Protective Effect of Vitamin C and N-Acetylcysteine Against Metsulfuron-methyl-Induced Oxidative Stress in Nile Tilapia (*Oreochromis niloticus)*

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By

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“In the Name of ALLAH, the most beneficent, the most Merciful”

Dedicated to

- *To my parents, who have always been my biggest supporters. I am grateful for your love, guidance, and sacrifices.*
- *To my brother Hussain Ahmad, who has always been there for me, through thick and thin. I am so lucky to have you in my life.*
- *To my family, who have always believed in me. I dedicate this thesis to you with all my love.*

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in the thesis is original work. I have not previously presented any part of this work elsewhere for any other degree.

Izhar Ahmad

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Izhar Ahmad

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACHE	Acetylcholinesterase
ALS	Acetolactate synthase
CAT	Catalase
CUP	currently used pesticides
FAO	Food and Agriculture organization
GSH	Reduced Glutathione
HCT	Hematocrit
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration,
MCV	Mean corpuscular volume
MSM	Metsulfuron methyl
NAC	N-acetylcysteine
OCP	organochlorine pesticides
POD	Peroxidase
POP	Persistent organic pesticides
RBCs	Red blood cells
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances,
WBCs	White blood cells
WHO	World health organization

ABSTRACT

Pesticide toxicity is a frequent occurrence within aquatic ecosystems, leading to detrimental impacts on aquatic organisms. Consequently, enhancing the antioxidant defense mechanisms of aquatic creatures becomes crucial in safeguarding them from the harmful effects of these noxious substances. In this study, simulative toxicity was established in the fish then the treatment process was followed. For this purpose, Nile tilapia *Oreochromis niloticus* exposed to Metsulfuron methyl (triazinylsulfonylurea herbicide) for 24 days and was treated with either N-acetyl cysteine (1.0 mM concentration) and dietary vitamin C 500 mg per kg feed (antioxidant) for 96 h. In this context, Reactive oxygen species (ROS), Thiobarbituric Acid Reactive Substances (TBARS), antioxidant enzymes (SOD, POD, CAT, GSH) were measured in fish gills and liver of the fish. Besides, hematology parameters were performed for all groups. At the result of MSM exposure, SOD, CAT, POD, CAT and GSH activities were reduced but ROS and TBARS were increased MSM treated groups in gills and liver tissues as compared to control group ($p < 0.05$). Treatment with NAC and vitamin C improved the overall antioxidant status and hematological parameters as well ($p < 0.05$). Considering the findings of the study, it was observed that dietary vitamin C was more effective than NAC administration at (1.0 Mm NAC) on herbicide toxicity. It was concluded that the most sensitive tissue was the gills.

INTRODUCTION

1.1 Introduction to Pesticides

According to the FAO and WHO (2014), “pesticide is any substance or a combination of chemical or biological ingredients designed to repel, eliminate, or manage pests, as well as to regulate plant growth (Eldridge, 2008)”. The term "pesticide" encompasses various substances like fungicides, insecticides, herbicides, and rodenticides, all employed to eradicate specific pests (Yadav *et al.,* 2015). Its primary purpose is to also hinder the transmission of diseases carried by vectors. This includes safeguarding crops, preserving food, and playing crucial roles in various commercial and industrial practices like aquaculture, agriculture, food processing, and storage (Sharma *et al.,* 2019).

On origin, pesticides can be categorized into two types of chemical pesticides and biopesticides. Biopesticides are specifically designed for the intended host, target specific organisms or closely related species. In contrast, chemical pesticides have a broader impact on non-target organisms (Rusch *et al.,* 2016).

Different types of chemical pesticides include carbamates, organophosphates, pyrethroids, and organochlorine pesticides. On the other hand, biopesticides are derived from natural sources such as animals, plants, and various microbes like bacteria, viruses, fungi, and nematodes. Biopesticides can be categorized as microbial pesticides, plant-based pesticides, and biochemical pesticides. Pesticides function through various mechanisms. Some act as growth regulators, stimulating or inhibiting pest growth, while others function as repellents to ward off pests, attractants to lure pests, or chemo sterilant to sterilize pests. Pesticides with broad-spectrum activities, capable of controlling multiple pest classes, can be challenging to classify. For instance, a pesticide like aldicarb, used in citrus production in Florida, can be classified as an acaricide (killing mites), insecticide (killing insects), or nematicide (killing nematodes) based on its target organisms (Fishel and Ferrell, 2013)

1.2 Toxicological classification of Pesticides

Pesticides exhibit distinct physical and chemical properties that differ among various classes. Thus, it is important to classify them based on these properties and investigate them within their respective groups. Synthetic pesticides, being manmade substances,

do not found naturally. They are categorized into different classes according to specific criteria. Currently, Drum, (1980) has put forward three commonly accepted approaches for the classification of pesticides:

(i) On the basis of mode of entry, which refers to how the pesticide enters the target organism or pest.

(ii) Based on the specific function of the pesticide and the type of pest, it is intended to control or eliminate.

(iii) On the basis of chemical composition or structure of the pesticide, providing insights into its molecular makeup and properties.

1.2.1 On the basis of Mechanism of entry

The methods by which pesticides encounter or enter the target organisms are referred to as modes of entry (Gerolt, 1970). These ways of entering comprise systemic action, contact application, ingestion of stomach poisons, use of fumigants, and application of repellents.

Systemic Pesticides

Systemic pesticides are a distinct pesticide type that can be absorbed by plants or animals and then spread to untreated parts within their structures. Systemic herbicides, for instance, can move throughout the plant, reaching untreated sections like leaves, stems, or roots not directly exposed to the pesticide application. This trait allows effective weed control even with incomplete spraying. Systemic pesticides easily penetrate plant tissues, traveling through their vascular systems to target and eliminate specific pests. Some systemic insecticides can likewise be given to animals, circulating through their bodies to combat pests such as warble grubs, lice, or fleas. The movement of systemic pesticides within plants can be one-way or two-way; some move only upward, while others move both upward and downward. When applied to the root zone, systemic pesticides can disperse throughout the entire plant, with the distance they travel varying based on the pesticide type. Examples of systemic pesticides include 2,4-Dichlorophenoxyacetic acid (2,4-D) and glyphosate (Holmwood and Büchel, 1983).

Non-systemic (Contact) pesticides

Contact pesticides are non-systemic pesticides that act on target pests when there is direct contact. They must physically touch the pests in order to be effective. Upon

contact, the pesticide enters the pests' bodies through their outer protective layer (epidermis) and causes poisoning, eventually leading to their death. Contact pesticides do not necessarily penetrate the tissues of plants and, as a result, are not transported through the plant's vascular system. Examples of contact pesticides include paraquat and diquat dibromide (Hrynko *et al.,* 2023).

Stomach poisoning and stomach toxicants

Stomach poisoning pesticides are ingested by pests and kill them by disrupting their digestive system. They are often applied to plant parts, such as leaves, where pests feed on them. Stomach poisons can also be applied to water, where they are ingested by filter-feeding larvae, such as mosquitoes. An example of a stomach poisoning pesticide is malathion (Abubakar *et al.,* 2020).

Fumigants

Fumigants are pesticides that kill pests by releasing poisonous gases. These gases enter the pests' respiratory system through small openings called spiracles. The gases then poison the pests and eventually kill them. Some fumigants are liquids that are pressurized and vaporize when released. Others are volatile liquids that vaporize at room temperature. Fumigants are commonly used to control pests in stored products, such as fruits, vegetables, and grains. They are also effective in controlling pests in soil. (Yadav and Devi, 2017).

Repellents

Repellents do not kill the pests. Repellents are formulated to be unpleasant or repellent to pests, making the treated environment or crop undesirable for them. They can also disrupt pests' ability to locate or identify the crop, further deterring their presence. The goal of repellents is to repel pests rather than eradicate them (Yadav and Devi, 2017).

1.2.2 On the basis of pesticide function and pest organism they kill

Pesticides can be classified according to the specific organism they target. This method of classification is called target-based classification. The group names of these pesticides typically include the suffix "-cide", which comes from the Latin word "cide" meaning "kill" or "killer". For example, insecticides are pesticides that kill

insects. It is important to note that not all pesticides necessarily end with the suffix "-cide"." (Mundeja and Rai, 2021).

Pesticides can also be categorized according to their specific functions. For instance, there are growth regulators that either stimulate or inhibit the growth of pests. Defoliants are leaf-shedding agents that cause plants to lose their leaves. Desiccants are drying agents that accelerate the drying process of plants. They are used to kill insects by dehydrating them. Repellents are designed to keep pests away. Attractants, on the other hand, lure pests, typically towards traps. Lastly, chemosterilants are employed to sterilize pests and inhibit their reproductive abilities (Rademacher, 2015).

1.2.3 On the basis of chemical composition or structure of pesticide

The most commonly used and suitable method for classifying pesticides is based on their chemical composition and the description of their active ingredients. This classification approach provides information on the efficacy, as well as the chemical and physical properties of specific pesticides. They can be grouped into four main categories based on their chemical structure, namely: organochlorines, organophosphates, carbamates, and pyrethrins/pyrethroids. (Kramer and Buchel, 1983).

Organochlorine pesticides

Organochlorines possess high chemical stability, allowing them to persist in the environment for extended periods. Furthermore, they can be deposited in adipose tissue (El Nemr *et al.,* 2016). Due to their lipophilic and persistent characteristics, most organochlorine pesticides (OCPs) have the potential to be stored in adipose tissue for extended periods. Under harsh environmental conditions, these compounds can be released into the circulatory system. Consequently, there may be a time lag between initial exposure and the manifestation of effects. For instance, DDT can persist in the human body for 50 years or even longer (Mrema *et al.,* 2013).

Organophosphate pesticides

Organophosphate pesticides are esters of phosphoric acid that can inhibit the enzyme acetylcholinesterase in the human body. This enzyme is responsible for breaking down acetylcholine, a neurotransmitter that plays a role in muscle contraction and other functions of the central nervous system. The inhibition of acetylcholinesterase

disrupts the normal transmission of nerve impulses by causing the phosphorylation of the hydroxyl (OH) group in the enzyme's active site (Vale and Lotti, 2015).

Carbamate pesticides

Carbamates are a group of organic compounds derived from dimethyl N-methyl carbamic acid. They are commonly used as herbicides, insecticides, nematicides, and fungicides. Some well-known carbamates include thiobencarb, propoxur, molinate, disulfiram (Antabuse), pyridostigmine, methiocarb, and carbaryl. The toxicity of carbamates varies depending on their molecular structure. However, they generally have a shorter duration of action than organophosphates and organochlorines, which inhibit acetylcholinesterase (Garcia *et al.,* 2012).

Pyrethroid pesticides

Pyrethroid insecticides find their origin in the natural extracts of pyrethrum, sourced from chrysanthemum flowers, with a specific focus on pyrethrin. These chemical compounds are primarily directed towards the central nervous system, orchestrating modifications in the behavior of cationic sodium channels situated within the cell membrane of nerves. This modulation consequently extends the duration during which sodium channels remain open. It's pertinent to highlight that the movement of sodium cations through the membrane is a common phenomenon shared by both vertebrates and insects. (Parry and Young, 2013).

1.2.4 Other Minor Classes of Pesticides

Classification based on mode of action

Pesticides are categorized into different groups based on their mechanism of action.

Physical poison

These types of pesticides cause the death of insects by exerting a physical action when they encounter them. for example, activated clay.

Protoplasmic poison

Pesticides such as arsenicals can cause protein precipitation.

Respiratory poison

Respiratory poisons are chemicals that can disable or obstruct respiratory enzymes. Hydrogen cyanide is an example of a respiratory poison.

Nerve poison

That inhibits impulse conduction. For example, malathion.

Chitin inhibition

Pesticides such as diflubenzuron can interfere with chitin synthesis in pests (Yadav and Devi, 2017).

Table 1.1 Pesticide name and its type and target pests (Fishel and Ferrell, 2013).

Type of pests	Pesticides example	Target pests/function
Avicides	Avitrol (aminopyridine)	Kill birds
Acaricides	Bifenazate	Eradicate mite populations
Algaecides	Copper sulfate	Prevent or control algal growth
Bactericides	Copper complexes	Inhibiting bacterial growth
Biopesticide	Bacillus thuringiensis	Variety of organisms
Bait	Anticoagulants	Diverse group of organisms
Desiccants	Boric acid	Desiccate plant tissues
Defoliant	Tribufos	Sheds plant leaves
Fungicides	Azoxystrobin, Chlorothalonil	Eradicate fungi (including blights, mildews, molds, and rusts)
Fumigant	Aluminum phosphide	Variety of organisms
Herbicides	Atrazine, glyphosate, 2,4-D	Control unwanted plant growth
Insecticides	Aldicarb, Carbaryl, imidacloprid	Kill insects and other arthropods
Insect growth regulator	Diflubenzuron	Control insects` growth
Larvicides	Methoprene	Controls larval growth
Molluscicides	Metaldehyde	Control snails that damage plants
Moth balls	Dichlorobenzene	Protect clothes from moth larvae or molds
Nematicides	Aldicarb, Ethoprop	Control nematodes that harm plants
Ovicides	Benzoxazin	Kills the eggs of insects and mites
Piscicides	Rotenone	Use to kill fishes
Plant growth regulator	Gibberellic acid, 2,4-D	Controls plant growth
Predacide	Strychnine	Against mammal predators
Repellents	Methiocarb	Deter pests by its taste or smell, vertebrates and invertebrates
Rodenticides	Warfarin	Eliminate mice and other rodents

1.3 History of pesticide production and application

The evolution of pesticides can be categorized into three distinct periods**.**

(1) During the initial phase (prior to the 1870s), pest control relied on the utilization of natural pesticides, such as sulfur which was employed in ancient Greece (Matthews, 2018).

(2) The second phase, spanning from the 1870s to 1945, marked the emergence of inorganic synthetic pesticides. During this era, the primary focus was on utilizing natural substances and inorganic compounds for pest control purposes (Ota, 2013).

(3) These pesticides have been instrumental in safeguarding and enhancing agricultural productivity. During the initial phase of the development of artificially produced pesticides, three primary types of insecticides were used, carbamated insecticides, organophosphorus insecticides, and organochlorined insecticides, soon after that, herbicides and fungicides also underwent significant advancements. It is predicted that the consumption of insecticides will gradually decrease, while herbicides are expected to gain popularity in the future (Muhammad Zulhilmi, 2017).

In 2007, the pesticide market in the United States was primarily influenced by the demand for maize and soybean pesticides, which together constituted 44.75% of total pesticide sales. Among these two crops, maize had nearly double the pesticide consumption compared to soybean. Herbicides were the predominant type of pesticides used for maize, accounting for 75.3% of its overall pesticide usage, followed by insecticides. Notably, there was a noteworthy surge in the consumption of fungicides/bactericides for maize, with expenditures increasing from 6 million US dollars in 2005 to 130 million US dollars in 2007 (Zhang *et al.,* 2011).

1.4 Global utilization of pesticides

The annual global utilization of pesticides amounts to approximately 2 million tonnes, with China being the primary contributor, followed by the USA and Argentina, both experiencing significant growth. However, projections suggest that Global pesticide usage is projected to reach 3.5 million tonnes by 2020 (Sharma *et al.,*2019). Currently, around the world, approximately 2 million tonnes of pesticides are used, with herbicides accounting for 47.5% of the total, insecticides for 29.5%, fungicides for 17.5%, and the remaining 5.5% comprising other types of pesticides (De *et al.,* 2014).

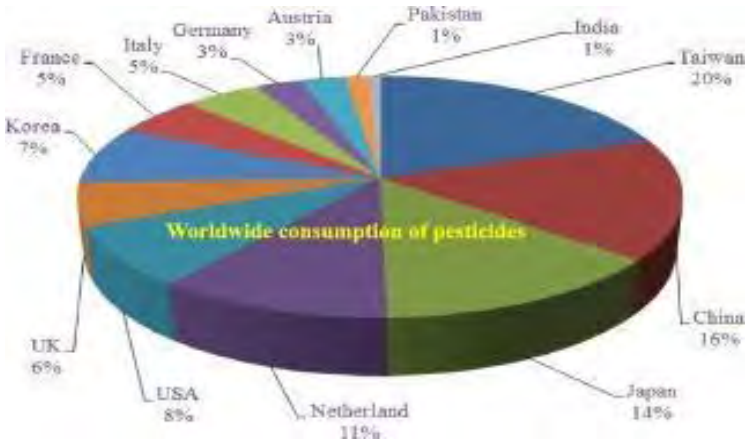

Figure 1.1: Global usage of pesticides (Yadav *et al.,* 2015)

1.5 Pesticide usage in Pakistan

Pesticide usage in Pakistan began in 1954 with an import of 250 metric tonnes. During the Green Revolution period in Pakistan, pesticides were imported from Europe in large quantities. and the USA. These pesticides were used to combat crop pest infections, control locusts, and suppress malaria (Ahad *et al.,* 2010). In 2003, Pakistan witnessed a rise in annual pesticide consumption, reaching a total of 78,132 tonnes (Syed and Malik, 2011)

1.6 Migration and behavior of pesticides in the ecosystem

When farmers use pesticides in a particular area or on plants, there is a risk of these chemicals spreading and breaking down in the environment. This process can be influenced by indigenous microbial strains and various physicochemical factors. Consequently, non-targeted plants and animal species within the ecosystem can be affected by the pesticides' entry and subsequent degradation (Tudi *et al.,* 2021). Within our ecosystem, pesticides undergo degradation through a range of physical and microbiological processes. These processes include exposure to light, temperature fluctuations, moisture levels, oxygen availability, and the activity of microorganisms. As a result of this degradation, pesticides transform into new chemical entities known as metabolites. The level of toxicity of these metabolites depends on their specific che mical composition and can vary from being hazardous to non-toxic (Marie *et al.,* 2017).

Pesticides and their breakdown products can move from their intended application sites to unintended areas through a variety of processes, including adsorption, leaching, volatilization, and surface runoff (Tudi *et al.,* 2021). Pesticides can adhere

to soil particles through a process called adsorption. The strength of this attraction is influenced by the soil's organic matter content and texture (Dhakal *et al.,* 2014).

1.7 Effect of pesticides

Pesticides are widely used in agriculture and public health, but their use can also have negative environmental and health consequences. Pesticides are unique among environmental contaminants because of their potent biological activity and toxicity. They can harm not only their intended targets, but also other organisms, including humans, animals, and the environment. Statistics indicate that approximately 5,000 to 20,000 fatalities occur annually, and around 500,000 to 1 million people suffer from pesticide poisoning each year. Approximately half of the individuals affected by pesticide poisoning and 75% of those who succumb to it are agricultural workers. The remaining cases of pesticide poisoning occur due to the consumption of contaminated food (Yadav *et al.,* 2015).

1.7.1 Impact of Pesticides on natural system

Pesticides have the potential to cause harm and accumulate in areas beyond just crops, primarily due to inadequate planning, execution, and communication regarding their use. Instances of misuse and overuse contribute to this problem. Users often fail to adhere to label instructions and guidance on safety, such as wearing gloves and protective eyewear, leading to incidents related to pesticide (Qu *et al.,* 2019). The use of pesticides can have diverse impacts on non-target organisms, leading to various environmental issues (Rosell *et al.,* 2008).

The presence of pesticides in the food chain, their uptake by organisms, toxic effects, distribution throughout the environment, breakdown processes, and elimination all affect different species. Pesticides are applied in excessive and indiscriminate amounts on different crops, leading to detrimental effects on beneficial organisms like microorganisms, honeybees, predatory species, birds, plants, and other small animals (Alengebawy *et al.,* 2021).

1.7.2 Pesticide impact on the aquatic system

Persistent organic pesticides (POPs) and certain currently used pesticides (CUPs) can reach water bodies through various processes. One common pathway is through atmospheric precipitation, where pesticides present in the air can be carried by rain or snowfall into water bodies. Additionally, chemical and pesticide manufacturing

industries can contribute to pesticide contamination by releasing untreated chemical waste directly into flowing water sources such as rivers or other water bodies. Once released, these pesticides can travel over long distances, sometimes for miles, through the water bodies. They can contaminate aquatic ecosystems, posing a huge threat to the organisms and overall health of the aquatic environment. The presence of pesticides in water bodies can have negative impacts on aquatic ecosystems, including disruption of aquatic food chains, harm to fish and other aquatic organisms, and long-term ecological consequences (Socorro *et al.,* 2016). Pesticides have the capacity to accumulate and transfer within the aquatic ecosystems, progressing from lower to higher trophic levels. This process directly impacts the aquatic flora and fauna. Consequently, the presence of these pesticides can ultimately affect human health through various routes, including consumption or other forms of exposure (Woodrow *et al.,* 2019).

1.7.3 Effects of pesticides on aquatic organisms

Pesticides encounter has detrimental effects not only on intended targets but also on a range of non-target organisms, particularly fish. Fish are particularly susceptible to mortality in certain cases when exposed to high levels of pesticides, but even lower levels of these chemicals can cause significant harm. Various fish species exposed to different pesticides experience alterations in hematological parameters, including RBCs, WBCs and plasma and serum levels. These changes lead to histological changes in vital organs such as the kidneys, brain, liver, gills, gut and muscles (Tahir *et al.,* 2021).

Several pesticides have been found to cause genotoxicity, posing a significant concern. As fish occupy the lowest level of the aquatic food chain, serve as indicators of water quality and contamination. They possess mechanisms that enable them to accumulate compounds such as heavy metals and pesticides, reflecting the level of contaminants in their environment. Fish primarily acquire pesticides through the consumption of contaminated algae, phytoplankton, and other aquatic plants, leading to the gradual accumulation of toxic substances in their tissues and organs. While some of these compounds can be broken down and removed from the fish's body, most of them build up in the fish's organs and organ systems. Pollutants are absorbed by the fish's gills, skin, and digestive tract, and then distributed throughout the fish's body, disrupting its natural processes. (Banaee *et al.,* 2011).

The gills, being directly exposed to water, are highly susceptible to pollution and are among the most affected organs. Toxic substances enter the fish's body through the gills, leading to an increased demand for oxygen. Therefore, monitoring the presence of any hazardous stress in the aquatic environment is crucial, as it serves as an important metric to assess the well-being of aquatic organisms (Panigrahi *et al.,* 2014).

1.7.4 Hematological abnormalities by pesticide in fish

The significance of fish hematological research has increased due to its reliability and sensitivity in evaluating biological and pathological alterations resulting from natural or human-induced factors like microbial infections or contamination levels in aquatic environments. Consequently, hematological indices are now recognized as a vital tool for assessing the overall health of fish under different stress conditions (Ali and Rani, 2009). The hematological parameters of fish can be rapidly altered by pesticides. Consequently, hematologic indices can be used to effectively monitor the health and response of fish and other aquatic organisms to different toxic substance. This approach not only provides insight into the ecological state of the environment but also offers a reliable method for assessing the sub-lethal effects of contaminants (Pimpao *et al.,* 2007).

Various genetic and environmental factors can lead to alterations in the blood parameters of fish. Among these factors, pesticides have been found to specifically impact a range of characteristics related to fish blood parameters. The focus of the impact of pesticides on fish is often directed towards the changes observed in their blood parameters (Rios *et al.,* 2002). The evaluation of hematological indices can serve as a valuable tool for assessing the impact of pesticides on the cellular components of blood, as well as the immune system of fish. By analyzing these hematological and biochemical parameters, it becomes possible to assess the overall health of the animals and gain insights into the conditions of their habitat. This approach offers a comprehensive understanding of the effects of pesticides and other toxicants on fish and their environment (Thrall *et al.,* 2012).

1.7.5 Behavioral alterations in fish caused by pesticides.

Pesticides can induce various behavioral and physiological changes in multiple fish species, including Mahasheer (*Tor putitora*) and Common carp (*Cyprinus carpio).* These alterations include aggregation behavior, the production of mucus by goblet cells in the skin (resulting in sliminess), reduced mobility, disruptions in migration patterns, a tendency to tumble towards the bottom, increased jumping behavior, decreased responsiveness accompanied by hyperexcitability, irregular activity patterns, elevated opercular rate (respiration rate), and changes in body coloration. Additionally, pesticides possess the ability to disturb and alter the swimming behavior of aquatic vertebrates such as fish and amphibians, as well as hinder their growth rates (Stehle and Schulz, 2015). Exposure to pyrethroids has been found to inhibit the function of the dopamine active transporter, leading to erratic or unpredictable fish behavior (Wang *et al.,* 2020).

1.8 Herbicides

Herbicide is a type of pesticide that is designed to control or eliminate unwanted plants, commonly known as weeds. In a variety of contexts, including agriculture, forestry, gardening, landscaping, and industrial locations, these chemicals are used to control vegetation.

Herbicides work by disrupting essential biological processes in plants, leading to their growth inhibition or death. They cause plants to wither, lose their capacity for photosynthesizing, or otherwise become incapable of surviving because they specifically target enzymes or biochemical processes important for plant development (US EPA, 2023).

Herbicides represent a significant portion of the global and national pesticide markets, surpassing the combined market share of 20 other pesticide types, including insecticides and fungicides. As of 2004, herbicides accounted for approximately 45% of total pesticide sales, while insecticides and fungicides constituted around 27% and 22%, respectively (Rutherford *et al.,* 2011).

Herbicides are a primary approach for controlling harmful weeds in both agricultural and non-agricultural lands worldwide, presenting for over 60% of all pesticides used in the agricultural sector (Elalfy *et al.,* 2017). There is a possible risk to a variety of aquatic biota, including fish, because of the widespread, ongoing, and intensive utilization of pesticides, especially herbicides, in agricultural lands (Guo *et al.,* 2008).

1.8.1 Metsulfuron-methyl

Metsulfuron-methyl is a white to pale-yellow solid herbicide with a chemical formula of $C_{14}H_{15}N_5O_6S$. It has a distinct ester-like odor and is a systemic herbicide that can be absorbed by the plant and move throughout its tissues. It can be applied before or after weeds emerge, making it a pre-emergent and post-emergent herbicide. It belongs to the triazinylsulfonylurea group of herbicides, which also includes iodosulfuron, ethametsulfuron, thifensulfuron, and their methylated forms. This group of herbicides works by inhibiting an enzyme that is essential for plant growth.

Metsulfuron-methyl is used to control a wide variety of broadleaf weeds, including dandelions, thistles, and chickweed. It is also used to control some grasses, such as crabgrass and barnyard grass. In Australia, metsulfuron-methyl has been approved for brush and broadleaf weed control on a range of crops including cereals (For example wheat, barley, canola, rye, triticale), linseed, chickpeas, mung beans and pastures, and in other activities such as forestry, commercial and industrial areas, and for *Mimosa pigra* control on floodplains (APVMA 2020).

Metsulfuron-methyl is a selective residual herbicide that retains its biological effectiveness in soil for a period of days to months. The half-life of metsulfuron-methyl in soil can vary depending on the soil moisture content, temperature, and acidity. In general, metsulfuron-methyl is degraded more rapidly in soils with high moisture content, high temperature, and low acidity. (Hertfordshire, 2013).

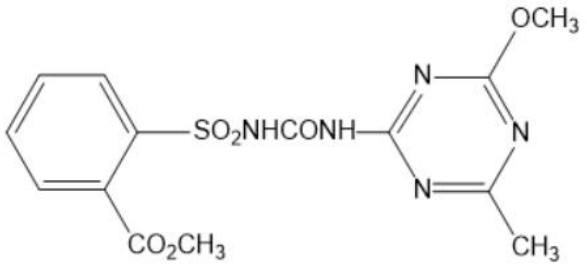

Figure 1.2 Chemical structure of Metsulfuron-methyl. ANZ Water Quality Guidelines (2021).

1.8.2 Effects on Aquatic Animals

Metsulfuron-methyl possesses high potential for leaching, and it can move from the area where it is applied to surface water during and after application. This can happen

through overland runoff or improper waste disposal (Sondhia, 2009). Changes in the aquatic ecosystems were observed with the rise in metsulfuron-methyl concentrations (Wendt-Rasch *et al.,* 2003). The effects of exposure to rice field herbicides, including Metsulfuron-methyl, on AChE activity in the brain and muscle tissues of silver catfish were investigated at environmentally relevant concentrations and it was reported that AChE activity was increased in the brain and muscles of Silver Catfish after short term exposure to these herbicides (dos Santos Miron *et al.,* 2005).

1.8.3 Mechanism of toxicity

Metsulfuron-methyl is primarily absorbed by plants through their roots and leaves, where it binds to the active site of acetolactate synthase, preventing the enzyme from catalyzing the synthesis of acetolactate (ALS) enzyme to cause harmful effects. The ALS enzyme is responsible for synthesis of amino acids. As a result of metsulfuron-methyl's effect, the biosynthesis of amino acid branching of affected plants experience gets inhibited. Exposure to the herbicide typically causes plant death within two to four weeks by stopping cell division and growth processes. (FAO UN 2015).

1.9 N-acetylcysteine

It functions as a plant antioxidant and glutathione precursor and is mostly found in Allium plant species, especially in onions (45 mg NAC/kg in Allium cepa) (Šalamon *et al.,* 2019). It has been used as a medication since 1960 and is included on the Model List of Essential Medicines of the WHO for treating toxicity. L-cysteine, an amino acid, serves as a precursor for NAC, which then transforms into the potent antioxidant glutathione (GSH) (Pieralisi *et al.,* 2016).

In addition to serving as a dietary supplement, N-acetylcysteine (NAC) can replenish intracellular glutathione (GSH). It functions as a non-toxic medicinal agent for treating neurotoxicity, immunotoxicity, lung infections, hepatotoxicity, and glutathione insufficiency in a variety of metabolic disorders (Atkuri *et al.,* 2007).

1.9.1 NAC as an antioxidant

The imbalance between antioxidants and oxidants in the cellular environment leads to oxidative stress. Reactive Oxygen Species (ROS) is a comprehensive term

encompassing molecules generated from oxygen, which can persist as active species or readily give rise to active species (Crespy and Williamson, 2004). Several components of ROS have been identified, which include superoxide, peroxides, hydroxyl radicals, alpha-oxygen, and singlet oxygen (Hayyan *et al.,* 2016). Superoxide is produced when molecular oxygen levels fall, and it is the precursor to the majority of ROS (Turrens, 2003). Superoxide is dismutated to H_2O_2 via a reaction. Hydrogen peroxide can be entirely reduced to water (H_2O) or reduced partially (hydroxyl radicals and hydroxide ions (Hayyan *et al.,* 2016).

ROS are energetic chemical mediators that are produced as a result of oxygen's partial regeneration. ROS molecules serve as a sign of severe oxidative stress since they are transitory and capable of causing DNA oxidative damage. (Cotter *et al.,* 2007). Antioxidants play an important role in preventing oxidation due to their specific chemical structures, which can be divided into two types: fat-soluble and water-soluble antioxidants. Water-soluble antioxidants interact with oxidants in blood plasma and cell cytosol, and vice versa, neutralizing their harmful effects. Conversely, fat-soluble antioxidants protect phospholipid membranes from lipid peroxidation, safeguarding cellular structures from oxidative damage (Vertuani *et al.,* 2004).

N-acetylcysteine (NAC) plays a significant role in mitigating hepato-renal oxidative damage. It finds widespread application in the treatment of pulmonary fibrosis, contrast-induced nephropathy, and chronic obstructive pulmonary disorder. The protective effects of NAC on the kidney and liver result from its ability to counteract oxidative stress and apoptosis. This is achieved through the release of L-cysteine and cysteine, which act as potent scavengers of free radicals (Vertuani *et al.,* 2004). NAC administration within 10-18 hours after an acetaminophen overdose and in individuals with alcoholism has demonstrated the ability to prevent liver damage and significantly reduce mortality rates. The potential mechanisms for its anti-toxic effects include enhanced blood flow in the liver, replenishment of glutathione levels, and scavenging of free radicals. Moreover, NAC proves beneficial in replenishing depleted glutathione levels in HIV-infected patients who have experienced acetaminophen overdose (Zafarullah *et al.,* 2003). These findings highlight the therapeutic potential of NAC in addressing liver-related toxicity and oxidative stress-induced damage in different clinical contexts.

1.10 Vitamin C (Ascorbic Acid) as an Antioxidant

Vitamin C is soluble in water. It has been demonstrated to directly counteract superoxide and hydroxyl radicals, reducing oxidative stress and neutralizing ROS (Medithi *et al.,* 2022). According to some theories vitamin C functions as a chain breaking antioxidant that halts the speed of peroxidative processes and lessens the amount of peroxidation brought on by pesticides. Via vitamin C E redox cycle, it also reduces lipid hydroperoxyl radicals by one electron (Medithi *et al.,* 2022).

In the erythrocytes of male albino Wistar rats, vitamin C stopped the oxidative damage caused by carbofuran (Rai *et al.,* 2009). Pretreatment of vitamin C with carbofuran resulted in a significant improvement in the altered levels of oxidative stress indicators. The levels of MDA, total thiols, GSH, and the activity of antioxidant enzymes such as SOD, CAT, and GST were found to be nearly identical to those of the control group. This suggests that vitamin C may offer substantial protection against pesticide poisoning in the rat heart (Jaiswal *et al.,* 2013).

El-Gendy *et al.,* (2020) investigated the protective impact of vitamin C (200 mg/kg b.w.) in male Swiss albino mice before and after treatment with imidacloprid, a neonicotinoid pesticide. Their study revealed that oral treatment of imidacloprid at a dose of 14.976 mg/kg significantly increased lipid peroxidation levels and the activities of antioxidant enzymes such as CAT, SOD, GPx, and GST (El-Gendy *et al.,* 2010). However, they suggested that vitamin C could mitigate imidacloprid-induced oxidative damage by reducing lipid peroxidation levels and modulating antioxidant defense mechanisms in the liver (El-Gendy *et al.,* 2010).

In the case of CPF-toxicated animals, vitamin C therapy reduced lipid peroxidation and GST activity while restoring CAT, SOD, and glucose-6-phosphate dehydrogenase activities to normal and increasing GSH levels (Aly *et al.,* 2010). Furthermore, when propanil and vitamin C were administered together, the negative effects of propanil on most assessed oxidative stress indicators in mouse liver tissues were mitigated. The investigation suggested that vitamin C could play a crucial role in reducing the hepatotoxicity caused by propanil, making it an important dietary component (Otuechere *et al.,* 2012) Overall, these findings strongly indicate the definite protective function of vitamin C against pesticide-induced damage.

1.11 Nile Tilapia (*Oreochromis niloticus*)

The origin of Nile tilapia (*Oreochromis niloticus*) can be traced back to Africa, more specifically to the Nile River basin and other water bodies in East Africa. The exact natural range of the species is not fully understood, evidence suggests that Nile tilapia is native to countries like Egypt, Sudan, Ethiopia, and other regions in the Nile River system (Geletu and Zhao, 2023)

The Nile tilapia, O. niloticus, is a cichlid freshwater fish native to northern parts of Africa and the south-western Middle East, but widely introduced. It is one of the world's most important food fishes, and the fourth most important species in global aquaculture production by weight, accounting for 8% of total global aquaculture production in 2016 (Bonham, 2022).

Tilapia has become a key priority in fishery and aquaculture due to their capacity to efficiently utilize natural feeds. Tilapia is herbivores in nature, predominantly feed on vegetation and algae, and are frequently reared in canals and manmade lakes to manage the growth of algae and vegetation (Suárez-Moreno *et al.,* 2012).

Using Nile tilapia (*Oreochromis niloticus*) as a model for MSM (Metsulfuron methyl) toxicology research in water is crucial due to its dual significance in aquaculture and as a food source. As one of the most widely cultivated and consumed freshwater fish globally, tilapia represents a valuable commodity, making it highly relevant to understanding potential toxicological risks associated with MSM exposure in water. Furthermore, its ease of maintenance, rapid reproduction, and similarity to other aquatic organisms make it an ideal model for studying MSM's effects on aquatic ecosystems, providing valuable insights into potential impacts on fish populations and the overall environment.

The present study offers a means to evaluate the impact of herbicides on fish, other aquatic species, aquatic systems, and the environment. The increasing use of the herbicide Metsulfuron methyl could potentially pose a threat to aquatic species in the future, making it crucial to investigate its toxicity in aquatic vertebrates. The study suggests that toxicities may be linked to oxidative stress, and ROS production. The discovery of effective antioxidants such as vitamin C and N-acetylcysteine has played an important role in attenuating Metsulfuron methyl-induced toxicities in fish`s hematological, gills, and liver antioxidant systems.

AIM AND OBJECTIVES OF THE STUDY

Aim

This study investigated the potential protective effects of vitamin C and N-acetylcysteine against oxidative stress induced by metsulfuron methyl in Nile tilapia (Oreochromis niloticus).

Objectives

- To evaluate the impact of Metsulfuron methyl exposure on oxidative stress markers in Nile tilapia.
- To determine the effects of administration of Vitamin C and N-Acetylcysteine on oxidative stress markers in Metsulfuron methyl-exposed Nile tilapia.
- To analyze the changes in Superoxide Dismutase (SOD) and Peroxidase (POD) and other antioxidant enzymes activity in fish subjected to Metsulfuron methyl exposure and antioxidant treatment.
- To compare the effectiveness of Vitamin C and N-Acetylcysteine in ameliorating oxidative stress.
- To assess the hematological parameters of Nile tilapia after exposure to MSM and protective effects of NAC and Vitamin C

MATERIALS AND METHODS

The present experiments were carried out at the Laboratory of Fish Physiology, Department of Zoology at Quaid-I-Azam University in Islamabad, Pakistan. All aspects of the experimental work were conducted and structured in accordance with the established protocols of the Bioethical Committee within the Department of Animal Sciences. The handling procedures adhered strictly to the guidelines set forth by the Ethical Committee.

2.1 Collection and transportation of experimental fish

About 150 fishes of the same species Nile Tilapia (*Oreochromis niloticus)* were procured and transported from NARC Islamabad to the Laboratory of Fish Physiology, Department of Zoology at Quaid-I-Azam University in Islamabad in close oxygenated tight plastics bags containing adequate amount of hatchery water and enough oxygen gas in order to reduce physiological stress on stock. After transportation, the fish were shifted to concrete tank for acclimatization. Fish were acclimatized for 14 days and fed at ad libitum with a basal feed having 32% crude protein. Prior to shifting, the tank was thoroughly cleaned and whitewashed with lime to prevent any bacterial disease, and fish were treated with 0.2% $KMNO_4$ solution for disease/parasite attack.

2.2 Chemicals

All chemicals were obtained from Sigma-Aldrich (Sigma, St. Louis, Missouri, USA). Heparin (Kota Bharu, Kelantan, Malaysia) was purchased locally. N acetyl cysteine (NAC) was purchased from Sigma-Aldrich (Germany). Metsulfuron methyl (Deft 10wp 40gm Metsulfuron Methyl Vantage) was purchased from Kissan Ghar Sargodha Pakistan. Formaldehyde, chloroform, NBT, ferrous sulphate, comassive blue, Triton-x100, BSA, Sodium chloride, Potassium chloride, SDS, PMSF, DEPPD, sodium azide, sodium hydroxide, hydrogen peroxide, Tris-HCL, TBA, TCA, riboflavin, potassium phosphate monobasic, L-methionine, potassium phosphate dibasic, sodium phosphate dibasic, guaicol, sodium phosphate monobasic, Tri-sodium Citrate, Eosin, DTNB, phosphoric acid, Hematoxylin, Methanol, etc.

2.3 Experimental design

After a 14-day acclimatization, Nile Tilapia (*Oreochromis niloticus*) was introduced into spacious Glass Aquaria. These specially selected aquaria, each with a 70-liter

capacity, were filled with aged and dechlorinated tap water. The fish, initially weighing (15.5g ± 2.0) and measuring (12.7cm ± 1.5) in length, were evenly distributed among four aquariums, housing 10 fish each. Each 40-liter aquarium maintained a stocking density of 1.08g/L. The assignment of aquaria to different groups was done randomly to ensure unbiased experimentation, resulting in four distinct experimental sets. The experiment followed a two-stage design, with four distinct groups: control, pesticide treatment, pesticide exposure with 1.0 mM NAC (N-acetylcysteine) treatment, pesticide exposure with Vitamin C treatment. Each group was replicated three times, and 10 fish were randomly assigned to each group.

In the initial stage, one group served as the control and remained unexposed to any pesticide or treatments (including N-acetyl cysteine and Vitamin C). The remaining three groups were subjected to Metsulfuron methyl, with a dose of 30 mg per liter (1/100 of the field rate) (Fathy *et al.,* 2019), in a simulated trial environment for a duration of 28 days.

In the subsequent stage, two of the pesticide-exposed groups were treated with cysteine at a concentration of 1.0 mM and Vitamin C at 500 mg/kg of feed. These treatments were administered to assess their effects for 4 days (Atamanalp *et al.,* 2021). (Table 2.1). At the end of the 24 days trial period, tissues samples were collected from all treatment fish.

Table 2.1 Experimental design.

Treatment Mark	Treatment Group	Treatment Detail
A	Control	No pesticide, NAC, or Vit. C
B	Pesticide Treatment	30 mg/L MSM
C	NAC Treatment	1.0 nM/L NAC
D	Vitamin C Treatment	500 mg/kg feed Vit. C

2.3.1 Dose selection And Calculation

Fish, Nile Tilapia (*Oreochromis niloticus*) were subjected to controlled exposure of Metsulfuron methyl (MSM) in a carefully designed aquatic environment. The chosen dosage of MSM was set at 30 mg per liter, which corresponds to 1/100 of the typical field application rate (Fathy *et al.,* 2019). This dosage was meticulously selected to mimic real-world conditions while ensuring the safety of the fish subjects. For a total

of 40 liters of water, this equated to a precise addition of 1200 mg (30 mg/L × 40 L) of MSM into the aquatic system. The calculation is given below.

Dosage per liter: 30 mg

Volume of water: 40 liters

Calculating the total dosage.

Total Dosage = Dosage per liter × Volume of water

Total Dosage = 30 mg/L × 40 L

Total Dosage = 1200 mg

Therefore, 1200 mg of Metsulfuron methyl was added to the aquatic system containing 40 liters of water to achieve a dosage of 30 mg per liter, which represents 1/100 of the typical field application rate.

2.3.2 Dosing time

The dosing time was kept constant throughout the experiment to avoid any possible stress or resistance from the animal. Dose synchronization with specific time is critical because it prepares the animal physiologically and psychologically for the dose.

2.3.3 Water quality parameters

Water quality management for Nile tilapia (*Oreochromis niloticus*) involved a routine replacement of 30% of the water volume every third day, aimed at waste reduction and maintenance of water freshness. Concurrently, a consistent dose of MSM was maintained accordingly. Removal of unconsumed feed and feces was carried out through siphoning, preventing organic matter accumulation. This integrated approach facilitated effective water quality maintenance.

The major water quality parameters like conductive property, concentration of dissolved ions sulphates, chlorides and nitrates etc., pH of water, and temperature were determined according to well-known protocol. properties as dissolved oxygen 7.7 mg/L, pH 6.3-7.3, temperature 25 ± 0.5ºC, and total hardness 220 mg/L were maintained.

2.4 Dissections

Prior to the day of sample collection, fish were starved for 24 hours. Following this, fish were instantly anesthetized with Clove oil. Fish weights were measured, and blood samples were collected by using a 3ml pre heparinized syringe by caudal vein. A heparinized fresh blood sample was stored in an EDTA tube for analysis of blood

hematology. Fish were sacrificed on an ice box. Liver and gills were removed using saline-rinsed sterilized surgical instruments. Half of the organs were wrapped in aluminum foil and stored at -20°C for biochemical analysis and half of them preserved in 10% buffered formalin for histological analysis.

2.5 Hematological parameter

Hematological indices like WBCs, RBCs, Hemoglobin, HCT, MCH, MCHC, and MCV were analyzed from fresh blood on an automatic hematology analyzer (Sysmex hematology analyzer). All the samples were run in duplicate with 15 sec interval time and mean values were calculated.

2.6 Biochemical analysis of tissue

The biochemical analyses of liver and gills was performed to analyze the activity of ROS, indirect measurement of lipid peroxidation through Thio barbituric-acid-reactive substances (TBARS), enzymes of antioxidant, the peroxide dismutase (POD), Catalase (CAT), superoxide dismutase (SOD) and non-enzymatic reduced Glutathione (GSH) and total protein concentration.

2.6.1 Preparation of Extract buffer (Lysis buffer)

For the preparation of extract buffer, 5.95 g of HEPES, 0.1 g of Sodium azide, 0.5 g of SDS, 4.38 g of NaCl were added into 495 ml of distilled water and 5 ml of Triton-x-100 was then added to take the final volume of extract buffer up to 500 ml.

2.6.2 Method of preparation of tissue homogenate

Liver and gills tissues were weighed (100 mg) and minced in frosted petri dishes using a hand-held manual homogenizer (GPE limited, UK). Tissues were homogenized in 1ml of extract buffer (Lysis buffer, pH 7.0), which also contained 0.1mg of PMSF. The homogenate was then centrifuged at 5031 g for 10 min to separate the supernatant and taken into the labelled autoclaved 1.5 ml Eppendorf tubes and stored at -20°C for biochemical studies.

2.7 Oxidative Profile Parameters

2.7.1 Estimation of Reactive oxygen species

The ROS concentration in tissue homogenates was determined using the protocol of (Hayashi *et al.,* 2007). To make 0.1M sodium acetate buffer, 4.1 g of sodium acetate was first dissolved in 500 ml of distilled water (PH-4.8). To make Reagent 1, 10 mg of N.N- Diethylpara phenyldiamine sulphate (DEPPD) was dissolved in 100 ml of buffer. 50 mg of ferrous sulphate dissolved in 10 ml of sodium-acetate buffer to

prepare stock solution of $FeSO_4$ and 62.5 µl of $FeSO_4$ from the stock solution was dissolved in 125 ml of sodium acetate buffer to prepare Reagent 2. Both Reagent 1and Reagent 2 were mixed in a 1:25 ratio and left in the dark for nearly 2 min. In a 3 ml cuvette, 1200 µl of sodium acetate buffer, 1680 µl of reagent mixture and 60 µl of homogenate sample were placed. A (UV-visible spectrophotometer) was used to measure absorbance at 505 nm (Agilent 8453, USA). Then three readings were taken. at the interval of 30 second for each sample.

2.7.2 Analysis of lipid peroxidation assay (TBARS)

Malondialdehyde (MDA) content within the homogenate was determined utilizing the technique outlined by Iqbal *et al.* (1996), which involves its reaction with Thiobarbituric acid (TBA). This approach provides an indirect evaluation of oxidative stress resulting from lipid peroxidation.

To conduct the analysis, a reaction mixture was prepared within a 15 ml Falcon tube. This mixture consisted of 0.1 ml of ascorbic acid (1.5 mM), 0.1 ml of 50 mM Tris-HCL, 0.1 ml of $FeSO_4$ (1mM), 0.6 ml of distilled water, and 0.1 ml of tissue homogenate. The components were homogeneously blended through vortexing, following which the mixture was subjected to an incubation period of 15 min at a temperature of 37°C.

After incubation, 1 ml of trichloroacetic acid (10%) and 1 ml of Thiobarbituric acid (0.375%) were introduced to the reaction mixture. This mixture was then subjected to boiling at 90°C for a duration of 15 min within a water bath. To facilitate the separation of components, the mixture was subsequently centrifuged at 3000 rpm for a duration of 10 min. The resulting supernatants were carefully transferred to cuvettes, and their absorbance was measured at a wavelength of 532 nm.

In order to achieve comprehensive data, three absorbance readings were recorded for each sample at 30-sec intervals. This rigorous procedure collectively allowed for the quantification of Malondialdehyde content, thereby providing insights into the extent of oxidative stress due to lipid peroxidation.

2.7.3 Antioxidant enzymes.

Enzymatic antioxidants are CAT, SOD, POD and GSH

2.7.4 Catalase Assays (CAT)

The activity of CAT in tissues was determined using a protocol modified slightly from (Aebi, 1984). In 3 ml of cuvette, 1000 µl of H_2O_2 (5.9 mM), 50 mM of

potassium phosphate buffer (1.99 ml) pH: 7.00 and 0.1 ml of homogenate sample were mixed. Absorbance at 240 nm was measured for each sample after every 30 sec and three readings were taken and then averaged.

2.7.5 Superoxide dismutase (SOD)

SOD activity was determined by using the (Kakar *et al.,* 1984). 4.5 mL of 9.9 mM L-Methione, 2.25 mL of Triton X-100 (0.025 percent) and 3 mL of 57µM Nitroblue Tetrazolium were combined to make the reagent (NBT). By adding 50 mM Phosphate buffer saline, the final volume was increased to 90 ml (PBS) The pH is 7.8. One ml of the above mixture was transferred to a cuvette and 20 µl of sample was then added to each cuvette. These were then exposed to a fluorescent lamp for 7 min before being incubated at 37°C for 5 min. Later, 10 µl of chilled Riboflavin was added to the reaction mixture to start the process and the contents were then incubated at 40°C for 8 min. Then, at 1-minute intervals, three readings at 560 nm were taken.

2.7.6 POD Assay

The concentration of POD in the tissue homogenate was determined using the (Chance and Maehly, 1955). 2.5 ml of 50 mM phosphate buffer, 0.1 ml of enzyme extract and 0.1 ml of 20 mM guaiacol were added to the reaction mixture. The contents were vigorously mixed to form a homogeneous solution and 0.3 ml of 40 mM H2O2 was added to the reaction mixture. After one minute, there was a change in absorbance of the reaction mixture at 470 nm.

2.7.7 Determination of Reduced glutathione (GSH)

Reduced Glutathione was determined using the method defined by (Jollow *et al.,*1974). 1 ml of disodium phosphate buffer (0.4 M), 0.1 ml of tissue homogenate and 0.5 ml of DTNB were combined to make the reagent mixture. DTNB (Ellman's reagent) was created by dissolving 40 mg of DTNB in 100 ml of 1% trisodium citrate. At 412 nm, the absorbance of the yellow colour appeared was measured.

2.7.8 Estimation of total protein

By the standard Bradford assay, total Protein was quantified in gills and liver.

Twenty-five mL methanol, 50 mL H_3PO_4, 100 mL distilled water and 50 mg Comassive blue were combined to make the stock solution. The solution was then stored until used it at 4°C in dark. Stock solution was diluted in a 1:4 ratio with distilled water to make the working solution. Similarly, 10 mg of BSA dissolved in 10 ml of phosphate buffer saline (PBS) to prepare stock solution of BSA. BSA dilutions

of 100x, 50x, 25x, 12.5x and 6.25x were prepared from the stock solution. 2900 µl of working solution was combined with 100 µl of BSA serial dilutions in a cuvette to generate the standardization curve and the absorbance change was measured at 595 nm. For protein estimation, 100 µl of tissue homogenate was mixed with 2900 µl of working solution and a change in absorbance at 595 nm was observed. Within a minute, three readings were taken.

2.9 Statistical Analysis

The results are given as mean ± SEM. Graph pad prism software 9.5.1 and Sigma plot 12.0 used for one-way analysis of variance (ANOVA). The level of significance was set at $p< 0.05$. The Tukey's post hock test was used to compare all groups with each other.

RESULTS

3.1 Oxidative stress Markers

ROS

3.1.1 Gills

Fish subjected to metsulfuron-methyl treatment (Group-II) displayed a noteworthy elevation in levels of Reactive Oxygen Species (ROS) with a significant increase ($p < 0.001$). Conversely, a substantial reduction in ROS was observed in Group III and Group IV when compared to the metsulfuron-methyl-treated group (Group-II). These findings are effectively elucidated in (Figure 3.1)

3.1.2 Liver

The activity of reactive oxygen species (ROS) increased significantly in the liver tissue of fish treated with metsulfuron-methyl (Group II) compared to the control group. The ROS value was significantly restored in Group III and Group IV. (Figure 3.2)

3.2 Activity of TBARS

3.2.1 Gills

The activity of TBARS increased significantly in the gills tissue of Nile tilapia treated with metsulfuron-methyl (Group-II) as compared to control. TBARS value was significantly restored by Treatment of NAC and vitamin C. (Figure 3.3)

3.2.2 Liver

The liver tissue of fish subjected to metsulfuron-methyl treatment (Group-II) exhibited a marked elevation in TBARS activity in contrast to the control group. However, the administration of NAC and Vitamin C effectively reinstated the TBARS values to a significant extent. These findings are thoroughly elucidated through the visual representation in Figure 3.4, presenting a comprehensive understanding of the outcomes.

3.3 Activity of SOD

3.3.1 Gills

In gills, the activity of SOD decreased significantly in metsulfuron-methyl (Group-II) treated group While in Group-III, and Group-IV have not significantly changed occurred as compared to control. In Group-III and Group-IV Significantly restored the SOD levels. Figure 3.7.

3.3.2 Liver

In liver, a substantial decrement in the enzymatic activity of Superoxide Dismutase (SOD) was evident within the metsulfuron-methyl treated group (Group-II) when compared to the control group (I). However, administration of N-acetylcysteine (NAC) and Vitamin C in Group III and Group IV exhibited a remarkable restorative effect on SOD levels Figure 3.8.

3.4 Activity of POD

3.4.1 Gills

In the gills of Nile Tilapia, there was a considerable decrease in the activity of Peroxidase (POD) within Group-II ($p < 0.001$). However, this trend was effectively countered by the treatment groups, where the POD levels were notably restored Figure 3.5.

3.4.2 Liver

In liver, the activity of Peroxidase (POD) in Group-II experienced a substantial reduction ($p < 0.001$) compared to the control group. Notably, the application of N-acetylcysteine (NAC) and Vitamin C treatments exhibited a commendable restoration of POD activity. A comprehensive insight into these observations is provided by the elucidating visual aid in (Figure 3.6).

3.5 Activity of CAT

3.5.1 Gills

The gills displayed a significant decline in Catalase (CAT) activity within Group-II compared to the control group. Notably, the administration of N-acetylcysteine (NAC) and Vitamin C treatments effectively reinstated and normalized CAT functions, particularly observed in Group-III and Group-IV. (Figure 3.9).

3.5.2 Liver

Catalase (CAT) activity in the liver of Group-II displayed a substantial decrease ($p < 0.001$) compared to the control group. However, in the NAC and Vitamin C treated groups (i.e., Group-III and Group-IV), there was a partial restoration of CAT activity. (Figure 3.10).

3.6 Activity of Reduced Glutathione-GSH

3.6.1 Gills

In Gills, the level of GSH decreased significantly in Group-II ($p < 0.001$) compared to control group. NAC and Vitamin C treatment however, restored GSH activity in the treatment Groups. (Figure 3.11).

3.6.2 Liver

In liver, there was a notable reduction in the level of Glutathione (GSH) within Group-II in comparison to the control group. Notably, the application of N-acetylcysteine (NAC) and Vitamin C treatments effectively reinstated the level of GSH. A comprehensive understanding of these outcomes is presented through the visual representation in figure 3.12. This graphical representation serves as a valuable tool in explaining the observed changes in GSH levels and highlighting the restorative influence of the applied NAC and Vitamin C treatments.

3.7 Hematological parameters

The hematological parameters of *O. niloticus* displayed alterations upon exposure to Metsulfuron methyl at 1/100 of the field rate concentrations, as detailed in Table 3.3. Notable changes were observed in nearly all hematological aspects among juvenile O. niloticus fish subjected to the herbicide. In the group exposed to metsulfuron-methyl, a significant decrease ($P < 0.05$) in the erythrocyte count and hemoglobin were evident compared to the control group. Leucocyte exhibited a significantly increased as well. Mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) demonstrated no significant changes in comparison to the control group. Fluctuations were observed in platelet count and hematocrit level in exposed fish, in contrast to the control group. Additionally, a non-significant increase was seen in mean corpuscular volume (MCV).

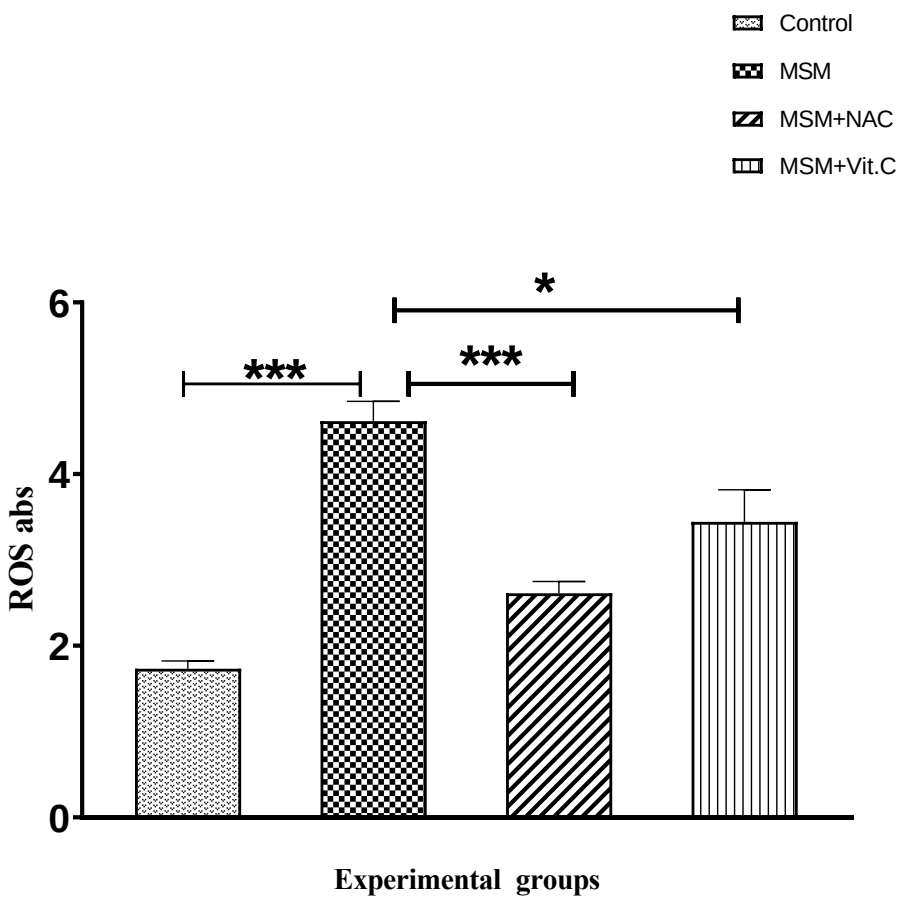

Fig.3.1 Mean ROS in Nile Tilapia (*Oreochromis niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

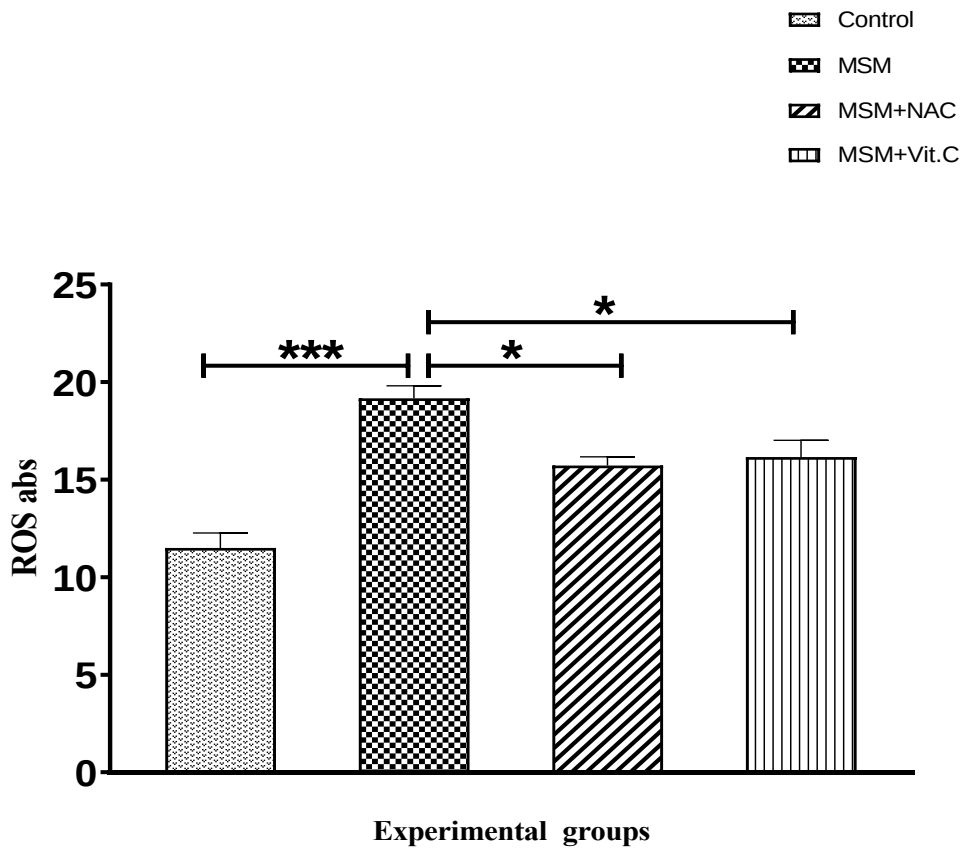

Fig.3.2 Mean ROS in Nile Tilapia (Oreochromis *niloticus*) liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per liter were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

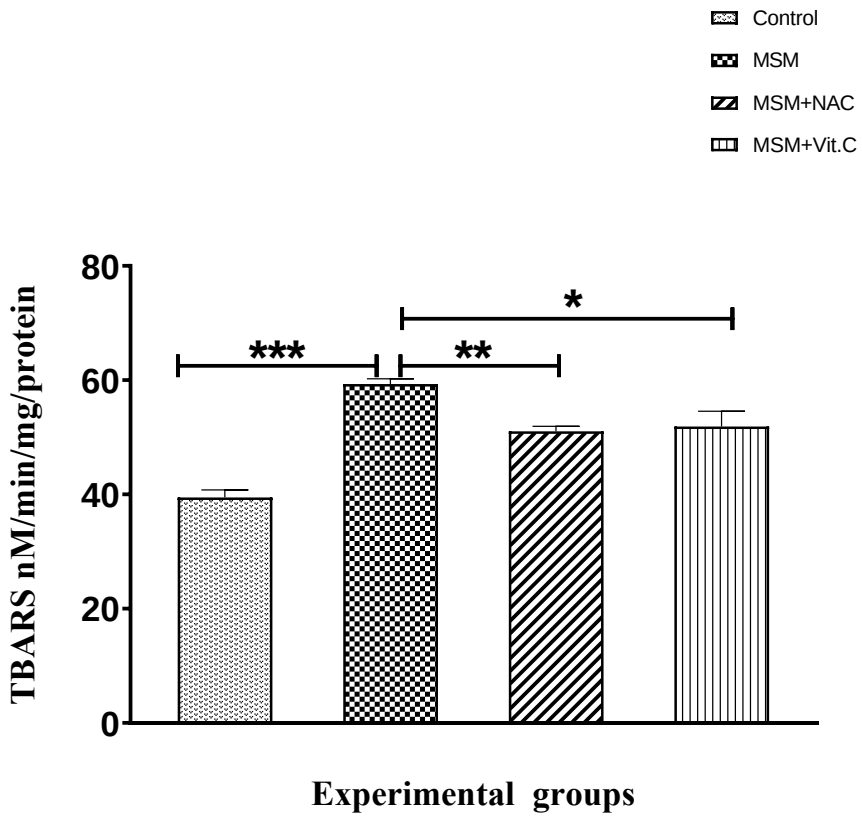

Fig.3.3 Mean of TBARS in Nile Tilapia (Oreochromis *niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

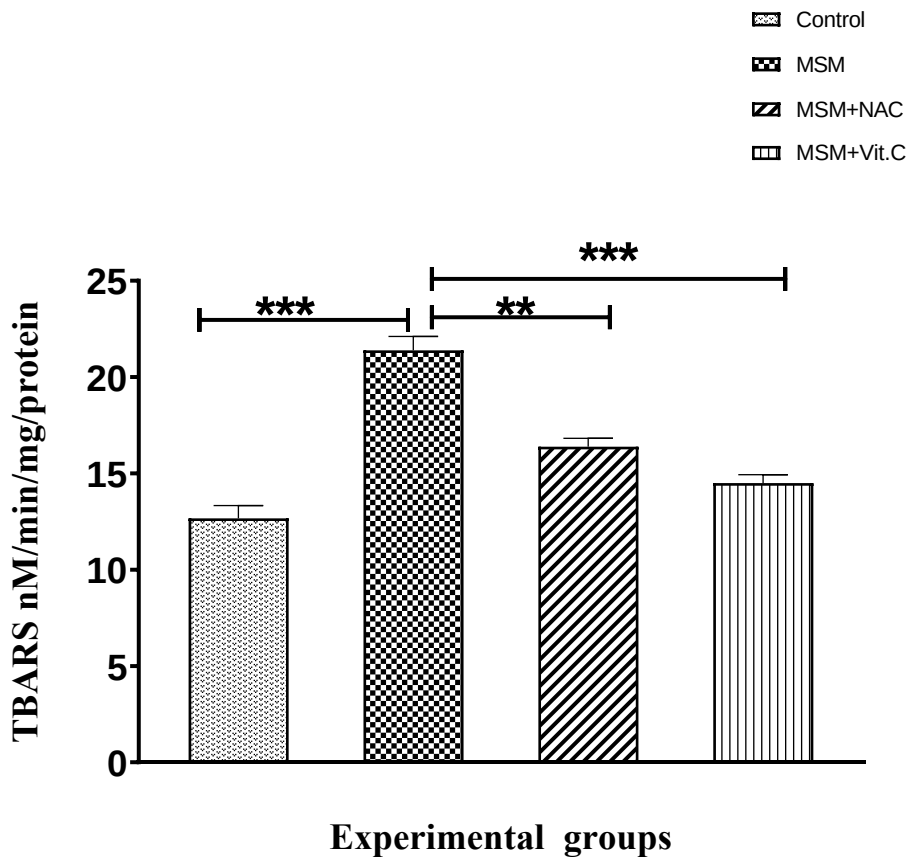

Fig.3.4. Mean of TBARS in Nile Tilapia (Oreochromis *niloticus*) Liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

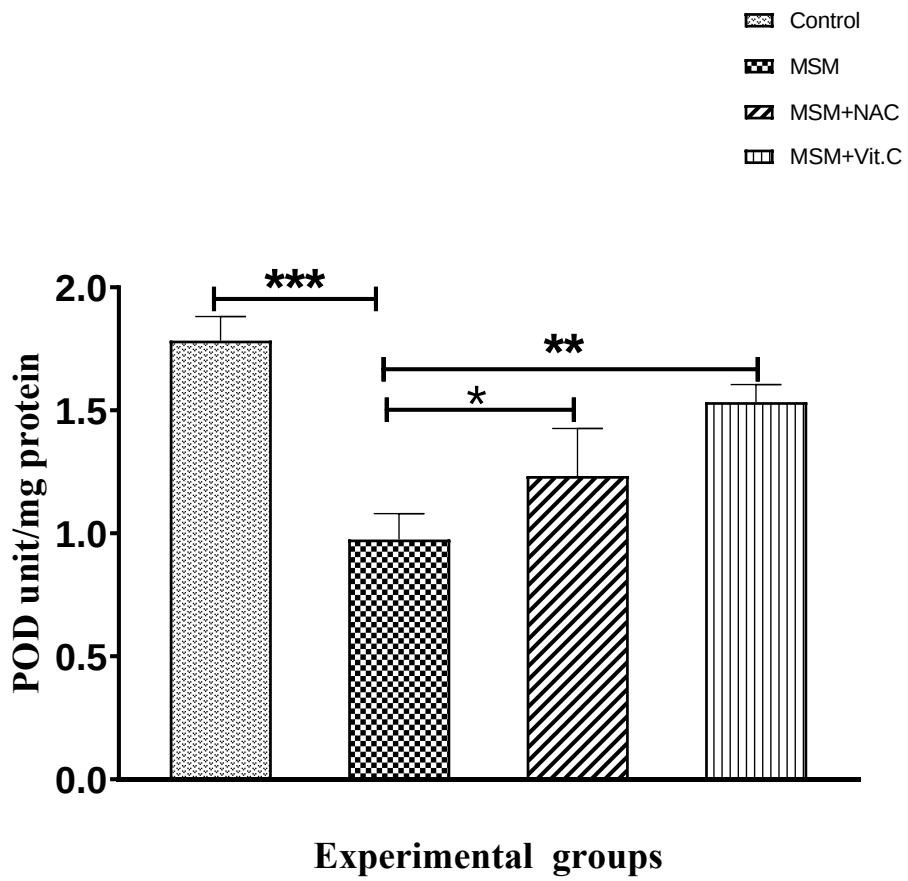

Fig.3.5. Mean POD in Nile Tilapia (Oreochromis *niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

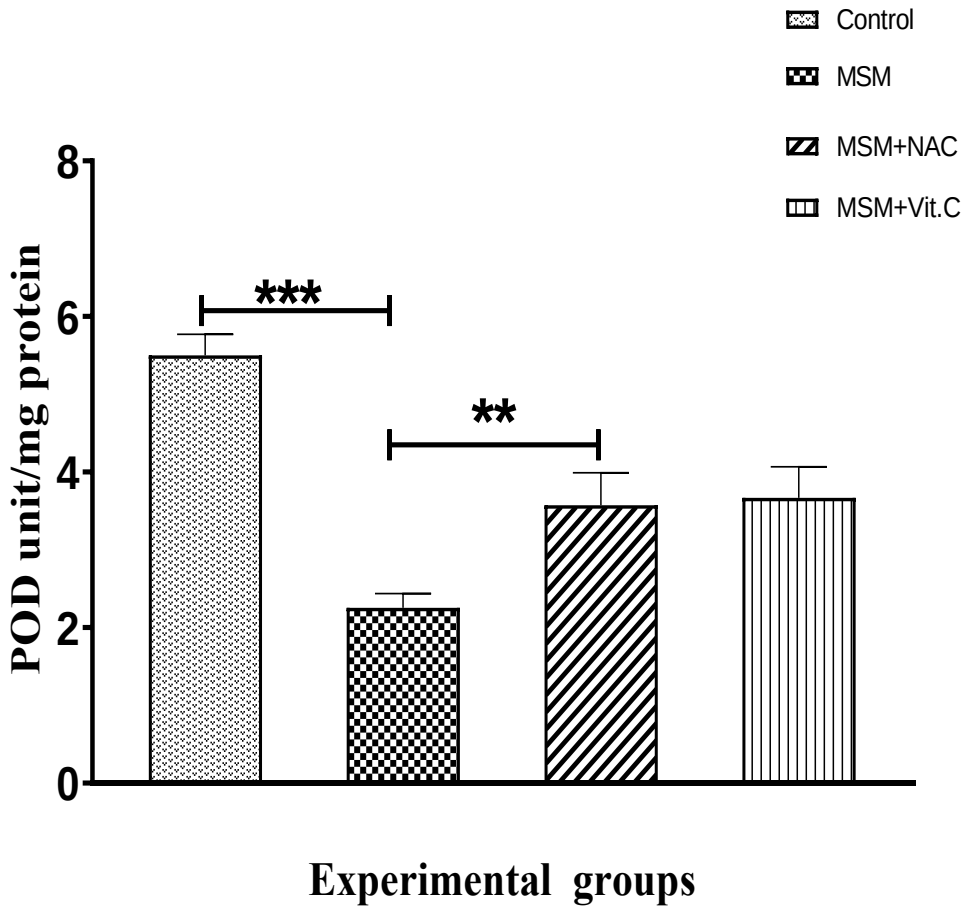

Fig.3.6. Mean POD in Nile Tilapia (Oreochromis *niloticus*) liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

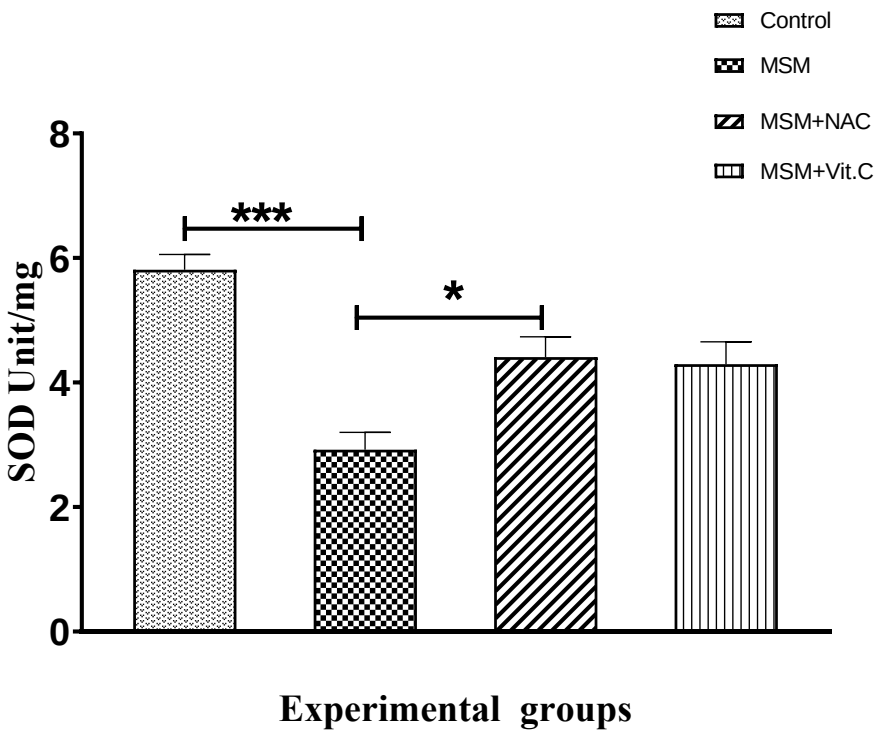

Fig.3.7. Mean SOD in Nile Tilapia (Oreochromis *niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$ & *** $p<0.001$ show significant difference between respective groups.

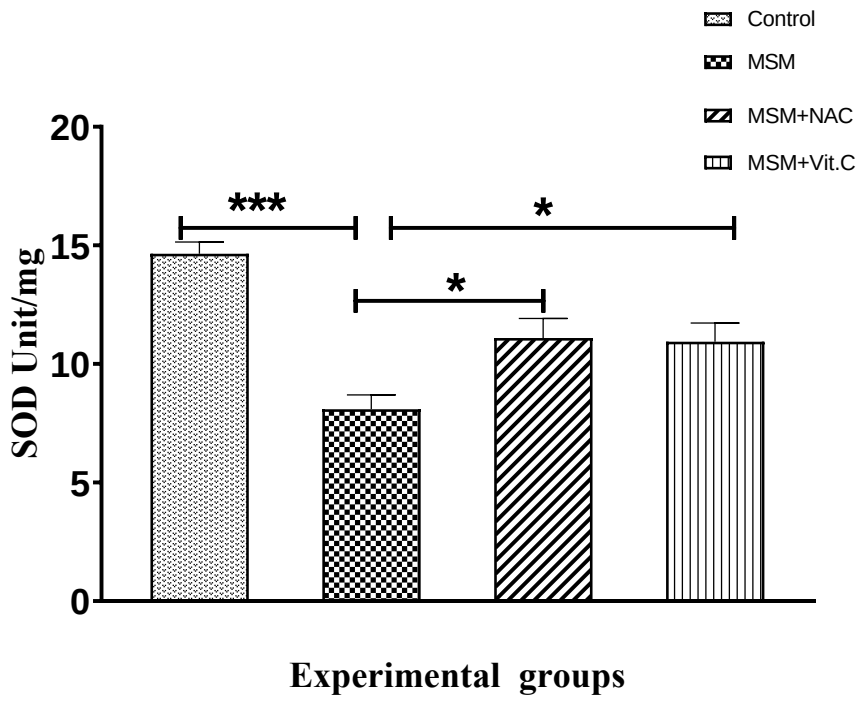

Fig.3.8. Mean SOD in Nile Tilapia (Oreochromis *niloticus*) liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$ & *** $p<0.001$ show significant difference between respective groups.

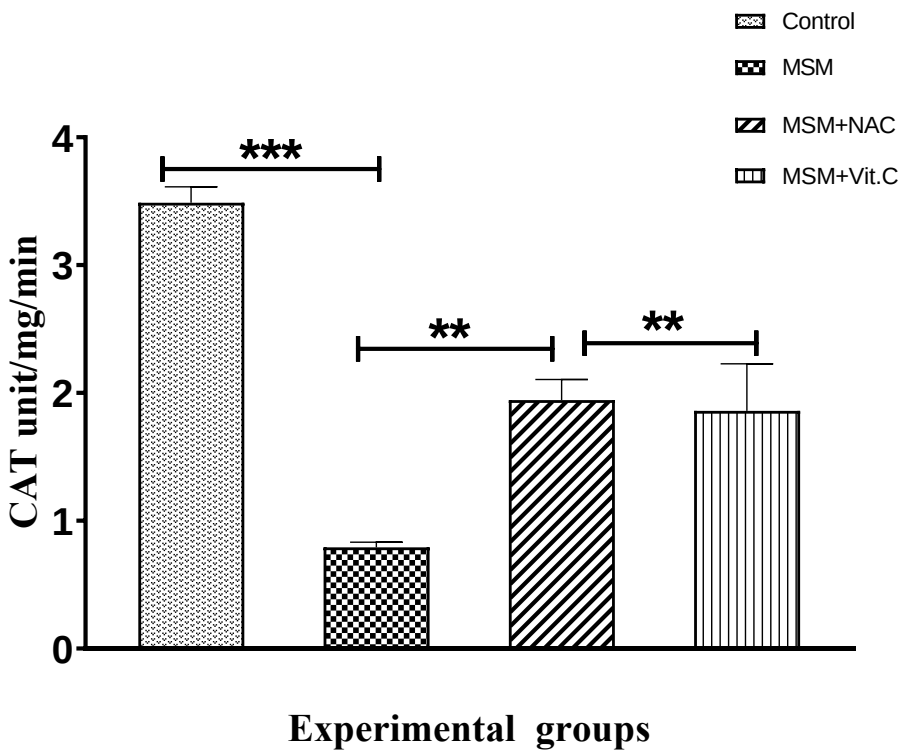

Fig 3.9. Mean CAT in Nile Tilapia (Oreochromis *niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (0.5 1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the groups exposed to pesticide for 4 days. **p<0.01 & *** p<0.001 show significant difference between respective groups.

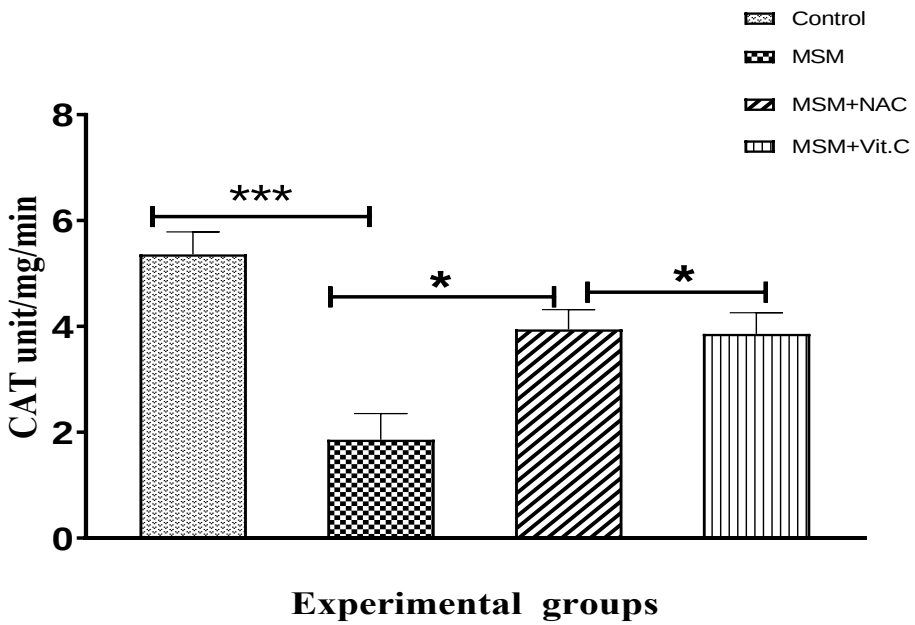

Fig.3.10.

Mean CAT in Nile Tilapia (Oreochromis *niloticus*) Liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (0.5 1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$ & *** $p<0.001$ show significant difference between respective groups.

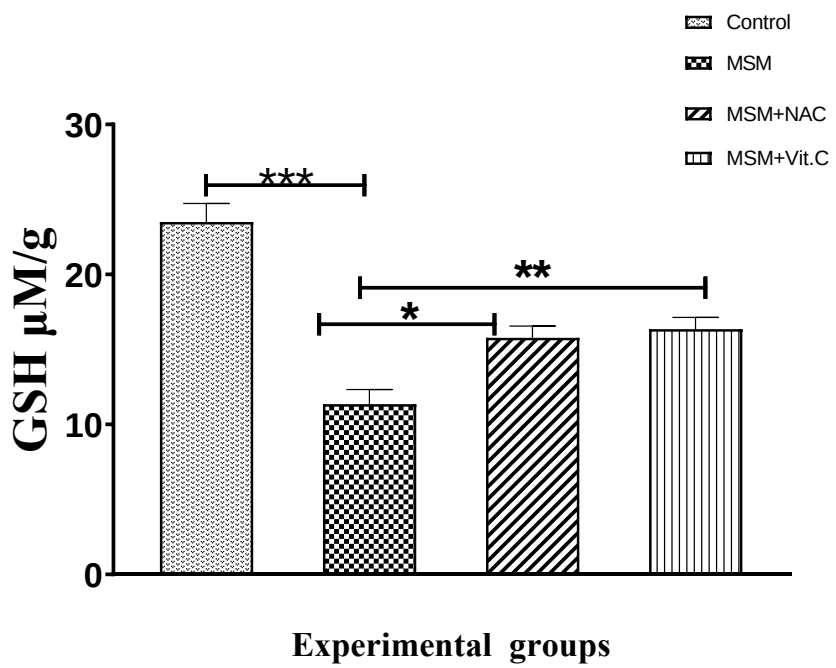

Fig.3.11. Mean GSH in Nile Tilapia (Oreochromis *niloticus*) gills (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

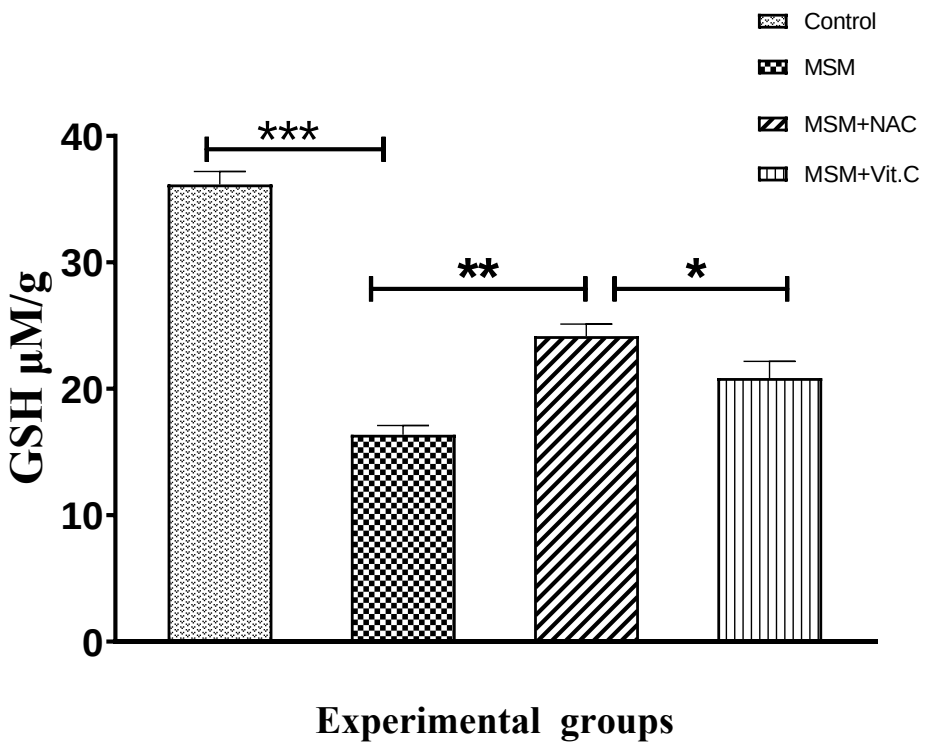

Fig.3.12. Mean GSH in Nile Tilapia (Oreochromis *niloticus*) Liver (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. * $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference between respective treatment groups.

TABLE 3.1 Antioxidant levels in gills of Nile Tilapia (Oreochromis *niloticus*) (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. Data expressed as mean±SEM.

Parameter	**G1**	**G2**	**G3**	**G4**
SOD unit/mg	5.812±0.60	2.923±0.67***	4.408±0.79*	4.295±0.87*
POD unit/mg protein	1.783±0.24	0.9750±0.25***	1.233±0.47*	1.533±0.17**
CAT unit/mg/min	3.488±0.30	0.7925±0.39***	1.945±0.09**	1.859±0.90*
GSH µM/g	23.50±3.01	11.36±2.38***	15.78±1.92*	16.36±1.89**
ROS abs	1.733±0.21	4.617±0.56***	2.610±0.33***	3.443±0.91*
TBARS nM/min/mg/protein	39.47±3.20	59.31±2.27***	51.03±2.20**	51.89±6.55*

* p<0.05, **p<0.01 & *** p<0.001 show significant difference as compared to control group. **(**SOD = Superoxide Dismutase, POD = Peroxidase, CAT = Catalase, GSH= Reduced Glutathione, ROS= TBARS = Thiobarbituric Acid Reactive Substances, ROS= Reactive oxygen species).

TABLE 3.2 Antioxidant levels in liver of Nile Tilapia (Oreochromis *niloticus*) (n=6/group) upon exposure to Metsulfuron methyl with doses of 30 mg per liter 1/100 of field rate (in simulative trial environment for 24 days), doses of N-acetyl cysteine (1.0 mM) and vitamin C 500 mg per kg of feed were administered to two of the experimental groups exposed to pesticide for 4 days. Data expressed as mean±SEM.

Parameter	**G1**	**G2**	**G3**	**G4**
SOD unit/mg	14.65±1.20	8.089±1.48***	11.10±2.02*	10.94±1.92*
POD unit/mg protein	1.783±0.24	0.9750±0.25***	1.233±0.47**	1.533±0.17
CAT unit/mg/min	5.362±1.03	1.859±1.21***	3.942±0.92*	3.859±0.98*
GSH µM/g	36.17±2.48	16.36±1.79***	24.17±2.31**	20.86±3.22*
ROS abs	11.50±1.87	19.17±1.57***	15.72±1.10*	16.16±2.10*
TBARS nM/min/mg/protein	12.67±1.63	21.39±1.76***	16.38±1.08**	14.50±1.04***

* $p<0.05$, **$p<0.01$ & *** $p<0.001$ show significant difference as compared to control group. **(**SOD = Superoxide Dismutase, POD = Peroxidase, CAT = Catalase, GSH= Reduced Glutathione, ROS= TBARS = Thiobarbituric Acid Reactive Substances, ROS= Reactive oxygen species).

Table 3.3 Hematological variables in *O. niloticus* exposed to 1/100 of field rate of MSM Data expressed as mean ± SEM.

Parameters	G1	G2	G3	G4
RBCs(million/mm3)	1.69±0.12	1.53±0.01**	1.57±0.04*	1.54±0.13
Hemoglobin (Hb) (g/dl)	8.05±0.1	7.65±0.44**	7.71±0.28*	7.67±0.25
HCT (PCV) (%)	23.35±0.94	23.05±1.06*	23.11±1.04*	23.08±0.98
MCV (µm3)	138.6±11.47	150.6±7.44	148.94±8.23	148.74±7.64
MCH (Pg)	47.81±3.72	49.98±2.55	48.88±3.43	49.45±2.98
MCHC (%)	34.52±1.67	33.38±3.09	33.78±2.94	33.51±3.14
Platelets (thousands/mm3)	315±3.82	313.25±4.27*	314.02±3.21*	313.59±4.15
WBCs (thousands/mm3)	835±18.27	841±9.45**	838±10.32*	840±9.25
Lymphocytes (%)	87.51±0.29	90.5±1.29**	88.43±0.94*	89.78±1.22
Monocytes (%)	3±0.81	2.25±0.95*	2.56±0.73*	2.39±0.99
Neutrophils (%)	7.5±0.57	6.25±0.5*	6.83±.61*	6.48±0.12
Eosinophils (%)	2.±0.00	1±0.00**	1.37±0.01*	1.24±0.01

***, **, * indicates significant p values < 0.001, 0.01 and 0.05 as compared to control (one-way ANOVA followed by Tukey post hoc test). (MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, HCT=hematocrit).

DISCUSSION

Understanding the effects of pesticides in aquatic environments is crucial. Herbicides near fishponds can cause significant changes in the health and functions of exposed fish, potentially affecting their growth, reproduction, and overall population (Qureshi *et al.,* 2016). Within aquatic environments, the infiltration of herbicides, fungicides, and insecticides from diverse origins is a prevalent occurrence. This phenomenon renders fish species a notably pertinent subject for investigating the effects of these noxious agents. The rationale behind this pertinence lies in the dual significance that fish hold—both in terms of economic implications and ecological equilibrium. This significance has been extensively elucidated in prior investigations, reinforcing the pivotal role of fish in comprehending the intricate interplay between contaminants and aquatic ecosystems (Moustafa *et al.,* 2016). The most sensitive biological alterations that have been reported with exposure to aquatic pollutants in fish were changes observed in biochemical, hematological, and cellular levels (El-Sayed *et al.,* 2015).

In the present study, Nile Tilapia was exposed to the field rate of herbicide Metsulfuron-methyl (triazinylsulfonylurea) and the oxidative stress caused by herbicide was treated with Vitamin C and NAC. The present study addresses the critical issue of oxidative stress induced by exposure to environmental stressors, specifically focusing on the herbicide Metsulfuron methyl, and evaluate the potential protective effects of Vitamin C and N-Acetylcysteine in mitigating its impact on Nile tilapia (*Oreochromis niloticus*). Present findings shed light on the intricate interplay between antioxidant mechanisms and herbicide-induced oxidative stress, hematological and histological responses in aquatic organisms, offering insights into the broader context of fish health and aquatic ecosystem management.

As oxidative stress continues to emerge as a significant concern in aquatic environments, the investigation of strategies to counteract its adverse effects are becoming increasingly relevant. How exogenous antioxidants, namely Vitamin C and N-Acetylcysteine, can modulate the oxidative stress triggered by Metsulfuron methyl exposure was the focus of the current study. By examining the alterations in key oxidative stress markers—such as Superoxide Dismutase (SOD), peroxidase (POD), Reduced Glutathione and Thiobarbituric Acid Reactive Substances (TBARS)—in response to antioxidant treatments, uncovering potential avenues for mitigating the detrimental impacts of herbicides on aquatic organisms.

The current investigation involved the exposure of Nile Tilapia (*Oreochromis niloticus*) to a diluted concentration (1/100) of the herbicide Metsulfuron methyl, reflecting field conditions. The study focused on assessing oxidative stress markers and antioxidant enzyme activity within the gills and liver, encompassing essential parameters such as SOD, POD, CAT, GSH, ROS, and TBARS. Additionally, hematological parameters including RBC, WBC, and hemoglobin were evaluated. Histological analyses of the liver and gills were also conducted.

Throughout the 24-day exposure period to the herbicide in a simulated trial, the potential impacts were closely examined. To ascertain potential protective mechanisms, the effects of NAC (N-acetylcysteine) and dietary Vitamin C were explored. This comprehensive investigation demonstrated an interplay between oxidative stress, antioxidant responses, hematological factors, and tissue histology in Nile Tilapia under herbicidal stress conditions.

The results of the study demonstrated that exposure to Metsulfuron methyl led to significant alterations in the oxidative stress markers and antioxidant enzyme activities within the liver and gills of the fish. Notably, the levels of ROS and TBARS were significantly elevated in response to the herbicide exposure. This aligns with (Ghaffar *et al.,* 2021) in which they exposed freshwater fish Rohu (*Labeo rohita*) to herbicide glyphosate and observed that the values of ROS and the oxidative stress parameter TBARS in the gills of herbicide glyphosate-treated fish were notably high in specific treated groups at various exposure intervals, when compared to the control group. (Tiwari and Vanage, 2017) have also documented elevated levels of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) due to the presence of toxic substances in rats, demonstrating a resemblance to our own research outcomes. This emphasizes the impact of Metsulfuron methyl on inducing oxidative stress and reinforces the significance of the study's findings.

The level of SOD and CAT were reduced significantly in MSM treated group compared to control group. In a parallel investigation, the functions of hepatic enzymatic antioxidants including SOD, CAT, glutathione peroxidase, glutathione and glutathione reductase were explored. Among these, catalase (CAT) emerged as a pivotal enzyme, crucial for cellular defense mechanisms against oxidative stress (Khare *et al.,* 2019). Various investigators have observed alterations in catalase (CAT) activity within the liver of fish exposed to pesticides. Consequently, this

enzyme has been regarded as a valuable indicator of chemical-induced oxidative stress in tissue (Clasen *et al.,* 2018). The reduction of SOD in liver and gills also aligns with (Li *et al.,* 2022). In this particular study, the exposure of fish to Pyriproxyfen (PPF) resulted in a reduction of superoxide dismutase (SOD) activity within the gill and liver tissues. This decline suggests an adaptive reaction of the fish to the presence of pesticides.

The POD and GSH level were reduced significantly in the herbicide (MSM) treated group. Glutathione reductase (GSH) proves to be a viable biomarker for assessing the effects of pesticides on aquatic organisms (Khare *et al.,* 2019). Our results, which closely resemble these findings of (Li *et al.,* 2022), and provided valuable support for the use of glutathione reductase as a robust biomarker when evaluating the effects of pesticides on aquatic organisms. This concurrence between present study and earlier research strengthens the credibility of glutathione reductase as a reliable indicator of pesticide impact. Along GSH, The POD enzymes remarkably lowered in liver of fish received high doses of PPF.

In current study vitamin C and NAC ameliorated the oxidative stress caused by MSM in gills and liver of Nile tilapia, ROS, TBARS were reduced in vitamin C and NAC treated groups compared to MSM treated groups, and SOD, POD, CAT and GSH were also recovered in both liver in liver in gills. Overall dietary vitamin C at the rate of 500 mg/kg of feed showed better ameliorative effect as compared to NAC. Present results align with (Atamanalp *et al.,* 2021) in which NAC 1.0 mM recovered all the above parameters up to a good extent in rainbow trout compared to self-healing group. Similarly (Feng *et al.,* 2015) also showed that the application of high doses of NAC demonstrates a notable ability to counteract oxidative stress and genotoxicity induced by the pesticide across all the examined tissues in rats. This application functions effectively as a remedy or antidote against adverse effects.

In the present study, it was observed that dietary vitamin C yielded impressive results as a remedy, effectively restoring the balance of antioxidant enzymes. This aligns closely with a study by Paduraru *et al.,* (2021), where they exposed zebrafish to a mixture of contaminants and treated them with vitamin C. Their findings showcased how vitamin C successfully mitigated both behavioral and biochemical disruptions induced by the contaminants. This further underscores the potential of vitamin C as a potent antioxidant intervention.

Hematological parameters serve as crucial and promising biomarkers in toxicological investigations for assessing the health condition of fish following their exposure to agrochemicals such as pesticides (for example, atrazine, penoxsulam, glyphosate, oxyfluorfen, and pendimethalin) as well as external environmental stressors like radiation (Hashemi *et al.,* 2017). In the present study, changes observed in all hematological parameters in MSM treated group, among these parameters, erythrocytes were reduced while leucocytes were increased significantly, while hemoglobin, MCV and MCHC were not significantly changed. These findings align with (Acar *et al.,* 2021), who found significant decreases in key hematological parameters, specifically RBC, Hb, and Hct, in fish subjected to exposure to GBH (Glyphosate-Based Herbicide).

These outcomes align with prior research, which has consistently reported adverse effects of herbicides and pesticides on the hematological profiles of fish.

Based on the findings, N-acetyl cysteine (NAC) and vitamin C known for their antioxidant properties, mitigated the harmful effects induced by pesticides across all examined tissues (gills and liver,). NAC functions by providing cysteine, which is believed to increase the levels of glutathione (GSH) in various tissues, thereby averting oxidative stress. However, when considering the effectiveness of two, the utilization dietary vitamin C showed more protection against toxicity and oxidative stress. NAC helped restore GSH better than vitamin C.

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