bioactivity, toxicity and off-target side effects of novel drug candidates, which also allows a thorough evaluation of new targets; thus, it may represent a suitable model for toxicity screening and the optimization of novel candidates (Abbate *et al.*, 2021). The priority list of pollutants compiled by the Environmental Protection Agency (EPA) of the United States contains eight widespread heavy metals, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc. Among these, arsenic is more significant from environmental and toxicological point of view. Fish appear to have evolved different mechanisms for

biotransformation of as to less toxic forms, which are then readily excreted (Beare*et al.* 2006).

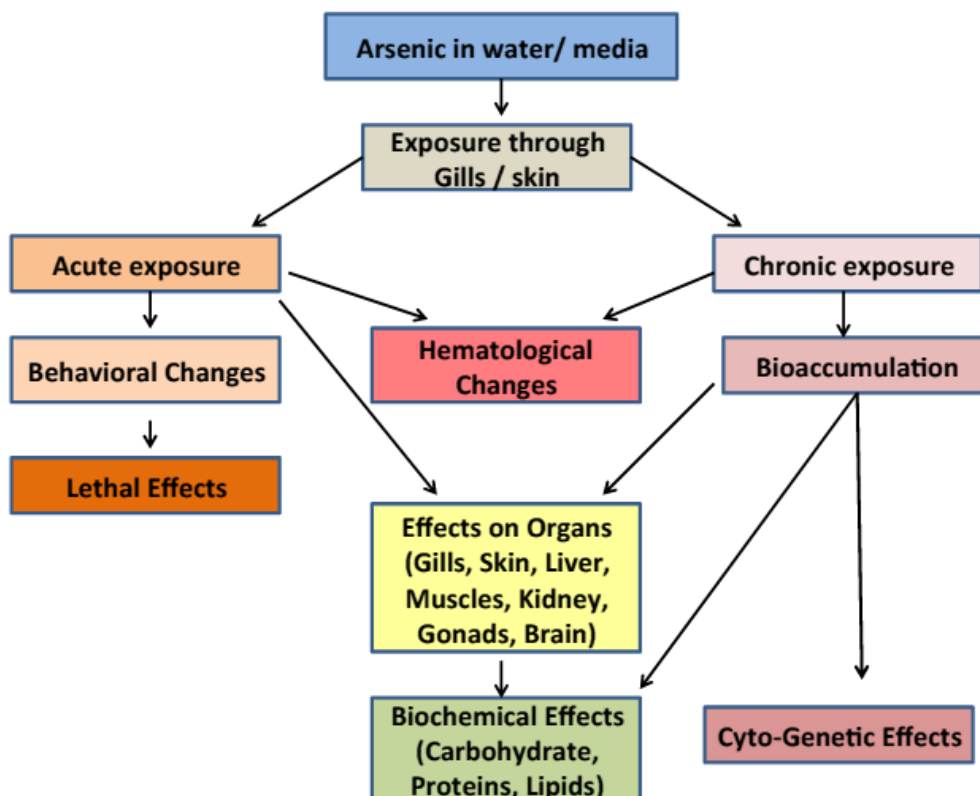


Fig. 5.0 Arsenic in water with acute and chronic exposure through skin/gills and their toxicological pathway in fish

The high level of as concentration, i.e., more than permissible limit as aquatic ecosystem affects various physiological systems such as growth, reproduction, ion regulation, smoltification, gene expression, immune function, enzyme activities and histopathology of fish (Figure 5.0)

Toxicity studies generally begin in *in vitro* studies, with many different cell lines at different sample concentrations. The substance is then tested in several animal models, especially in mice and rats, before using on patients. Zebrafish as an animal model could be extremely useful for intermediate toxicity tests (Chahardehi *et al.*, 2020). In the present study was to assess the histopathology, agarose gel electrophoresis in DNA, protein estimation and antioxidant enzymes in sodium arsenate heptahydrate exposed in Zebrafish (*Danio rerio*).

5.1 Effect of Sodium arsenate heptahydrate on body and liver weight of experimental Zebrafish (*Danio rerio*)

The effect of Sodium arsenate heptahydrate on body weight of experimental Zebrafish (*Danio rerio*) was investigated and represent in table 5.1 and figure 5.1. The body weight of control fish was 0.50g , 10ppm of Sodium arsenate heptahydrate exposed fish body weight was 0.30g and 20ppm of Sodium arsenate heptahydrate exposed fish body weight was 0.25g were observed. The body weight of the fish decreased with increased Sodium arsenate heptahydrate concentration as compared with control fish. Among the 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the body weight of Zebrafish was decreased in 20ppm exposed compared to control and 10ppm exposed of Sodium arsenate heptahydrate. Body weight is the important index of the toxicity studies. In this study decreased the body weight with increased Sodium arsenate heptahydrate indicated the toxicity of the Sodium arsenate heptahydrate.

Table 5.1 Effect of Sodium arsenate heptahydrate on body weight of experimental Zebrafish (*Danio rerio*)

CONTROL (g)	Na ₂ HAsO ₄ .7H ₂ O TREATED (10ppm)	Na ₂ HAsO ₄ .7H ₂ O TREATED (20ppm)
0.50(g)	0.30(g)	0.25(g)

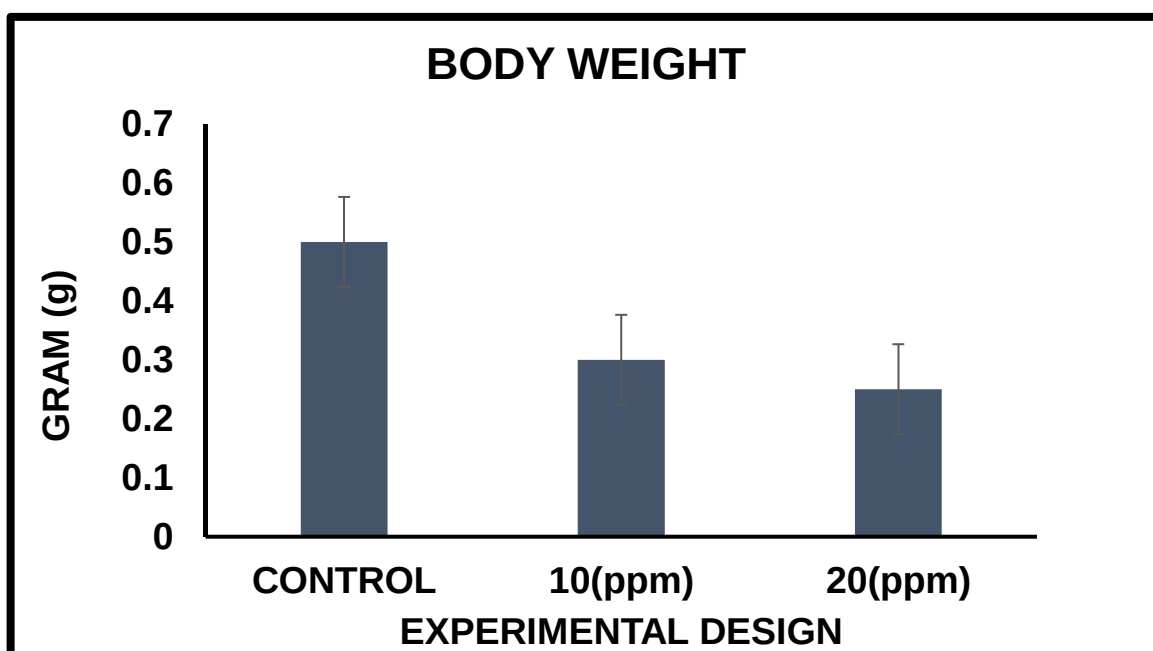


Figure 5.1 Effect of Sodium arsenate heptahydrate on body weight of experimental Zebrafish (*Danio rerio*)

The effect of Sodium arsenate heptahydrate on liver weight of experimental Zebrafish (*Danio rerio*) was investigated and represent in table 5.2 and figure 5.2. The liver weight of control fish was 0.70mg, 10ppm of Sodium arsenate heptahydrate exposed fish liver weight was 0.56mg and 20ppm of Sodium arsenate heptahydrate exposed fish liver weight was 0.40mg were noticed. The liver weight of the fish decreased with increased Sodium arsenate heptahydrate concentration as compared with control fish. Among the 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the liver weight of Zebrafish was decreased in 20ppm exposed compared to control and 10ppm exposed of Sodium arsenate heptahydrate. Many toxins specifically affect the liver, as this is the primary site of xenobiotic metabolism in vertebrates. Toxin-specific hepatic responses have been identified by decreased liver weight with increased Sodium arsenate heptahydrate concentration. Many studies showed the decreased liver weight in response to a range of toxins, including arsenic, acetaminophen, and ethanol (Xu *et al.*, 2013; Zhang *et al.*, 2014).

CONTROL	Na₂HAsO₄·7H₂O TREATED (10ppm)	Na₂HAsO₄·7H₂O TREATED (10ppm)
0.7(mg)	0.56(mg)	0.40(mg)

Table 5.2 Effect of Sodium arsenate heptahydrate on liver weight of experimental Zebrafish (*Danio rerio*)

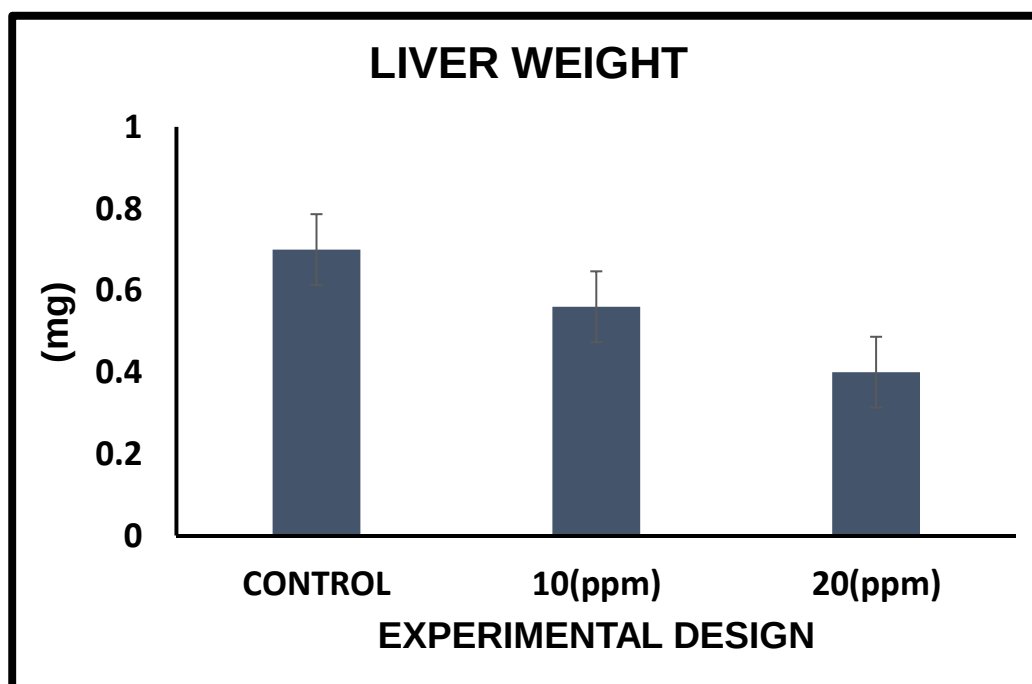


Figure 5.2 Effect of Sodium arsenate heptahydrate on liver weight of experimental Zebrafish (*Danio rerio*)

Proteins play a vital role in the physiology of living organisms. Proteins are highly sensitive to heavy metals and are one of the earliest indicators of heavy metal poisoning (Jacobs *et al.* 1977). Proteins are important substances required by organisms in tissue building and play an important role in energy metabolism (Yeragi *et al.* 2003). The effect of Sodium arsenate heptahydrate on protein content of experimental Zebrafish (*Danio rerio*) was investigated and represent in table 5.3 and figure 5.3. The protein content control fish was 2.548mg/ml, 10ppm of Sodium arsenate heptahydrate exposed fish protein content was 1.073mg/ml and 20ppm of Sodium arsenate heptahydrate exposed fish protein content was 0.857gm were noticed. The protein content of the fish decreased with increased

Sodium arsenate heptahydrate concentration as compared with control fish. Among the 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the protein content of Zebrafish was significantly decreased in 20ppm exposed compared to control and 10ppm exposed of Sodium arsenate heptahydrate. The depletion of protein in tissues after treatment with arsenic might be due to reduction in rate of protein synthesis and/or excessive proteolysis during stress condition. Present study agreement with Jana and Bondyopadhyay (1981) who observed a reduction in protein content has been noticed when the fish *Channa punctatus* was exposed to heavy metals such as mercury, arsenic and lead.

Table 5.3 Effect of Sodium arsenate heptahydrate on protein content in experimental Zebrafish (*Danio rerio*)

CONTROL	Na ₂ HAsO ₄ .7H ₂ O TREATED (10ppm)	Na ₂ HAsO ₄ .7H ₂ O TREATED (20ppm)
2.548(mg/ml)	1.073(mg/ml)	0.857(mg/ml)

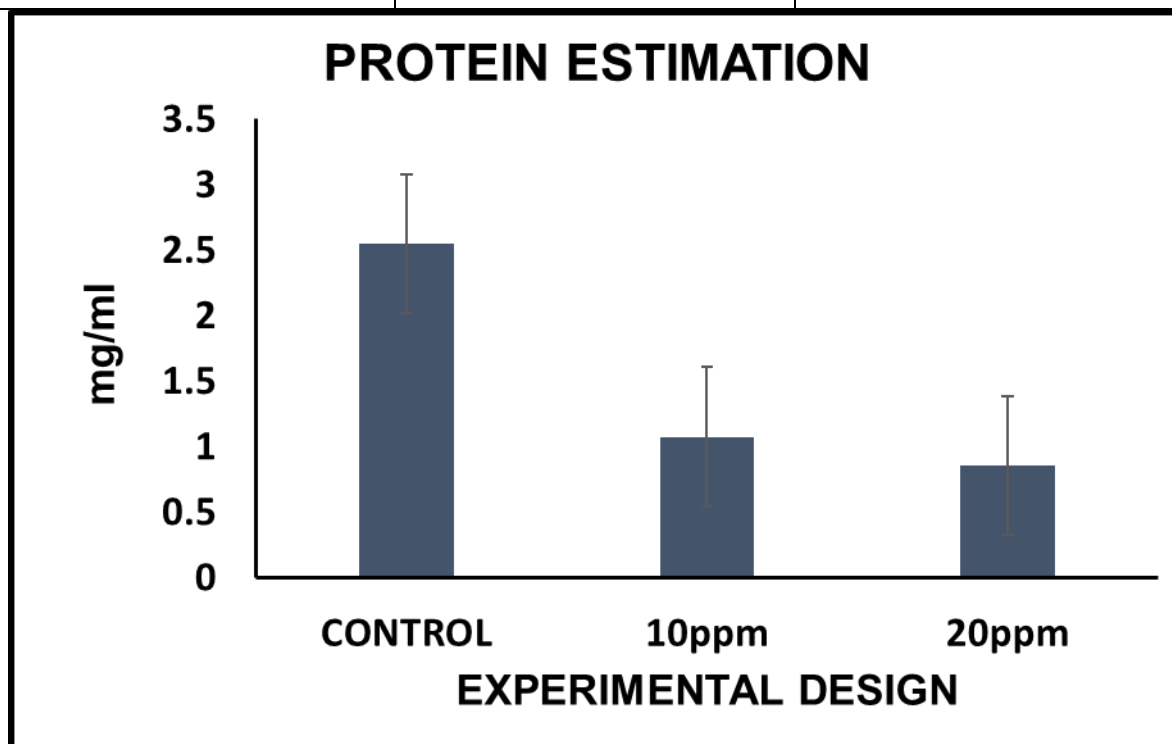


Figure 5.3 Effect of Sodium arsenate heptahydrate on protein content in experimental Zebrafish (*Danio rerio*)

5.4 Effect of Sodium arsenate heptahydrate on DNA of experimental Zebrafish (*Danio rerio*) using Agarose gel electrophoresis

Representative photographs of gel electrophoresis for arsenate induced DNA fragmentation analysis were showed in figure 5.4. The DNA fragmentation is increased in 20ppm of Sodium arsenate heptahydrate treated fish as compared with control. The significant alteration was observed in oxidative base-associated strand breaks. The DNA fragmentation indicates that Sodium arsenate heptahydrate induced sever DNA damage. Production of free radicals by arsenic exposure results in oxidative stress. Free radicals can react with macromolecules, including nucleic acids, proteins, and lipids. Free radicals attack DNA, causing oxidative DNA damage.

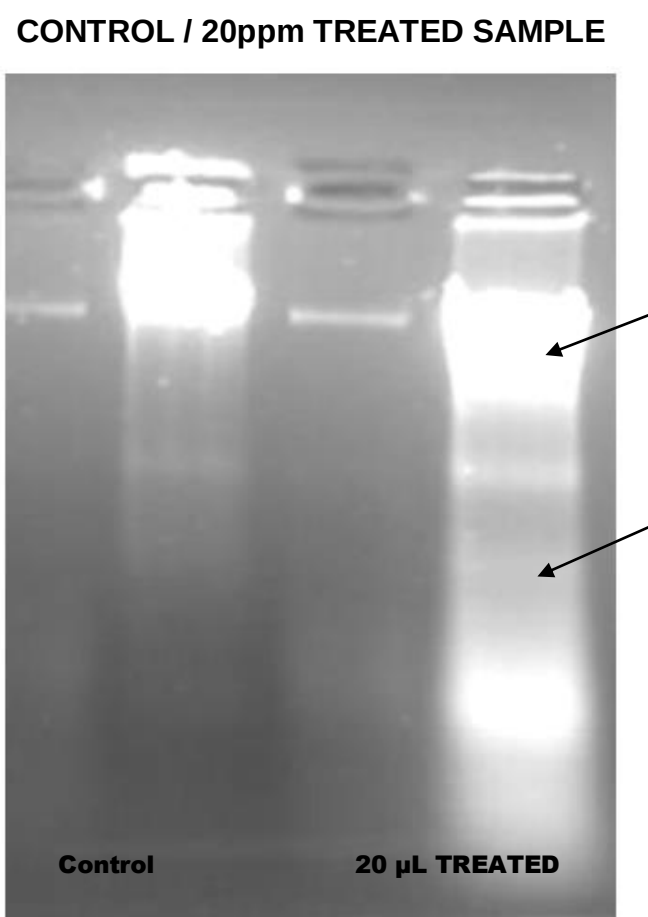


Figure 5.4 Photographs of gel electrophoresis for evaluation of DNA damage in Zebrafish (*Danio rerio*) exposed to Sodium arsenate heptahydrate. Gel electrophoresis was conducted on agarose gel in alkaline buffer for total strand break assay

5.5 Effect of Sodium arsenate heptahydrate on antioxidant status in experimental Zebrafish (*Danio rerio*)

The aquatic animal system has a complex system of natural enzymatic and non- enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants (Badarinath *et al.*, 2010). Free radicals are responsible for causing a large number of diseases including cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, aging and atherosclerosis (Velavan, 2011; Smith *et al.*, 2000). Protection against free radicals can be enhanced by ample intake of dietary antioxidants. Substantial evidence indicates that foods containing antioxidants and possibly in particular the antioxidant nutrients may be of major importance in disease prevention. The enzymatic antioxidants as SOD, Catalase, GST, GPX and non- enzymatic antioxidants as GSH, Vitamin E and Vitamin C are important to nullify the toxic effects. There is, however, a growing consensus among scientists that a combination of antioxidants, rather than single entities, may be more effective over the long term (Blokhina *et al.*, 2003).

Catalase is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Table 5.6 and figure 5.6 represents the effect of Sodium arsenate heptahydrate on catalase (CAT) activity in experimental Zebrafish (*Danio rerio*). The catalase activity of control was 0.097 μ /mg, 5ppm of Sodium arsenate heptahydrate exposed fish was 0.095 μ /mg, 10ppm of Sodium arsenate heptahydrate exposed fish was 0.092 μ /mg and 20ppm of Sodium arsenate heptahydrate exposed fish was 0.091 μ /mg were observed. Among the 5, 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the catalase activity of Zebrafish was decreased in 20ppm exposed fish as compared to control, 5 and 10ppm exposed Sodium arsenate heptahydrate. Excessive production of free radicals from detoxification of Sodium arsenate heptahydrate may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide.

Table 5.6 Effect of Sodium arsenate heptahydrate on catalase (CAT) activity in experimental Zebrafish (*Danio rerio*)

CONTROL	TREATED (5ppm)	TREATED (10ppm)	TREATED (20ppm)
0.097(μ/mg)	0.095(μ/mg)	0.092(μ/mg)	0.091(μ/mg)

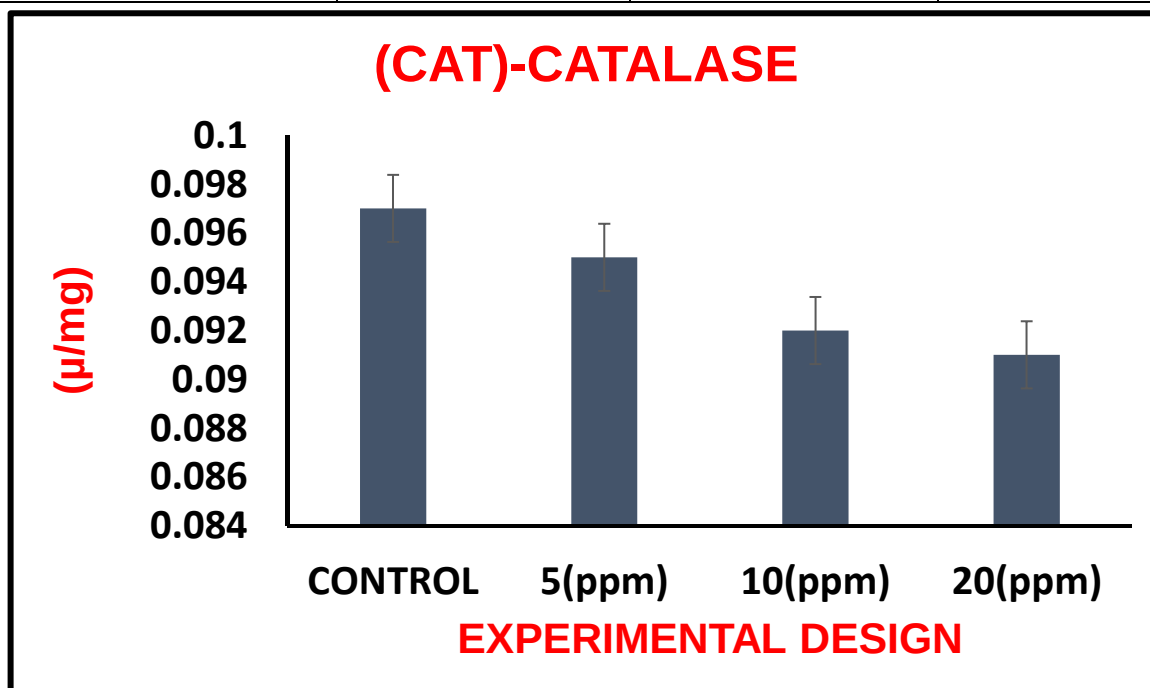


Figure 5.6 Effect of Sodium arsenate heptahydrate on catalase (CAT) activity in experimental Zebrafish (*Danio rerio*)

The SOD dismutase superoxide radicals O_2^- into H_2O_2 plus O_2 , thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. Table 5.7 and figure 5.7 represents the effect of Sodium arsenate heptahydrate on superoxide dismutase (SOD) activity in experimental Zebrafish (*Danio rerio*). The superoxide dismutase activity of control was 2.211μ/mg, 5ppm of Sodium arsenate heptahydrate exposed fish was 2.209 μ/mg, 10ppm of Sodium arsenate heptahydrate exposed fish was 2.199 μ/mg and 20ppm of Sodium arsenate heptahydrate exposed fish was 2.194μ/mg were observed. Among the 5, 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the superoxide dismutase activity of Zebrafish was decreased in 20ppm exposed fish as

compared to control, 5 and 10ppm exposed Sodium arsenate heptahydrate. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues.

Table 5.7 Effect of Sodium arsenate heptahydrate on superoxide dismutase (SOD) activity in experimental Zebrafish (*Danio rerio*)

CONTROL	TREATED (5ppm)	TREATED (10ppm)	TREATED (20ppm)
2.211(μ /mg)	2.209(μ /mg)	2.199(μ /mg)	2.194(μ /mg)

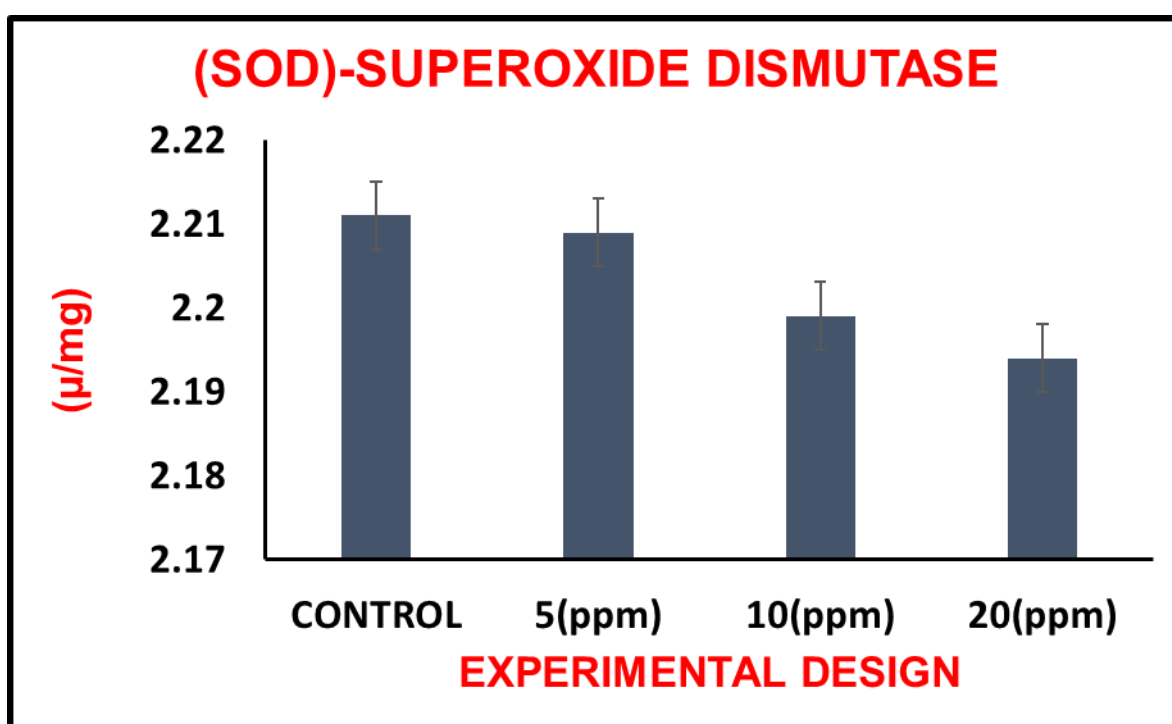


Figure 5.7 Effect of Sodium arsenate heptahydrate on superoxide dismutase (SOD) activity in experimental Zebrafish (*Danio rerio*)

Zebrafish is a widely used animal model in biomedical sciences and toxicology. Although evidence for the presence of phases I and II xenobiotic defense mechanisms in zebrafish exists on the transcriptional and enzyme activity level, little is known about the protein expression of xenobiotic metabolizing enzymes. Given the important role of glutathione S-transferases (GSTs) in phase II biotransformation (Christen *et al.*, 2014). GSTs are an enzyme family that plays a major role in phase II biotransformation processes by catalyzing the conjugation reaction of the tripeptide glutathione (GSH) with electrophilic substrates. This

reaction typically results in the formation of more hydrophilic and readily excretable products. Accordingly, GST activity is considered a critical contributor to detoxification and clearance of various intracellular metabolites, but also natural toxins and xenobiotic compounds, including drugs, and their reactive intermediates (Sau *et al.*, 2010). Table 5.8 and figure 5.8 represents the effect of Sodium arsenate heptahydrate on Glutathione-S-transferase (GST) activity in experimental Zebrafish (*Danio rerio*). The Glutathione-S-transferase activity of control was 28.32 μ /mg, 5ppm of Sodium arsenate heptahydrate exposed fish was 26.33 μ /mg, 10ppm of Sodium arsenate heptahydrate exposed fish was 26.29 μ /mg and 20ppm of Sodium arsenate heptahydrate exposed fish was 25.31 μ /mg were observed. Among the 5, 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the Glutathione-S-transferase activity of Zebrafish was decreased in 20ppm exposed fish as compared to control, 5 and 10ppm exposed Sodium arsenate heptahydrate. Decreasing our knowledge about GSTs therefore will help to better understand the xenobiotic defense mechanisms and their contribution to nullify the free radicals.

Table 5.8 Effect of Sodium arsenate heptahydrate on Glutathione-S-transferase (GST) activity in experimental Zebrafish (*Danio rerio*)

CONTROL	TREATED (5ppm)	TREATED (10ppm)	TREATED (20ppm)
28.32(μ /mg)	26.33(μ /mg)	26.29(μ /mg)	25.31(μ /mg)

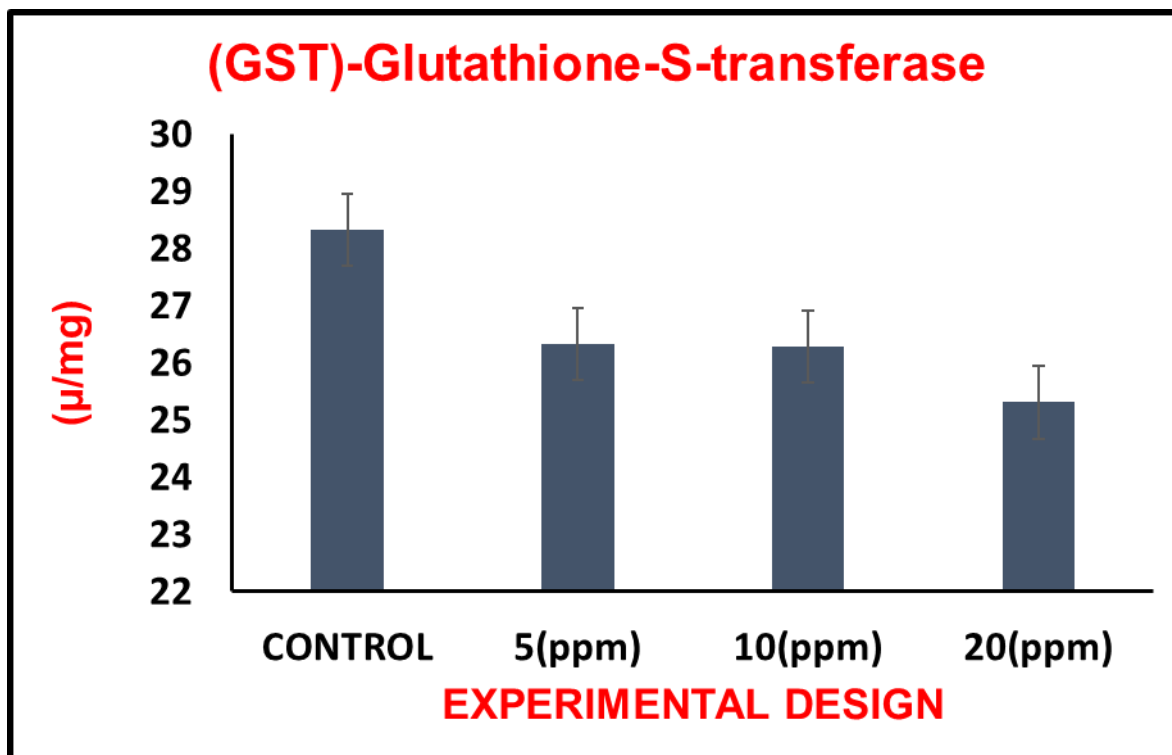


Figure 5.8 Effect of Sodium arsenate heptahydrate on Glutathione-S-transferase (GST) activity in experimental Zebrafish (*Danio rerio*)

Circulatory non-enzymatic antioxidant such as vitamin E is free radical scavengers. Their synergistic action in scavenging oxygen-derived free radicals is well documented (Wojacki *et al.*, 1995). Vitamin E reacts with lipid peroxy radicals acting as a chain terminator of lipid peroxidation while vitamin C helps to maintain the level of vitamin E at optimum concentrations. Table 5.9 and figure 5.9 represents the effect of Sodium arsenate heptahydrate on Vitamin E content in experimental Zebrafish (*Danio rerio*). The Vitamin E content of control was 7.96μ/mg, 5ppm of Sodium arsenate heptahydrate exposed fish was 7.85 μ/mg, 10ppm of Sodium arsenate heptahydrate exposed fish was 7.50 μ/mg and 20ppm of Sodium arsenate heptahydrate exposed fish was 7.38μ/mg were observed. Among the 5, 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the vitamin E content of Zebrafish was decreased in 20ppm exposed fish as compared to control, 5 and 10ppm exposed Sodium arsenate heptahydrate. The levels of vitamin E in the present study is significantly reduced in Sodium arsenate heptahydrate exposed fish as compared with control. This reduced content of vitamin E indicates that utilization for scavenging of free radicals which are

produced in Sodium arsenate heptahydrate.

Table 5.9 Effect of Sodium arsenate heptahydrate on Vitamin E content in experimental Zebrafish (*Danio rerio*)

CONTROL	TREATED (5ppm)	TREATED (10ppm)	TREATED (20ppm)
7.96(μ /mg)	7.85(μ /mg)	7.50(μ /mg)	7.38(μ /mg)

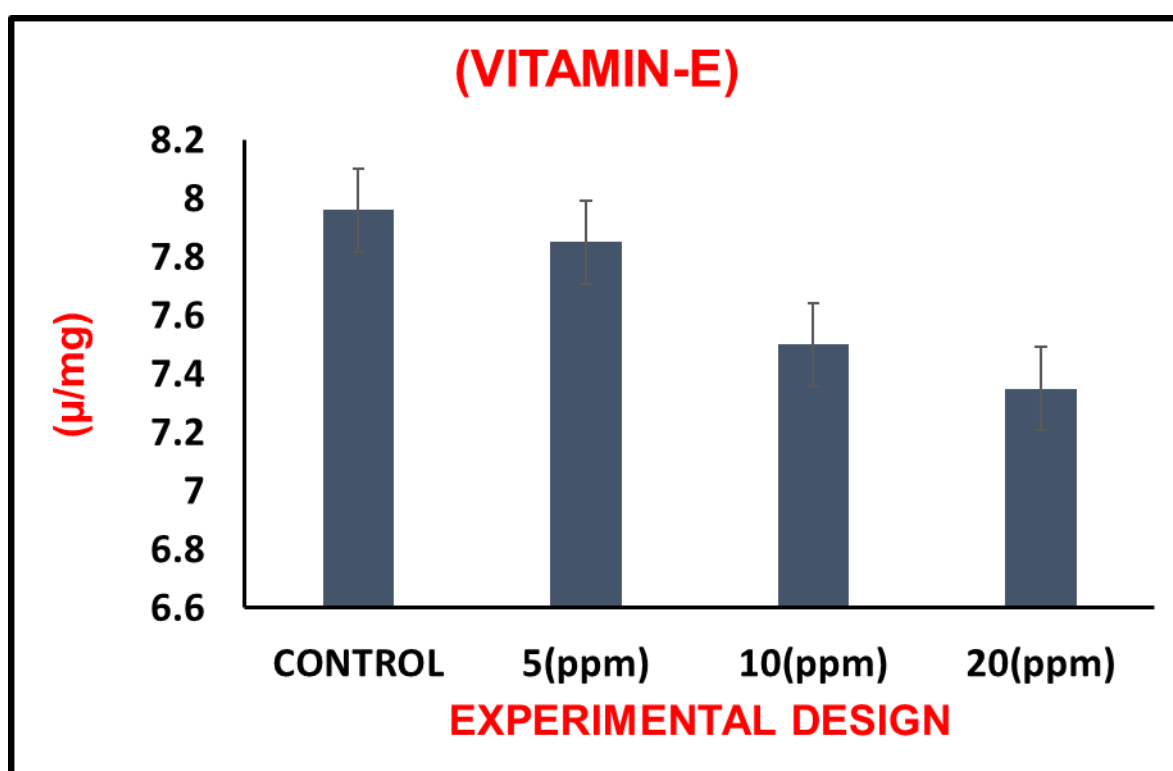


Figure 5.9 Effect of Sodium arsenate heptahydrate on Vitamin E content in experimental Zebrafish (*Danio rerio*)

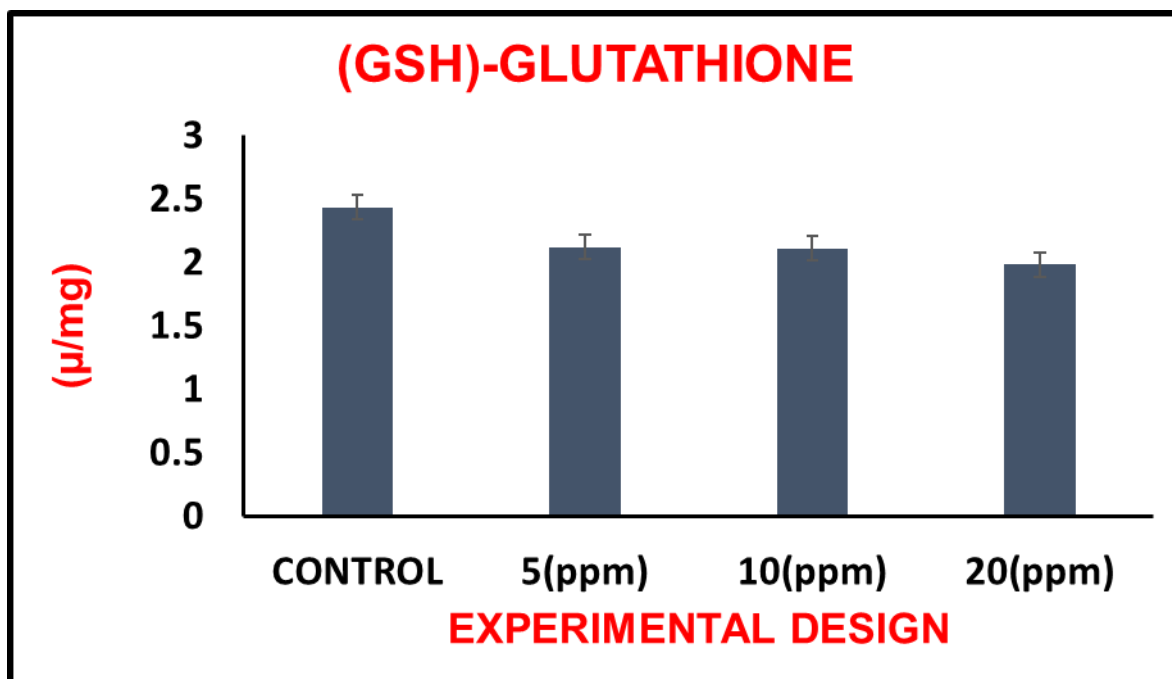
Glutathione is a ubiquitous thiol containing tripeptide, which plays a central role in cell biology. It is implicated in the cellular defence against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydro peroxides. Glutathione status is a highly sensitive indicator of cell functionality and viability. GSH depletion is linked to a number of diseases states including cancer, neurodegenerative diseases, kidney and cardiovascular diseases. Kidneys are exposed to various cytotoxic agents before the elimination of these agents in

urine. Thus, the GSH concentrations in liver cells are important (Pastore *et al.*, 2003). Table 5.10 and figure 5.10 represents the effect of Sodium arsenate heptahydrate on Glutathione (GSH) content in experimental Zebrafish (*Danio rerio*). The glutathione content of control was 2.43 μ /mg, 5ppm of Sodium arsenate heptahydrate exposed fish was 2.12 μ /mg, 10ppm of Sodium arsenate heptahydrate exposed fish was 2.11 μ /mg and 20ppm of Sodium arsenate heptahydrate exposed fish was 1.98 μ /mg were observed. Among the 5, 10 and 20ppm of Sodium arsenate heptahydrate exposed in fish, the glutathione content of Zebrafish was decreased in 20ppm exposed fish as compared to control, 5 and 10ppm exposed Sodium arsenate heptahydrate. In the present study, decreased content of GSH in Sodium arsenate heptahydrate as compared to control. The decreased plasma GSH content, implying increased consumption for oxygen radical cleansing activity.

Table 5.10 Effect of Sodium arsenate heptahydrate on Glutathione (GSH) content in experimental Zebrafish (*Danio rerio*)

CONTROL	TREATED (5ppm)	TREATED (10ppm)	TREATED (20ppm)
2.43(μ /mg)	2.12(μ /mg)	2.11(μ /mg)	1.98(μ /mg)

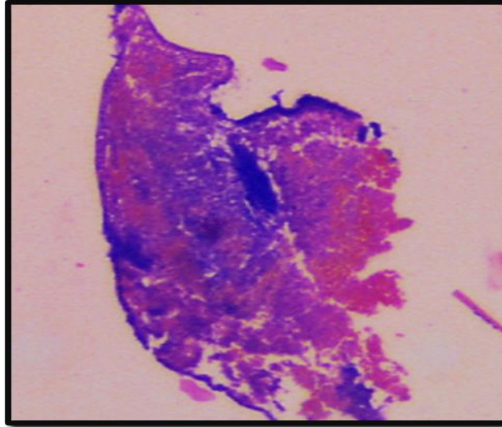
Figure 5.10 Effect of Sodium arsenate heptahydrate on Glutathione (GSH) content in experimental Zebrafish (*Danio rerio*)



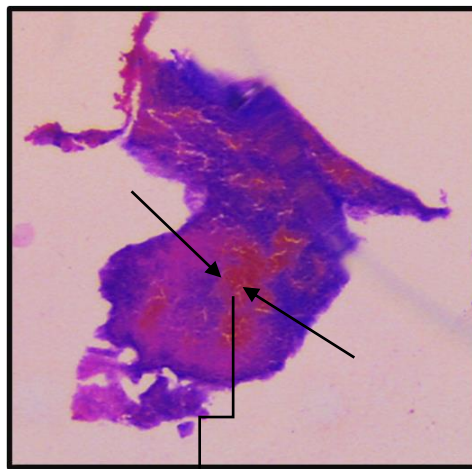
5.11 HISTOPATHOLOGY

Histopathological studies help in understanding the stress caused to the animal exposed to pollution. This is a universal method for the assessment of the impact of xenobiotics on the tissues of animal. The ability of any tissue to regulate its normal physiological function is extensively related to its structural integrity. Any damage to the tissues usually results in altered and frequently abnormal metabolic activities. Hence there is a need to study the histopathological changes in liver of Zebrafish in response to exposure of Sodium arsenate heptahydrate. Moderate alterations were observed in liver of Sodium arsenate heptahydrate exposed Zebrafish (Figure 5.11) as compared to control fish.

CONTROL – 500 μm



20 μL TREATED SAMPLE - 500 μm



Affected region

Figure 5.11 Histological observation of liver in control and experimental Zebrafish (*Danio rerio*)

CHAPTER 6

6. SUMMARY AND CONCLUSION

Zebrafish are a valuable tool for Environmental Health researchers as evidenced by a rapidly expanding body of research using zebrafish. A PubMed search using the terms “zebrafish environmental health” reveals that the use of zebrafish in this field has been steadily increasing over the past few decades. Zebrafish is a widely used animal model in biomedical sciences and toxicology. Although evidence for the presence of phases I and II xenobiotic defense mechanisms in zebrafish exists on the transcriptional and enzyme activity level.

Liver is the most important organ concerned with the biochemical activities in the human body. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. There is an increasing need of an agent, which could protect it from such damage. A number of pharmacological and chemical agents act as hepatotoxin and produce variety of liver ailments. Almost all the metals are toxic at higher concentrations and some are lethal even at a very low concentration. The priority list of pollutants compiled by the Environmental Protection Agency (EPA) of the United States contains eight widespread heavy metals, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc. Among these, arsenic is more significant from environmental and toxicological point of view. In the present study was to assess the histopathology, agarose gel electrophoresis in DNA, protein estimation and antioxidant enzymes in sodium arsenate heptahydrate exposed in Zebrafish (*Danio rerio*).

The following conclusion obtained from the study

- In the present experiment study, the sodium arsenate heptahydrate toxicity causes the physical aspects.
- The decreased in body and organ weight (liver) of sodium arsenate heptahydrate treated zebra fish were noticed due to the arsenate stress.
- A reduction in the protein content in the present investigation in zebra fish suggests that the tissue protein might have undergone proteolysis, during the stressful situation in the sodium arsenate heptahydrate intoxicated fishes.

- The DNA fragmentation was observed in 20μL of sodium arsenate heptahydrate intoxicated fish.
- The entire variable tested i.e., SOD, CAT, GST, reduced glutathione and vitamin E recorded a decrease on sodium arsenate heptahydrate intoxicated fishes indicates that the protective mechanism in response to ROS generated by arsenate.
- Histological observation showed the liver is a sign of damage was observed using microscopic examination of a biopsy.

Overall, it can be concluded that sodium arsenate heptahydrate induced the liver toxicity in dose dependent manner via oxidative stress confirmed by biochemical, antioxidants and histological studies.

REFERENCES

- Abbate F, Maugeri A, Laurà R, Levanti M, Navarra M, Cirimi S, Germanà A. Zebrafish as a Useful Model to Study Oxidative Stress-Linked Disorders: Focus on Flavonoids. *Antioxidants*. (2021), 10(5):668.
- Abel PD, Papoutsoglou SE. Lethal toxicity of cadmium to *Cyprinus carpio* and *Tilapia aurea*. *Bull. Environ. Contam. Toxicol*, 37, (1986), 382-386.
- Adami G. M. Barbieri, P. Fabiani, M. Piselli S., Predonzani S., and Resenhofer E. Levels of cadmium and zinc in hepatopancreas of reared *Mytilus galloprovincialis* from the Gulf of Trieste (Italy) chemosphere., 48, (2002), 671-677.
- Agarwal S. K. Bioassay evaluation of acute toxicity levels of mercuric chloride to an air breathing fish *Channa punctatus* (Bloch): Mortality and behavior study. *J. Environ. Biol*. 12: (1991) 99-106.
- Alabaster J. S. and Lloyd R. Water quality criteria for fresh water fish, 2nd Ed. FAO. By Butterworth Scientist, London, (1982), P.361.
- Aldavood, S. J., Abbott, L. C., Evans, Z. R., Griffin, D. J., Lee, M. D., Quintero-Arevalo, N. M., & Villalobos, A. R.. Effect of cadmium and nickel exposure on early development in zebrafish (*Danio rerio*) embryos. *Water*, 12(11), (2020), 3005.
- APHA/AWWAWPCF Standard method for the examination of water and waste water, 20th ed. Am. Public Health Assoc., New York. (1998).
- Arulmozhi, V., Krishnaveni, M., Karthiashwaran, K., Dhamodharan, G. and Mirunalini, S. Antioxidant and antihyperlipidemic effect of *Solanum nigrum* fruit extract on the experimental model against chronic ethanol toxicity, *Phcog. Mag.*, 6, (2010), 42-50.
- Ay, O. Kalay, M., Tamer, L. and Canli, M. Copper and Lead Accumulation in Tissue of a Fresh Water Fish *Tilapia zillii* and its effects on the Branchial Na, K-ATPase Activity. *Bull. Environ. Contam. Toxicol.*, 62: 1999, 160-167.
- Babu, S. Heavy metals (Cd, Cr, Cu, Hg, Ni, Pb & Zn) in the Ichthyofauna of Bay of Bengal. Ph.D. Thesis, Annamalai University, 80. (1995).
- Babu, S., Subramanian, A. N., Venugopalan, V. K., & Ayyakannu, K. Vellar Estuary and Surrounding Environment. In: Chemical Studies on the fate of pesticides and heavy metals in the tropical environment. *Ryo Tatsukawa*

- (ed.), (1990), 4 – 13.
- Badarinath, A. V., RAo, K. M., Chetty, C. M. S., Ramkanth, V., Rajan, T. V. S., & Gnanaprakash, K. A review on in-vitro antioxidant methods: comparisons, correlations and considerations, *Int. J. PharmTech Res.*, 2(2), (2010). 1276–1285.
- Baker H., Frank O., De Angeles B and Feinglod S. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. *Nutrition Reports International.*, 21, 1980, 531-565
- Baker, BI and Bird, D.J. Neuronal organization of the melanin — concentrating hormone system in primitive actinopterygians : evolutionary changes leading to the teleosts. *Comp. Neurol.*, 442(2): 2002,99-114.
- Banerjee S.K., Dastidar S. G., Mukopadhyay P.K., and Dehari P.V. :- Toxicity of Cadmium a comparative batrachus, in the non-air breathing one *Tilapia mossambica* (Peters) *Indian. J. Exp. Biol.*, 16: (1978), 1274-1277.
- Baur, W. H. Zur Kristallchemie der Salzhydrate. Die Kristallstrukturen von $\text{MgSO}_4 \cdot 4\text{H}_2\text{O}$ (leonhardtite) und $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ (rozenite). *Acta Crystallographica*, 15(9), (1962). 815-826.
- Bears H, Richards JG, Schulte PM Arsenic exposure alters hepatic arsenic species composition and stress-mediated gene expression in the common killifish (*Fundulus heteroclitus*). *Aquat Toxicol* 77: (2006), 257–266
- Beers R and Sizer I. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, 195, 1952, 133.
- Bhaskara Reddy, M. V., Arul, J., Angers, P., & Couture, L. Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearum* and improves seed quality. *Journal of Agricultural and Food chemistry*, 47(3), (1999). 1208-1216.
- Bhatkar, N., Vankhede, G. N., & Dhande, R. R. Heavy metal induced biochemical alterations in fresh water fish *Labeo rohita*. *J. Ecotoxicol. Environ. Monit.*, 14(1): (2004), 1-7.
- Bickers, D.R. and Athar, M., Oxidative stress in the pathogenesis of skin disease, *J. Invest. Dermatol.*, 126, (2006), 2565-2575.
- Blokhina O, Virolainen E and Fagersted KV Antioxidants, oxidative damage and

- oxygen deprivation stress: a review. *Annals Bot.* 91(2): (2003),179-94.
- Bosch, M.S. The role of α -tocopherol in plant stress tolerance, *J. Plant Physiol.*, 162, (2005), 743-748.
- Brezonik P. L., King, S. O., & Mach, C. E. (1991). The influence of water chemistry on trace metal bioavailability and toxicity to aquatic organisms. In *Metal Ecotoxicology: Concepts and Applications*, Newman MC, McIntosh AW (eds) Lewis Publishers Inc: *Michigan*, 1-26.
- Brown, D. A., & Parsons, T. R., (1978). Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (*Oncorhynchus kern*) exposed to mercury in a controlled ecosystem. *J. Fish. Res. Board Can.* 35, 880-884.
- Carginale, V., Scudiero, R., Capasso, C., Capasso, A., Kille, A., Di Prisco, G., & Parisi, E. (1998). Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish, which exhibits no basal metallothionein protein but high endogenous mRNA levels. *Biochemical Journal*, 332, 475–481.
- Chahardehi AM, Arsad H, Lim V. Zebrafish as a Successful Animal Model for Screening Toxicity of Medicinal Plants. *Plants (Basel)*. 2020 Oct 12;9(10):1345.
- Chakraborty, P., Kumar, S., Dutta, D. and Gupta, V. (2009) Role of antioxidants in common health diseases, *Research J. Pharm. Tech.*, 2, 238-244.
- Chen, W. Y., John, J. A. C., Lin, C. H., & Chang, C. Y., (2007). Expression pattern of metallothionein, MTF1 nuclear translocation, and its DNA-binding activity in zebrafish (*Danio rerio*) induced by zinc and cadmium. *Environmental Toxicology and Chemistry*, 26, 110–117.
- Cho, D. Shin, B., Jeon, B.W. and Kwak, J.M. (2009) ROS-mediated ABA signaling, *J. Plant Biol.*, 52, 102– 113.
- Choi CY, An KW, Nelson ER and HabibiHR, Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPX) mRNA in goldfish (*Carassius auratus*). *Comparative Biochemistry and Physiology*, 145(C), 2001, 595–600.
- Christen V., Fent K. (2014). Tissue-, sex- and development-specific transcription profiles of eight UDP-glucuronosyltransferase genes in zebrafish (*Danio*

- erio) and their regulation by activator of aryl hydrocarbon receptor. *Aquat. Toxicol.* 150, 93-94
- Christensen, G.M. 1971/72. Effects of metal cations and other chemicals upon the in vitro activity of two enzymes in the blood plasma of the white sucker, *Catostomus commersoni* (Lacepede). *Chem. Biol. Interactions.*, 4: 351-361.
- Chung, S.W., Chung, H.Y., Toriba, A., Kameda, T., Tang, N., Kizu, R. and Hayakawa, K. (2007) An environmental quinoid polycyclic aromatic hydrocarbon, acenaphthenequinone, modulates cyclooxygenase-2 expression through reactive oxygen species generation and nuclear factor kappa b activation in A549 cells, *Toxicol. Sci.*, 95, 348–355.
- Corpas, F. J., Fernández-Ocaña, A., Carreras, A., Valderrama, R., Luque, F., Esteban, F. J., Rodríguez-Serrano, M., Chaki, M., Pedrajas, J. R., Sandalio, L. M., Del Río, L. A., & Barroso, J. B. (2006). The expression of different superoxide dismutase forms is cell-type dependent in olive (*Olea europaea* L.) leaves, *Plant Cell Physiol.*, 47, 984-994.
- Crist, R. H., Oberholser, K., Schwartz, D., Marzoff, J., Ryder, D., & Crist, D. R. (1988). Interactions of metals and protons with algae. *Environ. Sci. Technol.*, 22, 755-760.
- Deepak, K., Nayak, G., Rajalingam, D., Sengupta, P., & Maity, K. (2007). Evaluation of hepatoprotective and antioxidant activity of *Ichnocarpus frutescens* on paracetamol-induced hepatotoxicity in rats, *Tropical J. Pharmaceut. Res.*, 6, 755-765.
- Dimari G. A., Abdulkarim, F. I., Akan, J. C., & Garba, S. T. (2008). "Metal concentrations in tissues of *Bagrus bayad*, *Clarias lazera* and *Osteoglossides* caught from Alau Dam, Maiduguri, Borno state, Nigeria", *American Journal of Environmental Sciences.* 4, 379-473.
- Eisler R. (1973):- Acute toxicity of organochlorine and organophosphorous insecticides to estuarine fishes. W.S. Bar.Spot.Fish Wild. Tech. Paser. 46: 1-12.
- Elaiyaraja C., S. Subha, A. Sobana Kumari, and A. Arunachalam. (2018) Effects of zinc sulphate on the biochemical changes in the fish *Cyprinus carpio*. *International Journal of Zoology and Applied Biosciences*, 3(5), 390-395.
- Farombi, E. O., Adelowo, O. A., & Ajimoko, Y. R. (2007). Biomarkers of oxidative

- stress and heavy metal levels as indicators of environmental pollution in African Cat fish (*Clarias gariepinus*) from Nigeria ogun river. *Int. J. Environ. Res. Public Health*, 4(2): 158-165.
- Ferraris, G., Jones, D. W., & Yerkess, J. (1971). Determination of hydrogen atom positions in the crystal structure of pharmacolite, $\text{CaHAsO}_4 \cdot 2\text{H}_2\text{O}$, by neutron diffraction. *Acta Crystallographica Section B: Structural Crystallography and Crystal Chemistry*, 27(2), 349-354.
- Finney. D.J. (1971) 'Probit analysis' third edition Cambridge University, press.PP.333.
- Firat, O., & Kargin, F. (2010). Individual and combined effects of heavy metals on serum biochemistry of Nile tilapia *Oreochromis nilotica*, *Arch. Environ. Contam. Toxicol.*, 58, 151-157.
- Fisher R.A. (1964) Graphical method for research works oliver and body London.
- Foulkes, E. C. (1988). On the mechanism of transfer of heavy metals across cell membranes. *Toxicol.*, 52, 263-272.
- Gao, L. Cheng,M., Chou, H. and Yang, Y. (2009) Ineffective GSH regeneration enhances G6PD-knockdown HepG2 cell sensitivity to diamide-induced oxidative damage, *Free Rad. Biol. Med.*, 47, 529– 535.
- Gautam P.P. and Gupta A.K.(2008) Toxicity of cypermethrin to the juveniles of freshwater fish peculiar reticulate in relation to selected environmental variables, *Natural Products radiance* Vol.7(4) 2008 pp 314-319.
- Geetha, R., Kumaraguru, A.K. and Thatheyus, A.J. 1996. Toxic effects of heavy metal copper on fish *Lepidocephalichthys thermalis* in short term exposure. *Poll. Res.*, 15(2): 151-153.
- George, S. G., Hodgson, P. A., Tytler, P., & Todd, K. (1996). Inducability of Metallothionein mRNA expression and *Cadmium tolerance* in larva of teleosts the turbot (*Scophthalmus maximus*). *Fundamental Application of Toxicology*, 33, 91-99.
- Ghazaly, K.S. 1992b Hematological and physiological response to sublethal concentrations of cadmium in a freshwater teleost, *Tilapia zilli*. *Water Air Soil Pollut.*, . 64: 551-559.
- Gill, T.S. Parisib, E. and Pant, J.C. 1990. Effects of sublethal concentrations of mercury in a teleost *Punctius conchoni*, biochemical and haematological

- parameters. *Indian J. Exp. Biol.*, 19: 571-573.
- Guedenon P., Edorh A.P., Hounkpatin A.S.Y., Alimbac G., Ogunknmi A., Nwokejiegbe E. G. and Boko M.(2012) :- Acute toxicity of (Hgcl₂) to African catfish, *Clarias gariepinus*. *Research J. Chem. Sci.* Vol 2(3) 41-45.
- Habig, W.H., Pabst, M.J. and Jokoby, W.B. Glutathione transferase : A first enzymatic step in mercapturic acid III formation, *J. Biol. Chem.*, 249, 1974, 7130-7139.
- Hameed, S. V. S. A, & Muthu, K. K. (2006). Impact of cadmiun on the biochemical constituents of freshwater fish *Oreochromis mossambicus*. *Indian J Environ Sci.*, 10(1), 63-65.
- Hand Sharma., 1990. Database on medicinal plants used in Ayurveda. Delhi: Documentation and Publication Division, Central Council for Research in Ayurveda and Siddha. 3, 404-424.
- Heath, A. G. (1987). Water Pollution and Fish Physiology. CRC Press Inc. *Boca Raton*, 145.
- Hilmy, A. M., El-Domiaty, N. A., Daabees, A. Y., & Abdel Latife, H. A. (1987). Some physiological and biochemical indices of zinc toxicity in two freshwater fishes, *Clarias lazera* and *Tilapia zilli*. *Comp Biochem Physiol*, 87, 297–301.
- Hogstrand C., & Haux, C. 1996). Naturally high levels of zinc and metallothionein in liver of several species of the squirrelfish family from Queensland, Australia. *Marine Biology*, 125(1), 23–31.
- Holden A.V. (1972):- The effect of pesticides on the life in freshwater, *proc.R. Soc. London*. B-180-383-394.
- Huy, L.P., He, H.G. and Huy, C.P. (2008) Free radicals, antioxidants in disease and health, *Int. J. Biomed. Sci.*, 4, 89-96.
- Irukayama, K., Kondo, T., Kai, F., & Fujiki, M. (1961). Studies on the origin of causative agent of Minamata disease. 1. Organic mercury compound in the fish and shellfish from Minamata Bay. *Kumamoto Med. J.*, 14, 157 -169.
- Jacobs, J.M., Carmichael, N. and Cavanagh, J.B. 1977. Ultrastructural chages in the nervous system of rabbits poisoned with methyl mercury. *Toxicol Appl. Pharmacol.*, 39: 249-261.
- Jain S. and Salman S. (1995) Heavy metal concentration in highly eutrophic lake

- sediments and overlying water. *Pollution. Res.*, 14(4)471-476.
- James, R. V., Sampath, K., Siva Kumar, V., & Manthiramoorthy, S. (1991). Individual and combined effects of heavy metals on survival and biochemistry of *Oreochromis mossambicus*. *Indian J Fisheries*, 38(1), 49-54.
- Jana S. Bandopadhyaya N. (1987) Effect of heavy metals on some biochemical parameters in the fresh water fish *Clarias punctatus*. *Environ. Eco*, (3);488-493.
- Jana, S. and Bondyopadhyay, N. 1981. Effect of heavy metals on some biochemical parameters in the freshwater fish, *Channa punctatus*. *Environ. Ecol.*, 5(3): 488-493.
- Janani, P., Sivakumari, K., Geetha, A., Ravisankar, B. and Parthasarathy, C. (2010) Chemopreventive effect of bacoside A on *N*-nitrosodiethylamine induced hepatocarcinogenesis in rats, *J. Cancer Res. Clin. Oncol.*, 136, 759-770.
- Janardhan R. and Ramamurthy R.R. (1987):- Identification of perturbations in respiratory metabolism of prawn toxicity. *J. Ecotoxicol Environ Monit.*, 7,145-149.
- Janardhan Readdy S. (1999):- Impact o phosalone toxicity on energy metabolism of prawn, *Macrobrachium rosenbergii*. *J. Ecotoxicology Environ. Monit* 9 (4) 231-235.
- Johansson J. and Rasmusen L. (1977):- Retrospective study (1944-1976) of heavy metals in the epiphyte *pterogonium gracile* collected from one phorophyte, *Byologist*, 80 (3) 625-629.
- John Prentera, Calum Mac Neila, Jaimie T.A. Dicka, Gillian E. Riddella and Alison M. Dunnb, (2004) :- Lethal and sub-lethal toxicity of ammonia to native, invasive and parasitized Freshwater amphipods *Water Res.*, 38:2847-2850.
- Kakkar P, Das B and Viswanathan PN. A modified spectrophotometric assay of SOD. *Ind J Biochem Biophy*, 21, 1984, 130-132.
- Karbassi R. Rayati I. and Moattar F. (2006):- Origin and chemical partitioning of heavy metals in riverbed sediments *Int.J. Environ and Econ* 18: 143-151.
- Katz, M. 1975. The effects of heavy metals on fish and aquatic organisms. In: 'Heavy metals in the Aquatic Environment'. Supplement of progress in water

- technology. Proceedings of International Conference of Nachville Tennessee, December, 1973. (Ed.), Krenkel, P.A. Penguin Press, Oxford, U.K. pp.25-33.
- Khan A.T. Atkinson A., Graham T.C. Thompson S., Ali S. Shireen (2004):- Effect of inorganic mercury on reproductive performance of mice food chemo toxicol.(2004)
- Khlifi Rim., & Hamza-Chaffai Amel. (2010). Head and neck cancer due to heavy metal exposure via tobacco smoking and professional exposure: A review. *Toxicology and applied pharmacology*, 248, 71-88.
- Khurshid S., Abdul B. ZaheeruddinA. and Usman S.M. (1998) :- Effect of Waste disposal on water quality in parts of Cochin, Kerala, Indian J. Environ. Health 40(45-50).
- Kiffin, R., Bandyopadhyay, U. and Cuervo, A.M. (2006) Oxidative stress and autophagy, *Anti. Redox Sig.*, 8, 152-162.
- Klee, M., Eilertson, C., & Sheridan, M. A. (1990). Nutritional state modulates hormone-mediated hepatic glycogenolysis in chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Experimental Zoology*, 254(2), 202-206.
- Kling, P., & Olsson, P. E., (1993). Metal regulation of the rainbow trout metallothionein-A gene. In: *proceedings of the Seventh International Symposium on Responses of Marine Organisms to Pollutants*.
- Kobayashi, J. (1970). Relation between the 'Itai-itai' disease and the pollution of river water by cadmium from a mine. In: *Advances in water pollution research*. S.H. Jenkine (ed.). 1, 1-7.
- Kothari, S., & Soni, R. (2004). Role of poly-unsaturated fatty acids against zinc sulphate toxicity in the gills of a cat fish *Heteropneustes fossilis* (Bloch). *Journal of Tissue Research*, 4, 253-256.
- Krishna A.K. and Govil P.K. (2004):- Heavy metal contamination of soil around Pali industrial area Rajsthan India *Environmental Geology* 38-44.
- Kumar CH, Ramesh A, Suresh Kumar JN, Mohammed Ishaq B. A review on hepatoprotective activity . of medicinal plants. *Int J Pharmaceu Sci Res* 2011; 2(3):501-515.
- Lakshmidevi, S. and Anuradha, C.V. (2010) Mitochondrial damage, cytotoxicity

- and apoptosis in iron-potentiated alcoholic liver fibrosis: amelioration by taurine, *Amino Acids*, 38, 869–879.
- Lange, A., Ausseil, O., & Segner, H. (2007). Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc. *Comparative Biochemistry and Physiology*, 131, 231–243.
- LeFauve, M. K., & Connaughton, V. P. (2017). Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish. *Current zoology*, 63(2), 221-227.
- Liu, R.M. and Pravia, K.A.G. (2010) Oxidative stress and glutathione in TGF- β -mediated fibrogenesis, *Free Rad. Biol. Med.*, 48, 1-15.
- Lloyd, R. (2008). "*Pollution and Fresh Water Fish*, Cornwalls press, Britain, 1192, 77-86.
- Lloyd R. H. (1965):-Factors that effect the tolerance of fish to heavy metal poisoning. Biological Problems in the Water Pollution, 3rd Seminar, U.S. Dept., Health Education and Welfare., pp.181.
- Mali R.P, (2002):- Studies on some aspects of physiology of fresh water female crab *Brytelphusa guerini* with special reference to inorganic pollutants. Ph.D. Thesis Submitted to Swami Ramanand Teerth Marathwda, University, Nanded, Maharashtra.
- Manian, R., Anusuya, N., Siddhuraju, P. and Manian, S. (2008) The antioxidant activity and free radical scavenging potential of two different solvent extracts of *Camellia sinensis*(L), *Ficus bengalensis* L and *Ficus Racemosa* L, *Food Chem.*, 107, 1000-1007.
- Martinez, C. B, R., Nagae, M. Y., Zaia, C. T. B. V., Zaia, D. A. M. (2004). Morphological and physiological acute effects of lead in the neotropical fish *Prochilodus ineatus*. *Braz. J. Biol.*, 64(4), 1797-807.
- Mohammad Reza R., & Alipoor, V. (2012). The determination of LC50 and bioconcentration of mercury chloride (HgCl₂) in (*Esox lucius*). *World Applied Sciences Journal*, 17(6), 735-738.
- Morais, S., Costa, F. G., & Pereira, M. L. (2012). Heavy metals and human health, in Environmental health – emerging issues and practice, *Oosthuizen J ed.*, 227–246.

- Morata, J.W. and Ramamoorthy, S. Duston. 1982. Copper. In: "Heavy Metals in Natural Waters Applied Monitoring and Impact Assessment". (Eds.), Desanto, R.S., Springer-Verlag, New York, Berlin, pp.77-99.
- Moron M.S., DsePierre J.W and Manerwik K.B. Levels of glutathione, glutathione reductase and glutathione-s-transferase activities in rat lung and liver *Acta of Biochemica, biophysica* 582, 1979, 67-68.
- Natarajan and Sreenivasan (1962):- Proximate to mineral composition of freshwater fishes. *Ind. Jour.Fish.*8-324.
- Nevalenyyi, A. N., & Bednyakov, D. A. (2004). Influence of cadmium ions on the activities of enzymes responsible for membrane digestion in carp. *Russian Journal of Ecology.*, 35(2), 128-130.
- Olsson, RE., Kling, P. and Hogstrand, C. 1998. Mechanisms of heavy metal accumulation and toxicity in fish. In: 'Metal Metabolism in Aquatic Environments'. (EDS.), Langston, W.J. and Bebianno, M.J. Chap. 8. Chapman and Hall, London, Upm pp. 321-350.
- Oyedemi, S.O., Bradley, G. and Afolayan, A.J. (2010) *In -vitro* and *-vivo* antioxidant activities of aqueous extract of *Strychnos henningsii* Gilg, *Afr. J. Pharm. Pharmacol.*, 4, 70-78.
- Pandey Govind P. Hepatogenic effect of some indigenous drugs on experimental liver damage. PhD thesis. Jabalpur, MP, India: JNKVV; 1990.
- Pandey Govind P. Pharmacological studies of Livol with special reference to liver function. MVSc & AH thesis. Jabalpur, MP, India: JNKVV; 1980.
- Panigrahi A. K. and B. N. Mishra (1978):- Toxicological effect of mercury on a freshwater fish, *Anabus scandes*, CUV and VAL and their ecological implications. *Environ. Pollut.*1978, 16:31-39.
- Pickering, A.D. and Duston, J. 1983. Administration of cortisol to brown trout, *Salmo trutta* (L.), and its effects on the susceptibility to saprolegunia infection and furunculosis. *J. Fish Biol.*, 23: 163-175.
- Polit, E.S., Drezkiwicz, M. and Krupa, Z. (2009) Lipid peroxidation and antioxidative response in *Arabidopsis thaliana* exposed to cadmium and copper, *Acta Physiol. Plant*, 32, 169-175.
- PrabhaS., and Selvapthy. P. (1997):- Heavy metal pollution in Indian rivers. *Indian J. Environ. Prot.* 17(6), 641-649.

- Prosser Ladd L. (1973):- Respiratory function of blood pp. 271-261. In comparative animal physiology W.B. Saunders Company. Philadelphia.
- Qixiao Zhai, Arjun Narbad and Wei (2015). Dietary strategies for the treatment of cadmium and toxicity Vol.7, p.552-571.
- Rani, U. A. (2000). Cadmium induced bioaccumulation in tissue of freshwater teleost *Oreochromis mossambicus*. *Ann. N.Y. Acad.* 919(1), 318-320.
- Rao, D. B., Kiran, C. H., Madhavi, R., Rao, P.K., & Rao, T. R. (2009). Evaluation of antioxidant potential of *Clitoria ternatea* L. and *Eclipta prostrata* L., *Indian J. Biochem. Biophys.*, 46, 247-252.
- Reza Jalilvand, M, (2012). The effect of electronic word of mouth on brand image and purchase intention: An empirical study in the automobile industry in Iran. *marketing intelligence & planning*, 30(4), 460-476.
- Riggioa, M., Filosaa, S., Parisib, E., & Scudieroa, R. (2003). Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 135(2), 191–196.
- Rish V. and Rish M. (2001):- Ecological effect of urbanization on swan river watershed district Und, Himachal Pradesh. *International Journal of Pharmacy Life Science*. 2011,2(5);723-779.
- Robbins and Cotran pathologic basis of disease (2004). 7th ed. Pennsylvania: Saunders, an imprint of Elsevier;. p. 877–937.
- Rovira, M. S., Fernández-Díaz, C., Canavate, J. P., & Blasco, J. (2005). Effects on metallothionein levels and other stress defenses in Senegal sole larvae exposed to cadmium. *Bulletin of Environmental Contamination and Toxicology*, 74, 597–603.
- Rubin, A.J. 1974. Aqueous environmental chemistry of metals. Sci. Publ. Ann Arbor .Michigan, U.S.A.
- Sambasivarao K. Khan, A.K., Alan, S. M. and Nagbhushanam R. (1988):- Heavy metal toxicity to the marine crab, *Ozius rugulosus*. *Environment and Ecology*, 6(1): ISSN 0970-0420.
- Sanchez-Venegas, J. R., Dinamarca, J., Moraga, A. G., & Gidekel, M. (2009). Molecular characterization of a cDNA encoding Cu/Zn superoxide

- dismutase from *Deschampsia Antarctica* and its expression regulated by cold and UV stresses, *BMC Res. Notes*, 2, 1-7.
- Sandu, C., Farkas, A., Musa-Iacob, R., Ionica, D., Parpala, L., Zinevici, V., Dobre, D., Radu, M., Presing, M., Casper, H., Buruiana, V., Wegmann, K., Stan, G., Bloesch, J., Triebkorn, R., & Köhler, H. R. (2008). Monitoring pollution in River Mures, Romania, part I: the limitation of traditional methods and community response. *Large Rivers*, 18(1–2), 91–106.
- Sarkar, A., Dash, S., Barik, B.K., Muttigi, M.S., Kedege, V., Shetty, J.K. and Prakash, M. (2010) Copper and ceruloplasmin levels in relation to total thiols and GST in type 2 diabetes mellitus patients, *Ind. J. Clin. Biochem.*, 25, 74-76.
- Sau A., Tregno F. P., Valentino F., Federici G., Caccuri A. M. (2010). Glutathione transferases and development of new principles to overcome drug resistance. *Arch. Biochem. Biophys.* 500, 116–122.
- Schroder, H.A. 1973. The trace elements and man, some positive and negative aspects. Davin — Adair Co., New York, U.S.A.
- Selvanathan, M. Suresh Kumar, S. Vincent (2011). Determination of median tolerance limit (LC50) of *Clarias batrachus* for cadmium chloride and mercuric chloride. *Rec. Res. Sci. and Tech.* 3(11):84-86.
- Sesha, S. V., & Rao, B. M. (1999). Chromium induced histological alteration in the gill of the fresh water teleost fish *Labeorohita* (Ham). *Indian J Comp. Animal Physiol.*, 17(1), 31-33.
- Shao, H.B., Chu, L.Y., Lu, Z.H. and Kang, C.M. (2008) Primary antioxidant free radical scavenging and redox signaling pathway in higher plant cell, *Int. J. Biol. Sci.*, 4, 8-14.
- Sheridan, M.A. 1987. Effects of thyroxine, cortisol, growth hormone and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. *Gen. Comp. Endocrinol.*, 64: 220-238.
- Shieh, P., Chen, Y., Kuo, D., Chen, F., Tsai, M., Chang, I., Wu, H., Sang, S., Ho, C. and Pan, M. (2010) Induction of apoptosis by [8]-Shogaol via reactive oxygen species generation, glutathione depletion, and caspase activation in human leukemia cells, *J. Agric. Food Chem.*, 58, 3847-3854.
- Siddiqui A.A. Shah I.H. Bhat T.A Bhat M.M. Shah Y. (2009):- Effect of copper

- sulphate on lipid in some vital organs of freshwater crab *Barytelphusa guerini*. *Journal of the Indian Society of Toxicology* Vol. 5(2) pp 1-3.
- Smet, H. D., Wachter, B. D., Lobinski, R., & Blust, R. (2001). Dynamics of (Cd, Zn)-metallothioneins in gills, liver and kidney of common carp (*Cyprinus carpio*) during cadmium exposure. *Aquatic Toxicology*, 52, 269–281.
- Smith, D.L. (2000). Anemia in the elderly. *Am Fam Physic*; 62(7): 1565–72.
- Sohini, V.S. and Rana, S. (2007) Protective effect of ascorbic acid against oxidative stress induced by inorganic arsenic in liver and kidney of rat, *Indian J. Exp. Biol.*, 45, 371-375.
- Souza, A. D., Kurien, T. B., Rodgers, R., Shenoi, J., Kurono, S., Matsumoto, H., Hensley, K., Nath, S. K., & Scofield, R. H. (2008). Detection of catalase as a major protein target of the lipid peroxidation product 4-HNE and the lack of its genetic association as a risk factor in SLE, *BMC Medical Genetics*, 9, 62.
- Spencer, K., D.J. Shafer, R.W. Gauldie and EH De Carlo 2000. Stable lead isotope ratios from distinct anthropogenic sources in fish otoliths; a potential nursery ground stock marker. *Comp. Biochem. Physiol. A mo. Integr. Physiol.*, 127(3): 273-284.
- Srinivasa Gowd. Pradip. K. Govil (2007):- National Geophysical Research Institute, Hyderabad , India.2007.
- Stephenson T. (1987):- Sources of heavy metals in wastewater. Heavy metal in wastewater and sludge treatment.Sources.Analysis and Legislation. JN Lester (ed). 1; 13-64, CRC Press, Cleveland. DH.
- Subash, S., & Subramanian, P. (2009). Morin a flavonoid exerts antioxidant potential in chronic hyperammonemic rats: a biochemical and histopathological study, *Mol. Cell Biochem.*, 327, 153-161.
- Tabrez, S., and Ahmad, M. (2009) Some enzymatic/non-enzymatic antioxidants as potential stress primarkers of trichloroethylene, heavy metal mixture and ethyl alcohol in rat tissue, Wiley Periodicals Inc., pp.1-10.
- Tewari, M., Gill, T.S. and Pant, J. 1987. Impact of chronic lead poisoning on the haematological and biochemical profiles of fish, *Barbus conchoni* (Ham.). *Bull. Environ. Contam. Toxicol.*, 38: 748-752.
- Thiele, D. J. (1992). Metal Regulated Transcription in Eukaryotes. *Nucleic Acid Research*, 210, 1183-1188.

- Thomas, P. 1990. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. *Am. Fish. Soc. Sym.*, 8: 9-28.
- Tucker B.W. (1997):- Overview of current sea food nutritional issueformation of potential toxic products. In Shahidi F.,Y. Jones and D.D. Kitts (Eds) sea food safety, processing and biotechnology (PP:5- 10). Technomic Publishing Co.Inc.
- Turkseven, S., Kruger, A., Mingone, C. J., Kaminski, P., Inba, M., Rodella, L. F., Ikehara, S., Wolin, M. S., & Abraham, N. G. (2005. Antioxidant mechanism of heme oxygenase-1 involves as increase in superoxide dismutase and catalase in experimental diabetes, *Am. J. Physiol. Heart Circ. Physiol.*, 289, 701-707.
- Uluturhan, E., & Kucuksezgin, F. (2007). Heavy metal contaminants in Red Pandora (*Pagellus erythrinus*) tissues from the Eastern Aegean Sea, Turkey. *Water Res.*, 41, 1185-1192.
- Ural M.S. and Simsek, S. (2006) :- Acute toxicity of dichlorvos offingerling European catfish, *Silurus glanis* *Bull. Envir.Contom.Toxicol.* 76:871-876.
- Valko, M., Leibfritz, D., Momel, J., Cromin, M.T.D., Mazur, M. and Telses, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease, *Intl. J. Biochem., Cell Biol.*, 39, 44-84.
- Vanden Belt, K. Vanpkymbroeck, S., Witters, H. 2000. Toxicity of cadmium — contaminated clay to the zebrafile *Donio rerio*. *Arch. Environ. Contam. Toxicol.*, 38: 191-196.
- Vasanthi N., Muthukumaravel, K., Sathick, O., & Sugumaran, J. (2019). Toxic effect of mercury on the freshwater fish *Oreochromis mossambicus*. *RJLBPCS*, 5(3), 364-371.
- Veena, K., Shanthi, P. and Sachdanandam, P. (2007) Therapeutic efficacy of Kalpaamruthaa on reactive oxygen/nitrogen species levels and antioxidative system in mammary carcinoma bearing rats, *Mol. Cell. Biochem.*, 294, 127-135.
- Velavan, S. (2011). Free radicals in health and diseases- A Mini Review. *Pharmacologyonline Newsletter*, 1, 1062-1077.
- Vinodhini R. and Narayan M. (2009):- The impact of toxic heavy metals on the

- hematological parameters in common carp (*Cyprinus carpio*) Iran. *J. Environ Health. Sci. Eng.* 6(1).23-38
- Virk and Kaur, 1998. Biochemical alterations in the gonads of *Cyprinus carpio* Linn. Exposed to mixture of Nickel and chromium. *Indian J. Ecol.*, 25(2): 159-164.
- Virk, S., & Sharma, A. (2003). Changes in the biochemical constituents of gills of *Cirrhinus mrigala* (Ham) following exposure to metals. *Indian J Fish.*, 50(1), 113-117.
- Wu, S. M., Weng, C. F., Yu, M. J., Lin, C. C., Chen, S. T., Hwang, J. C., & Hwang, P. P. (1999). Cadmium-inducible metallothionein in tilapia (*Oreochromis mossambicus*). *Bulletin of Environmental Contamination and Toxicology*, 62, 758–768.
- Wüst, P. (Ed.). (1997). *Die Lilie: eine mittelfränkische Dichtung in Reimprosa und andere geistliche Gedichte aus der Wiesbadener Handschrift* (Vol. 15). Weidmann.
- Xu H, Lam SH, Shen Y, Gong Z. Genome-wide identification of molecular pathways and biomarkers in response to arsenic exposure in zebrafish liver. *PLoS One*. 2013;8:e68737.
- Yeragi, S.G., Rana, A.M. and Koli, V.A. 2003. Effect of pesticide on protein metabolism of mudskipper, *Boleophthalmus dussumieri*. *J. Ecotoxicol. Environ. Monit.*, 13(3): 211-214.
- Zaki, M. S., Elbattrawy, N., Fawzi, O. M., Awad, I., & Atta, N. S. (2011). Effect of mercuric oxide toxicity on some biochemical parameters on African cat fish *Clarias gariepinus* present in the River Nile. *Life Sci J.*, 8, 363-368.
- Zhang X, Li C, Gong Z. Development of a convenient in vivo hepatotoxin assay using a transgenic zebrafish line with liver-specific DsRed expression. *PLoS One*. 2014;9:e91874.
- Zhang, W., Zhang, Y., Zhang, L., & Lin, Q. (2016). Bioaccumulation of metals in tissues of seahorses collected from Coastal China. *Bull Environ Contam Toxicol*, 96(3), 281–288.