EFFECTS OF SILVER AND COPPER NANOPARTICLES IN FRESH WATER FISH *ONCORHYNCHUS MYKISS* (WALBAUM, 1792)

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Zoology

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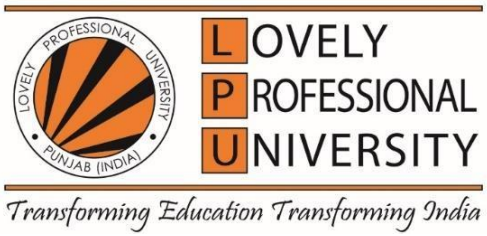

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2025

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**EFFECTS OF SILVER AND COPPER NANOPARTICLES IN FRESH WATER FISH *ONCORHYNCHUS MYKISS* (WALBAUM, 1792)**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Joydeep Dutta, working as Associate Professor, in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. And Co-Supervisor of Dr. Mohd Ashraf Rather, Assistant Professor, Division of Fish Genetics and Biotechnology, Faculty of Fisheries, Rangil, SKUAST-K. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**EFFECTS OF SILVER AND COPPER NANOPARTICLES IN FRESH WATER FISH *ONCORHYNCHUS MYKISS* (WALBAUM, 1792)**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Zoology, School of Bioengineering and Biosciences, is a research work carried out by Saba Khursheed, Registration No. 12211506, is Bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Nanotechnology is revolutionizing diverse fields, including biomedical, electronic, energy, and environmental industries, owing to the unique properties of engineered nanomaterials (ENMs). Among these, silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) are widely utilized due to their antimicrobial, catalytic, and electrical properties. However, the increasing release of these nanoparticles into aquatic environments has raised concerns about their ecological impact, especially their toxicity to non-target organisms such as fish. Aquatic ecosystems are particularly vulnerable to nanoparticle exposure, as these materials can persist in the water column, sediment, and biota, leading to potential bioaccumulation and toxicity. Despite extensive research on the individual effects of AgNPs and CuNPs, studies examining their combined toxicity remain scarce. This study examines the toxicological impacts of AgNPs, CuNPs, and their combined exposure on the freshwater fish *Oncorhynchus mykiss* (rainbow trout), with an emphasis on oxidative stress markers, lipid peroxidation, and histopathological alterations.

Rainbow trout were subjected to AgNPs exposure at concentrations of 0.2, 0.8, and 1.4 mg/L over a 21-day period to assess their impact. The biochemical evaluation revealed significant changes in the activity levels of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST). SOD and CAT activities increased in moderate exposure groups (T1 and T2), peaking on the 14th day, but declined in the highest exposure group (T3) by day 21, indicating oxidative stress. Lipid peroxidation levels showed no significant damage, while glutathione reductase (GR) activity increased with concentration. Lower concentrations of AgNPs induced an upregulation of these enzymes, reflecting an adaptive response to oxidative stress. However, prolonged exposure at higher concentrations led to a decline in enzyme activity, indicating oxidative damage and the exhaustion of antioxidant defences. Histopathological analysis showed dose-dependent gill damage. Control fish had normal gill architecture, while the T1 group exhibited mild hypertrophy and cellular infiltration. The T2 group showed increased blood vessel congestion and proliferation of goblet and chloride cells. Severe damage, including necrosis and lamellar fusion, was observed in the T3 group. Liver histology revealed mild congestion in the T1 group, hemorrhages, and hepatocyte vacuolation in the T2 group, while the T3 group exhibited severe necrosis, hepatic degeneration,

and Kupffer cell proliferation. These findings highlight the dose- dependent toxicity of AgNPs, with higher concentrations leading to significant oxidative-stress and alterations in the tissue.

The study on CuNPs toxicity examines the biochemical and histopathological impact on *Oncorhynchus mykiss* when treated to different concentrations (0.2, 0.6, and 1.0 mg/L) for up to 21 days. Biochemical analysis of gills and liver showed no significant change in superoxide dismutase activity across all treatments, indicating that SOD may not play a central role in the antioxidant defense against CuNPs exposure. However, catalase (CAT) activity improved significantly at higher concentration, particularly at 1.0mg/L on day 14, reflecting an adaptive response to oxidative stress, but declined on day 21 due to potential enzyme depletion. Lipid peroxidation (LPO) levels decreased across all sampling periods, suggesting the activation of compensatory antioxidant mechanisms. Glutathione-S-transferase and glutathione reductase activities were largely unaffected, with a transient increase in GR activity at 0.6 mg/L on day 14. Histopathological analysis revealed dose-dependent damage to both gills and liver. In the control group, gills showed normal architecture with well-developed primary and secondary lamellae. However, exposure to CuNPs led to several gill deformities, including lamellar fusion and hypertrophy, particularly in the T1 and T2 groups. In the T3 group, more severe changes, such as necrosis, hypertrophy, and cellular degeneration, were observed. Liver histology also showed varying degrees of damage, with the T1 group exhibiting mild liver deformities such as hepatocyte shrinkage and the presence of Kupffer cells. The T2 group showed blood aggregation and necrosis, while the T3 group exhibited severe necrosis, hepatocellular degeneration, and pyknotic nuclei. These findings highlight the toxicological impact of CuNPs on aquatic organisms, with higher concentrations causing significant oxidative stress and histopathological alterations.

To explore the combined toxicity of AgNPs and CuNPs, fish were exposed to three treatment groups: T1 (0.2+0.2 mg/L), T2 (0.8+0.6 mg/L), and T3 (1.4+1.0 mg/L) for 7, 14, and 21 days. Combined exposure produced more severe oxidative stress and histopathological damage compared to individual nanoparticle exposures. Antioxidant enzyme activity showed significant increases at intermediate exposure levels (T2) but declined at the highest concentration (T3) after prolonged exposure.

Lipid peroxidation levels rose sharply in both gills and liver, indicating heightened oxidative damage. Histological analysis confirmed extensive damage to critical organs, including gill lamellar fusion, hyperplasia, and liver vacuolation and necrosis. The most pronounced effects were observed in the T3 group, emphasizing the synergistic and dose-dependent nature of combined nanoparticle toxicity. This study provides one of the first comprehensive analyses of the combined toxicological effects of AgNPs and CuNPs on rainbow trout, an ecologically and economically significant model species. The combination of biochemical assays and histopathological analyses provides insights into the mechanisms driving oxidative stress and tissue damage caused by nanoparticles. The findings highlight that the combined exposure to AgNPs and CuNPs exacerbates toxicity compared to their individual effects, emphasizing the need for a deeper understanding of nanoparticle interactions in real-world aquatic environments.

The study's implications are significant for both scientific research and environmental policy. It underscores the necessity for stricter regulatory frameworks to monitor and control the release of nanoparticles into aquatic ecosystems. Furthermore, the insights gained can guide the development of safer nanomaterials with reduced ecological impacts and inform wastewater treatment practices to minimize nanoparticle contamination. Ultimately, this research contributes to safeguarding aquatic biodiversity and promoting the sustainable use of nanotechnology.

ACKNOWLEDGEMENTS

First and foremost, I extend my heartfelt gratitude to Almighty Allah for blessing me with the strength, resilience, and courage to undertake this research project. His divine guidance enabled me to overcome the challenges I encountered during this journey and brought this endeavor to fruition.

I am deeply indebted to my esteemed supervisor, ***Prof. Joydeep Dutta***, Head of the Department of Zoology, whose visionary outlook and unwavering support served as the cornerstone of this project. Dr. Dutta's ability to inspire calm amidst challenges and his exemplary work ethic have been a continuous source of motivation throughout my academic pursuit. His encouragement and insight have guided me through the highs and lows of this journey, and for that, I am profoundly grateful.

I extend my sincere thanks to my co-supervisor, ***Dr. Mohd Ashraf Rather***, Assistant Professor at SKUAST-K, for his invaluable guidance, expertise, and steadfast faith in my abilities as a scholar. His encouragement during moments of doubt, especially when experiments were going, was instrumental in helping me persist. His unwavering support has been a beacon of hope throughout my research work.

I am deeply grateful to ***Dr. Neeta Raj Sharma***, Dean and Head of the School of Bioengineering and Biosciences, for her kind support and encouragement. I also express my heartfelt appreciation to my panel members ***Dr. Najitha Banu***, ***Dr. Lovleen***, and ***Dr. Yachana Mishra, Dr. M.A. Kham (HOL)*** for their valuable guidance, consistent support, and thoughtful suggestions. Additionally, I sincerely acknowledge ***Professor. Irfan Ahmad Khan, Professor Gohar Bilal Wani***, ***Mr. Tariq Ahmad***, and ***Irshad Ahmad*** for their cooperation and for facilitating my experimental work at SKUAST-K.

Above all, I extend my deepest gratitude to my family for their unwavering love, support, and encouragement throughout my Ph.D. journey. I owe everything to my parents, **Mr. Khursheed Ahmad Khan** and **Mrs. Shakeela Khan**, whose wisdom, sacrifices, and steadfast belief in my potential have been the foundation of all my accomplishments My mother's endless love and heartfelt prayers have been greatest

source of comfort and strength, while my father's guidance and encouragement have continually inspired me to persevere and excel. I also wish to express my heartfelt appreciation to my brother, **Majid Khan**, and my sisters, **Ms. Shazia** and **Dr. Masarat**, whose constant support, and affection have been a pillar of strength throughout this endeavor. My loving niece and nephews have filled my life with joy. With deep reverence, I remember my late grandmother, whose prayers and blessings continue to illuminate my path. Her memory remains a lasting source of inspiration and faith in my life. To my entire family thank you for being my anchor, my strength, and my reason to strive for excellence.

Special thanks to ***Dr. Ishtiyaq Ahmad*** for his constant guidance, patience, and invaluable support throughout my Ph.D. His dedication and assistance during experiments and sampling processes, till the end for sampling until O-dark hundred hours on many occasions have been invaluable.

I also extend my gratitude to my friend Dr. Junaid Nazir and Suhail Ashraf for their insightful advice and for spotting my tendency to write 'however' more often than necessary. I would also like to acknowledge Dr. Irfan Ashraf Badroo for his camaraderie and moral support, which made this journey more enjoyable.

I am immensely thankful to my dear friends, Dr. Sumaira, Dr. Atif, Amita, Asma, Snoubar Mir, Dr. Irtizah, Dr. Hudisa, Dr. Sajad, Basharat, Yasir, Dr. Suhail, Dr. Kaiser, Dr. Muzaffar, and my lab mates for their support, cooperation, and affection. Their companionship made my research work less arduous and more memorable. I also acknowledge the non-teaching staff of the university for their assistance and cheerful cooperation.

Saba Khursheed Khan

"And my success is not but through Allah. Upon Him I have relied, and to Him I return."
(Surah Hud, 11:88)

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LIST OF ABREVATIONS

AgNPs	Silver nanoparticles
CuNPs	Copper nanoparticles
ACHE	Acetylcholinesterase
CdS/CdTe QD	Cadmium sulfide/cadmium telluride core-shell quantum dots
MCV	Mean Corpuscular Volume
HCT	Hematocrit
WBC	White blood cells
RBC	Red blood cells
SOD	Superoxide dismutase
CAT	Catalase
LPO	Lipid peroxidation
ROS	Reactive oxygen species
ATP	Adenosine triphosphatase.
TBARS	Thiobarbituric acid reactive substances
GPX	Glutathione peroxidase
PUFAs	Polyunsaturated fatty acids
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

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Chapter 1

1. INTRODUCTION

The rapid advancements in nanotechnology have revolutionized various scientific and industrial sectors, leading to the widespread production and application of nanoparticles (NPs). Nanoparticles are materials with dimensions ranging from 1 to 100 nano meters, possessing special physico-chemical characteristics that distinguish them from their bulk counterparts (Joudeh & Linke, 2022; Imani et al., 2015). These properties, such as a high surface area-to-volume ratio and enhanced reactivity, have enabled their utilization in a diverse range of applications, including electronics, medicine, cosmetics, food packaging, textiles, and environmental remediation (Jeevanandam et al., 2018; Khan et al., 2022).

Nanotechnology has emerged as one of the most important fields of the 21st century, offering innovative solutions across healthcare, environmental management, food safety, and industrial production (Pushparaj et al., 2022; Kakakhel et al., 2021). Significant advancements have been made in the synthesis of nanoparticles with precise shapes, sizes, and functional coatings. These characteristics play a critical role in influencing their reactivity and stability, thereby enabling their application in targeted and efficient ways (Dhand et al., 2015; Jurasin et al., 2016). The enhanced surface area and chemical reactivity of nanoparticles have drawn considerable attention from engineers, chemists, and biologists (Ji et al., 2024) Among the various types of engineered nanoparticles (ENPs), metals, nonmetals, and composites are commonly utilized. Silver, silica, copper oxide, titanium dioxide, and zinc oxide are among the most frequently used materials due to their versatility and effectiveness in various applications (Panhwar et al., 2021). However, the increasing prevalence of ENPs in aquatic systems has raised concerns about their potential ecological and biological risks. Aquatic ecosystems are particularly vulnerable to nanoparticle pollution, serving as the ultimate sink for many anthropogenic contaminants (Bathi et al., 2022). The presence of ENPs in these environments poses substantial risks to aquatic organisms, especially fish, which are directly exposed to these pollutants through water, sediment, and food (Rex et al., 2023).

Among them most extensively utilized nanoparticles are silver nanoparticles and copper nanoparticles. AgNPs are particularly prominent due to their exceptional antimicrobial

activity, high catalytic potential, and stability, making them indispensable in products like medical devices, water treatment systems, and textiles (Zhang et al., 2016; Bruna et al., 2021). Similarly, CuNPs are highly valued for their superior catalytic, electrical, and antimicrobial properties, driving their application in coatings, electronics, textiles, and environmental management (Molahalli et al., 2024; Edis et al., 2019). As nanotechnology has grown exponentially over recent decades, the number of nano-embedded consumer products has exceeded 5,000, as documented in the (Nanodatabase, 2024). The global nanomaterial market, estimated at €20 billion in 2019, is projected to grow at an annual rate of 13.1% between 2020 and 2027 (Pandey and Jain, 2020). Despite these advancements, the widespread applications of nanoparticles have increased significant concerns regarding their potential environmental and ecological implications. The global production of AgNPs has surged, with an estimated annual output of approximately 500 tonnes, a figure expected to grow significantly by 2024 (Buric et al., 2015; Inshakova and Inshakov, 2017). Similarly, CuNPs production, though currently lower at 200 metric tons as of 2015, is anticipated to increase sharply due to escalating industrial demand (Rajput et al., 2020). These nanoparticles unavoidable discharge into aquatic ecosystems occurs through industrial discharges, agricultural runoff, wastewater treatment plant (WWTP) effluents, and atmospheric deposition (Turan et al., 2019; Mat Lazim et al., 2023). Marine environments face additional contamination from antifouling paints, which release 3 to 27 μg/cm²/day of CuNPs, equivalent to 0.2–1.8% of the applied amount over 180 days (Adeleye et al., 2016). Similarly, the agricultural application of nano pesticides and biosolids derived from WWTPs further exacerbates nanoparticle contamination (Gardea-Torresdey et al., 2014; Lazareva et al., 2014). These concerns underscore the need for comprehensive research to understand the environmental impact of nanoparticles and to develop strategies for mitigating their adverse effects (Scown et al., 2009; Khan et al., 2021; Khan et al., 2025).

Furthermore, reactive oxygen species produced by AgNPs and CuNPs are known to cause oxidative distress, damage to cells, including disturbance of essential physiological functions in aquatic organisms (Wang et al., 2015; Amani et al., 2022). Their small size and high reactivity allow them to penetrate biological membranes and accumulate in vital organs, such as the gills and liver, exacerbating their toxic effects (Griffitt et al., 2007; Ostaszewska et al., 2016). For example, CuNPs have been shown to cause histopathological changes in the gills, including necrosis and altered sinusoidal gaps, as

well as structural and functional damage in liver (Griffitt et al., 2007; Al-Bairuty et al., 2013; Khan et al., 2025).

As the demand for nanoparticles continues to grow, understanding their environmental impact becomes increasingly important. The interaction of nanoparticles with biological systems often leads to complex toxicological effects, including genotoxicity, immune suppression, and bioaccumulation. The potential risks associated with AgNPs and CuNPs necessitate comprehensive studies on their ecotoxicological effects, particularly in model organisms such as fish. Fish, including species like rainbow trout (*Oncorhynchus mykiss)*, are critical components of aquatic ecosystems and serve as valuable bioindicators for assessing environmental contamination (Authman et al., 2015; Abdallah et al., 2024). The potential for nanoparticles to disrupt cellular homeostasis, impair detoxification processes, and induce oxidative stress highlights the need for stringent regulatory guidelines on their use and disposal. In order to fill important information gaps and support the sustainable advancement of nanotechnology, this study will investigate the environmental destiny and toxicological impacts of AgNPs and CuNPs.

1.1 Overview of nanoparticles and their applications

Nanotechnology has emerged as a transformative field, enabling the creation of materials with unique properties at the nanoscale. Metallic nanoparticles, including AgNPs and CuNPs, have gained prominence because of their distinctive physicochemical and biological characteristics. These nanoparticles exhibit high reactivity, antimicrobial activity, and catalytic efficiency, making them indispensable in various industrial, medical, and environmental applications. However, their increasing production and widespread use have raised concerns about their environmental and ecological impacts, particularly in aquatic ecosystems.

1.1.1 Silver Nanoparticles (AgNPs)

Silver, a metallic element known for its brilliant white color, high luster, and excellent conductivity, has been extensively utilized in various nanoscale applications. The distinctive characteristics of AgNPs arise from their face entered cubic (FCC) crystalline structure, which contributes to their stability and enhanced reactivity. There have been notable developments in the manufacturing of metallic nanoparticles, especially carbon and

silver nanoparticles. According to (Yusuf et al., 2023), the global market for these nanoparticles was expected to be worth trillions of dollars by 2018.

Among the diverse range of engineered nanomaterials, AgNPs stand out as one of the most extensively utilized, accounting for nearly 34% of global nanoparticle production. With over 445 silver-based products currently in commercial use, their widespread application underscores the urgent need to understand their environmental behavior and potential ecological risks (Mohsenpour et al., 2020). Their antimicrobial properties have led to their inclusion in a variety of products, such as washing machines, paints, toothpaste, water purification systems, and textiles (Wijnhoven et al., 2009; Tanasa et al., 2023). The demand for AgNPs is projected to increase by 63% by 2024 (Inshakova et al., 2017). Their antibacterial efficacy against a wide spectrum of bacteria, fungi, and viruses has been extensively documented in various studies (Mahmoudabadi et al., 2021; Vali et al., 2020; Rashidian et al., 2021; Vijayakumar et al., 2019). Consequently, AgNPs are widely applied in medical, industrial, and environmental sectors. However, the release of silver nanoparticles during processes such as washing or product disposal raises environmental concerns due to potential contamination of aquatic ecosystems (Waalewijn et al., 2014; Kang et al., 2023).

The adverse effects of AgNPs in aquatic environments are well documented. These nanoparticles can penetrate cells through endocytosis, disrupt mitochondrial function, and generate reactive oxygen species (ROS), leading to protein degradation and tissue damage (Li et al., 2013; Ramzan et al., 2022). Even at minimal concentrations, AgNPs are toxic to aquatic life, including freshwater and marine organisms, primarily due to silver ions inducing oxidative stress (Waalewijn et al., 2014). Research has highlighted various toxic effects of AgNPs, such as embryotoxicity, genotoxicity, and mitochondrial impairment (Huang et al., 2022). The toxicity of AgNPs is affected by many characteristics like size of particle, structure, surface oxidation, and physicochemical properties of the surrounding medium (Walters et al., 2016). Model organisms frequently used in nanoparticle toxicity studies include medaka (*Oryzias latipes*) (Wise et al., 2010; Amin et al., 2021), Nile tilapia (*Oreochromis niloticus*) (Kakavand et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Johari et al., 2014), and common carp (*Cyprinus carpio*) (Vali et al., 2020).

1.1.2 Copper Nanoparticles (CuNPs)

Copper nanoparticles (CuNPs) are recognized for their affordability, optical properties, and

broad-spectrum antimicrobial activity. These nanoparticles possess distinctive chemical and physical attributes, such as superior thermal and electrical conductivity, corrosion resistance, and the ability to form alloys. These features make CuNPs essential in industries involving coatings, textiles, and plastics (Naika et al., 2015; Rohit Guin et al., 2015). Additionally, CuNPs are extensively utilized in healthcare applications, including antimicrobial, dermatological creams, hospital surface coatings, and medical textiles (Crisan et al., 2021). Their large surface area and remarkable electrochemical properties have facilitated their use in areas like sensors, catalysts, photovoltaic systems, and nanofluids for heat transfer (Molahalli et al., 2024). Despite these advantages, CuNPs exhibit significant toxicity compared to many other nanoparticles, primarily due to the release of copper ions, which leads to DNA destruction and cytotoxicity (Karlsson et al., 2008; Boyles et al., 2015; Khan et al., 2025). This toxicity is largely driven by the generation of reactive oxygen species, which cause oxidative stress and cellular harm (Zou et al., 2021). Oxidative stress occurs when there is an imbalance between the cell's antioxidant defenses and prooxidant activity, resulting in the excessive production of reactive oxygen species (ROS). This imbalance destabilizes cellular functions by inducing the oxidation of vital biomolecules, including proteins, lipids, carbohydrates, and nucleic acids. Consequently, critical biological processes such as metabolism, respiration, and detoxification become compromised (Topal et al., 2017; Lushchak, 2016). For CuNPs, the production of ROS significantly amplifies their toxicity by exceeding the cell’s natural antioxidant defenses. Moreover, CuNPs gets accumulated in critical tissue organs, such as the liver and gills, thereby intensifying their harmful effects (Ostaszewska et al., 2016; Khan et al., 2025).

As industrial applications of CuNPs continue to grow, their persistence in aquatic environments necessitates a thorough assessment of their short-term and long-term impacts on fish health. Their bioaccumulation within the aquatic food chain can lead to significant ecological and public health concerns, especially at higher concentrations where their toxic effects become more pronounced (Malhotra et al., 2020). Fish, being key indicators of aquatic ecosystem health, are particularly susceptible to CuNPs exposure due to their constant interaction with contaminated environments. These nanoparticles can enter the body through inhalation or ingestion, leading to bioaccumulation in organs like the liver and gills, cause oxidative damage, and tissue impairment (Ameh and Sayes, 2019).

1.2. Comparative toxicological effects of AgNPs and CuNPs

Both silver (AgNPs) and copper nanoparticles (CuNPs) have been reported to generate reactive oxygen species (ROS), leading to oxidative stress that compromises the integrity of key biomolecules, including DNA, lipids, and proteins (Rajkumar et al., 2016; Zou et al., 2021). This oxidative imbalance disrupts cellular homeostasis, affecting vital physiological processes, including respiration, metabolism, and detoxification (Topal et al., 2017; Lushchak, 2016). Fish, as sentinel species, show significant histological and physiological changes when exposed to nanoparticles. The gills, which play a crucial role in respiration and osmoregulation, represent one of the most sensitive targets of nanoparticle exposure. Such exposure can lead to pronounced structural alterations and functional impairments, compromising the overall physiological performance of aquatic organisms. Similarly, the liver, a key organ for metabolism and detoxification, undergoes structural and functional changes under nanoparticle exposure (Griffitt et al., 2007; Al-Bairuty et al., 2013; Scown et al., 2010).

Rainbow trout (*Oncorhynchus mykiss*), a species of significant ecological and commercial importance in cold-water ecosystems, is widely employed as a model organism for assessing nanoparticle toxicity owning to its high sensitivity and environmental relevance (Authman et al., 2015; Nabi et al., 2022). While substantial research has focused on the individual effects of AgNPs and CuNPs, little is known about their combined impacts, which are critical for understanding real-world pollution scenarios where multiple nanoparticles often coexist (Griffitt et al., 2009; Scown et al., 2009). Addressing this gap in knowledge is vital for advancing aquatic nanotoxicology and supporting regulatory initiatives aimed at reducing nanoparticle pollution while fostering the safe and sustainable application of AgNPs and CuNPs in industrial and environmental contexts.

1.3. Model organisms in toxicological research

Non-human species, known as model organisms, are extensively studied in laboratory environments for their distinct advantages in exploring biological mechanisms (Ankeny and Leonelli, 2021). In recent decades, fish have become integral to ecotoxicological research due to their sensitivity to various contaminants in aquatic systems (Naigaga et al., 2011). Widely used species include zebrafish, rainbow trout, medaka, cod, Atlantic salmon, and killifish. Zebrafish, used since the 1950s, have been pivotal in toxicology,

genetics, and developmental studies. Similarly, species like medaka, rainbow trout, and fathead minnow have been employed, with the latter chosen by the Environmental Protection Agency for standardized toxicity tests (Ballatori and Villalobos, 2002; Brain et al., 2018). Despite their acceptance, the necessity of fish models compared to rodent models is occasionally questioned. However, fish serve unique roles in ecotoxicology due to their ecological and practical significance (Yancheva et al., 2015). As a key link in the human food chain, any disruption in fish populations impacts broader ecosystems and human health. Additionally, fish models are favored for their high reproductive rates, ease of care, and well-defined physiological systems, including immune and endocrine systems (Song et al., 2012). Their natural exposure to chemical mixtures further validates their reliability in studying multiple contaminants under controlled conditions. Among various fish species, rainbow trout (*Oncorhynchus mykiss*) has gained prominence for toxicological assessments, often replacing other freshwater species. This study uses rainbow trout to examine the toxic effects of nanoparticles, highlighting their importance in understanding aquatic pollution and its broader implications.

1.3.1. Taxonomic and morphological features of rainbow trout

Kingdom: Animalia
Phylum: Chordata
Superclass: Gnathostomata
Class: Actinopterygii
Order: Salmoniformes
Family: Salmonidae
Genus: *Oncorhynchus*
Species: *mykiss*

Figure 1. Morphological features of rainbow trout

1.3.2. Characteristics and conservation of rainbow trout *(Oncorhynchus mykiss)*

The rainbow trout (*Oncorhynchus mykiss*) is a versatile fish species capable of inhabiting a wide range of aquatic environments, including oceans, lakes, and streams. It thrives in cold waters with temperatures below 21°C, making such conditions ideal for its cultivation. Rainbow trout typically attain sexual maturity at 3 to 4 years of age, with their growth and development shaped by environmental factors such as climate and food availability. They are distinguished by an elongated, fusiform body, a bluish-green to olive-green back, a silvery-white underside, and a distinct pink stripe along their lateral line. Black spots are scattered across its body, including its fins, and the adipose fin is outlined in black. Variations in coloration and morphology are influenced by species, subspecies, and environmental factors (Hardy, 2002).

Despite its adaptability, rainbow trout populations have faced significant declines in their native habitats. Factors such as overfishing, habitat destruction, pollution, climate change, disease, and hybridization with introduced subspecies have contributed to these declines. Many trout species are now listed as endangered, vulnerable, or threatened. To combat these challenges, various governmental and non-governmental organizations are implementing conservation measures. These include habitat restoration, public education campaigns, and targeted strategies to support the long-term survival of trout populations.

1.3.3. Toxicological effects of nanoparticles on Rainbow trout

The increasing presence of nanoparticles in aquatic environments represents a significant threat to freshwater species, especially fish. The accumulation of silver and copper nanoparticles in fish tissues can interfere with cellular and biochemical functions, resulting in toxic effects. Understanding the individual and combined impacts of these nanoparticles is essential for assessing their ecological risks. This research examines the toxicological effects of chemically synthesized silver and copper nanoparticles on rainbow trout, focusing on biochemical and histological alterations. By exploring these effects, the study aims to enhance understanding of the ecological risks associated with nanoparticle exposure and support efforts to mitigate their negative impacts on aquatic ecosystems.

Chapter 2

2. REVIEW OF LITERATURE

Nanoparticles are unintentionally introduced into the environment as a result of activities in the nanotechnology sector. Products developed using nanotechnology play a significant role in numerous industries, including nano electronics, bacteria-resistant medical and personal items, odor control systems, fuel additives, and materials such as ceramics and paints. Among these, inorganic nanoparticles like silver and copper nanoparticles are frequently detected in municipal wastewater and in densely populated or industrialized regions (Khan et al., 2021; Gagnon et al., 2021). These nanoparticles reach aquatic ecosystems through various sources, including municipal wastewater (both treated and untreated), road dust caused by tire erosion (Guhananthan et al., 2023), and atmospheric deposition from vehicle exhaust. As a result, aquatic organisms are exposed to mixtures of nanoparticles along with other dissolved pollutants. The climate change further exacerbates this issue by increasing the intensity and frequency of precipitation events, which often lead to sewer overflows. During such events, untreated wastewater mixes with rain water and is discharged into water bodies due to the limited capacity of sewage systems. For example, silver and copper oxide nanoparticles (nAg and nCuO), commonly embedded in fabrics to prevent microbial odors, are often found in municipal effluents (Kour et al., 2022). In addition to their use in textiles, the antimicrobial properties of nAg and nCuO have made them valuable in cosmetics, clothing, and, more recently, surgical masks designed to prevent the transmission of airborne diseases like COVID-19 (Pollard et al., 2021). The improper disposal of these masks, often discarded on streets, allows them to enter sewage systems or accumulate in landfills, leads accidental discharge of CuO and polymer based nanoparticles into aquatic ecosystem.

Because of these issues, it is critical to comprehend the toxic effects of metals and associated NPs before they cause harm to aquatic environment and human health. Human societies life standards have been drastically altered since the arrival of contemporary technologies. New feature of these technologies is the constant emergence of new nanoproducts. According to a recent estimate, 1,358 companies are actively involved in the fabrication of 6928 nanoproducts (Inshakova and Inshhakov, 2017). Global manufacture of nanomaterials is estimated to exceed $ 75.8 billion by 2020. (RNCOS,

2015). Nevertheless, the widespread use of nanotechnology has prompted questions about its safety and environmental impact, owing mostly to the use of zinc, silver, carbon, silica, copper, gold, and various other consuming nanoproducts (Glisovic et al., 2017). Furthermore, the designed NPs 1have at least one dimension shorter than 100 nm, resulting in exceptionally high surface areas and increased percentages of their surface component atoms. Many nanoparticles exhibit remarkable reactivity as a result, leading to their employment in consumer products, environmental and biology. The distinctive parameters that add value to NPs for industrial uses have raised concerns that nanomaterials may have exclusive biological properties, resulting in possible toxicity in the case of inadvertent usage into the environment (Moore, 2006; Lovern and Klaper, 2006). Engineered nanoparticles are deposit in aquatic ecosystem after being released into the environment, posing a risk to marine life (Moore, 2006).

2.1. Sources and pathways of nanoparticles into aquatic environment

The environment has always contained nanoparticles (NPs) (Bundschuh et al., 2018). Both natural (atmospheric, geogenic, biogenic, and pyrogenic) and man-made (engineered or by product) sources can produce nanoparticles. Nanoparticles can occur naturally in the atmosphere generated by volcanic eruptions, desert surfaces, and from dust and cosmic sources (Rahman, 2021). Carbon black or soot is the best example of fossil fuels and vegetation, which is the partial combustion product (Long et al., 2013). Volcanic eruption also produces nanoparticles into the environment by Aiken-mode nucleation process (Senapati and Kumar, 2018). Minerals and ores of metals, forest fires, pollen fragments, and meteorites are the other few examples of the natural source of nanoparticles. Nanoparticles can occur naturally or be engineered. Naturally occurring nanoparticles include metals such as silver, gold, and iron oxides, along with humic and fulvic acids, organic acids, carbon nanotubes, and nanospheres. Engineered nanoparticles, in contrast, comprise materials such as platinum, metal phosphates, functionalized fullerenes, carbon black, polyethylene glycol, zeolites, ceramics, and others (Abdullaeva, 2017; Guerranti and Renzi, 2015). Nanoscale materials are present in organisms like magneto tactic bacteria, molluscs, arthropods, fish, birds and even in human brain (Banaclocha et al., 2011). Besides the natural sources, humans have created nanoparticles as by-products of simple combustion, chemical manufacturing, oil refining and smelting, combustions of

treated sewage sludge, coal, airplane engines and during welding process (Carter et al., 2013). These are collectively referred as nanoparticles of anthropogenic origin.

Nanoparticles enter the environment through both intentional and unintentional releases. Intentional release primarily occurs during their application in engineered processes such as drug development, groundwater remediation, biomedical imaging, and various other technological innovations (Turan et al., 2019). On the other hand, inadvertent releases result from mining, burning fossil fuels, automobile emissions, and building demolition. Once introduced into the environment, nanoparticles begin accumulating in aquatic systems (Iavicoli et al., 2017). According to (Abbas et al., 2020) nanoparticles can enter water bodies directly or indirectly through nonpoint sources such water infiltration and air deposition. Nanoparticles present in wastewater or sewage effluents pose environmental risks when they mix with aquatic ecosystems. On terrestrial surfaces, nanoparticles contaminate air, soil, and solid waste, and can indirectly enter water systems through runoff from rain or wind-driven dispersion (Singh et al., 2023). Industrial waste and materials discarded in landfills further contribute, as they often get washed into nearby water bodies, creating another pathway for nanoparticles to enter aquatic environments (Moore et al., 2006; Zahra et al., 2022).

The introduction of nanoproducts and their byproducts into aquatic habitats has become practically inevitable due to the growth of nano-based businesses and the large-scale manufacture of nanomaterials (Rogers and Pidgeon, 2007; Bellanthudawa et al., 2023). About 60% of wastewater systems are utilised in industrial, pharmaceutical, and medical applications, making them a major source of nanomaterial discharge (Kurwadkar et al., 2015). The main sources of metallic nanoparticles, especially copper and silver, are industrial processes, consumer goods, and waste products from manufacturing and cleaning operations in industries like electronics, textiles, and photography (Turan et al., 2019; Azimzada et al., 2021). The main sources of metallic nanoparticles, especially copper and silver, are industrial processes, consumer goods, and waste products from manufacturing and cleaning operations in industries like electronics, textiles, and photography (Gottschalk et al., 2009; Palani et al., 2023). Both methods show the widespread influence of nanoparticles on natural ecosystems by facilitating their direct or indirect release into the environment.

2.2. Fate and behaviour in aquatic environments

Above and beyond the applications of nanoparticles, there are many hazards for the environment right from the production site to the disposal of particles. Thus, along with promoting the use and benefits of nanoparticles in various fields, proper measure should be taken to ensure that no harmful effects result from the use of nanoparticles. Nanoparticles can enter the environment through three primary pathways, during the manufacturing of raw materials and nano-enabled products, during their application or use, and through the release of untreated wastewater effluents (Tolaymat et al., 2017). Direct or indirect emissions from the wastewater treatment plants discharge nanoparticles into the environmental compartments, mainly on soil, landfills, sediments, air and groundwater. Some of the nanoparticles undergo aggregation leading to the increased concentration in groundwater and soil. Global estimation of nanoparticles discharge has reported as 63-91% in the landfills followed by 8-25% in soil and 7% and 1.5% in aquatic environment and air, respectively (Hennebert et al., 2017). The production of metal oxide nanoparticles rose dramatically from 0.27 million tonne in 2012 to 1.66 million tonne in 2020, according to the Global Market for Metal Oxide Nanoparticles (Rajput et al., 2020).

Risk assessment of nanoparticles in the environment mainly based upon the reactivity, mobility, ecotoxicity and persistent nature of the particles. Detection of environmental concentrations of nanoparticles in the natural ecosystem can be measured by several analytical methods and computational modelling. By using fractionation techniques in conjunction with light scattering and elemental detection, or single particle inductively coupled plasma mass spectrometry, metal-based nanoparticle concentration and size can be ascertained (Philippe and Schaumann, 2014). Nanoparticles evade most of natural barriers and may turn even reserved rare elements into ubiquitous ones. Prolonged exposure of organisms to nanoparticles may potentially lead to unforeseen health and environmental hazards. The organisms like algae, fungi, plants, prokaryotes, planktons etc., which are included in the primary consumer groups that interact immediately with the environment are the primary targets of nanoparticles exposure. Bioaccumulation of nanoparticles inside the body of the primary consumers enables the trophic transfer of nanoparticles through the food chain and finally results in biomagnifications in higher organisms, including human (Cedervall et al., 2012)

2.3.Toxicological effects of nanoparticles in aquaculture

The use of nanoparticles in aquaculture has raised concerns regarding their potential toxicological effects on aquatic organism's results in the emergence of a novel form of waste known as nano wastes, posing significant environmental hazards. Thus, it is critical to look into how NPs build up and have negative impacts on various trophic phases of food chain (Asztemborska et al., 2014). Nanoparticles can enter aquatic environments through various routes, such as waste water from nanomaterial production facilities or the use of nanoparticles containing products in aquaculture. When released into water, nanoparticles can interact with aquatic organisms such as fish, crustaceans, and molluscs, potentially causing harmful effects on their health and disrupting the aquatic ecosystem. A major concern about the toxic effects of nanoparticles in aquaculture is their tendency to bioaccumulate within aquatic species. Nanoparticles can be taken up by fish through gills, ingestion, or skin absorption, and once inside the organ ism, they can accumulate in various tissues and organs (Scown et al., 2010). This bioaccumulation can lead to long-term exposure of aquatic organisms to nanoparticles, increasing the risk of adverse effects.

Various studies have explored the toxic effects of nanoparticles on farmed fish (Table 1). For instance, silver nanoparticles, widely valued for their antimicrobial properties, have been found to trigger oxidative stress, DNA damage, and alterations in gene expression in fish (Griffitt et al., 2008; Ahamed et al., 2010). Similarly, titanium dioxide nanoparticles have been observed to cause oxidative stress, inflammation, and behavioral changes in fish. According to (Scown et al., 2010), AgNPs smaller than 10 nm caused significant harm to the kidneys and gills of rainbow trout compared to larger AgNPs exceeding 35 nm in size. (Johari et al., 2013) found that the toxicity of AgNPs decreased as rainbow trout progressed from the eleuthero stage of embryo development to the larval and juvenile stages, resulting in lower mortality rates. The study also revealed that during the initial ninety-six hours of exposure, even at low concentrations such as 0.25, 0.71, and 2.16 mg/L, AgNPs were lethal to eleuthero eggs, larvae, and juveniles, respectively. Additionally, a three-hour exposure of juveniles to AgNPs led to a decrease in chloride and potassium concentrations and an increase in adrenaline and cholinesterase concentrations. The freshwater fish *Mystus gulio's* liver, muscles, and gills lost protein and carbohydrates after being exposed to AgNPs for 15 days, on the other hand, these tissues had higher lipid content (Abirami & Sudharameshwari, 2017) suggest that the decline in energy content could be attributed to

increased glucose utilization under stress, the reduction in protein to heightened protein utilization under stressful circumstances, and the rise in lipids to inappropriate lipid utilization by the tissues.

In fish species like *Mystus gulio,* silver nanoparticles (AgNPs) have been linked to detrimental effects on the main gills lamellae and resulting in problems including tissue necrosis, hypertrophy, and fusing of the main membranes (Abirami & Sudharameshwari, 2017). AgNPs have also been found to decrease the pace at which aquatic organisms feed (Raissy & Ansari, 2011). Furthermore, it was reported that silver nanoparticles (AgNPs) can influence the haematological, serum metabolite, and enzymatic levels in (*Oncorhynchus mykiss*). Additionally, exposure to AgNPs triggers a widespread oxidative stress response in these fish (Mirghaed et al., 2018). Moreover, they may impede the development of micro crustaceans and phytoplankton, which are vital parts of the aquatic food chain and aid in their purification of tainted aquaculture systems (Pham, 2019). Furthermore, AgNPs have the capacity to increase oxidative metabolism in aquatic species tissues, which causes cypla-2 expression in the gills (Scown et al., 2010). (Hedayati et al., 2022) demonstrated that exposure to iron oxide nanoparticles (IoNPs) resulted in increased levels of cortisol, glutathione S-transferase, and malondialdehyde, along with significant liver damage in fish, characterized by hyperemia, hepatocyte vacuolation, and necrosis. However, incorporating *Lactobacillus casei* into the diet offered protective effects, mitigating the adverse impacts of IoNPs on the common carps physiological functions.

Moreover, it has been reported that Mozambique tilapias (*Oreochromis mossambicus*) may experience abnormalities in their liver, brain, and gill functioning when exposed to copper nanoparticles (NPs), a major water pollutant (Riber et al., 2024). Exposure to nanoparticles has a negative impact on organisms' growth and developing pace, which eventually raises mortality (Al Ghais et al., 2019). For example, zinc oxide nanoparticles (ZnO NPs) have been shown to disrupt *Oreochromis niloticus* antioxidant defence system, leading to toxicity and physiological stress; these negative effects can be substantially mitigated by vitamin E supplementation (Farsani et al., 2017). Additionally, research has been done on the toxicity of nickel nanoparticles and are harmful to a variety of aquatic species, including fish, planktonic crustaceans, and algae. (Sadeghi & Peery, 2018) studied that fish size was reduced and toxicity was enhanced when the impacts of silver and selenium nanoparticles on *Tenualosa illish* larvae, fry, juveniles, and fingerlings. Some reports are

on the detrimental effects of different iron-containing nanoparticles on Japanese medaka fish due to the growing usage of nanoparticle zerovalent iron-mediated oxidation processes for wastewater treatment. As per there results, Japanese medaka mortality rates were higher during their developmental stages than they were throughout their adult stages due to issues resulting from nanoparticle toxicity (Chen et al., 2013). Green-synthesized zinc nanoparticles have been reported to exhibit lower toxicity, even at higher concentrations, compared to chemically synthesized alternatives, making them a suitable option as nutrient supplements for aquatic species (Ghafarifarsani et al., 2024). Mahboub et al. (2022) evaluated the impact of *Allium hirtifolium* extract (AHE) on enhancing the antioxidant responses of common carp (*Cyprinus carpio*) exposed to zinc oxide nanoparticles (ZnO-NPs) through their diet. Results indicated that AHE significantly improved blood indices, stress biomarkers, and antioxidant parameters, while also reducing oxidative stress markers. These findings suggest that AHE supplementation offers notable protective benefits against the toxic effects of ZnO-NPs in common carp. Some scientists have gone deeper to investigate ways to reduce the toxicity of nano particles in response to their significant value in treating aquaculture effluents. Unfortunately, it has been found that even plant-mediated nanoparticles which are thought to be more ecologically benign than chemically synthesized ones have harmful impacts on aquatic ecosystem membranes (Abirami & Sudharameshwari, 2017).

Moreover, silver nanoparticles synthesized using a metronidazolium-based ionic liquid with citrate as a reducing agent show promise as an antibacterial component in wound dressings, offering enhanced efficacy against both aerobic and anaerobic bacteria (Farjadian et al., 2020). Nevertheless, numerous approaches have been identified to mitigate the toxicity associated with nanoparticles. One such finding reveals that elevating the salinity of the ambient surroundings diminishes the toxicity of silver nanoparticles by promoting the formation of carbonate pellets within fish intestines. This process contributes to the decontamination of pollutants (Kalbassi et al., 2011). Moreover, direct effects on fish health, nanoparticles can also impact the aquatic ecosystem by affecting other organisms. According to (Navarro et al., 2015), phytoplankton, an essential component of the aquatic food chain, may be damaged by copper nanoparticles, which are frequently employed in aquaculture as an antifungal agent. Understanding the fate and behaviour of nanoparticles in aquatic environments is crucial for reducing their potential toxic effects in aquaculture. Their toxicity can be influenced by various factors, including

size, shape, surface coating, and concentration (Jovanovic & Palic, 2012). Additionally, the development of environmentally friendly nanomaterials and sustainable aquaculture practices can help reduce the risk of nanoparticle contamination in aquatic environments.

Table 1. Impact of nanoparticles on different fish species.

Types of nanoparticles	Fish species	Concentration	Effect	References
AgNPs	*Rhamdia quelen*	0.03, 0.3, and 3 mg g^{-1}	Injuries in the kidneys and liver, as well as steatosis and vascular congestion was observed.	(Lopez-Barrera et al., 2012)
	Cyprinus carpio	0.03, 0.06, 0.09 mg/L	Most of the sections under study experienced alteration and damage at 0.09 mg/L.	(Kakakhel et al., 2021)
	Clarias garepinus	10 and 100 mg/L (10nm and 100 nm)	Significant damage was observed in the liver that improves after the recovery time, particularly for 10 nm at 100 mg/L.	(Naguib et al., 2020)
	Oreochromis niloticus and Tilapia zillii	2 and 4 mg L^{-1}	Elevated concentrations of silver nanoparticles, such as 4 mg/L, negatively affect the antioxidant system in the brain.	(Afifi et al., 2016)
	Oncorhynchus mykiss	0.1,0.2,0.4mg/L	It was reported that the development slowed, Major Histocompatibility Complex (MHC) and Mean Corpuscular Volume (MCV) values dropped, hepatic enzyme levels rose, and plasma indices changed.	(Khursheed et al., 2023)
	Common Molly (*Poecilia sphenops*)	0, 5, 15, 25, 35, 45, 60mg/L	The hematocrit, white blood cells, red blood cells values drastically dropped, and the reproductive physiology changed.	(Khursheed et al., 2023)
ZnO NPs	*Cyprinus carpio* L	--	ZnO NPs may have an impact on liver and renal function.	(Chupani et al., 2016)
	Oreochromis mossambicus	20 ppb	Modification in histopathology, morphology and haematological parameters.	(Nascimento et al., 2018)
	Carassius auratus	0.5, 1, and 1.5 mg L^{-1}	Significant histological changes in the organs of the liver, kidneys, and gills as well as oxidative stress.	(Ghafarifarsani et al., 2023)

	Oreochromis niloticus	1mg/L and 2 mg/L	Antioxidant enzyme, mRNA expression was dramatically reduced.	(Abdelazim et al., 2018)
	Cyprinus carpio	0.382, 0.573 and 1.146 mg L	changes to the gill structure, as well as liver and muscle cell dysfunction.	(Rajkumar et al., 2022)
Cu NPs	*Danio rerio*	0,25,0.5,1,2,4,8 mg/L	According to the particulate size of the copper suspensions under examination, various anomalies were seen in the structure and behaviour of the zebrafish embryos.	(Hua et al., 2016)
	Rutilusrutilus caspicus	0.1 to 0.5 mgL	Hepatocellular and renal histological alterations observed. The study's findings demonstrated that copper nanoparticles could kill fish and harm the tissues of the Caspian roach, *Rutillus rutillus caspicus*.	(Aghamirkarimi et al., 2017)
	Oncorhynchus mykiss	--	Damaged gill paving cells and filaments; the species of fish tested varied in their susceptibility to these effects.	(Song et al., 2015)
CuO-NPs	*Oreochromis niloticus*	10mg/L, 20mg/L, and 50 mg/L. (68.92 ± 3.49 nm)	Histological impairment of the hepatopancreatic tissues, distal kidneys, and gills.	(Abdel-Latif et al., 2021)
TiO_2 NPs	*(Oreochromis mossambicus)*	0, 0.5mg/L, 1.0mg/L, and 1.5 mg/L.	TiO_2-NPs induced DNA damage.	(Shahzad et al., 2022)
	Prochilodus lineatus	1mg/L to 50 mg L	Ti accumulates in the brain, liver, and muscles and suppressed the activity of Acetylcholinesterase (ACHE) in the muscles.	(Carmo et al., 2019)
Al2O3-NPs	*Tilapia zillii* and *Oreochromis niloticus*	120 to 180 mgL	The brain's system for antioxidants is negatively impacted by Ag-NPs at greater concentrations, such as 4 mg/L.	(Afifi et al., 2016)

As NPs	*Labeo rohita*	1mg/L, 10mg/L, and 20 mg/L	Injury to the urinary tract, liver, and lungs was reported.	(Raza et al., 2021)
CdTeQD/CdS	*Oncorhynchus mykiss*	Increasing concentrations system.	cadmium sulfide/cadmium telluride core-shell quantum dots (CdS/CdTe QD) toxicity depended on both concentration and size. Greater than smaller nanoparticles, bulky CdS/CdTe QD masses (25 nm < size < 100 nm) decreased the phagocytosis.	(Bruneau et al., 2013)
CdS-NPs	*Channa punctatus*	50 mg/L	Loss of mucous, pillar, and epithelium cells; the micro ridges and microbridges disintegrate, particularly after 30 days.	(Verma et al., 2020)
SiO2-NPs	*Oreochromis niloticus*	20, 40, and 100 mg/L	liver and kidney damage.	(Rahman et al., 2022)

2.4. Toxicological effect of nanoparticles in human health

The toxicological effects on aquatic life and potential human health risks raise significant concerns (Tortella et al., 2020). Nanoparticles can enter aquaculture systems through industrial waste, agricultural runoffs, and direct applications, leading to bioaccumulation in fish and other seafood, which humans ultimately consume (Saravanakumar et al., 2022). Studies show that exposure to NPs in aquatic species results in oxidative stress, cellular damage, inflammation, endocrine disruption, and reproductive impairment, with metals like silver and copper nanoparticles demonstrating pronounced toxicity. These toxic effects are exacerbated by the nanoparticles small size and ability to penetrate cellular barriers, increasing the risk of DNA damage and genotoxicity (Brohi et al., 2017; Malhotra et al., 2020; Tortella et al., 2020). In humans, dietary exposure to contaminated seafood can lead to cytotoxic, genotoxic, and potentially carcinogenic effects (Marques et al., 2011; Vignardi et al., 2015). Research has linked prolonged NP exposure to respiratory, neurological, and reproductive health risks, particularly through biomagnification in the food chain (Karimi et al., 2018). As a result, current regulatory frameworks in the U.S. and Europe are reevaluating the safety standards and risk assessment protocols for NPs in aquaculture to mitigate these impacts (Maldonado-Siman ´ et al., 2018). Despite existing guidelines, more comprehensive studies are essential to fully understand nanoparticles

long-term effects and to establish stricter regulations to protect both environmental and human

2.5. Mechanism of nanoparticles accumulation in aquatic organism

Nanoparticle toxicity in fish is a complex process influenced by their unique physicochemical characteristics, such as a high surface area-to-volume ratio, nanoscale size, and inherent reactivity. These properties enable nanoparticles (NPs) to traverse biological membranes, disrupt cellular equilibrium, and impair essential physiological functions. Key toxicological pathways include oxidative stress and histopathological damage, often resulting in organ dysfunction and cellular disturbances. The gills and liver, crucial organs for respiration, metabolism, and detoxification, are particularly vulnerable. Toxicity arises primarily due to excessive reactive oxygen species (ROS), which surpass the organism's antioxidant defenses under normal conditions (Bashri et al., 2018). NPs, similar to other harmful substances, can promote ROS production at elevated levels, thereby inducing oxidative stress in exposed organisms (Figure 4). Research highlights that even small quantities of CuO or ZnO NPs can generate substantial ROS within cells (Chang et al., 2012). Upon contact with organelles, especially mitochondria, NPs may disrupt the electron transport chain, leading to the formation of superoxide radicals (Zhang & Gutterman, 2007). Moreover, the large surface area of NPs facilitates interactions with biomolecules, granting CuO and ZnO NPs a high electronic density (Pisanic et al., 2009).

Research on metallic and metal oxide nanoparticles highlights the critical role of oxygen in reactive oxygen species (ROS) generation, particularly for silver nanoparticles (AgNPs) and nano-zero-valent iron. In contrast, ROS production by TiO_2 and ZnO nanoparticles depends on light exposure (Yang et al., 2013). The toxicity of AgNPs is primarily linked to the release of silver ions and the enhanced production of ROS (Zhang et al., 2016). An overproduction of ROS, especially superoxide radicals (O_2^-), results in oxidative stress (Bashri et al., 2018). These superoxide radicals are converted into hydrogen peroxide through the enzymatic activity of superoxide dismutase. Hydrogen peroxide then undergoes Fenton's reaction, where transition metals catalyze its transformation into highly reactive hydroxyl radicals (Yamakoshi et al., 2003). ROS interact with biomolecules, leading to lipid and protein oxidation, which disrupts biological systems and compromises cellular functions (Xia et al., 2008; Xiong et al., 2011). Studies on zebrafish have shown that metal oxide nanoparticles can induce biomolecular damage due to

oxidative stress even in the absence of light (Yang et al., 2009). This phenomenon is a well-documented mechanism of nanoparticle-induced cellular damage, as supported by numerous studies (Pulskamp et al., 2007; Yang et al., 2009). Oxidative stress occurs when ROS production overwhelms the cell's antioxidant defenses, causing an oxidative burst. This imbalance leads to the release of intracellular calcium ions (Ca^{2+}), disrupting mitochondrial function and triggering apoptosis (Xia et al., 2008). Excessive ROS generated by nanoparticles can also compromise DNA integrity, impair protein synthesis (Yang et al., 2009), and induce DNA point mutations within lysosomes (Singh et al., 2009).

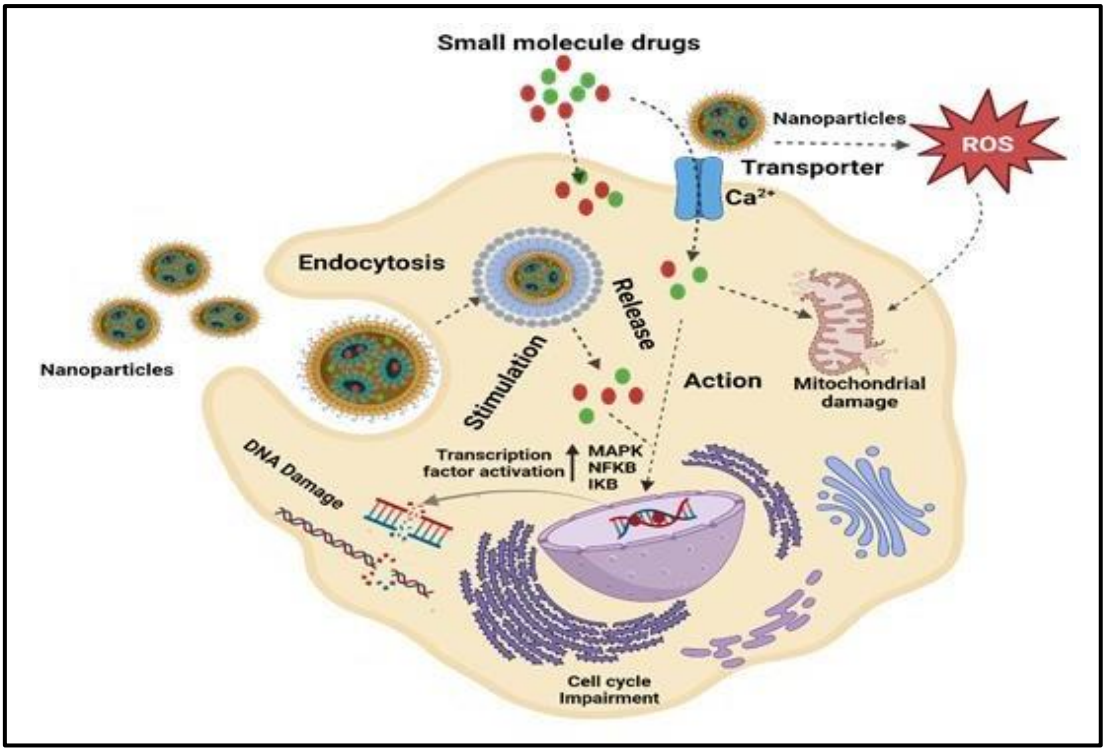

Figure 2. Systematic mechanism illustrating how nanoparticles get accumulated.

2.6. Toxicological studies and applications of AgNPs and CuNPs in fish

2.6.1. Silver nanoparticles (AgNPs) applications

Due to their antimicrobial and antibacterial qualities, silver nanoparticles are frequently utilized in products such as medical devices, wound dressings, water treatment systems, textiles, consumer goods, catalysts, sensors, odour resistant clothing, food packaging, cosmetics, washing products (Zhang et al., 2016; Bruna et al., 2021). According to (Keller et al., 2013), nano silver materials like AgNPs, Ag nanowires, and Ag nanorods are now the most significant nanomaterials listed in consumer product inventories. Annually,

around 60,000 tons of nanomaterials are produced globally (Jovanovic et al., 2011), with 1,628 nanotechnology-based products reported across 30 countries (Khan et al., 2015). Among these, silver nanoparticles represent the fastest-growing segment in the nano industry, with an estimated annual production of 320 tons (Wang et al., 2018). This rapid growth is driven by their unique properties and high consumer demand. Currently, approximately 30% of all registered nanoproducts claim to incorporate nano-silver (Project on Emerging Nanotechnologies, 2013). The annual turn out entering of about more than 60 tons of AgNPs into the water bodies was reported and also the presence of 0.03 to 0.32µg/L AgNPs in the marine environment was reported (Batley et al., 2013). As a result, various researchers worldwide are investigating the potential effects of AgNPs on aquatic and terrestrial animals (Mansouri and Johari, 2016; Johari et al., 2016; Kalbassi et al., 2011; Sharifian et al., 2013; Sohn et al., 2015; Falanga et al., 2020). Many of these metals are harmful to aquatic organisms in their soluble forms, meaning that nanoparticle preparations have the potential to cause toxicological consequences in aquatic organisms (Khan et al., 2024). There are rules in place to safeguard marine life from dissolved forms of these metals, it is uncertain if they are acceptable for usage with metal nanomaterials which have different toxicity (Wang et al., 2016). The combination effects of particle accumulation increase particle size and release dissolved metallic ions (Zhang et al., 2023). These considerations make adequate characterization of exposure conditions critical, as well as the need of including the influence of dissolved metals in any study of nanoparticle toxicity to aquatic organisms.

One of the most beneficial results of nanotechnology is colloidal silver, which works well against a wide range of harmful microorganisms, including bacterial fish pathogens also silver nanoparticles were used directly in reducing fungal infection in trout eggs (Ozil et al., 2022). However, there are currently no clear safety rules or toxicity evidence for the commercial application of soluble AgNPs to fish (Santos et al., 2014). It was reported that AgNPs with a diameter of around 10nm found in wastewater from washing clothes with AgNPs accounting for 30-80% of the released Ag in the form of particles more than 450nm (Clarke et al., 2017). Ionic silver is one of the metals that is known to kill aquatic animals, with lethal concentrations in the low gL^{-1} range. This is another reason why AgNPs have garnered a lot of interest in the aquatic environment (Bohme et al., 2015).

2.6.2. Toxicity of silver nanoparticles in aquatic organisms

The extensive use of silver nanoparticles has led to major concerns regarding their potential impact on aquatic animals as well as their wider effects on the environment and public health. Exposure to silver nanoparticles can occur through multiple pathways, including water, food, cosmetics, and pharmaceuticals, and is associated with various adverse effects (Ferdous and Nemmar, 2020). Despite being widely used in many different industries, little is known about how nanoparticles affect the environment and human health. Because of their unique physicochemical characteristics and nanoscale size, silver nanoparticles (AgNPs) may be hazardous to ecosystems (Sharma et al., 2019). Their toxicity is mostly determined by parameters like particle size and surface area (Abbasi et al., 2023) Numerous biological reactions, including apoptosis, reactive oxygen species generation, and cell activation, can be triggered by nanoparticles (Canaparo et al., 2020). Recent studies suggest that AgNPs exposure may impair mitochondrial function (Mello et al., 2022). Additionally, research has demonstrated that AgNPs are harmful to freshwater algae like *Chlamydomonas reinhardtii* (Navarro et al., 2008) and marine diatoms such *Thalassiosira weissflogii* (Mishra et al., 2020). Numerous studies have examined the consequences of acute and chronic exposure to AgNPs in fish, identifying oxidative stress, behavioral abnormalities, and programmed death cell (Kakakhel et al., 2021; Mahboub et al., 2021). The release of AgNPs into terrestrial and aquatic environments has been steadily increasing (Tortella et al., 2020) and their environmental toxicity is predominantly attributed to the release of silver ions (Ag^{+}), which are readily absorbed by organisms and can interact with other nanoparticles within ecosystems (Sharma et al., 2019). Smaller aquatic species are particularly vulnerable due to their high surface area-to-body mass ratio and large gill surface area, which play crucial roles in ion exchange, osmoregulation, and chemical absorption (Silver and Doini, 2021). In fish, gills are vital for gas exchange, osmoregulation, acid-base balance, and nitrogen excretion, making them primary targets for waterborne pollutants. Although the production of mucus in response to contaminants provides temporary protection, excessive mucus accumulation can impair gill function (Lead et al., 2018).

Recent studies have extensively examined the toxic effects of AgNPs on aquatic organisms, particularly fish. These effects include developmental deformities and increased mortality during early life stages (Rather et al., 2018), elevated mortality rates in adult fish (Hedayati et al., 2022; Rajkumar et al., 2016), disruptions in blood parameters

(Shaluei et al., 2013; Rajkumar et al., 2016), and alterations in metabolic enzyme activity (Rajkumar et al., 2016). Additional effects include oxidative stress (Bacchetta et al., 2017), lipid peroxidation (Xiang et al., 2020), silver bioaccumulation (Bruneau et al., 2015; Clark et al., 2019), DNA damage, and changes in gene expression (Pham et al., 2012; Johari et al., 2016; Massarsky et al., 2014; Thummabancha et al., 2016). For example, (Valerio-Garcia et al., 2017) demonstrated that a 21-day exposure to AgNPs caused oxidative stress in adult goodeid fish (*Chapalichthys pardalis*), along with reductions in antioxidant enzyme activity and glucose levels, and increases in oxidative biomarkers such as thiobarbituric acid reactive substances (TBARS) and oxidized proteins. Fish mitigate oxidative damage through enzymatic and non- enzymatic antioxidant defenses (Veisi et al., 2021; Jung et al., 2016). AgNPs are poisonous and harmful to ecosystems due to their changeable features and small size (Yaqoob, 2020). In the aquatic environment, silver can be found in different oxidation states (Ag^{3+}, Ag^{2+}, Ag^{+}, Ag) (Mazej et al., 2015). Furthermore, due to a lack of familiarity, the precise evaluation of AgNPs toxicity is still delayed (Ale et al., 2018). The toxicity of AgNPs is usually assumed to be caused by the release of Ag^{+} into the environment, which is then absorbed by aquatic organisms because of their potent antibacterial properties (Zhao et al., 2021). Moreover, the mechanism explaining how these silver nano particles accumulate the cell membrane is depicting in (Figure.3).

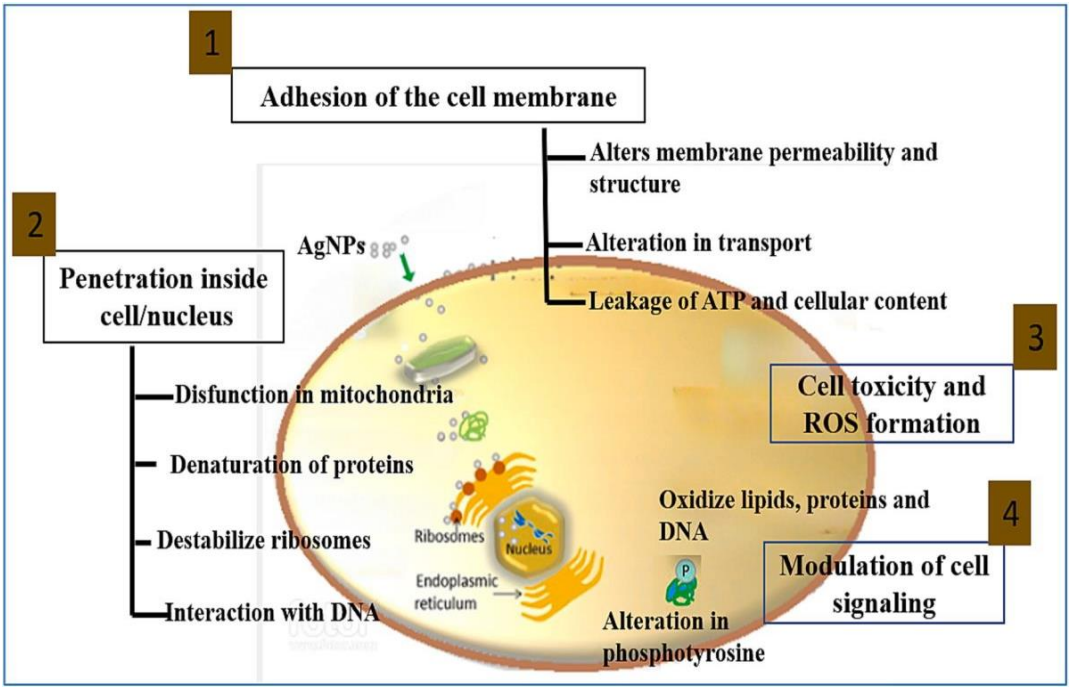

Figure 3. Silver nanoparticles accumulation into cell membrane

2.6.3. Effect of silver nanoparticles on fish growth

The effect of silver nanoparticles on fish growth can vary based on various factors, including nanoparticle concentrations, fish size and the duration of exposure. Studies have shown that exposure to silver nanoparticles can have both positive and negative impacts on fish growth (Table 2). On the one hand, silver nanoparticles have been shown to improve the growth and survival of certain fish species by improving their immune function and enhancing their ability to resist disease (Ochoa-Meza et al., 2019). On the other hand, exposure to high concentrations of silver nanoparticles can also have toxic effects on fish, including reduced growth rates, decreased feeding behavior, and changes in the fish's metabolism.

Low concentrations exposure of silver nanoparticles (10 µg/l) improved the growth performance of common carp. The study showed that the fish exposed to silver nanoparticles had significantly higher body weight, length, and condition factor in comparison to the control group (Mabrouk et al., 2021). The researchers suggested that the silver nanoparticles may have acted as a dietary supplement and enhanced the fish's growth (Pirani et al., 2021).

2.6.4. Impact of silver nanoparticles on fish health

Silver nanoparticles (AgNPs) have emerged as a promising tool in aquaculture, offering innovative solutions to combat various fungal, bacterial, and viral infections, particularly in the face of rising antibiotic resistance. AgNPs have demonstrated bactericidal properties against *Aeromonas* species, which are among the most common pathogens affecting the aquaculture industry (Mahanty et al., 2013). For instance, Shaalan et al. (2018) observed that short-term exposure of *Oncorhynchus mykiss* infected with *Aeromonas salmonicida* to silver nanoparticles (100 g/Lh-1) resulted in no mortality or clinical symptoms, whereas the untreated control group exhibited severe effects. Similarly, AgNPs have shown potent antibacterial activity against *Aeromonas hydrophila* and *Vibrio harveyi* in various aquatic organisms (Adeniji, Nontongana, Okoh, & Okoh, 2022). Moreover, dietary supplementation of silver nanoparticles in appropriate quantities has been linked to improved survival rates, enhanced zootechnical performance, better metabolic efficiency, and reduced stress from both abiotic and biotic sources in aquatic species (Kumar & Rajeshkumar, 2018).

However, the increasing application of AgNPs across different industries raises concerns about their potential environmental impact (Islam, Jacob, & Antunes, 2021). Silver nanoparticles can enter aquatic ecosystems during production processes or through the disposal of nanoparticle-containing products. The effects of AgNPs exposure in fish depend on factors such as exposure duration, concentration, and nanoparticle size. High levels of exposure have been associated with various adverse outcomes, including inflammation, immune suppression, metabolic disturbances, biochemical imbalances, and impaired growth (Mahanty et al., 2013). Consequently, even at low levels resulting from environmental contamination or therapeutic use, it is crucial to conduct comprehensive assessments of AgNPs toxicity in aquatic environments.

Table 2. Silver nanoparticles toxicity in different fishes.

S.NO.	Model organism	Toxicity effect	Size of NPs	References
1.	Zebrafish (*Danio rerio*)	Defects in regeneration and penetration into different organelles	10nm-20nm	(Yeo and Pak, 2008)
2.	Zebrafish (*Danio rerio*)	High binding of Ag NP with gills and other organs1	26.6nm+8.8nm	(Griffitt et al., 2009)
3.	Zebrafish (*Danio rerio*)	Differential toxicity of Ag NPs at different phases of development1	20-30nm	(Griffitt et al., 2008)
4.	Zebrafish (*Danio rerio*)	Particle size dependent toxicity morphological anomalies	3, 10, 50,100nm	(Bar-Ilan et al., 2009)
5.	Perch *(Perca flavescens*)	Ag NPs bind to the gills and reduce tolerance to hypoxia.	81nm	(Bilberg et al., 2010)
6.	Brown trout (*Salmo trutta)*	Uptake varies with size Ag nanoparticles are abundant in the gill70-s and liver. Oxidative stress has increased.	10nm-35nm	(Scown et al., 2010)
7.	Zebrafish embryo	Ag NP transport through chorion channels	11.6 + 3.5	(Lee et al., 2007)
8.	Zebrafish embryo	Deposition in the nucleus of a cell, the brain, the neurological system, and the blood	5nm-20nm	(Asharani et al., 2008)

2.6.5. Applications of copper nanoparticles

Copper nanoparticles are also valued for their antimicrobial and catalytic properties, finding applications in electronics, agriculture, antimicrobial coatings, and industrial applications (Mitrea et al., 2016; Inkinen et al., 2016; Lushina et al., 2005; Salem et al., 2019) construction materials (Gupta et al., 2019), antimicrobial agents, formation of alloys with other metals. Copper contamination is often prevalent near copper mining sites. Aquatic ecosystems, including lakes, rivers, and oceans, are particularly susceptible to metal pollution due to the discharge of industrial waste, urban mining activities, and soil weathering into these water bodies, adversely affecting aquatic life (Eisler, 2000). Fish and shellfish farming have made substantial use of nanoparticles for nanofiltration applications in recent years. It is concerning that nanoparticles are also employed in the manufacture of fish meals (Handy et al., 2012). The increased popularity of NPs causes increase in their concentration in the environment, especially aquatic settings. Unfortunately, understanding of the dangers associated with the use of nanoparticles is inadequate. The presence of NPs in the environment can have major environmental ramifications as well as adverse effects on human and animal health (Handy et al., 2008).

2.6.6. Toxicity of copper nanoparticles in aquatic systems

When Oncorhynchus mykiss was exposed to copper sulfate and copper nanoparticles in a semi-static aquatic system, 85% mortality was recorded by the fourth day at a concentration of 100 g/L of copper sulfate. This is higher than the 14% mortality caused by CuNPs with the same settings. Because of their large surface area, the gills were discovered to be the primary copper collection location. (Shaw et al., 2012). Furthermore, CuNPs elicited ion regulatory toxicity (decreased Na^+/K^+ ATPase activity), rendering them less hazardous than copper sulphate at the same concentration. CuNPs at 10 g/L exposure to fresh and brackish water euryhaline kill fish (*Fundus heteroclitus*) increased consumption of oxygen and aerobic choice in salty water to kill fish but decreased $Na^+/K^{+,}$ ATPase action by more than 40% (Black et al., 2017).

The toxicity of CuNPs to crucian carp was assessed by exposing the fish to a copper solution with specific properties 2.91 mg Ca^{2+}/L, approximately 300 µg Cu^{2+}/L, pH 6.6, and conductivity of 25 µS/cm. At concentration of 300 µg Cu^{2+}/L, CuNPs were not considered hazardous to fish (96-h LC50), as mortality rate was only seen after 10 days of

the exposure period. Notably, CuNPs concentration used in this study was ten times higher than the copper levels 10–20 μg/L that have been shown to be highly toxic to freshwater teleost's in saline environments (McGeer et al., 2002). CuNPs tend to produce $Cu2^{+}$, which can generate hydroxyl free radicals and damage the fish's membranes. Furthermore, the results of the toxicity of both AgNPs and CuNPs nanoparticles for fish at various life stages is referred to as "toxic" or "very toxic." (Ramyadevi et al., 2012; Johari et al., 2013).

2.6.7. Toxicity mechanism action of copper nanoparticles

The toxic effects of copper oxide nanoparticles (CuNPs) arise from several interconnected mechanisms, as reported in the literature. These include oxidative stress, DNA degradation, lipid peroxidation, damage to cellular membranes, disruption of mitochondrial function, release of metal ions, and nanoparticle dissolution. Together, these pathways reveal the intricate ways CuNPs interact with biological systems (Ameh and Sayes, 2019). A key contributor to CuNPs toxicity is the generation of reactive oxygen species (ROS) and the subsequent oxidative stress they cause (Liu et al., 2024). Copper nanoparticles (CuNPs), even at low concentrations, can generate substantial levels of reactive oxygen species, like superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). These reactive molecules have the potential to cause significant harm to cellular components, including lipids, proteins, and DNA (Sielska et al., 2024). The mitochondria are particularly susceptible, with CuNPs disrupting their membranes and further enhancing ROS production, leading to intensified cellular oxidative damage (Yang et al., 2009; Xia et al., 2007). The dissolution of CuNPs, coupled with the release of copper ions ($Cu2^{+}$), also plays a major role in the toxicity (Razmara, 2021). This dissolution process is affected by various factors, like the size and surface area of the nanoparticles, their chemical makeup, environmental pH, temperature, and the presence of organic substances. Smaller particles with larger surface areas tend to dissolve more readily, releasing higher amounts of $Cu2^{+}$ ions, which disrupt cellular balance and add to oxidative stress (Conway et al., 2015).

Another important aspect of their toxicity is their interaction with genetic material. When CuNPs enter the nucleus, they release Cu^{2+} ions, which can lead to oxidative damage to DNA. This damage manifests as strand breaks and structural alterations, hindering proper cellular function and replication (Wang et al., 2019). The combined effects of oxidative stress and direct damage to genetic material highlight the complexity of CuNPs-induced

toxicity. The CuNPs toxicity involves a combination of ROS production, mitochondrial disruption, nanoparticle dissolution, and DNA damage (Abdelsattar et al., 2024). A thorough understanding of these mechanisms is essential for assessing the risks posed by CuNPs and for devising strategies to minimize their effects on biological systems.

Table 3. Copper nanoparticles toxicity in different fishes

S.NO.	Model organism	Toxicity effect	Size of NPs	References
1.	*Danio rerio*	Toxicity in gills, 48h LC50 and 1.5 mg/L	80 nano meters	(Griffitt et al., 2007)
2.	*Oncorhynchus mykiss*	Reduction in branchial	20 and 100 87 ± 27 nm, µg/L	(Shaw et al., 2012)
3.	*Fundulus heteroclitus*	>40% reduction in Na in FW	5–10 nm and 10 µg/L	(Black et al., 2017)
4.	*Ctenopharyngodon idella*	Growth reduction	100 and 200 mg 50 nm, /L	(Abdollahzadeh et al., 2018)
5.	*Cyprinus carpio*	Cell swelling, aourtism, edema	10, 40, 80 mg/L	(Malhotra et al., 2020)
6.	*Apistogramma agassizii*	Oxidative stress and histological alterations.	58.31; 69.6 µg/L	(Noureen et al., 2021)
7.	*Plotosus lineatus*	Increase in H^+ and increase in Na+/K^+ pump activity.	12.16 40–60 nm, ± 1.77 µg/L	(Tesser et al., 2020)
8.	*Rutilus rutilus caspicus*	Fish became anemic Condition.	40 nm, 0.1, and 0.5 mg/L^{-1}	(Aghamirkarimi et al., 2017)
10.	*Rutilus rutilus caspicus*	Deformation of nuclei and cellular degeneration occurs.	40 nm, 0.1, and 0.5 mg/L^{-1}	(Aghamirkarimi et al., 2017)
11.	*Rutilus rutilus caspicus*	Blood congestion in veins occurs.	0.1, 0.2, and 0.5 mg/L^{-1}	(Aghamirkarimi et al., 2019)
12.	*Cyprinus carpio*	Increase in oxidative stress markers	<50 nm, 20, and 100 µg/L^{-1}	(Gupta et al., 2016)
13.	*Trachidermus fasciatus*	Increase in malondialdehyde (MDA), and decrease in Na+/K+- ATPase,	10–30 nm, 20 and 100 µg/L1	(Liu et al., 2021)
14.	*Oreochromis niloticus*	Increase in SOD, CAT, GPX	<50 nm, 10, 50 and 100 µg/L^{-1}	(Tunçsoy and Erdem, 2018)

15.	*Rutilus rutilus caspicus*	Degeneration in the tubule cells, and other tissues.	40nm, 0.1, 0.5 mg/L^{-1}	(Aghamirkarimi et al., 2017)
16.	*Oreochromis niloticus*	Excess copper is seen bound to tissues.	10, 50, and 100 µg Cu/L	(Tunçsoy and Erdem, 2018)
17	*Cyprinus carpio*	Tubular vacuolization	40 nm, 0.25, 25 mg/L^{-1}	(Hoseini et al., 2016)
18.	*Tilapia mossambica*	Reduction in oxidative stress.	15 mg/L^{-1}	(AlGhais et al., 2019)
19	*Tilapia mossambica*	Reduced lateral line neuromasts.	20 ± 9 nm, 50, 225 $\mu g/L^{-1}$	(McNeil et al., 2014)
20	*Oncorhynchus mykiss*	Oxidative stress	<50 nm, 50 $\mu g/L^{-1}$	(Sovova et al., 2014)

2.7. Combined effect of silver and copper nanoparticles

The combined exposure of silver nanoparticles and copper nanoparticles in fish presents a multifaceted toxicological challenge, as these nanomaterials often co-exist in aquatic environments due to their widespread industrial applications and subsequent release into water bodies. While extensive studies have elucidated the individual toxicological effects of AgNPs and CuNPs, their combined effects remain inadequately understood, despite their relevance to real-world environmental scenarios where multiple contaminants interact. The co-exposure of fish to AgNPs and CuNPs amplifies their toxicological impact through several mechanisms, primarily mediated by oxidative stress, cellular damage, and disruption of physiological processes. Both nanoparticles are known to generate reactive oxygen species (ROS), which play a central role in their toxicity. When combined, the generation of ROS is often synergistically elevated, overwhelming the antioxidant defense mechanisms of the fish. This imbalance leads to oxidative stress, characterized by extensive lipid peroxidation, protein denaturation, and DNA damage, all of which disrupt cellular integrity and homeostasis (Rajkumar et al., 2016; Zou et al., 2021).

At the cellular level, the simultaneous presence of AgNPs and CuNPs exacerbates the cytotoxic effects by interfering with mitochondrial function and inducing apoptotic pathways. Both nanoparticles release their respective ions, silver and copper, into the cellular milieu. The elevated ion concentrations increase toxicity, promoting mitochondrial

dysfunction, oxidative phosphorylation disruption, and excessive ROS production. These processes culminate in structural damage to cellular membranes and organelles, thereby intensifying the toxic effects compared to individual nanoparticle exposure (Hussain et al., 2016; Li et al., 2017).

The combined effects of AgNPs and CuNPs are particularly evident in critical organs such as the gills and liver, which serve as primary sites for nanoparticle accumulation and toxicity. The gills, being the principal respiratory and osmoregulatory organs, are highly susceptible to damage due to their extensive surface area and direct contact with the aquatic medium. Co-exposure to AgNPs and CuNPs impairs gill function by disrupting ion transport mechanisms, causing epithelial cell necrosis, and reducing gas exchange efficiency. The liver, a central organ for detoxification and metabolism, also accumulates significant quantities of nanoparticles. Histopathological studies reveal hepatocellular degeneration, vacuolization, and inflammatory responses in fish liver when exposed to AgNPs and CuNPs. These structural and functional impairments compromise the liver's ability to metabolize and eliminate toxicants, thereby exacerbating systemic toxicity (Griffitt et al., 2007; Scown et al., 2010).

Moreover, organ-specific toxicity, the co-exposure to AgNPs and CuNPs profoundly affects oxidative parameters. Fish exposed to a combination of nanoparticles show significant reductions in the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), indicating the oxidative stress induced by the nanoparticle mixture. The immune system also suffers adverse effects, with decreased lysozyme activity and impaired phagocytic efficiency, rendering the fish more vulnerable to pathogenic infections (Sumana et al., 2023). The ecological implications of the combined toxicity of AgNPs and CuNPs are substantial, as these nanoparticles not only affect individual fish but also have cascading effects on aquatic ecosystems. The accumulation of nanoparticles in sediments, their bioavailability to aquatic organisms, and their potential to integrate into the food web present risks to higher trophic levels, including humans. The interaction of AgNPs and CuNPs with natural organic matter, other contaminants, and environmental factors further modulates their toxicity, complicating efforts to predict and mitigate their impact (Vali et al., 2020).

Despite these findings, there remains a significant gap in understanding the long-term effects of chronic co-exposure to AgNPs and CuNPs in aquatic organisms. The majority

of research has concentrated on acute toxicity, mainly ignoring the cumulative and interaction effects under long-term exposure circumstances. Investigating these aspects is essential for accurately assessing the environmental risks posed by nanoparticles and informing regulatory strategies to limit their release and promote their safe application in industrial and environmental contexts (Griffitt et al., 2009)

2.7.1. Oxidative stress and antioxidant responses

Oxidative stress plays a significant role in nanoparticle toxicity, arising from an imbalance between reactive oxygen species (ROS) production and the cellular antioxidant defense mechanisms in aquatic organisms. ROS, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, are natural by-products of metabolic activities. However, exposure to nanoparticles, especially AgNPs and CuNPs, intensifies ROS production through processes like surface oxidation, ion release, and disruption of mitochondrial function (Rajkumar et al., 2016; Zou et al., 2021). Excessive ROS accumulation results in oxidative damage to critical cellular macromolecules, including lipids, proteins, and DNA. Lipid peroxidation compromises membrane stability, leading to increased permeability and ion imbalance. Similarly, protein oxidation affects enzymatic activity and structural integrity, while DNA damage, such as strand breaks and base alterations, undermines genomic stability (Topal et al., 2017; Lushchak, 2016).

Fish counteract ROS through a complex antioxidant defense system. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), neutralize specific ROS species, while non-enzymatic antioxidants like GSH scavenge free radicals. However, exposure to high concentrations of nanoparticles overwhelms these defenses, leading to oxidative stress. Studies indicate that nanoparticle exposure initially induces an upregulation of antioxidant enzymes as a compensatory mechanism, which is subsequently followed by their suppression due to prolonged oxidative burden and enzyme exhaustion (Lushchak, 2016). Oxidative stress caused by nanoparticles triggers signalling pathways that regulate inflammatory and antioxidant responses, such as mitogen-activated protein kinases (MAPKs) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Zou et al., 2021). Nevertheless, chronic stimulation of these processes may lead to death, inflammatory disorders, intensifying tissue damage and impairing function in fish.

2.7.2. Histopathological Effects in Fish

Histopathological damage provides a direct measure of nanoparticle toxicity in fish. Organs like the gills and liver, which are vital for survival, are most severely affected due to their roles in environmental interaction and detoxification.

Gills: The gills, being the primary site for gas exchange and osmoregulation, are highly exposed to nanoparticles in the aquatic environment. Nanoparticles adhere to the gill surface, penetrate epithelial cells, and accumulate within the tissues. This leads to structural changes such as epithelial lifting, lamellar fusion, and necrosis, impairing gas exchange and ionic balance (Griffitt et al., 2007). Morphological changes, including increased mucus secretion, thickening of the epithelium, and reduction in interlamellar spaces, have been documented in fish exposed to AgNPs, reflecting the respiratory distress caused by these nanoparticles (Vali et al., 2020). Moreover, CuNPs induce oxidative damage in gill tissues by promoting lipid peroxidation and protein denaturation, further aggravating respiratory dysfunction. The accumulation of nanoparticles in the gills triggers local inflammation, characterized by leukocyte infiltration and cytokine release, which compromises the structural integrity and functional capacity of the gill tissues (Scown et al., 2010).

Liver: The liver, as the primary organ for metabolism and detoxification, is another critical target of nanoparticle toxicity. Nanoparticles accumulate in hepatocytes through endocytosis and are often metabolized, releasing ions that perpetuate ROS generation. Histopathological changes in the liver include hepatocellular vacuolization, sinusoidal congestion, inflammation, and necrosis (Al-Bairuty et al., 2013). For instance, studies on CuNP exposure have demonstrated mitochondrial dysfunction in hepatocytes, leading to impaired ATP production, excessive ROS generation, and activation of apoptotic pathways (Li et al., 2013). AgNPs have been shown to alter liver enzyme activity, including transaminases and alkaline phosphatase, which are critical indicators of hepatotoxicity and metabolic disruption (Mirghead et al., 2018). Beyond the gills and liver, nanoparticles also affect other tissues, including the kidneys, spleen, and intestine. In the kidneys, nanoparticles induce glomerular damage and tubular necrosis, impairing excretory functions and causing systemic imbalances in ion and water homeostasis (Scown et al., 2010). The spleen, a key immune organ, exhibits lymphoid depletion and increased macrophage activity upon nanoparticle exposure, reflecting compromised immune

responses. The intestinal tract, as another site of nanoparticle exposure through ingestion, exhibits villous atrophy, enterocyte damage, and inflammatory cell infiltration, disrupting nutrient absorption and overall metabolic health. The histopathological effects observed in fish serve as indicators of the systemic toxicity of nanoparticles, highlighting their potential to disrupt physiological functions at multiple levels. These effects are particularly concerning in ecologically and economically important species, where nanoparticle exposure could have cascading impacts on aquatic ecosystems and food webs.

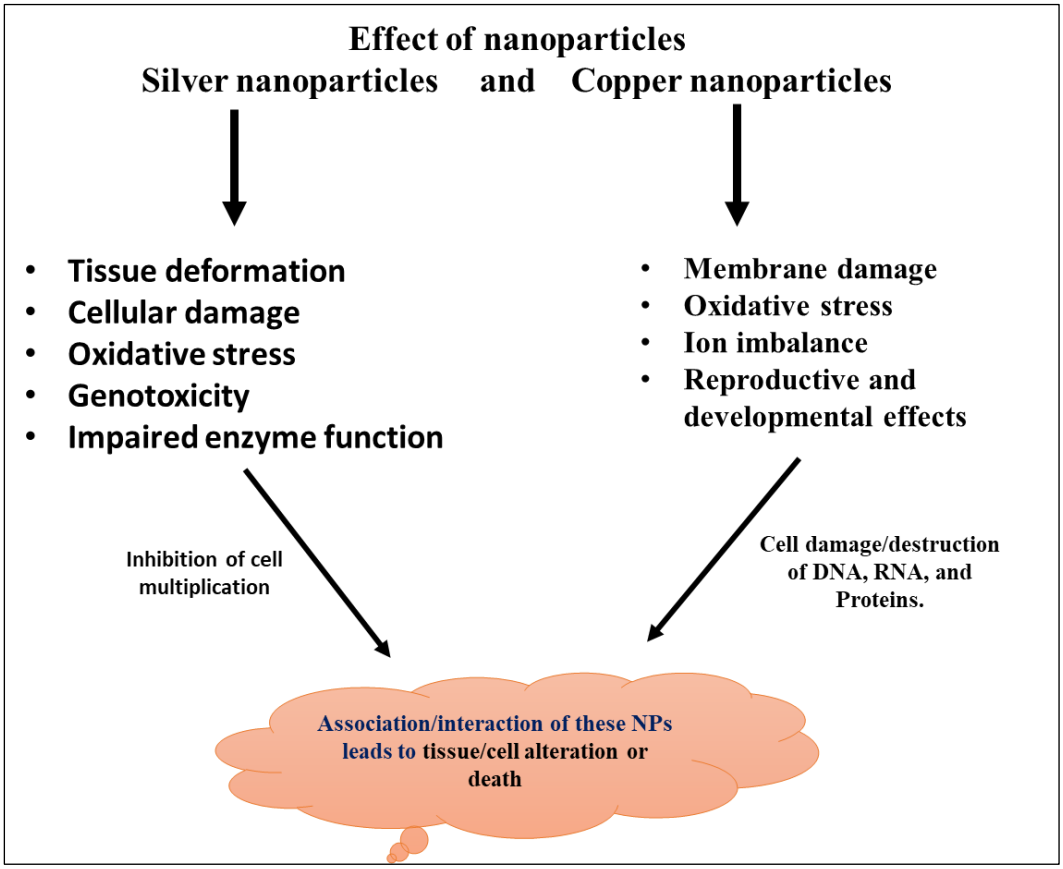

Figure.4. Combined effect of silver and copper nanoparticles

Chapter 3

3. HYPOTHESIS

The hypothesis of this study is that the exposure of rainbow trout (*Oncorhynchus mykiss*) to silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) will result in significant biochemical and histopathological alterations, with combined exposure to both nanoparticles inducing more severe toxicological effects compared to individual exposures. Specifically, it is hypothesized that:

Exposure to AgNPs and CuNPs in rainbow trout leads to oxidative stress, as evidenced by alterations in the activities of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione S-transferase. At higher concentrations of AgNPs and CuNPs, antioxidant enzyme activity, particularly in SOD and CAT, increases up to a certain threshold, indicating an adaptive response to the oxidative stress. However, prolonged exposure, particularly to higher concentrations, will lead to the depletion of these enzymes, indicating oxidative damage and exhaustion of the antioxidant defense system. Additionally, it is expected that combined exposure to AgNPs and CuNPs will exacerbate oxidative stress and further reduce the activity of these enzymes compared to individual nanoparticle exposures. It is hypothesized that lipid peroxidation (LPO), measured by TBARS levels, will increase as a result of oxidative stress induced by nanoparticle exposure. However, the study anticipates that in lower concentration treatments, compensatory antioxidant mechanisms may counteract lipid damage, leading to a decline in LPO. In higher concentrations and during combined exposure, a more significant rise in LPO levels is expected, reflecting increased oxidative damage to cellular membranes and tissues.

Histopathological analysis is expected to reveal dose-dependent damage to key organs, particularly the gills and liver. It is hypothesized that exposure to AgNPs and CuNPs will cause structural damage to gill and liver tissues, including cellular infiltration, lamellar fusion, necrosis, and tissue degeneration. The severity of these changes will be greater with higher nanoparticle concentrations, and the combined exposure to AgNPs and CuNPs will produce more extensive tissue damage compared to individual exposures. Gills are anticipated to show hypertrophy, hyperplasia, and lamellar fusion, while liver tissues will exhibit vacuolation, necrosis, and hepatocellular degeneration.

The hypothesis also points that the combined exposure to AgNPs and CuNPs will have synergistic effects, causing more severe oxidative stress and histopathological damage than the sum of the individual nanoparticle exposures. This will be reflected in higher levels of lipid peroxidation, more significant biochemical alterations, and more pronounced histopathological changes. In summary, this study hypothesizes that both AgNPs and CuNPs induce oxidative stress and tissue damage in rainbow trout, with combined exposure resulting in enhanced toxicity. The findings of this study will enhance our understanding of the environmental risks linked to the release of engineered nanomaterials into aquatic ecosystems and guide the development of strategies to manage nanoparticle toxicity in aquatic organisms.

Chapter 4

4. OBJECTIVES

The objectives selected for present study steadily followed the approved guidelines for fish acute toxicity; OECD-203 (OECD, 1992) and the objectives are as follows

1. Synthesis and characterization of Ag and Cu nanoparticles
2. To study the biochemical effect of single and combined Ag and Cu nanoparticles in rainbow trout.
3. To study the histological alterations in gill and liver of rainbow trout, exposed to the single and combined effect of Ag and Cu nanoparticles.

Chapter 5

5. METHODS AND MATERIALS

Objective 1: Synthesis and characterization of Ag and Cu nanoparticles.

5.1. Synthesis and Characterization of Silver Nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were synthesized following a chemical reduction method, as described by (Rashid et al., 2013) with slight modifications to ensure reproducibility. A solution of 0.1M silver nitrate (Ag NO_3) was reduced using 0.8g trisodium citrate (Na_3 $C_6H_5O_7$) and 0.1g sodium borohydride (Na BH_4) in distilled water (final pH 8.0). The reaction mixture was stirred continuously for 3 hours at room temperature, during which the colour change to yellow-brown indicated successful nanoparticle formation. To ensure batch reproducibility, multiple batches of nanoparticles were synthesized, and the resulting suspensions were centrifuged at 6,000 rpm for 20 minutes to isolate the nanoparticles. The pellet was washed with distilled water, freeze-dried, and stored at 4°C for further experiments (Figure 5.1).

The synthesized AgNPs were characterized by several techniques to confirm their size, shape, and stability. UV-visible spectroscopy (Lasany, India, Model L1-2800 Ex) was used to monitor the absorbance of the nanoparticle suspension between 200 and 800 nm, with a peak at ~418 nm confirming surface plasmon resonance. X-ray diffraction (XRD) was performed using a Rigaku Smart Lab diffractometer to determine the crystalline structure and purity and Fourier-Transform Infrared Spectroscopy (Nicolet FTIR-IS50) was used to identify functional groups involved in nanoparticle stabilization. Scanning electron microscopy (Apreo LoVac Analytical-SEM) was employed to examine particle morphology and size distribution, confirming uniform spherical particles with an average size of 50 nm. Zeta potential measurements (-28 mV) were conducted using the Litesizer TM 500 to confirm the colloidal stability of the nanoparticles.

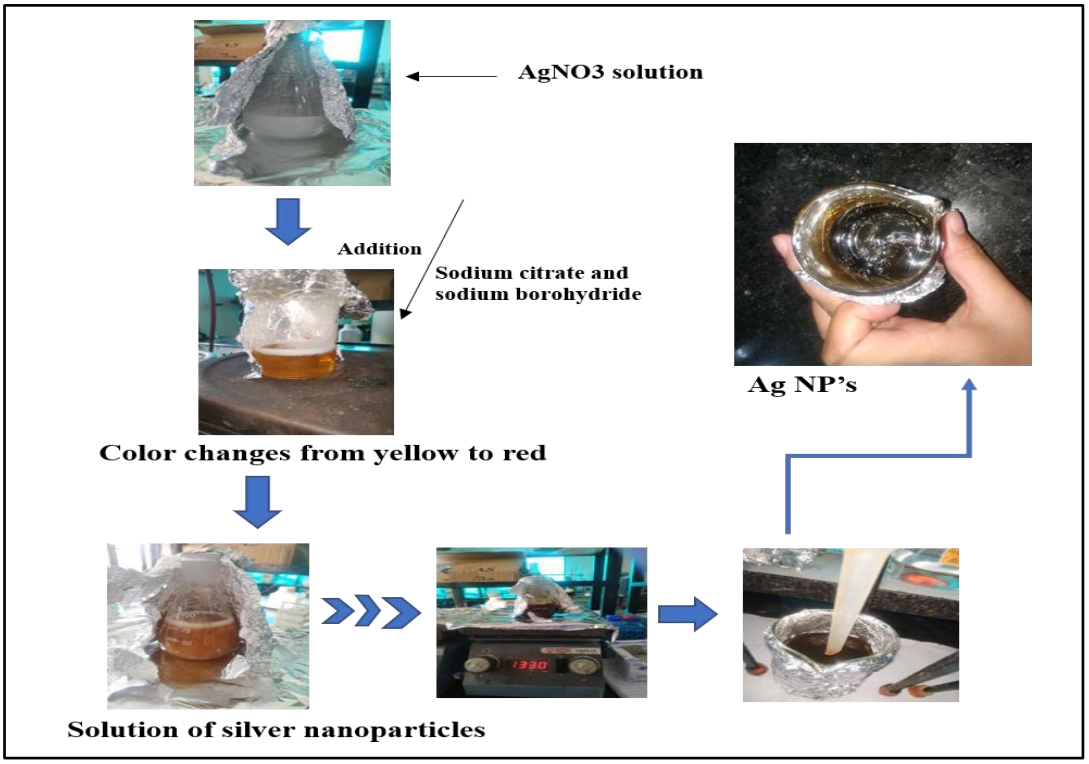

Figure.5.1 Synthesis of silver nanoparticles

5.1.1. Synthesis and Characterization of Copper Nanoparticles (CuNPs)

Copper nanoparticles (CuNPs) were synthesized using the sol-gel technique as described by (Dorner et al., 2019) with slight modifications. 0.1 M copper nitrate solution was prepared by dissolving copper nitrate in 100 mL of deionized water, followed by the gradual addition of 0.2M NaOH under constant ultrasonic stirring at 80°C for 2 hours. The resulting black precipitate was filtered, washed extensively with methanol, and dried at 400°C in a hot air oven as shown in (figure 5.2).

The synthesized CuNPs were characterized using multiple techniques, UV-Vis Spectroscopy was performed to measure absorbance in the range of 200-800 nm using a Microprocessor UV-Vis Double Beam Spectrophotometer Model: L1-2800 Ex; (Lasany, India) to confirm nanoparticle formation and surface plasmon resonance. X-ray Diffraction (XRD) analysis was conducted using a Rigaku Smart Lab diffractometer system (AMU India) to determine crystalline structure. Fourier Transform Infrared Spectroscopy (FTIR) was used to demonstrate the functional groups on CuNPs surfaces, with spectra recorded from 500 to 4000 cm^{-1} using a (Nicolet™ iS50 FTIR Spectrometer) for and structure of the

nanoparticles. Zeta Potential analysis was conducted using a (Litesizer^{TM}500) to measure the surface charge and colloidal stability of CuNPs.

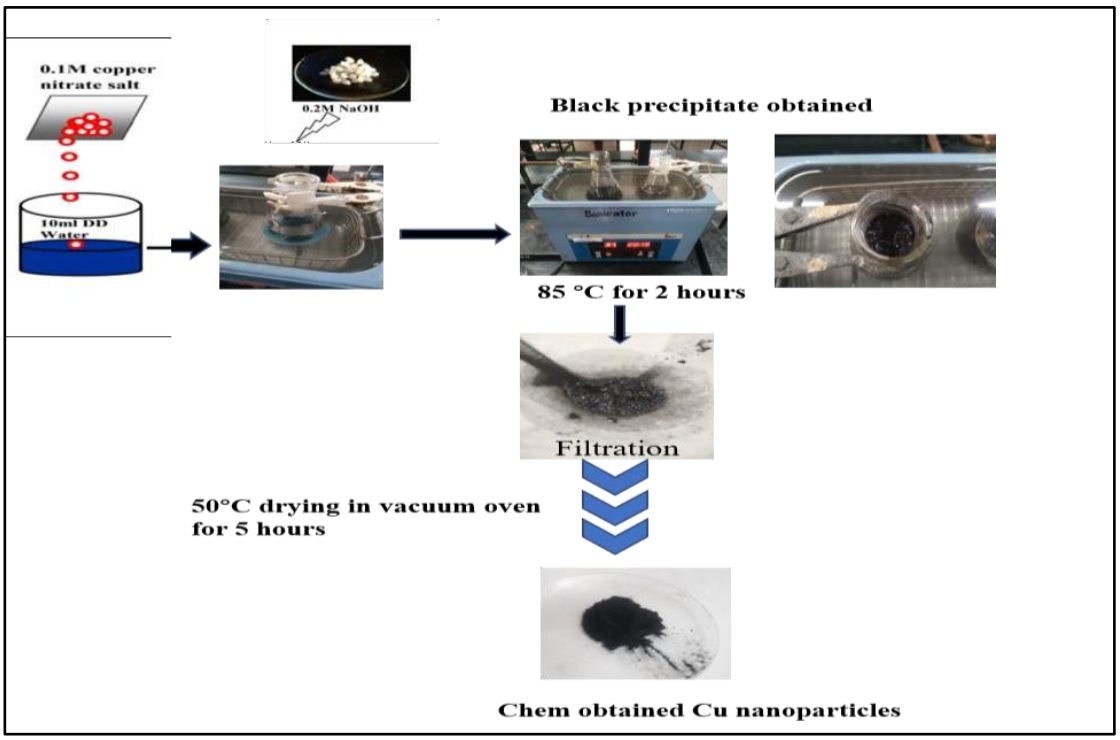

Figure.5.2 Synthesis of copper nanoparticles

Objective 2: To study the biochemical and histological effect of silver nanoparticles in rainbow trout.

5.2. Fish collection and acclimatization

Rainbow trout fingerlings (*Oncorhynchus mykiss*) (n=312, average weight 4.5g, length 5.20 ± 0.30 cm) were obtained from the State Government Fishery Department hatchery located at Dachigam, Laribal in Srinagar, Jammu and Kashmir, India (Figure 5.3). Fish were transported in oxygen-filled double polythene bags. Upon arrival at the Division of Fish Genetics and Biotechnology, SKUAST-K, the fish were acclimated for 14 days under controlled laboratory conditions, with 5cm^3 air stones and water temperature was maintained between 15°C and 18°C and a 12h light:12h dark photoperiod. Fish were housed in aerated tanks with a water exchange rate of 50% every 48 hours, and water quality parameters were maintained within acceptable limits (pH 7.2-7.8, DO 7-7.3 mg/L, CO_2 6-6.5 mg/L). During acclimatization, fish were fed a commercial diet at 5% of their body weight daily.

Prior to the experiment, fish were treated with potassium permanganate ($KMNO_4$) as a prophylactic measure (Ahmed and Ahmad, 2020).

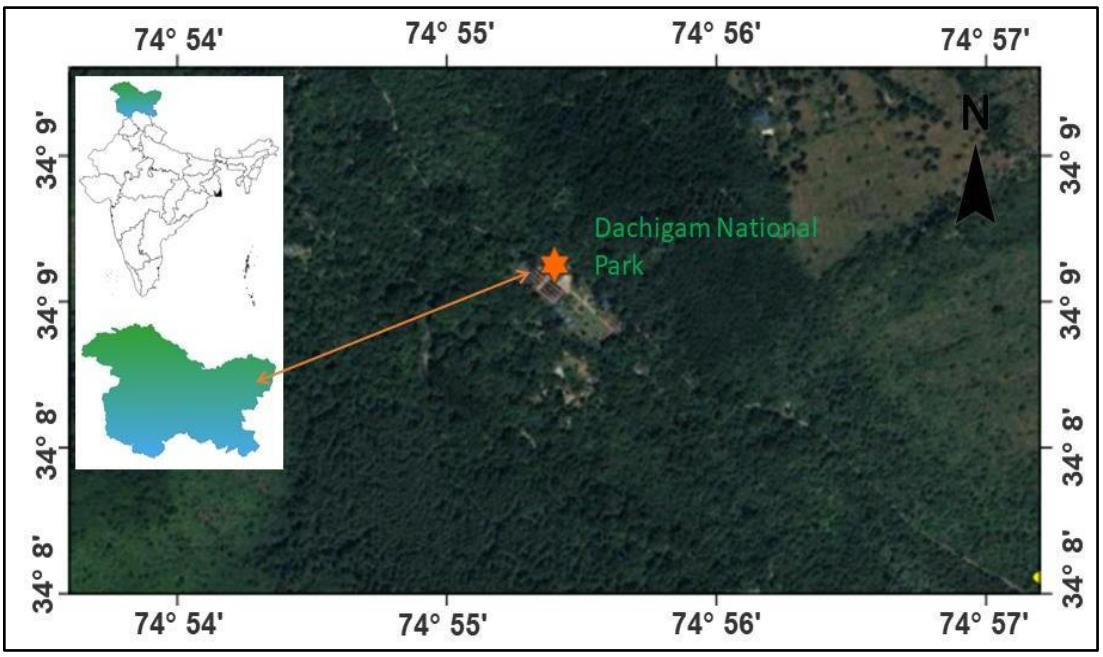

Figure 5.3. A map depicting the positioning and layout of the fish farm region located in Dachigam, Kashmir, India.

5.2.1. Experimental design and AgNPs exposure

The experimental design included a 96-hour acute toxicity test followed by a 21-day chronic exposure. A total of 192 fish were used for the acute toxicity test, where fish were exposed to AgNPs concentrations of 0.2, 0.8, 1.4, 2.0, and 2.6 mg/L, with 12 fish per replicate in triplicate. Based on the LC50 value calculated at 2.0 mg/L, by Probit method (Finney, 1971). Sublethal concentrations of 0.2 mg/L (T1), 0.8 mg/L (T2), and 1.4 mg/L (T3) were used in the chronic exposure experiment. In the chronic experiment, 120 fish were exposed to AgNPs for 7, 14, and 21 days, with samples taken at each interval. Fish were fed commercial feed daily, and water renewal and quality monitoring were conducted every 48 hours. To maintain consistent exposure, AgNPs concentrations were re-established during each water renewal, implementing a static renewal system. Treatment solutions were prepared by diluting AgNPs stock solutions to target concentrations with distilled water to ensure uniform nanoparticle dispersion before addition to each tank. Each AgNPs concentration was tested in triplicate, with each replicate containing 12 fish to account for variability and enhance statistical robustness.

5.2.2. Histological examination

At the 7th, 14th, and 21st day of exposure, fish tissue samples from the livers and gills were collected and the tissues were processed for histological analysis primarily following the guidelines provided by (Martoja and Martoja, 1967). Tissue samples from the gills and liver were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 5- 7µm using microtome (Labotech, B.D. Instrumentation, India). Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX21, Model: CX21FS1, Tokyo, Japan) to assess histological changes.

5.2.3. Antioxidant enzyme assays

Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and glutathione reductase (GR), were measured in liver and gill tissue samples using established protocols (Bradford, 1976; Atli et al., 2016; Lartillot et al., 1988; McCord and Fridoyich, 1969; Habig et al., 1974). Lipid peroxidation (LPO) was quantified using the thiobarbituric acid reactive substances (TBARS) method (Uchiyama and Mihara, 1978).

5.2.4. Statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (Duncan, 1955) to determine significant differences between groups at a significance level of $p < 0.05$. Before conducting ANOVA, the data were checked for normality using the Shapiro-Wilk test and for variance homogeneity using Levene's test. Results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software version 28.0.1 (IBM Corp). Sample size adequacy and statistical power were evaluated prior to the experiments to ensure the reliability of the conclusions.

Objective 3: **To study the biochemical and histological effect of copper nanoparticles in rainbow trout.**

5.3. Experimental fish, collection and study design

Fingerling rainbow trout (*Oncorhynchus mykiss*), with an average weight of 4.5g and length of 5.20 ± 0.30 cm, were obtained from the State Government Fishery Department Hatchery (Dachigam Laribal, Srinagar, Jammu & Kashmir). Fish were transported in double polythene bags supplied with oxygen. Upon arrival at the Division of Fish Genetics and Biotechnology, SKUAST-Kashmir, the fish were acclimatized for two weeks in aerated water with 5 cm^3 air stones at 15°C-18°C, maintaining dissolved oxygen levels of 7.0-7.3 mg/L. They were fed a commercial diet of dry pellets at a rate of 5% of body weight twice daily.

Out of 360 healthy fish, 216 were stocked in 16 troughs, with toxicity tests conducted in triplicate for each CuNPs concentration, and 12 fish were placed per 20-L trough. Copper nanoparticle (CuNPs) concentrations of 0.2, 0.6, 1.0, 1.4, and 1.8 mg/L were used for a 96-hour exposure period, alongside a control group without CuNPs. To minimize the confounding effects of food intake on copper bioaccumulation and metabolism, food deprivation was implemented. This ensured that the fish were in a baseline metabolic state, which allowed for a clearer interpretation of the impact of CuNPs exposure on their physiological and biochemical parameters. The fish were not fed during the exposure, and the CuNPs solution in each trough was renewed every 24 hours. During the 96-hour exposure, dead fish were counted, and the LC_{50} (96 hours) of CuNPs for rainbow trout was determined as 1.1 mg/L, calculated using the Probit method (Finney, 1971).

Subsequently, fish (n=144) were exposed to three sub-lethal CuNPs concentrations: 0.2 mg/L, 0.6 mg/L, and 1.0 mg/L, based on the 96-hour LC_{50}. A control group was maintained without CuNPs exposure. Exposure durations were set at 7^{th}, 14^{th}, and 21^{st} days, with the experiment conducted in triplicate using 12 fish per treatment group. Water troughs were renewed every 48 hours, and parameters such as temperature, pH (7.2-7.8), dissolved oxygen, and dissolved carbon dioxide were monitored regularly, with constant aeration maintained throughout. Fish were fed twice daily (at 9 am and 5 pm) with dry pellets at 5% of their body weight prior to exposure. Fish waste was removed by siphoning from the

bottom of the troughs. The experimental troughs were exposed to a natural photoperiod (12 hours of light and 12 hours of darkness).

5.3.1. Sample collection

Fishes were sampled after 7^{th}, 14^{th}, and 21^{th} day of exposure. On each sampling day, 4 fishes from each replicate were sampled from each experimental group. At each sampling time, fishes were sacrificed and livers and gills were removed from control and CuNPs treated fish using cervical sectioning on ice for histological analysis, extra subsamples were collected and preserved in Bouin's fixative solution. Subsamples of tissue were extracted and kept at -80°C for subsequent antioxidant examination.

5.3.2. Histopathological examination

Liver and gill tissues were fixed in Bouin's solution, processed, and cut into sections 5-7 µm thick using a microtome. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX21, Model: CX21FS1, Tokyo, Japan) to evaluate histological alterations, following the methods described by (Martoja and Martoja, 1967) and (Soliman et al., 2022).

5.3.3. Antioxidant enzyme activity assays

Liver and gill tissues were excised from sacrificed fish and immediately homogenized in phosphate-buffered saline (PBS, pH 7.4). Homogenates were centrifuged at 10,000×g for 30 minutes at 4°C, and the supernatant was used for enzyme assays in accordance with the enzyme characterizations that were previously reported (Atli et al., 2016). Protein content was normalized using the Bradford assay (Bradford, 1976). The following antioxidant enzyme activities were measured: Catalase (CAT) activity following the method of (Lartillot et al., 1988). Lipid Peroxidation (LPO) levels measured via thiobarbituric acid reactive substances (TBARS) assay (Uchiyama & Mihara, 1978). Superoxide Dismutase (SOD) activity using the method of (McCord and Fridovich, 1969). Glutathione reductase (GR) and Glutathione-S-Transferase (GST) activities following (Habig et al., 1974).

5.3.4. Statistical analysis

Data analysis was conducted using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to compare mean values among the treatment groups. A

significance threshold of $p < 0.05$ was used. Statistical evaluations were carried out using SPSS software version 28.0.1.

Objective 4: To study the biochemical and histological effect of combined silver and copper nanoparticles in rainbow trout.

5.4. Experimental fish, collection and study design

The test fish fingerling rainbow trout (*Oncorhynchus mykiss*) with an average weight of 4.5g/fish and length of 5.20 ± 0.30 cm was obtained from the State Government Fishery Department Hatchery (Dachigam Laribal, Srinagar, Jammu & Kashmir). Fishes were transported in double polythene bags supplied with oxygen. The experiments were carried out in the Division of Fish Genetics and Biotechnology, SKUAST-Kashmir. Physical and chemical parameters of water were measured daily (during the experiment). fish were acclimated for 14 days under controlled laboratory conditions, with $5cm^3$ air stones and water temperature maintained between 15°C and 18°C and a 12h light:12h dark photoperiod. Fish were housed in aerated troughs with a water exchange rate of 50% every 48 hours, and water quality parameters were maintained within acceptable limits (pH 7.2-7.8, DO 7-7.3 mg/L, CO_2 6-6.5 mg/L). During acclimatization, fish were fed a commercial diet at 5% of their body weight daily. Prior to the experiment, fish were treated with potassium permanganate ($KMnO_4$) as a prophylactic measure (Ahmed and Ahmad, 2020).

Acute toxicity test lasting 96h was performed to calculate median lethal concentrations (96h LC50) of AgNPs and CuNPs that was already performed in our previous study (Khan et al., 2024a;b). Based on the 96-h LC50 values, that is 2.0 mg/L for AgNPs and 1.1 mg/L for copper nanoparticles, the following sublethal concentrations of silver 0, 0.2, 0.8, 1.4 mg L^{-1} and copper 0, 0.2, 0.6, 1.0 mg L^{-1} were used for toxicity test.

To perform the bioassays, fish (n=144) were exposed to NPs suspensions to exposure troughs, containing previously dechlorinated tap water, to obtain the following nominal test concentrations: T1 (0.2+0.2 mg L^{-1} AgNPs+ CuNPs), T2 (0.8+0.6 mg L^{-1} AgNPs+ CuNPs) and T3 (1.4+ 1.0 mg L^{-1} AgNPs+ CuNPs). The fish were exposed in triplicate with 12 fishes per treatment group for each concentration of (AgNPs + CuNPs), and were stocked into 12 tanks of 20 L water at the density. Experiment duration was set for

7th, 14th and 21st days under static conditions (50 % of water was changed after 48hours with re-dosing after each change). A control group was maintained without (AgNPs+ CuNPs) exposure. Water parameters such as temperature, pH (7.2-7.8), dissolved oxygen, and dissolved carbon dioxide were monitored regularly and a constant aeration was maintained throughout the experimental period. The fish were fed daily 2 times a day at 9am and 5pm with a commercial diet in the form of dry pellets at the rate of 5% of their body weight prior to the exposure. Fish feces were removed by siphoning from the bottom of the troughs. The experimental troughs were maintained under a natural photoperiod of 12 hours light and 12 hours darkness.

5.4.1. Sample collection

On the 7th, 14th, and 21st days of the experiment, 12 fish (four fish from each of three tanks) were sampled from each experimental group. The fish were euthanized using MS-222 (ethyl 3-aminobenzoate methane sulfonic acid, 1:5000, pH 7.5 adjusted with NaHCO3, Sigma Aldrich, UK). Livers and gills were carefully dissected through cervical sectioning on ice for histological analysis and were preserved in Bouin's solution and 4% paraformaldehyde. Tissue subsamples were extracted and stored at -80°C for subsequent antioxidant analysis.

5.4.2. Histopathological Examination

Liver and gill tissues were preserved in Bouin's solution, processed, and sectioned at 5-7 µm thickness using a microtome. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX21), following the method outlined by (Martoja and Martoja, 1967).

5.4.3. Antioxidant enzyme activity assays

Liver and gill tissues were excised from sacrificed fish and immediately homogenized in phosphate-buffered saline (PBS, pH 7.4). Homogenates were centrifuged at 10,000×g for 30 minutes at 4°C, and the supernatant was used for enzyme assays in accordance with the enzyme characterizations that were previously reported (Atli et al., 2016). Protein content was normalized using the Bradford assay (Bradford, 1976). The following antioxidant enzyme activities were measured: Catalase (CAT) activity following the method of (Lartillot et al., 1988). Lipid Peroxidation (LPO) levels measured via thiobarbituric acid

reactive substances (TBARS) assay (Uchiyama & Mihara, 1978). Superoxide Dismutase (SOD) activity using the method of McCord and Fridovich (1969). Glutathione reductase (GR) and Glutathione-S-Transferase (GST) activities following (Habig et al., 1974).

5.4.4. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to compare means between the different treatment groups. A significance level of $p < 0.05$ was applied. Statistical analysis was conducted using SPSS software (version 28.0.1).

5.4.5. Ethical statement

This study followed all applicable international, national, and institutional guidelines for the care and use of animals. The protocols used were approved by the Institutional Animal Ethics Committee (IAEC) in compliance with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under registration number 1809/GO/ReBi/S/15/CPCSEA. Furthermore, the study was conducted in alignment with the ARRIVE guidelines.

CHAPTER 6

6. RESULTS AND DISCUSSION

Objective 1: Synthesis and characterization of Ag and Cu nanoparticles.

6.1. Characterization of silver nanoparticles (AgNPs)

6.1.1. The UV-Vis spectroscopy of synthesized silver nanoparticles (AgNPs)

The UV-Vis spectroscopy analysis of the synthesized silver nanoparticles (AgNPs) revealed a characteristic surface plasmon resonance (SPR) peak at 418 nm as shown in (Figure 6.1). This sharp and distinct peak confirms the successful formation of monodisperse nanoparticles, indicating their uniform size and spherical shape. The spectrum, which measures absorbance across the wave length range of 200–800 nm, demonstrates strong light absorption at 418 nm, attributed to the collective oscillation of conduction band electrons in response to incident light. Beyond the SPR peak, the gradual decline in absorbance at longer wave lengths suggests a reduced interaction of the nanoparticles with light in the visible region, indicative of their well-dispersed nature and the absence of significant aggregation. The UV-Vis spectrum thus confirms the optical properties and successful synthesis of silver nanoparticles.

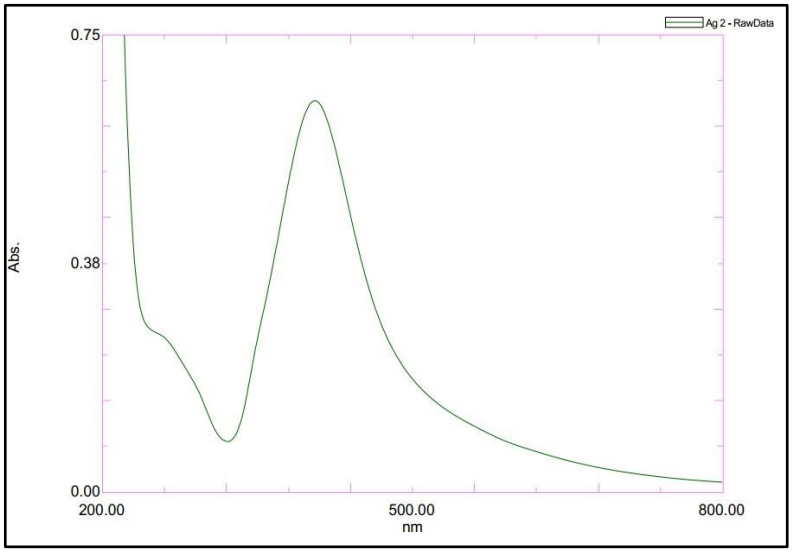

Figure.6.1. The confirmation of UV-visible spectrum of silver nanoparticles (AgNPs).

6.1.2. The X-ray diffraction (XRD) pattern of the synthesized silver nanoparticles (AgNPs)

The X-ray diffraction (XRD) analysis of the synthesized silver nanoparticles (AgNPs) confirmed their crystalline nature and structural integrity as shown in (Figure 6.2). The diffraction pattern displayed distinct peaks at 38°, 46°, 64.4°, and 77°, corresponding to the (111), (200), (220), and (311) crystal planes, respectively. These planes are characteristic of silver's face-centered cubic (FCC) crystal structure. The sharpness and high intensity of the observed peaks indicate a high degree of crystallinity, reflecting well-ordered atomic arrangements within the nanoparticles. Additionally, the absence of significant noise or broad peaks in the pattern suggests that the AgNPs are largely free from amorphous content and impurities. This confirms the successful synthesis of high-purity crystalline silver nanoparticles.

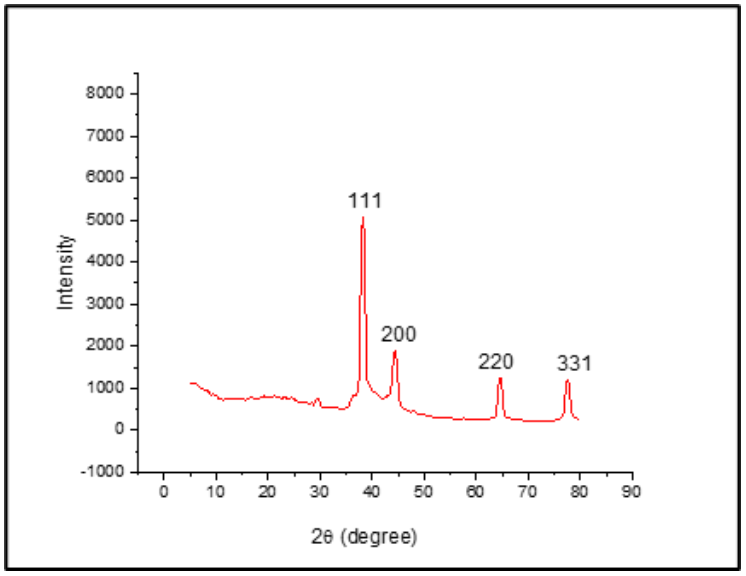

Figure 6.2. The XRD analysis of silver nanoparticles (AgNPs)

6.1.3. The FTIR spectrum of the synthesized silver nanoparticles (AgNPs)

The FTIR spectrum of the synthesized silver nanoparticles (AgNPs) reveals the functional groups responsible for their reduction and stabilization as shown in (Figure 6.3). Key peaks are observed at ∽3434.6, 2922.6, 1637.3, 1384.6, 1026.6, and 613.3 cm^{-1}, indicating the involvement of specific chemical groups. The broad peak at ∽3434.6 cm^{-1} corresponds to NH stretching of heterocyclic amines, while the peak at ∽2922.6 cm^{-1} is

attributed to asymmetrical and symmetrical stretching of methylene C-H groups. The strong absorption at ∽1637.3 cm^{-1} corresponds to nitrate ion (NO_3^-) stretching vibrations, confirming the presence of nitrates as part of the capping agents. Additionally, the intense peaks at ∽1384.6 cm^{-1}, ∽1026.6 cm^{-1}, and ∽613.3 cm^{-1} are associated with primary CN stretching and alkylene C-H bending, further highlighting the functional groups involved in nanoparticle stabilization.

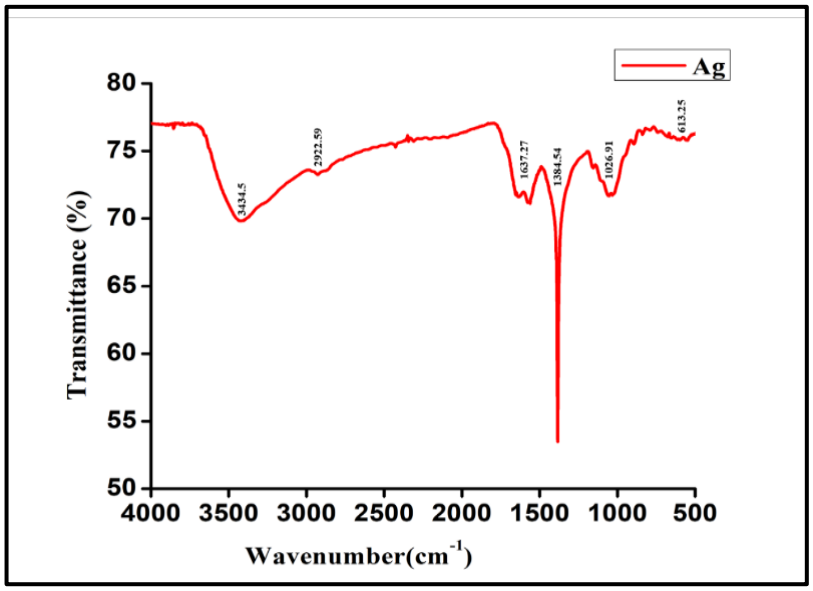

Figure 6.3. FTIR spectroscopy identified in silver nanoparticles (AgNPs)

6.1.4. Scanning Electron Microscopy (SEM)

The morphology and size of the synthesized nanoparticles were examined using scanning electron microscopy (SEM), as shown in (Figure 6.4). The SEM image reveals that the nanoparticles are predominantly spherical with a uniform size distribution. The average particle diameter was determined to be approximately 50 nm, corroborating the findings from the size distribution analysis. The particles exhibit smooth surfaces and well-defined boundaries, indicating the efficacy of the synthesis process in producing high-quality nanoparticles.

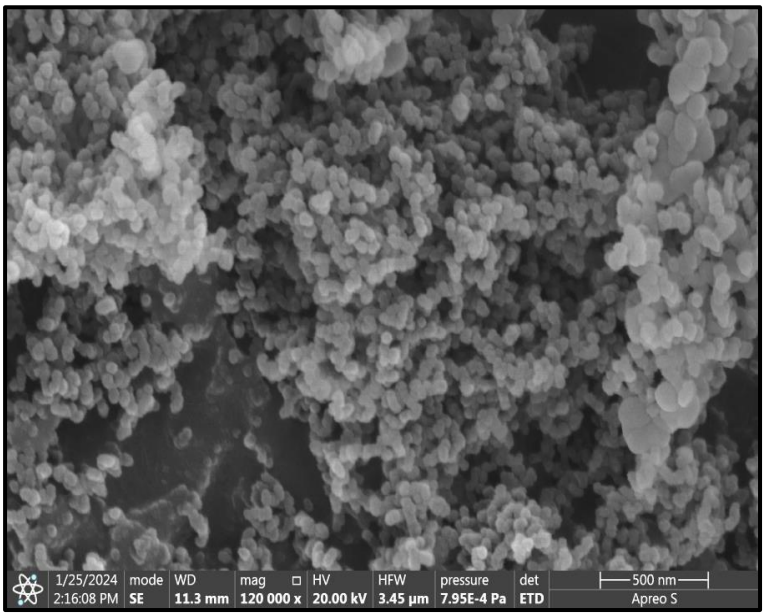

Figure 6.4. SEM analysis of silver nanoparticles (AgNPs)

6.1.5. Zeta potential analysis of silver nanoparticles

The size distribution function depicted in (Figure 6.5) represents the particle size distribution within the sample. The x-axis corresponds to particle size, while the y-axis indicates the relative frequency or intensity of particles within a given size range. The graph shows a sharp peak at approximately 50 units, indicating a predominantly monodisperse sample with most particles concentrated around this size. The steep decline beyond the peak suggests a minimal presence of larger particles, while the absence of a significant distribution toward smaller sizes implies negligible fragmentation or smaller particles in the sample. Furthermore, zeta potential measurements of -28 mV confirm the nanoparticles excellent colloidal stability. The high negative surface charge generates strong electrostatic repulsion, effectively preventing aggregation and ensuring the nanoparticles remain well-dispersed in the colloidal system.

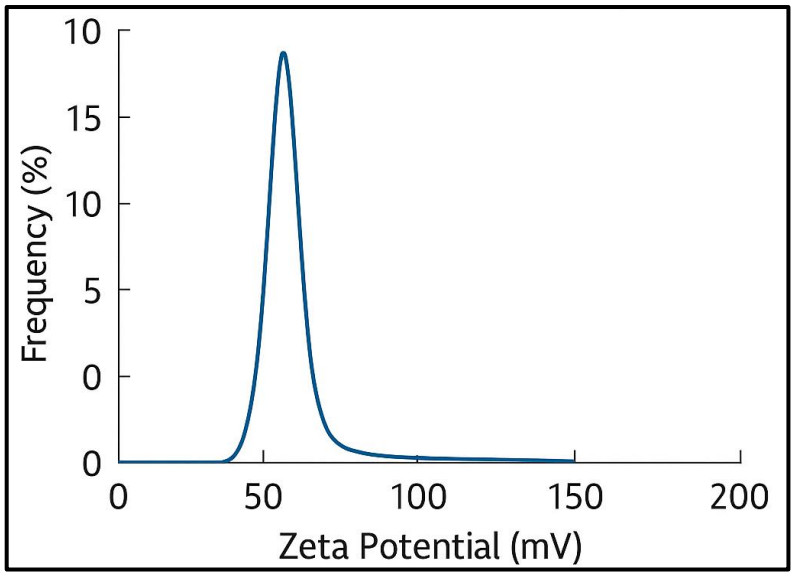

Figure 6.5. Zeta analysis of silver nanoparticles (AgNPs).

6.2. Characterization of copper nanoparticles (CuNPs)

6.2.1. UV-Vis Spectroscopy of Copper nanoparticles

The UV-Vis absorption spectrum reveals a sharp peak at approximately 400 nm, characteristic of copper nanoparticles (CuNPs) exhibiting surface plasmon resonance (SPR). This confirms the successful formation of monodisperse CuNPs with uniform size and spherical shape. The peak at 400 nm is due to the collective oscillation of conduction band electrons on the nanoparticle surface in resonance with incident light as shown in (Figure 6.6). The well-defined peak indicates minimal aggregation and supports the nanoparticles mono dispersity. The spectrum also shows a gradual decrease in absorbance at higher wave length, indicating reduced interaction with light. This confirms the synthesis of CuNPs.

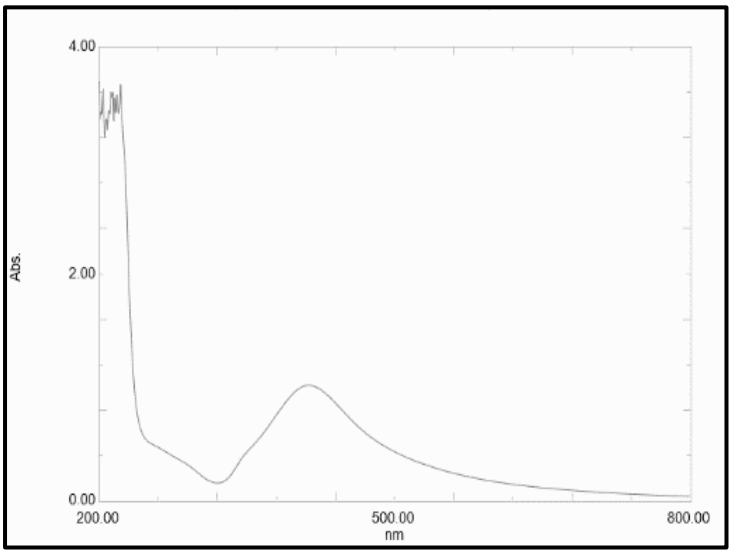

Figure 6.6 UV-visible absorption spectrum of copper nanoparticles (CuNPs).

6.2.2. XRD of copper nanoparticles (CuNPs)

The XRD pattern displayed in (Figure 6.7) reveals distinct peaks at 2θ values of approximately 43.4°, 50.5°, and 74.2°, which correspond to the (111), (200), and (220) planes of copper (Cu), respectively. The intense peak at 43.4° represents the (111) plane, indicating a preferential orientation and higher atomic density in this direction. The peaks at 50.5° and 74.2° confirm the presence of (200) and (220) planes, respectively. The sharpness and intensity of these peaks highlight the material's high crystallinity and purity, with the absence of additional peaks indicating minimal impurities or secondary phases, confirming the successful formation of the material.

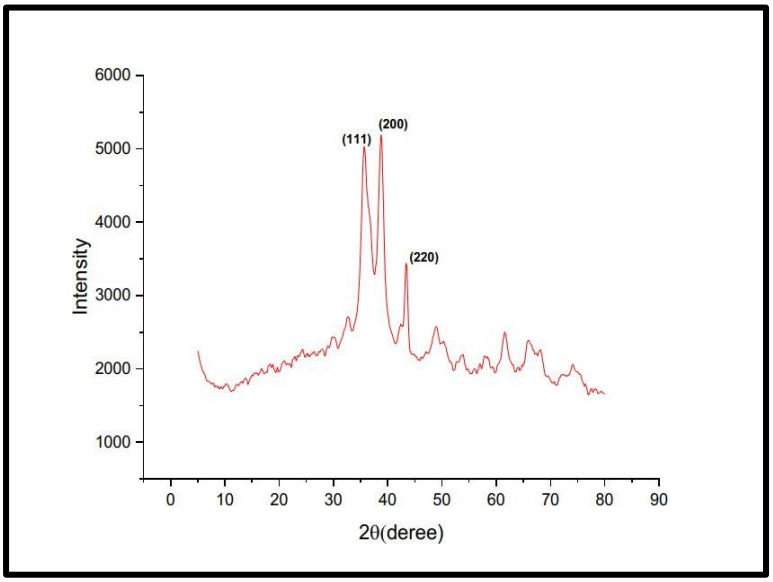

Figure 6.7 XRD analysis of copper nanoparticles (CuNPs)

6.2.3. Fourier transform infrared (FTIR) analysis of copper nanoparticles

The FTIR spectrum shown in Figure (6.8) highlights the functional groups and chemical bonds present on the surface of copper nanoparticles (CuNPs). The spectrum exhibits key absorption peaks at 3427, 1469, 1378, 844, and 526 cm^{-1}, corresponding to different functional groups associated with the CuNPs surface. The prominent peak at 3427 cm^{-1} is attributed to O-H stretching vibrations, suggesting the presence of hydroxyl groups, likely from surface hydroxylation or adsorbed water. A strong absorption peak at 1469 cm^{-1} corresponds to N-H bending vibrations, indicating the presence of amine groups. The peak at 1378 cm^{-1} is associated with C-H bending, signifying the existence of organic moieties on the nanoparticle surface. Additionally, peaks at 844 cm^{-1} and 526 cm^{-1} are attributed to Cu-O vibrations, confirming the formation of copper-oxygen bonds. The FTIR analysis confirms the successful surface functionalization of CuNPs, as evidenced by the observed functional groups.

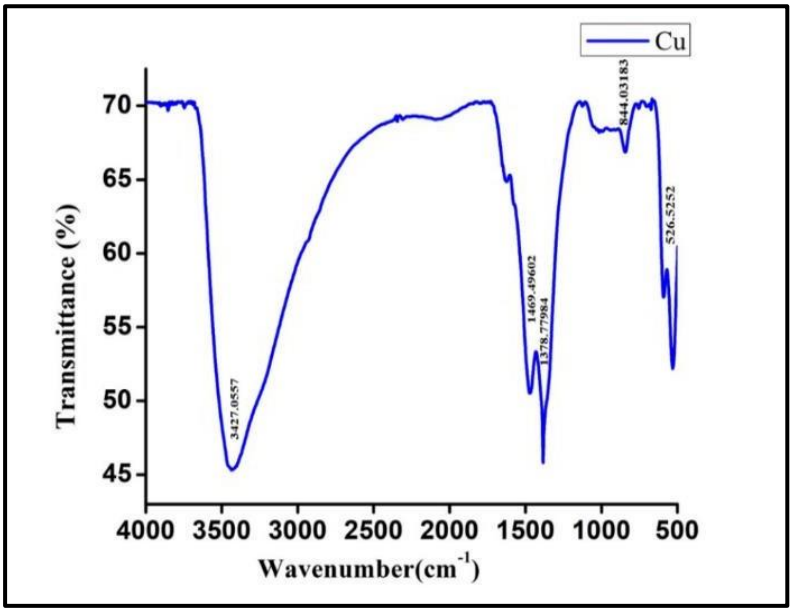

Figure 6.8 FTIR analysis of copper nanoparticles (CuNPs)

6.2.4. Scanning electron microscopy (SEM) analysis

The SEM image in (Figure 6.9) provides a high-resolution view of the morphology and surface characteristics of the synthesized copper nanoparticles (CuNPs). The nanoparticles are predominantly spherical with an average size of approximately 60 nm, and the size distribution is uniform. The image also shows good dispersion with minimal agglomeration, indicating effective stabilization during synthesis, likely due to the use of

capping agents. The absence of significant clustering further suggests well-controlled nanoparticle formation. Additionally, the fine granular texture observed on the nanoparticle surfaces may enhance surface area, improving their interactions with the surrounding environment. The magnification and scale bar (200 nm) confirm the nanoscale dimensions of the particles. Overall, the SEM analysis confirms the successful synthesis of well-dispersed, spherical CuNPs with controlled size and minimal agglomeration.

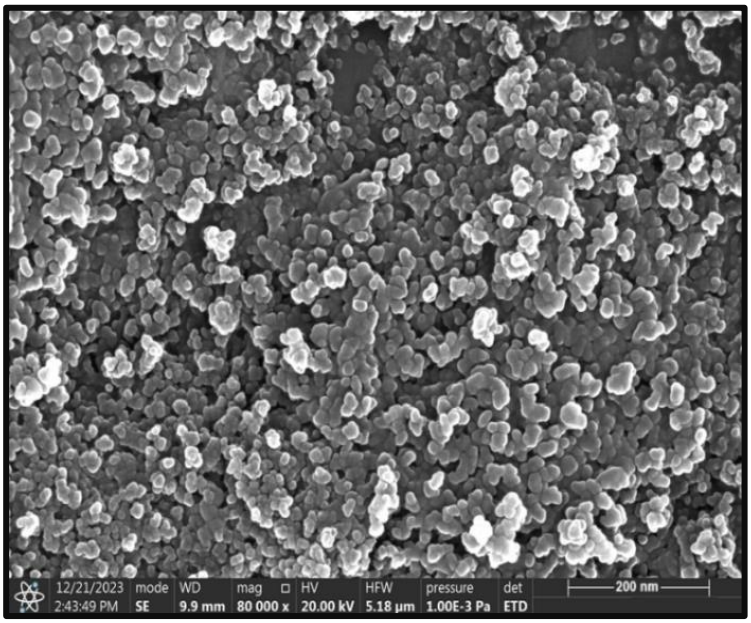

Figure 6.9 SEM analysis of copper nanoparticles (CuNPs).

6.2.5. Zeta potential analysis of copper nanoparticles

The Zeta potential measurements, shown in (Figure 6.10) indicate a surface charge of -28 mV for the synthesized copper nanoparticles (CuNPs). This negative zeta potential value suggests that the CuNPs exhibit sufficient electrostatic repulsion, preventing aggregation and promoting colloidal stability. The negative charge is likely a result of surface functionalization, which imparts stability by preventing particle coalescence through electrostatic repulsion. The measured zeta potential confirms that the CuNPs are colloidally stable and exhibit minimal aggregation under standard conditions, ensuring their stability in solution.

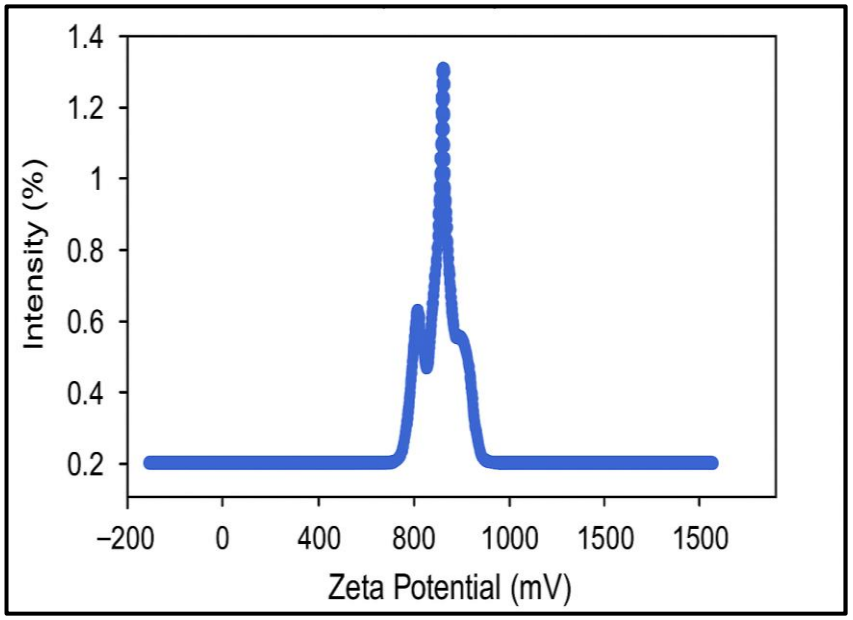

Figure 6.10. ZETA of copper nanoparticles (CuNPs)

6.3. Objective 2: To study the biochemical and histological effect of silver nanoparticles in rainbow trout.

6.3.1. Biochemical Enzyme assay in gill and liver.

The results of biochemical enzyme assays demonstrated significant alterations in superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities as shown in (Figure 6.11 and 6.12). SOD and CAT activities were significantly enhanced in the T1 and T2 groups, peaking at day 14th day as compared to control group before declining in the T3 group at day 21th. The highest CAT activity was observed in the gills of the T2 group (16.9 U/mg protein, $p < 0.05$). Lipid peroxidation (LPO), measured by TBARS, showed a declining trend in both gills and liver throughout the sampling period, indicating that AgNPs did not induce significant oxidative damage to lipids. GST activity decreased ($p < 0.05$) significantly in gills and liver over the sampling period, while an incremental increase was observed with increased AgNPs concentrations. Glutathione reductase (GR) activity displayed a dose-dependent increase, with the highest activity recorded in the gill and liver of the T2 group (10.2 U/mg protein). However, a decline in antioxidant activity in the T3 group at day 21th day suggested that prolonged exposure to higher AgNPs concentrations overwhelmed the antioxidant defense system.

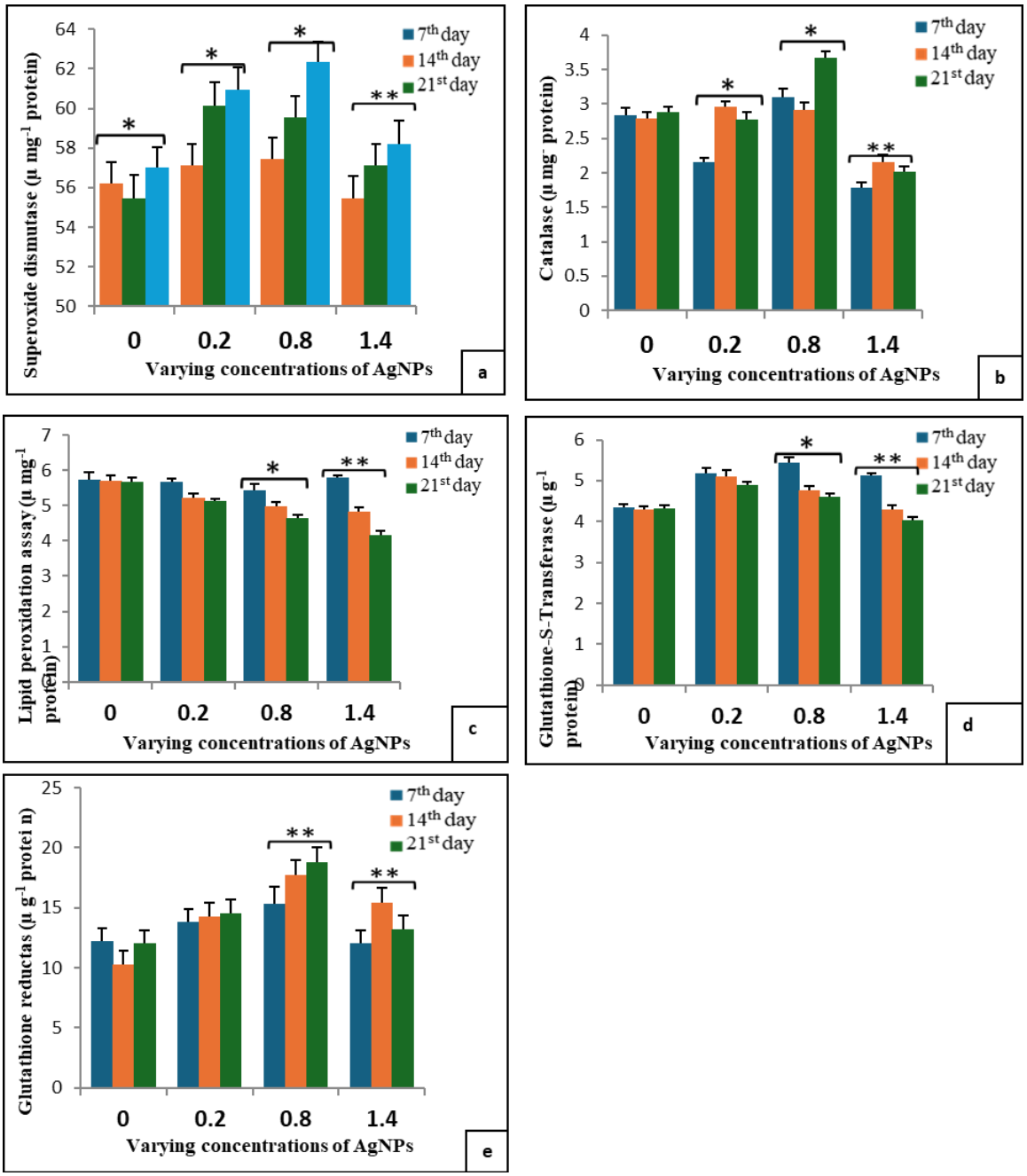

Figure 6.11. Biochemical enzyme assay of gills in rainbow trout (*Oncorhynchus mykiss*) after exposure to varied concentrations of AgNPs compared to the control (without AgNPs). Figures (a–e) represent the activities of (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) lipid peroxidation (LPO), (d) glutathione-S-transferase (GST), and (e) glutathione reductase (GR), respectively. Statistical significance among groups was analyzed using one-way ANOVA followed by a suitable post hoc test ($p < 0.05$).

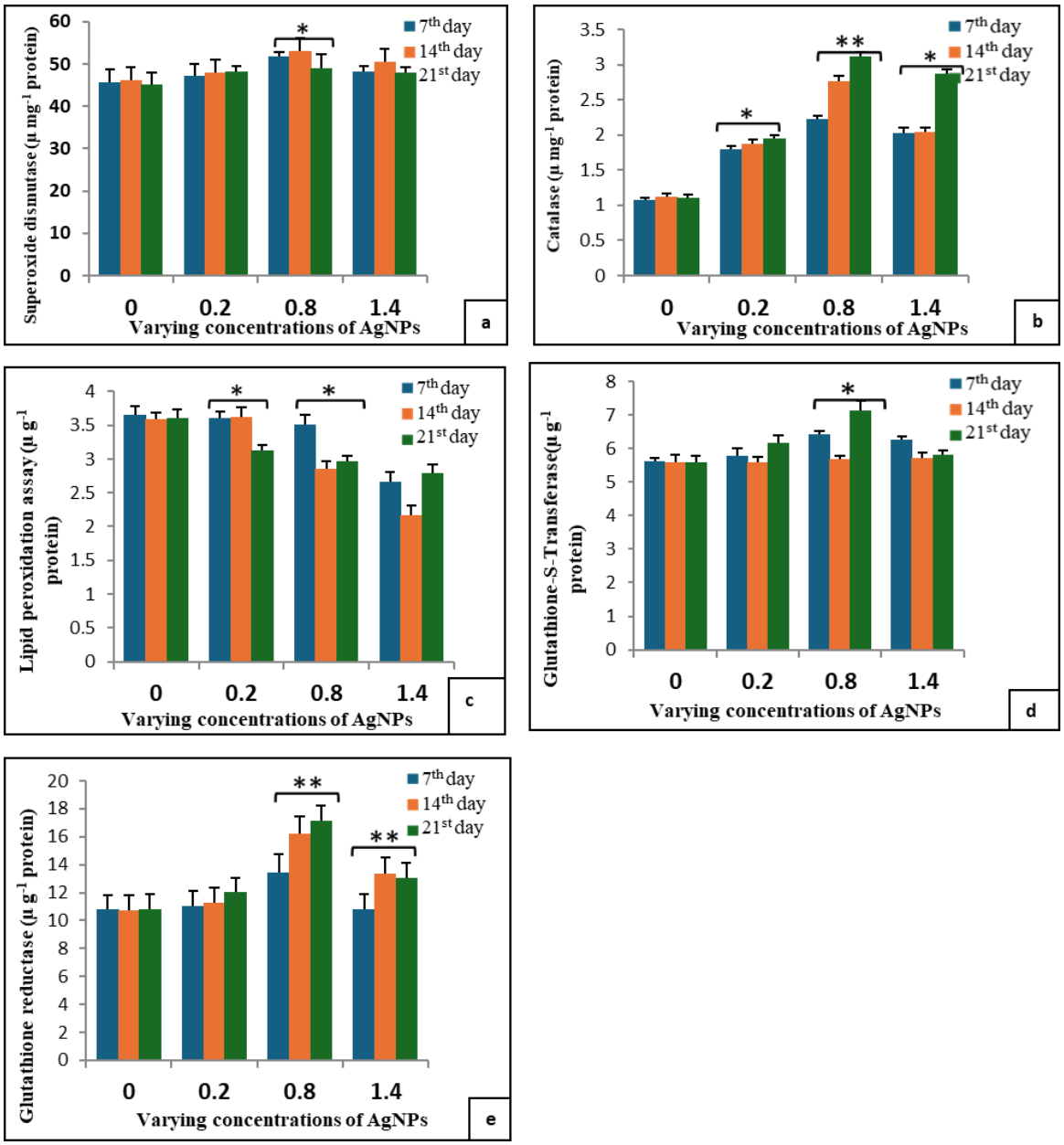

Figure 6.12. Biochemical enzyme assay of liver in rainbow trout (*Oncorhynchus mykiss*) following exposure to varying concentrations of silver nanoparticles (AgNPs) compared to the control group (without AgNPs). Panels (a–e) represent the activities of (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) lipid peroxidation (LPO), (d) glutathione-S-transferase (GST), and (e) glutathione reductase (GR), respectively, in liver tissue of experimental fish. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with significance considered at ($p < 0.05$).

6.3.2. Histopathological changes in gill and liver

Histological examination of the gills revealed dose-dependent morphological changes as demonstrated in (Figure 6.13). The control group exhibited normal gill architecture, with well-defined lamellae and minimal cellular infiltration. Moreover, stratified epithelium and mucous cells border the primary lamellae between secondary lamellae, and faintly pigmented chloride cells are dispersed throughout the base of lamellae and the interlamellar region. In the T1 group (0.2 mg/L), mild hypertrophy and cellular infiltration and adjoining

of gill lamellae were observed by day 14th and 21th day of AgNPs exposure. Fish in the T2 group (0.8 mg/L) showed more pronounced congestion of blood vessels in primary as well as secondary lamellae and proliferation in goblet and chloride cells throughout the sampling period. The most severe damage was observed in the T3 group (1.4 mg/L), where fish exhibited necrosis**,** hyperplasia of epithelial cells, uplifting of cellular elements, and lamellar fusion by day 21.

Histological sections of the liver also demonstrated significant pathological changes (Figure 6.14). In the control group, hepatocytes appeared normal with intact nuclei and well-organized sinusoids. By the day 14th and 21st, fish in the T1 group exhibited mild congestion in the hepatic blood vessels and sinusoids, while the T2 group showed hemorrhages and vacuolation of hepatocytes. The T3 group exhibited severe necrosis**,** degeneration of hepatic tissue**,** and increased kuffer cell proliferation**,** indicating extensive liver damage due to high AgNPs exposure observed at 14th and 21th day.

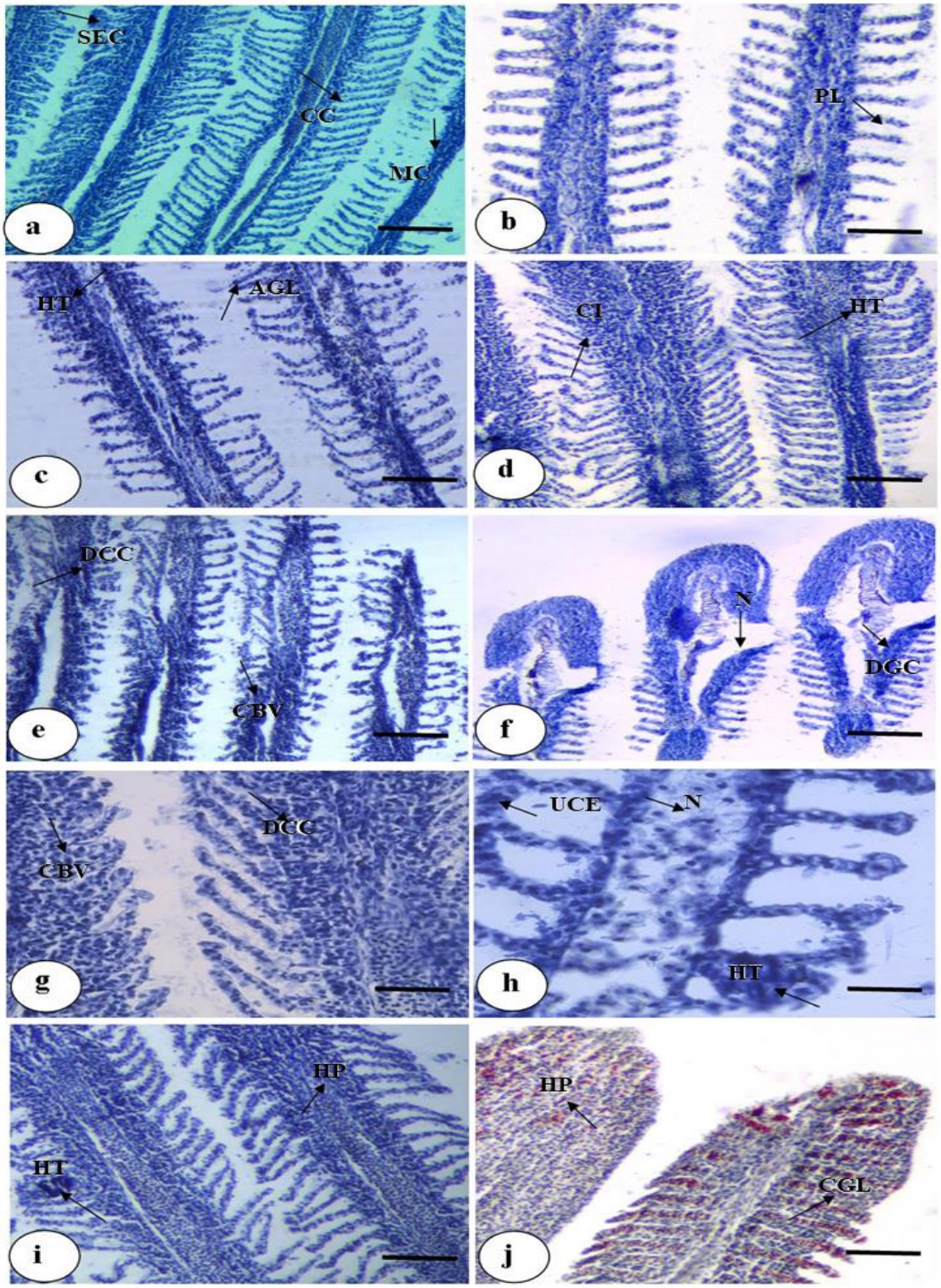

Figure 6.13. Histopathological images of AgNPs treated gills of *Oncorhynchus mykiss* fingerlings. Control (a) CC: chloride cells; MC: mucous cells; SEC: stratified epithelium cells, 0.2 mg/L concentration (b, c and d) PL: primary lamellae; HT: hypertrophy; AGL: adjoining gill lamellae; CI: cellular infiltration, 0.8 mg/L concentration (e, f and g) CBV: congested blood vessels; DCC: deformities in chloride cells; N: necrosis, 1.4 mg/L concentration (h, i and j) HT: hypertrophy; HP: hyperplasia; CGL: congested gill lamellae; UCE: uplifting of cellular elements; N: necrosis. Scale bar: 10 µm

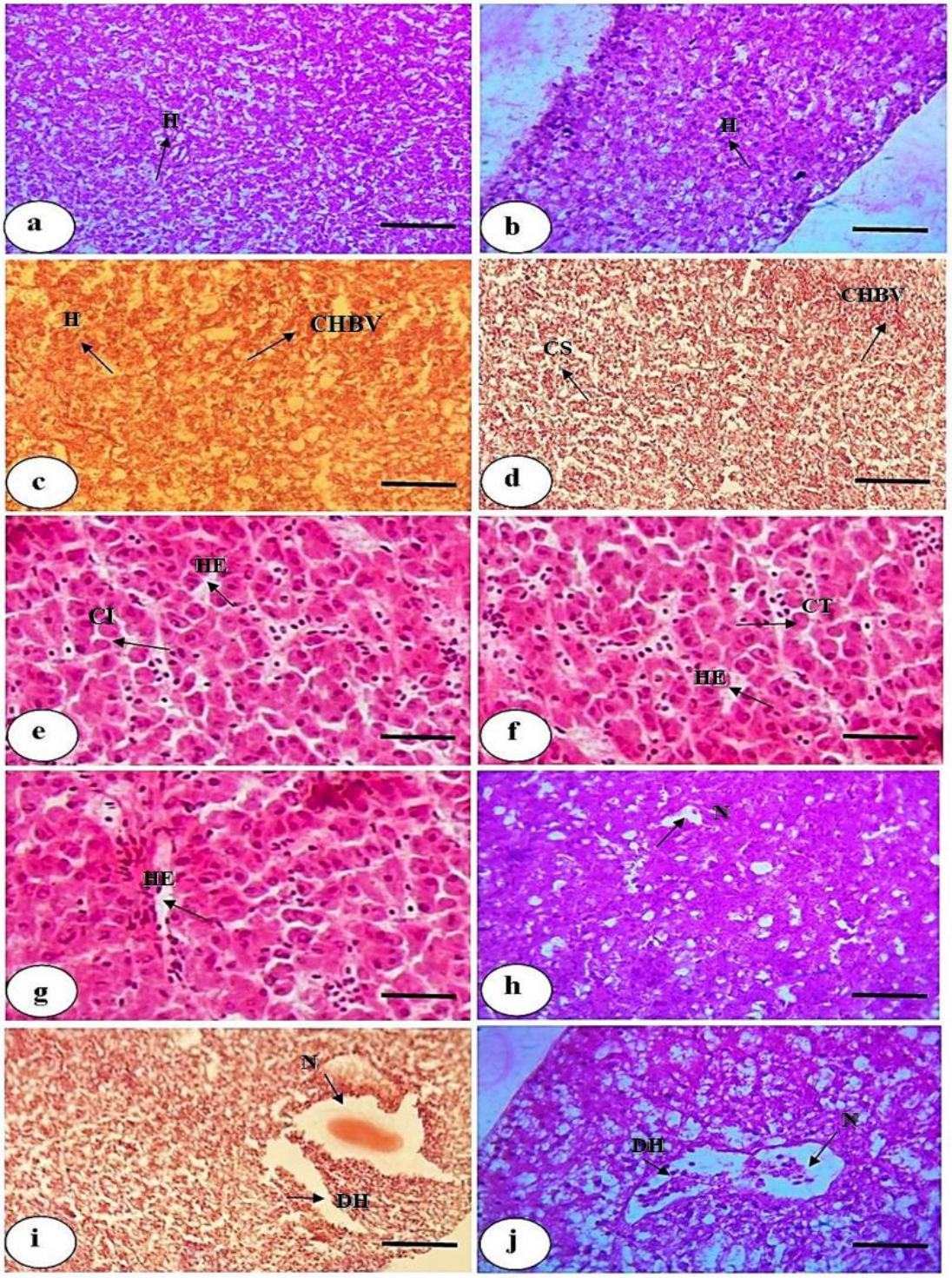

Figure 6.14. Histopathological images of AgNPs treated liver of *Oncorhynchus mykiss* fingerlings. Control (a) H: hepatocytes; 0.2 mg/L concentration (b, c and d) H: hepatocytes; CS: congested sinusoids; CHBV: congested hepatic blood vessels, 0.8 mg/L concentration (**e, f** and g) HE: hemorrhages; CI: cellular infiltration, 1.4 mg/L concentration (h, i and j) N: necrosis; DH: degeneration of hepatic tissue. Scale bar: 10 μm

6.4. Discussion

The escalating production and utilization of silver nanoparticles (AgNPs) have led to their accumulation in aquatic ecosystems, raising concerns regarding their potential toxicity to aquatic organisms (Ottoni et al., 2020; Schlich et al., 2013). Despite the increasing prevalence of AgNPs, the toxicological impacts of their exposure, particularly in fish, remain inadequately explored. The effects of AgNPs on rainbow trout (*Oncorhynchus mykiss*), highlighting a dose-dependent response across biochemical stress, and histopathological parameters. The findings extend the current understanding of AgNPs toxicity. Moreover, the nanoparticle size plays a crucial role in determining toxicity levels. In this study, the AgNPs had an average size of around 50 nm, which aligns with the common size range used in industrial applications (10–100 nm). This size range is especially relevant for toxicology research, as it reflects the nanoparticle sizes frequently found in consumer products and various industrial environments (Matzke et al., 2014; Bacchetta et al., 2017).

The biochemical enzymes activity results further elucidate the oxidative stress mechanisms involved in AgNPs toxicity. The study provides crucial insights into the impact of AgNPs exposure on the oxidative damage and antioxidant defense system of the organisms under investigation (Liu et al., 2020). Catalase and superoxide dismutase are crucial enzymes in regulating reactive oxygen species (ROS) levels in organisms, serving as bioindicators of elevated ROS generation (Akter et al., 2018). Initially, after the 7^{th} and 14^{th} day the upregulation of enzymes such as superoxide dismutase (SOD) and catalase (CAT) in both gill and liver was observed compared to the control groups, suggests an adaptive response aimed at mitigating oxidative stress induced by AgNPs exposure. Same results for these parameters were noted in Nile Tilapia, *Oreochromis niloticus* after AgNPs exposure (Mabrouk et al., 2021). Besides, an increased activity of these enzymes may be a fish's attempt to counteract the increased production of reactive oxygen species (ROS) generation by AgNPs. However, the eventual decline in these enzyme activities in the highest concentration group (1.4 mg/L) indicates that prolonged or high-dose exposure overwhelms the fish's antioxidant defense. This pattern of initial enzyme overexpression followed by depletion is in line with recent research on the toxicity of nanoparticles, which shows that oxidative stress is a major factor in cellular damage (Tee et al., 2016).

The study found that during the sample periods, the amount of lipid peroxidation (LPO) in the liver and gills decreased. The decrease in AgNPs content raises the possibility that exposure to AgNPs may not result in considerable oxidative damage to lipids or extensive oxidative damage to cell membranes. The study discovered a substantial decrease in glutathione S-transferase (GST) activity in the gills over the course of the sampling period, which implies that AgNPs exposure may be inhibiting the activity of this detoxification enzyme. Similar results were found in the gill tissues of *O.mossambicus* experimented with AgNPs (Sibiya et al., 2022). The liver showed a significant incremental increase in GST activity with an increase in AgNPs concentrations. The difference in responses between gills and liver may be due to the varying responses of different tissues to AgNPs exposure. The study showed a significant improvement in glutathione reductase activity after exposure to AgNPs (0.8 mg/L) after 7-14 days, followed by a reduction on all sampling days. The nonlinear response suggests a complex interaction between AgNPs exposure and GR activity, an enzyme responsible for maintaining the reduced form of glutathione for antioxidant defense (Liu et al., 2020).

Histopathological examination of the gills and liver corroborates the physiological and molecular findings, highlighting the tissue-specific damage caused by AgNPs exposure. The gills, as the primary site of gas exchange and nanoparticle entry, exhibited marked pathological changes, including necrosis, lamellar fusion, hypertrophy, uplifting of cellular elements and hyperplasia of epithelial gill filaments. AgNPs exposure in the T1 group showed early alterations, including hypertrophy, cellular infiltration, and gill lamellae adjoining, indicating stress and structural modifications in gill tissues even at the lowest concentration. The T2 group displayed significant gill tissue deformities, including changes in chloride and goblet cells, and blood vessel congestion in primary and secondary lamellae during the sampling period. (Ostaszewska et al., 2016) found dilated blood vessels, epithelial necrosis, and epithelium hypertrophy in Siberian sturgeon (*Acipenser baerii's)* gills, consistent with our findings. Goblet cells indicate increased mucus production due to irritants or stress, while blood vessel congestion suggests gill circulatory system disruption, potentially affecting oxygen exchange. The T3 group showed the most significant changes in gill tissues throughout all sampling days, leading to extensive damage, cell death and disruptions in the structural integrity of the gills, with severe damage observed at higher doses. Gill damage was also found to be severe at greater doses of AgNPs compared to the lowest concentration in a different investigation conducted on

Cyprinus carpio (Kakakhel et al., 2021). These structural alterations likely contributed to the reduced hematological parameters, as impaired gill function would directly impact oxygen uptake.

The histopathological examination of liver tissues exposed to AgNPs shows a dose-dependent effect on hepatic morphology. The control group, with typical healthy liver features, serves as a baseline, displaying normal hepatocytes, sinusoidal architecture, and spherical nuclei, indicating intact liver integrity. This observation is consistent with (Naguib et al., 2020) who reported similar hepatocyte organization in *Clarias gariepinus*, with a cord-like arrangement interspersed with blood vessels and sinusoids. The liver, a key organ for detoxification and metabolism, displayed signs of severe damage, ranging from congestion and vacuolation to necrosis, particularly in the higher AgNPs concentration group. In the T1 group, AgNPs exposure caused congestion in hepatic blood vessels and sinusoids at 14 and 21 days, indicating an early liver response. The intact hepatocyte structure suggests adaptive changes in the liver's vasculature. These findings highlight the liver's resilience to foreign nanoparticles. Fish livers exposed to 0.1 and 0.5 mg/L of silver nanoparticles (AgNPs) showed signs of hepatocyte vacuolation and increased size reported by (Ostaszewska et al., 2016). The study found severe histological changes in the T2 group, including hemorrhage and infiltration of round cells, indicating a heightened immune response and potential hepatic microenvironment disruption, emphasizing the need for a closer examination of the underlying mechanisms. The T3 group exhibited marked degeneration and necrosis of hepatic tissue, including cellular vacuolation, dilated sinusoids, and an increase in Kupffer cell populations at 14–21 days post-exposure to AgNPs. The observed proliferation of Kupffer cells in the liver likely reflects an inflammatory response, potentially triggered by nanoparticle accumulation and oxidative stress. These findings align with previous studies indicating that nanoparticle accumulation in fish liver tissues induces toxic effects (Kakakhel et al., 2021). Overall, this histological evidence emphasizes the severe, tissue-specific toxicity of AgNPs and highlights the central role of oxidative damage in nanoparticle-induced hepatic pathophysiology. A study on Nile tilapia, (*Oreochromis niloticus),* found congested central vein, sinusoids, and engorged portal blood vessels in the liver section after exposure to AgNPs (Mabrouk et al., 2021). These severe alterations signify a sustained and detrimental impact, indicating a critical threshold beyond which the liver's adaptive mechanisms are overwhelmed, leading to extensive tissue damage and loss of cellular viability.

6.5 Objective 2: To study the biochemical and histological effect of copper nanoparticles in rainbow trout.

6.5.1 Biochemical enzyme activity response mediated by copper nanoparticles (CuNPs)

The results of biochemical parameters of gill and liver are showed in (Figure 6.15 and 6.16). In both gill and liver superoxide Dismutase (SOD) there were no significant changes ($p > 0.05$) in SOD activity across all treatments and sampling days i.e 7^{th}, 14^{th} and 21^{nd} days of exposure, indicating SOD may not play a central role in the antioxidant defence against CuNPs exposure. Catalase (CAT) activity increased significantly ($p < 0.05$) with CuNPs concentration, particularly at 1.0 mg/L by day 14, reflecting an adaptive response to elevated oxidative stress. However, a decline in CAT activity was observed on day 21 in the 1.0 mg/L group, possibly due to enzyme depletion. However, lipid Peroxidation (LPO) Contrary to expectations, LPO levels showed a declining trend in both gills and liver across all sampling i.e. at 7^{th}, 14^{th} and 21^{st} days of exposure periods, suggesting that compensatory antioxidant mechanisms may have been activated (Figure 6.15c and Figure 6.16c). The Glutathione-S-Transferase (GST) and Glutathione Reductase (GR) both activities remained largely unaffected except for a transient increase in GR activity in the 0.6 mg/L group on day 14^{th}, indicating limited involvement of these enzymes in the oxidative stress response (Figure 6.15d, 6.16d). GST activity for both gills and liver were insignificant ($p > 0.05$) throughout the experiment. While the activity of GR in both gills and liver showed a positive response up to a certain level and thereafter reduction in this parameter was noted.

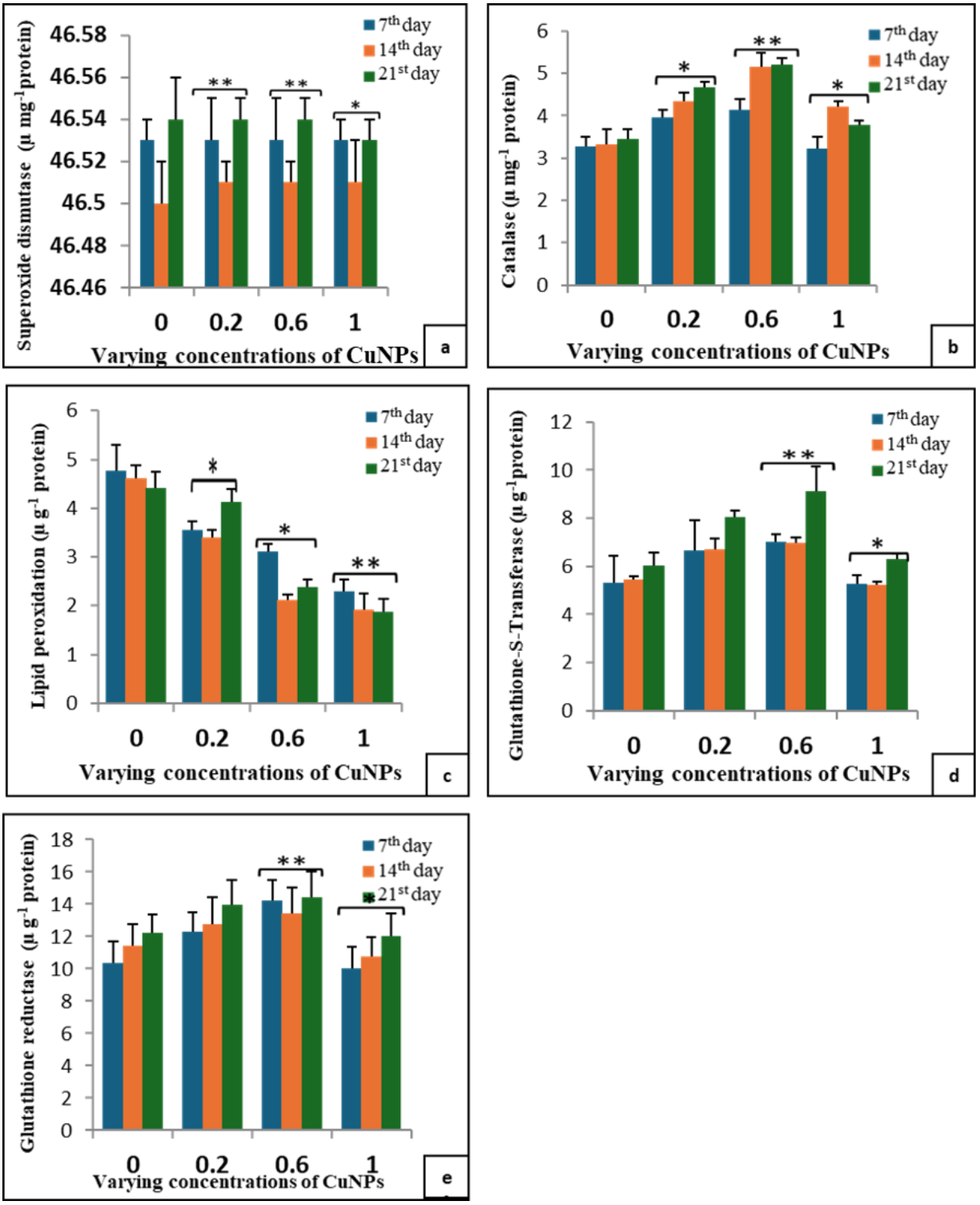

Figure 6.15. Antioxidant status of gills in rainbow trout (*Oncorhynchus mykiss*) after exposure to varied concentrations of CuNPs, with the control group unexposed to CuNPs. Figures (a), (b), (c), (d), and (e) represent the activities of SOD, CAT, LPO, GST, and GR, respectively, in gill tissues of *O. mykiss* exposed to different CuNPs concentrations. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test, with significance considered at ($p < 0$ 0.05).

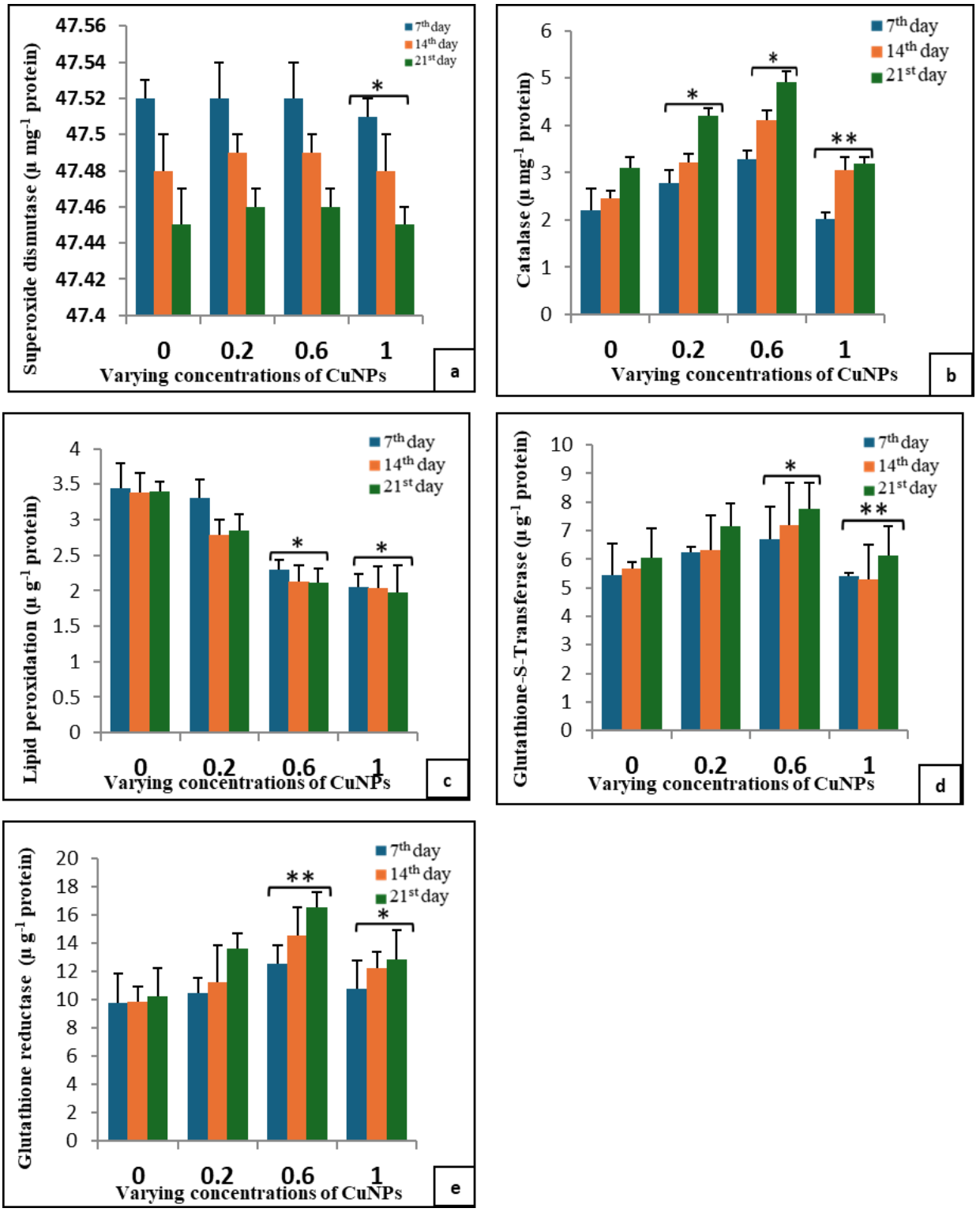

Figure 6.16. Antioxidant status of liver in rainbow trout (*Oncorhynchus mykiss*) exposed to different concentrations of CuNPs compared to the control. Figures (a, b, c, d, e) represent SOD, CAT, LPO, GST, and GR activities, respectively. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with significance considered at ($p < 0.05$).

6.5.2 Histopathological analysis of gill and liver exposed to copper nanoparticles (CuNPs)

The histological results of gills and liver are illustrated in (Figure 6.17 and 6.18) respectively. In control group, histological section of gills was without any deformities and showed well developed architecture of primary and secondary gill lamellae. However, a series of histopathological changes were observed in the gills after the exposure of different CuNPs concentrations. Likewise, in all the treatment groups, non-developed and fusion of gill lamellae were noted. Moreover, in T1 group, histological sections showed hypertrophy and hyperplasia during 14^{th} and 21^{st} days of CuNPs exposure. In addition to this, several deformities were recorded in T2 group which includes reduction in primary and secondary lamellae, fusion of secondary lamellae and cellular hyperplasia. However, at higher concentration i.e. at T3 group, more severe changes were observed throughout the experiment including uplifting of outer epithelium, congestion of blood in primary and secondary filaments, necrosis, hypertrophy, hyperplasia and cellular degeneration of epithelial tissues.

Liver sections of control group showed hepatocytes with normal cytoplasm along with a nucleus. After 7^{th} day of sampling, T_1 group showed various deformities in the liver parenchyma which includes presence of kuffer cells, hyaline degeneration and shrinkage of hepatocytes. In T_2 group, there was blood aggregation in the blood vessels. Besides, necrosis and cytoplasmic vacuolation were seen throughout the sampling period. While at higher concentration of CuNPs i.e. at T_3 group, liver sections showed blood sinusoids, hepatocellular necrosis and pyknotic nuclei.

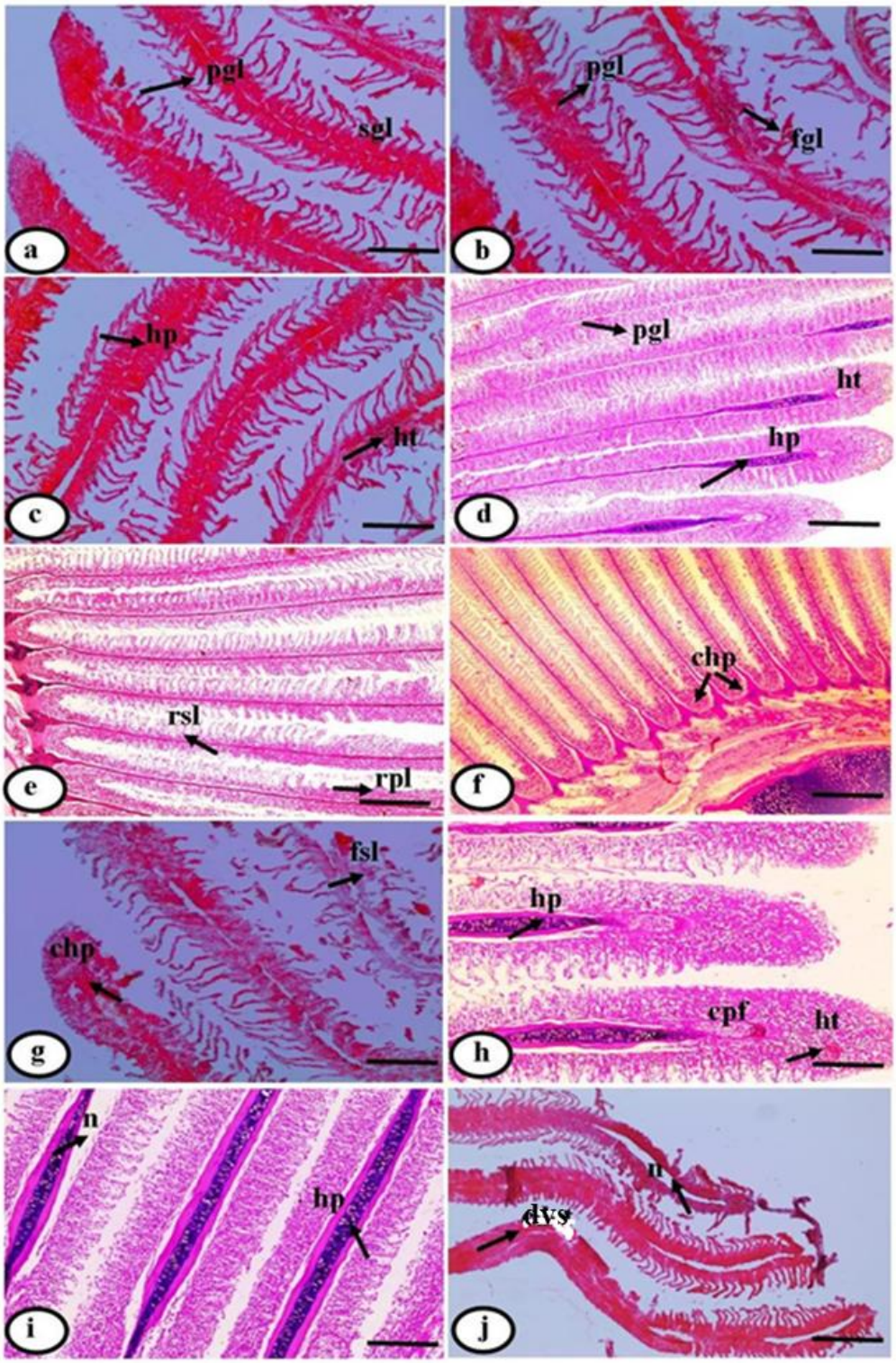

Figure 6.17. Histopathological images of CuNPs treated gills of *Oncorhynchus mykiss* fingerlings. Control (a) PGL: Primary gill lamellae, SGL: Secondary gill lamellae, 0.2 mg/l concentration (b, c and d) PGL: Primary gill lamellae; FGL: Fused gill lamellae; HP: Hyperplasia; HT: Hypertrophy, 0.6 mg/L concentration (e, f and g) RSL: Respiratory surface lamellae, SGL: Secondary gill lamellae; CHP: Chloride cell hyperplasia; FSL: Fusion of secondary gill lamellae, 1.0 mg/l concentration (h, i and j) HP: hyperplasia; CPF: chlorpyrifos; HT: Hypertrophy; N: Necrosis; DVS: dilation of venous sinus. scale bar: 10 µm.

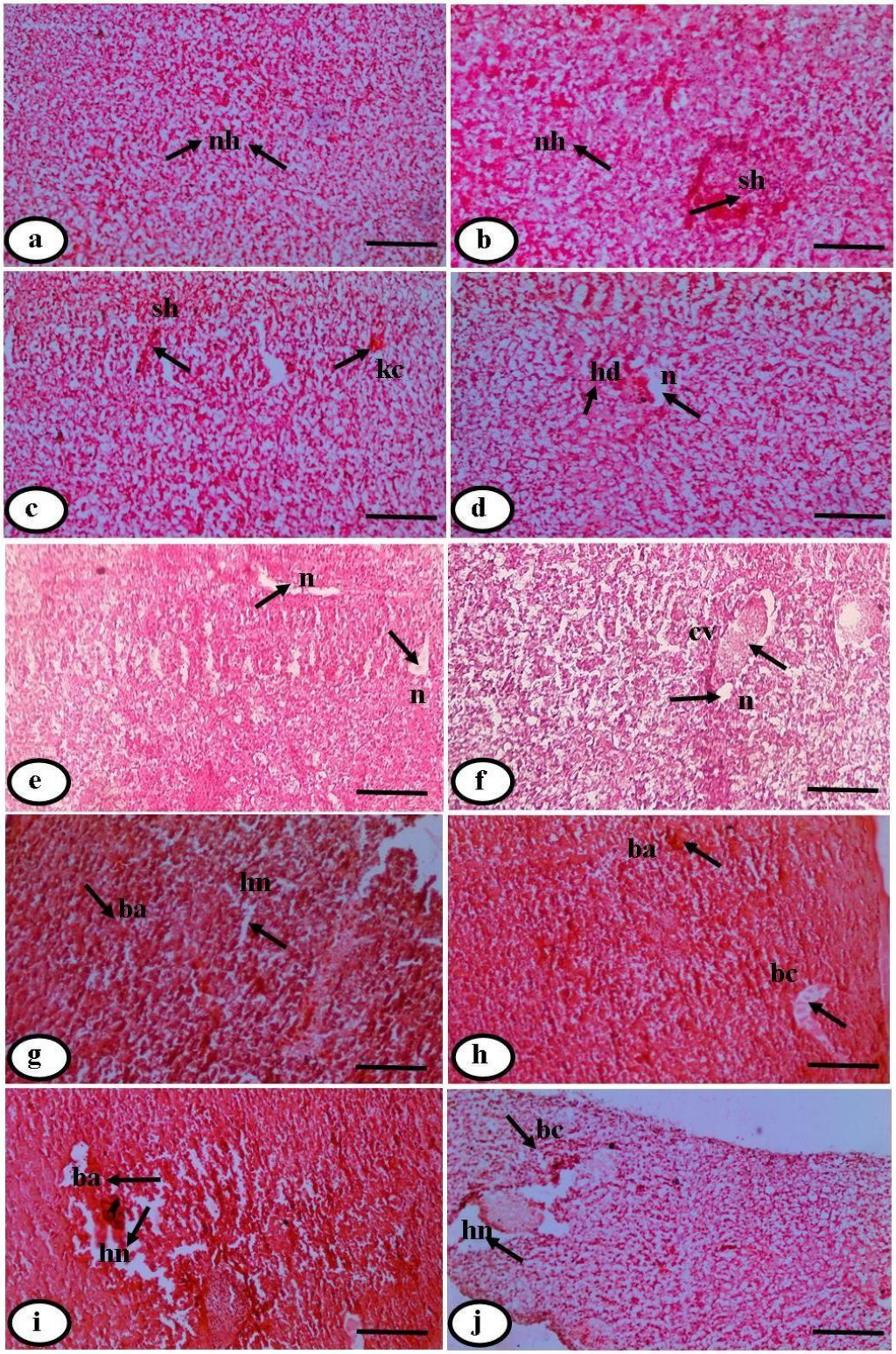

Figure 6.18. Histopathological images of CuNPs treated liver of *Oncorhynchus mykiss* fingerlings. Control (a) NH: Normal hepatocytes; 0.2 mg/L concentration (b, c and d) NH: hepatocytes; SH: sustained hypoxia; KC: Kuffer cells, 0.6 mg/L concentration (e, f and g) N: necrosis; CV: Central vein; BA: Biliary atresia; HN: Hepatocyte necrosis, 1.0 mg/L concentration (h, i and j) BA: Biliary atresia; BC: Blood congestion; HN: Hepatocyte necrosis. Scale bar: 10 µm

6.6 Discussion

This study provides important insights into the toxic effects of co-exposure to silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) in rainbow trout (*Oncorhynchus mykiss*). It highlights significant biochemical stress and physiological disruptions resulting from extended exposure to these nanoparticles.

In the present study, CuNPs were synthesized using the sol-gel method and initially confirmed by the color change of the reaction mixture from colorless to brownish and black. The blackish colour precipitate confirmed the formation of CuNPs (Muthuvel et al., 2020). The UV-Vis spectroscopy peak at 400 nm, indicating surface plasmon resonance, aligns with previous findings suggesting that monodisperse CuNPs are highly suitable for catalytic processes due to their consistent particle size and high surface area (Alahdal et al., 2023; Shankar and Rhim, 2014). X-ray diffraction (XRD) patterns confirming the monoclinic crystalline structure with sharp peaks further support their stability and uniformity, which are critical for electronic and sensing applications (Kasthuri et al., 2024; Wang et al., 2015; Waseda et al., 2011). Additionally, FTIR spectra showed functional groups such as O-H and N-H, which indicate successful surface functionalization, enhancing their reactivity and colloidal stability (Wu et al., 2004). The SEM analysis was used to study surface morphology of synthesized nanoparticles, it displayed spherical nanoparticles with an average size of 60 nm, corroborating previous studies that associate such morphology (Jabbar, 2016). These results highlight the polymers crucial stabilising function. It is well known that the strong bonding tendency of copper nuclei causes copper nanoparticles to clump together during synthesis. The increased surface area of the copper nanoparticles could perhaps be the cause of the aggregation (Yang et al., 2021; Usman et al., 2012). Finally, the zeta potential of -28 mV confirmed excellent colloidal stability, critical for maintaining nanoparticle dispersion and preventing aggregation in aqueous environments (Muthuvel et al., 2020).

Biochemical enzymes are essential in protecting cells from oxidative damage induced by reactive oxygen species (ROS) (Jedli et al., 2022; Saoudi et al., 2021). Their sensitivity to environmental pollutants makes them effective bioindicators for assessing aquatic

environmental health (Topal et al., 2017). ROS are highly reactive molecules that can induce significant damage to critical biomolecules such as proteins, polyunsaturated fatty acids (PUFAs), and DNA, leading to oxidative stress. This stress disrupts cellular integrity and impairs vital biological processes, ultimately compromising overall cellular functionality (Atamanalp et al., 2023). To counteract these effects, organisms rely on a strong antioxidant defense system, which includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These enzymes work synergistically to neutralize ROS and maintain cellular equilibrium. However, when the antioxidant defence is weakened, ROS levels increase, resulting in oxidative damage that affects lipids, proteins, and DNA, with detrimental consequences for cellular and physiological functions (Atamanalp et al., 2023).

In the present study, the activities of superoxide dismutase (SOD) in both gills and liver did not show significant changes across all sampling days, suggesting that SOD might not be the primary antioxidant enzyme responding to CuNPs induced stress under the conditions tested. This observation contrasts with some studies where SOD activity increased in response to nanoparticle exposure, potentially reflecting species-specific differences in antioxidant responses (Shekh et al., 2019). However, the antioxidant response in rainbow trout, showed the increase in catalase (CAT) activity, particularly up to the 21st day, after which a declining trend was noted, points to the elevated production of reactive oxygen species during CuNPs exposure. Catalase plays a key role in breaking down hydrogen peroxide, a harmful byproduct of oxidative stress, and its increased activity suggests an adaptive response to neutralize the toxic effects of ROS (Jedli et al., 2022; Saoudi et al., 2021; Baker et al., 2004). However, the observed decline in CAT activity after 21 days of exposure at the highest CuNPs concentration (1.0 mg/L) may indicate enzyme depletion or inhibition due to prolonged oxidative stress. This suggests that while fish may initially cope with oxidative stress, extended exposure to high levels of CuNPs could overwhelm their antioxidant defences.

Interestingly, lipid peroxidation (LPO) levels showed an unexpected decline throughout the exposure period in both gill and liver. This could be attributed to the activation of compensatory mechanisms such as increased antioxidant enzyme activity or the presence of cellular repair processes that mitigate oxidative damage (Li et al., 2015). Alternatively, it is possible that LPO levels peaked earlier in the exposure period and then declined as

damaged cells were replaced. Future studies should include more frequent time-point measurements to capture the full dynamics of LPO during CuNPs exposure. Glutathione-S-transferase (GST) and glutathione reductase (GR) activities did not show significant differences, except for a temporary increase in GR activity, indicating a limited role for these enzymes in the response to CuNPs exposure under the study conditions.

Histological analysis provides direct evidence of tissue damage and cellular alterations caused by toxicants. In this study, gill tissues of the control group exhibited normal architecture, Whereas, fish exposed to higher CuNPs exposure groups (0.6 mg/L and 1.0 mg/L) revealed severe structural damage in the gills and liver, depending on the concentration and duration of exposure. Particularly in the gills, which are the primary site of nanoparticle entry in fish, exhibited lamellar fusion, hypertrophy, hyperplasia, cellular degeneration and necrosis, all of which impair respiration and osmoregulation, with the severity increasing at higher CuNPs concentrations. Such alterations in gill structure are consistent with previous studies, where nanoparticles have been shown to cause gill damage, leading to impaired respiration and osmoregulation in fish (Khan et al., 2021; Nwani et al., 2013). Liver histology also revealed significant damage in fish exposed to CuNPs, with the most severe changes observed at the highest concentration. Liver tissues showed necrosis, vacuolation, and the presence of kupffer cells, indicating a strong immune response and impaired detoxification capacity. The liver is a primary detoxification organ, and its damage is a clear sign of the toxic effects of CuNPs. These findings align with previous studies that have reported similar histopathological changes in fish exposed to metal nanoparticles, highlighting the liver tissue vulnerability to such stressors including copper nanoparticles (Chatterjee et al., 2015; Griffitt et al., 2007). Kidney function is another critical area to assess in CuNP toxicity studies, as kidneys play a vital role in metal excretion and maintaining ionic balance. Future studies should include renal markers to gain a fuller understanding of CuNP effects on fish physiology.

6.7 Objective 3: To study the biochemical and histological effect of combined silver and copper nanoparticles in rainbow trout.

6.7.1 Oxidative stress in gills exposed to co exposure of silver and copper nanoparticles

The antioxidant responses in the gills of (*Oncorhynchus mykiss)* were evaluated over 21 days under co-exposure to AgNPs and CuNPs (Fig. 6.19). Superoxide dismutase (SOD), activity results show an increase ($p<0.05$) in the percentage of inhibition in gills at14^{th} days of exposure to all tested concentration of co exposure of nanoparticles followed by the dose-dependently, peaking in the AgNPs 1.4 + CuNPs 1.0 mg/L group (45.62 ± 3.10 U/mg protein) on day 21^{st}, compared to the control (38.31 ± 2.07 U/mg protein). Catalase (CAT) activity declined significantly in treated groups, with the lowest levels observed in the AgNPs 1.4 + CuNPs 1.0 mg/L group (3.18 ± 0.04 U/mg protein) on 21^{st} day compared to stable levels in the control (4.61 ± 0.02 U/mg protein). However, there was an increase in the enzyme activity to near the control values, in T2 group exposed to combined nanoparticles. Lipid peroxidation (LPO) was elevated, indicating oxidative damage, with the highest levels in the AgNPs 1.4 + CuNPs 1.0 mg/L group (4.39 ± 0.01 nmol MDA/mg protein), compared to the control (2.79 ± 0.01 nmol MDA/mg protein). Glutathione S-transferase (GST) activity was concentration-dependent, peaking at 6.46 ± 0.05 U/mg protein in the AgNPs 0.8 + CuNPs 0.6 mg/L group after the 7^{th} and 14^{th} of the exposure, there was a significant increase ($p<0.05$) as compare to the control group with a decrease in enzyme activity at 21^{st} days in the highest exposure (4.95 ± 0.04 U/mg protein). Glutathione reductase (GR) activity increased progressively, peaking at 9.71 ± 1.07 U/mg protein in the AgNPs 1.4 + CuNPs 1.0 mg/L group, compared to the control (8.47 ± 0.07 U/mg protein). These results demonstrate that co-exposure induces oxidative stress, evidenced by increased SOD, GR, and LPO levels and decreased CAT and GST activities ($p < 0.05$).

6.7.2 Oxidative Stress in liver exposed to co exposure of silver and copper nanoparticles

The results indicate significant variations in oxidative stress biomarkers (SOD, CAT, LPO, GST, and GR) across treatments and over time (Fig.6.20). SOD activity increased in all treated groups compared to the control, with the highest activity observed in the high-dose group (AgNPs 1.4 + CuNPs 1.0 mg/L), reaching 42.12 ± 2.12 on the 21^{st} day compared to

36.14 ± 2.07 in the control, The SOD analysis in livers shows a significant increase($P<0.05$) after 14th and 21st of exposure of mixture of nanoparticles, indicating an enhanced oxidative stress response.CAT activity showed a dose-dependent decrease, with the high-dose group declining from 3.64 ± 0.03 on the 7th day to 3.55 ± 0.02 on the 21st day, compared to relatively stable values in the control. LPO levels, reflecting lipid oxidative damage, increased significantly in treated groups, peaking in the high-dose group at 5.05 ± 0.02 on the 21st day, compared to 3.12 ± 0.02 in the control. GST activity, a marker of detoxification capacity, increased in low- and medium-dose groups, peaking at 6.56 ± 0.03 in the medium dose exposure of (0.08mg/L AgNPs+0.06mg/L CuNPs) on the 21st day, while declining in the high-dose group to 5.39 ± 0.02, suggesting impaired detoxification at higher doses. The statistical analysis showed a significant increase ($p < 0.05$) in GST levels after 14 days of exposure to mixed nanoparticles. GR activity exhibited a similar trend, with an initial increase in low- and medium-dose groups, peaking at 8.17 ± 0.05 in the medium dose, but declining significantly in the high-dose group to 5.77 ± 0.05 on the 21st day, indicating enzymatic inhibition under severe oxidative stress. These findings highlight the dose- and time-dependent oxidative stress and antioxidant response induced by co exposure of AgNPs and CuNPs.

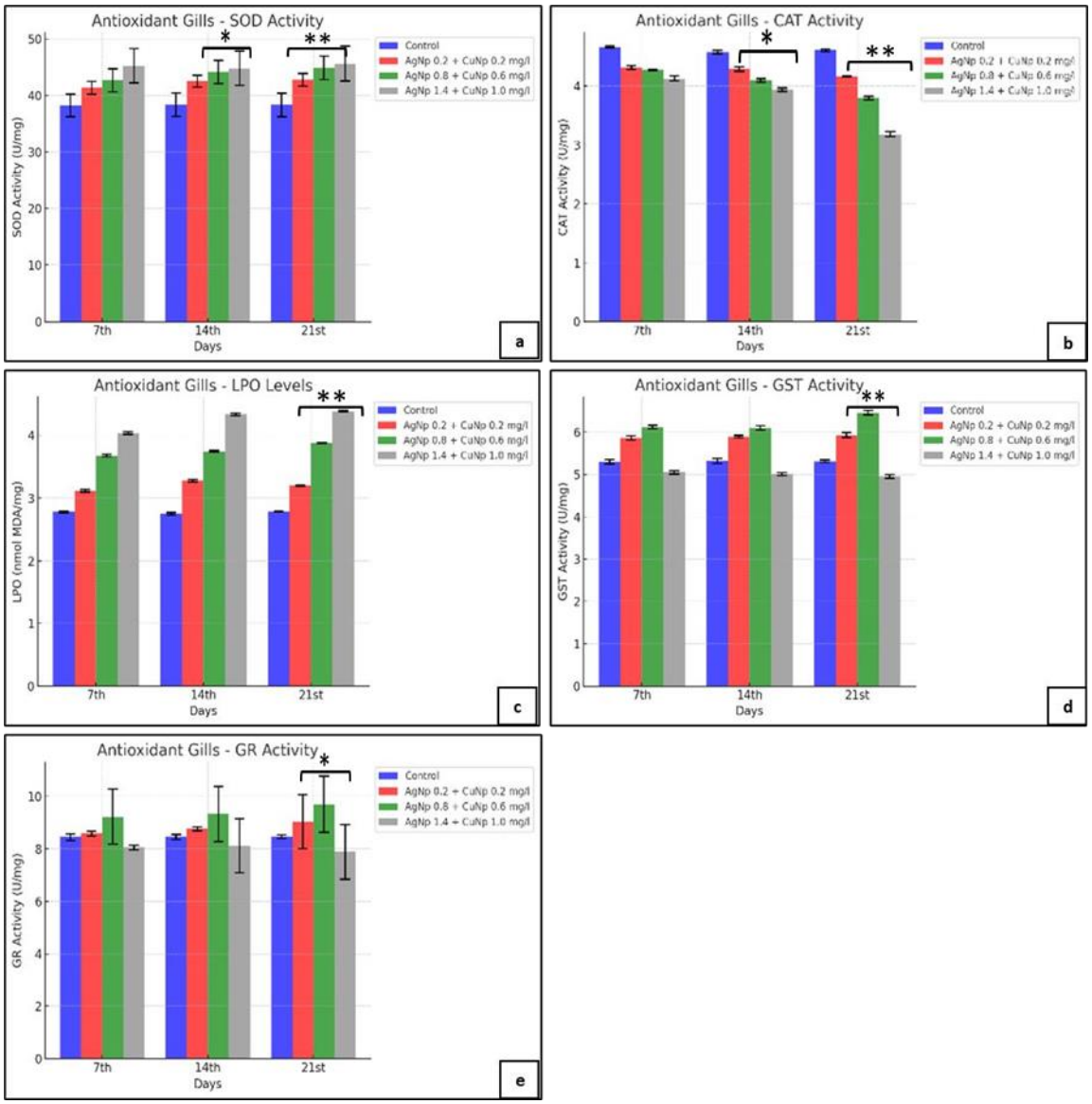

Figure 6.19. The antioxidant status of gill tissues in rainbow trout (*Oncorhynchus mykiss*) was evaluated following exposure to varying concentrations of a combined solution of silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs), with a control group unexposed to the nanoparticle mixture. Figures (a, b, c, d, and e) present a comparative analysis of lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) levels, respectively, across experimental groups exposed to different concentrations of AgNPs + CuNPs. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with significance considered at ($p < 0.05$).

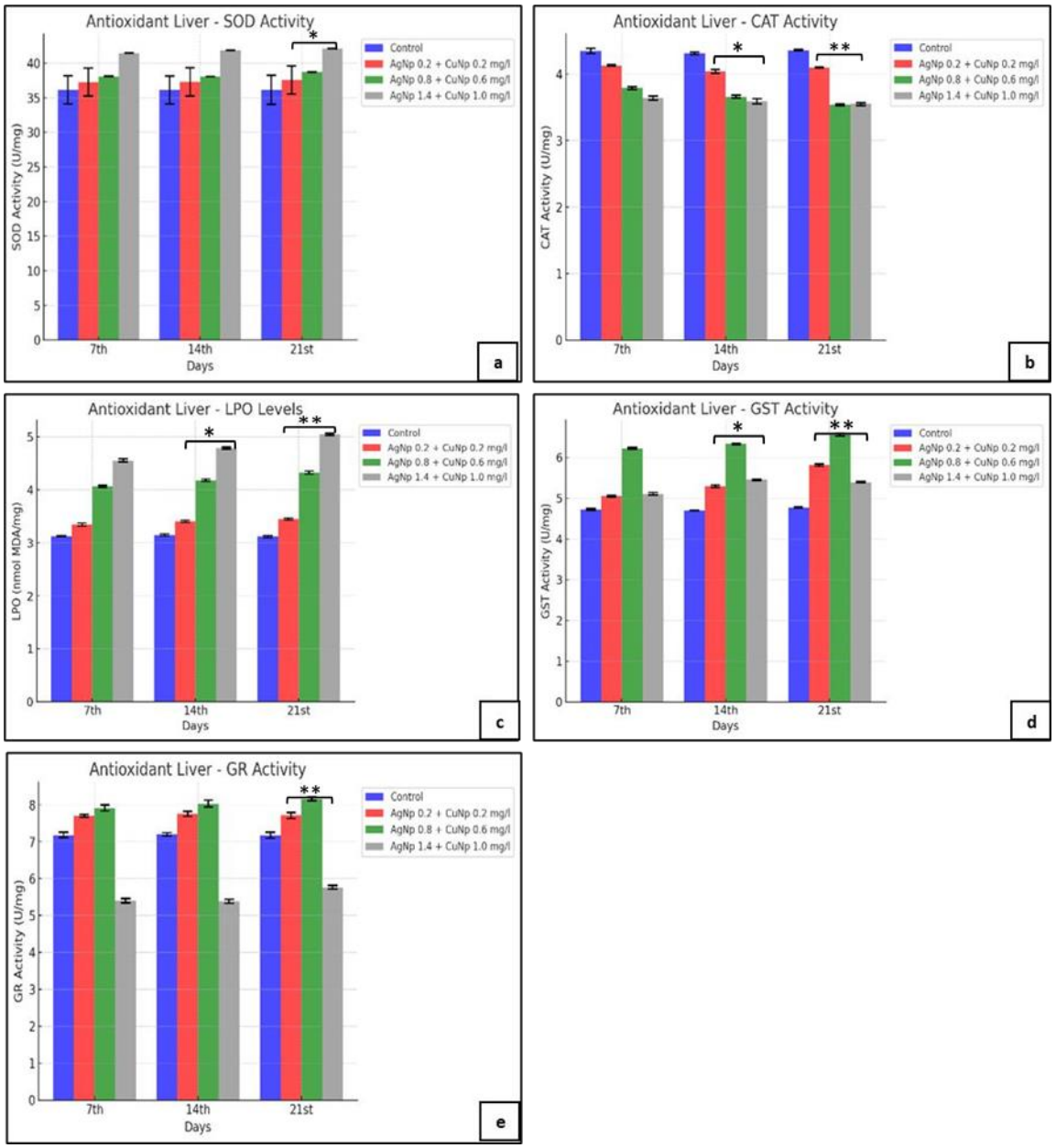

Figure 6.20. The antioxidant status of the liver in rainbow trout (*Oncorhynchus mykiss*) was assessed after exposure to varying concentrations of a combined solution of silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs), alongside a control group unexposed to the nanoparticle mixture. Figures (a, b, c, d, and e) illustrate a comparative analysis of lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) levels across experimental groups exposed to different concentrations of AgNPs + CuNPs. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with significance considered at ($p < 0.05$).

6.7.3 Histopathology changes in gill exposed to co exposure of silver and copper nanoparticles

The histopathological analysis of gills in *Oncorhynchus mykiss* fingerlings co-exposed to AgNPs and CuNPs demonstrated a progressive, dose-dependent deterioration in gill structure and function (Figure 6.21). The control group exhibited normal gill architecture

with intact chloride cells, mucous cells, and stratified epithelium (figure 6.21a). In the T1 group (0.2 mg/L AgNPs + 0.2 mg/L CuNPs), mild pathological changes, including hypertrophy, cellular infiltration, and slight swelling of adjoining lamellae, were observed, indicating early stress responses. The T2 group (0.8 mg/L AgNPs + 0.6 mg/L CuNPs) showed more severe damage, characterized by pronounced hyperplasia, congested blood vessels, deformities in chloride cells, and necrosis, reflecting significant inflammation and functional impairment. At the highest concentration in the T3 group (1.4 mg/L AgNPs + 1.0 mg/L CuNPs), extensive histological damage was evident, including severe hyperplasia, lamellar congestion, uplifting of cellular elements, and widespread necrosis, resulting in near-complete loss of structural integrity following 21 days exposure for all tested nanoparticles concentrations.

6.7.4 Histopathology changes in liver exposed to co exposure of silver and copper nanoparticles

The histopathological examination of the liver in *Oncorhynchus mykiss* fingerlings co-exposed to AgNPs and CuNPs over 7^{th}, 14^{th}, and 21^{th} days revealed dose-dependent hepatic damage (Figure 6.22). In the control group, the liver exhibited normal architecture with healthy hepatocytes (Figure 6.22a). At a low concentration T1 group (0.2 mg/L AgNPs + 0.2 mg/L CuNPs), mild pathological changes, including congested sinusoids, pyknotic nuclei, and hyaline degeneration, indicated early signs of cellular stress. Exposure to a moderate concentration T2 group (0.8 mg/L AgNPs + 0.6 mg/L CuNPs) led to more pronounced damage, such as dilated sinusoids, hemorrhages, cellular infiltration, necrosis, and kuffer cell activation, suggesting progressive inflammation and tissue disruption. At the highest concentration T3 group (1.4 mg/L AgNPs + 1.0 mg/L CuNPs), severe histological alterations were observed, including congested hepatic blood vessels, extensive hemorrhages, dilated sinusoids, necrosis, and widespread degeneration of hepatic tissue, indicative of advanced and irreversible liver damage. These findings demonstrate the hepatotoxic effects of AgNPs and CuNPs, with escalating severity linked to higher concentrations and longer exposure durations.

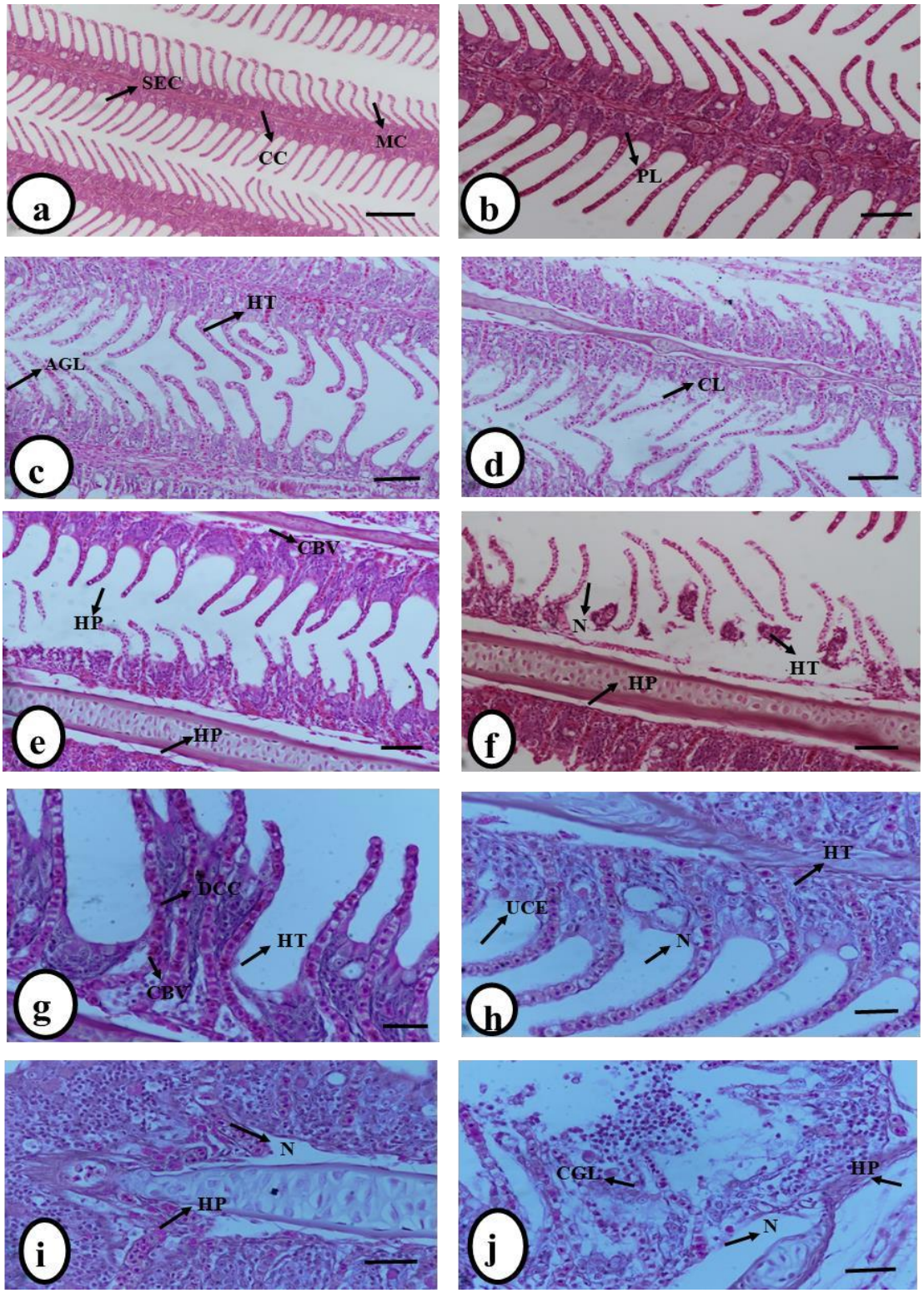

Fig. 6.21. Histopathological images of co exposure of (AgNPs + CuNPs) treated gills of *Oncorhynchus mykiss* fingerlings after 7th, 14th, 21days. Control group (a) CC: chloride cells; MC: mucous cells; SEC: stratified epithelium cells, (0.2 mg/L AgNPs+ 0.2mg/l CuNPs)concentration (b, c and d) PL: primary lamellae; HT: hypertrophy; AGL: adjoining gill lamellae; CI: cellular infiltration, (0.8mg/L AgNPs + 0.6mg/L CuNPs) concentration (e, f and g), HP: Hyperplasia; HT: Hypertrophy; CBV: congested blood vessels; DCC: deformities in chloride cells; N: necrosis, (1.4 mg/L AgNPs+ 1.0mg/L CuNPs) concentration (h, i and j) HT: hypertrophy; HP: hyperplasia; CGL: congested gill lamellae; UCE: uplifting of cellular elements; N: necrosis. Scale bar: 10 µm.

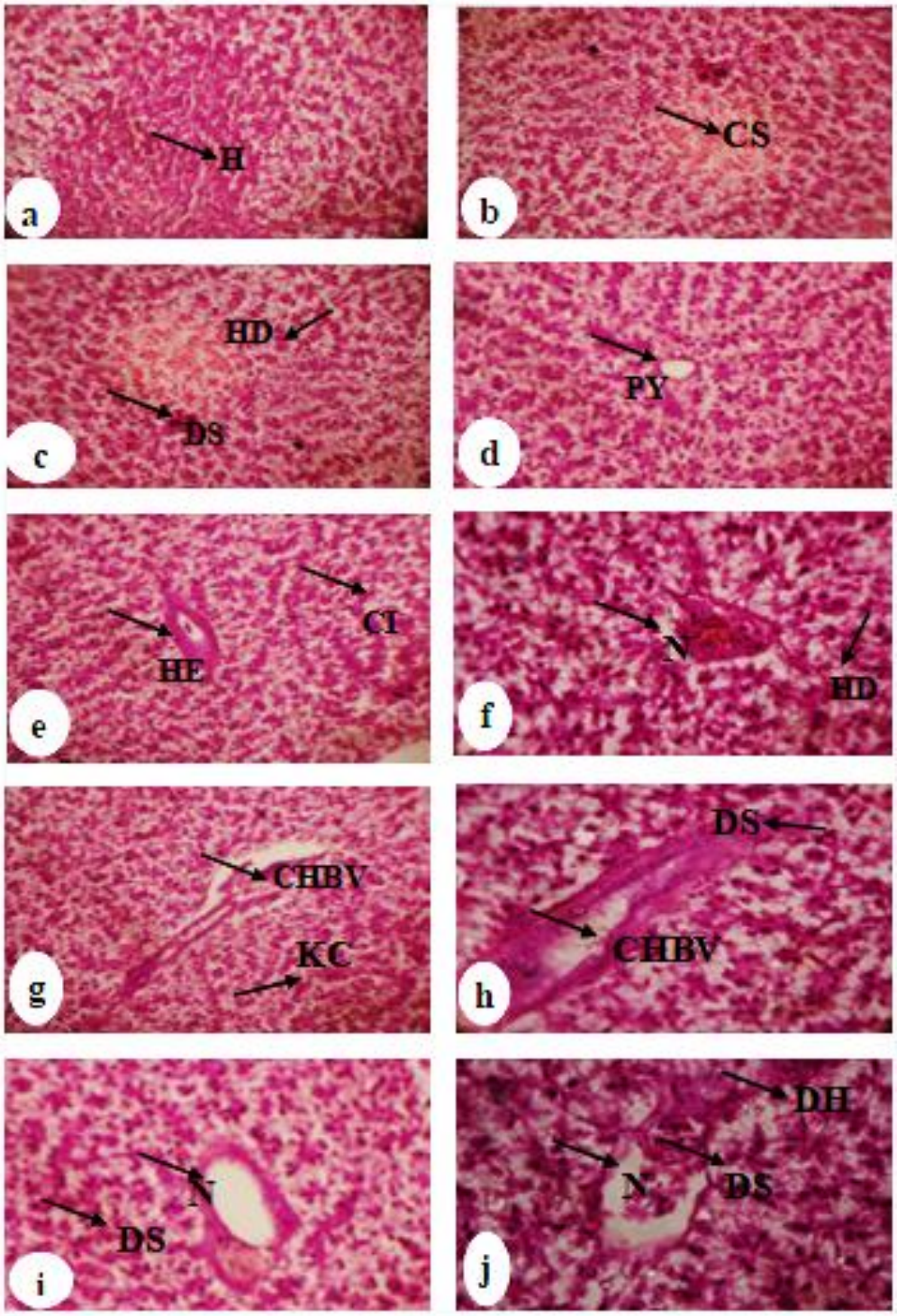

Fig.6.22. Histopathological images of co exposure of (AgNPs + CuNPs) treated liver of *Oncorhynchus mykiss* fingerlings after 7th, 14th, 21days. Control group (a) H: hepatocytes; (0.2 mg/L AgNPs + 0.2mg/L CuNPs) concentration (b, c and d) H: hepatocytes; CS: congested sinusoids; DS: Dilated sinusoid; HD: hyaline degeneration PY: pyknotic nucleus (0.8mg/L AgNPs + 0.6 mg/L CuNPs) concentration (e, f and g) HE: hepatic degeneration; CI: cellular infiltration; N: necrosis; HD: hyaline degeneration; CHBV: congested hepatic blood vessels; KC: Kupffer cell. (1.4mg/L AgNPs + 1.0 mg/L CuNPs) concentration (h, i and j) CHBV: congested hepatic blood vessels; DS: dilated sinusoid; N: necrosis; DH: degeneration of hepatic tissue. Scale bar: 10 µm

6.8 Discussion

This study provides critical insights into the toxicological effects of co-exposure to silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) in rainbow trout (*Oncorhynchus mykiss*). The detrimental effects of these nanomaterials on living things have been shown by nanotoxicological research conducted both in vivo and in vitro, which has raised worries about the possible health dangers to humans (Ferdous and Nemmar, 2020; Ostaszewska et al., 2016; Pohanka, 2019). One important but little-researched aspect of these nanoparticles' toxicity is their simultaneous presence. Ag and Cu NPs are commonly combined in consumer products, which raises questions regarding how they might interact in biological systems (Vance et al., 2015). There are several possible results from co-exposure to these nanoparticles, such as additive, synergistic, or antagonistic cytogenotoxic effects. Even though AgNPs and CuNPs are widely used and becoming more prevalent in consumer goods and the environment, little is known about their genotoxic and physiological consequences when exposed together in aquatic models. Copper and silver nanoparticles were found to be among the most dangerous when (Kovriznych et al., 2013) investigated the toxicity of 31 different nanoparticles in zebrafish. In order to provide a better understanding of their possible harmful interactions and to illuminate the underlying processes underlying these effects, this work explores the combined effect of Ag and Cu NPs on the physiology and biochemical expression. The results highlight the potential risks these nanoparticles represent to aquatic ecosystems, underscoring the urgent need for stronger regulation and prudent use and disposal management.

The co-exposure of *Oncorhynchus mykiss* to AgNPs and CuNPs for 21 days elicited a dose-dependent oxidative stress response in both gills and liver tissues, highlighting the synergistic toxicity of these nanoparticles. Superoxide Dismutase (SOD) activity significantly increased across all treated groups, particularly at higher concentrations (AgNP 1.4 + CuNP 1.0 mg/L). This upregulation indicates an adaptive response to mitigate elevated levels of reactive oxygen species (ROS), consistent with prior findings on

nanoparticle-induced oxidative stress (Asharani et al., 2011; Hussain et al., 2016). Conversely, Catalase (CAT) activity declined progressively with increasing nanoparticle concentrations, particularly in high-dose groups, reflecting the enzymatic system's inability to neutralize accumulated hydrogen peroxide. This impaired detoxification mechanism suggests that the antioxidant defense system becomes overwhelmed under prolonged nanoparticle exposure (Chupani et al., 2018). Lipid Peroxidation (LPO), a critical marker of oxidative damage to cell membranes, increased significantly in both gills and liver tissues, with the highest levels observed in high-dose groups. This indicates severe ROS-mediated damage to lipid components of cellular membranes, corroborating earlier reports of nanoparticle-induced lipid peroxidation (Sadeghi et al., 2015). It is in line with research showing that metal nanoparticles can catalyze lipid oxidation and pass-through biological membranes (Khan et al., 2021; Sharma et al., 2019). By impairing membrane integrity, this oxidative damage may interfere with cellular homeostasis and liver metabolism.

Glutathione S-transferase (GST) and Glutathione Reductase (GR) activities exhibited contrasting trends. GST activity initially increased in low- and medium-dose groups, suggesting enhanced detoxification efforts; however, it declined at higher doses, likely due to glutathione depletion or enzymatic inhibition. Ahmed et al. (2021) showed a similar dose-dependent suppression of GST at higher nanoparticle concentrations, emphasizing its dual role as a susceptible and adaptive biomarker. Similarly, GR activity showed a concentration-dependent increase, reflecting an effort to regenerate reduced glutathione under oxidative stress. However, at high nanoparticle concentrations, enzymatic activity likely became insufficient to restore redox balance (Handy et al., 2008; Johnston et al., 2010). The decline in GR activity in the high-dose group may reflect enzymatic inhibition due to excessive ROS or direct interactions with nanoparticles, as suggested by studies on metal nanoparticle toxicity (Chen et al., 2020; Xu et al., 2022). Overall, the oxidative stress response was characterized by elevated SOD and GR activities, increased LPO levels, and disrupted CAT and GST activities. These findings underline the complex interplay between antioxidant activation and oxidative damage under nanoparticle exposure, emphasizing the cumulative impact of AgNPs and CuNPs on aquatic organisms.

Histopathological investigation offers vital information about the mechanisms driving tissue damage and the sub-lethal effects of xenobiotics on target tissues (Gyimah et al., 2020). Given their vital roles in gas exchange, osmoregulation, and detoxification,

histological alterations are frequently seen in the liver and gill tissues of fish exposed to toxicants and are important biomarkers in toxicological studies. The effects of different nanoparticles on fish have been the subject of numerous in vitro (Connolly et al., 2015; Wang et al., 2016) and in vivo (Al-Bairuty et al., 2013; Ostaszewska et al., 2018; Wang et al., 2015) investigations. Histopathological analyses revealed a dose- and time-dependent progression of structural damage in both gills and liver tissues, consistent with the biochemical indicators of oxidative stress. As the main organs for gas exchange and osmoregulation in our investigation, the gills gradually deteriorated structurally as the concentration of nanoparticles increased. In the control group, gills exhibited normal architecture with intact chloride cells, mucous cells, and stratified epithelium, reflecting baseline physiological conditions. However, at the lowest concentration T1 group (0.2 mg/L AgNPs + 0.2 mg/L CuNPs), mild changes such as hypertrophy, cellular infiltration, and slight lamellar swelling were observed. These alterations suggest an early stress response triggered by ROS and mild inflammation (Khan et al., 2015). According to Martinez et al. (2004), these alterations might be the consequence of pillar cell and blood vessel destruction, which would raise lamellar blood flow.

Both Japanese medaka (*Oryzias latipes*) and Siberian sturgeon (*Acipenser baerii*) treated to AgNPs and CuNPs have been shown to exhibit similar effects, including telangiectasia, epithelial detachment, and lifting of the epithelium (Wu and Zhou, 20213). T2 group (0.8 mg/L AgNPs + 0.6 mg/L CuNPs) led to more pronounced damage, including hyperplasia, vascular congestion, deformities in chloride cells, and necrosis. These pathological changes reflect progressive inflammation and compromised osmoregulatory function (Handy et al., 2008). These results are consistent with other research that found inflammation and oxidative stress to be important factors in tissue damage brought on by nanoparticles (Ahmed and Ahmad, 2020; Khan et al., 2021). At the highest concentration T3 group (1.4 mg/L AgNPs + 1.0 mg/L CuNPs), severe structural disruptions such as extensive hyperplasia, lamellar congestion, and widespread necrosis were observed, resulting in near-complete loss of gill integrity underscores the compounded toxicity of co exposure to AgNPs and CuNPs. Such damage indicates significant respiratory and ionic regulatory impairments, which are critical for fish survival (Al-Bairuty et al., 2013).

Similar results were published by (Ostaszewska et al., 2016; Khan et al., 2024), emphasising that metal nanoparticle aggregation on gill surfaces is a major cause of oxidative stress and substantial tissue damage. The surface area accessible for gas and ion exchange was decreased by gill lamellae shortening and fusion, as well as epithelial hyperplasia. Several fish species exposed to nanoparticles, including AgNPs, have shown histopathological abnormalities include epithelium hypertrophy, hyperplasia, and lifting (Wu and Zhou, 2013). Zinc oxide (ZnO), aluminium (Al2O3), and other aquatic contaminants (Boran et al., 2012) (Benavideset al., 2016). In this work, blood cell aggregation and lamellar blood vessel dilatation were brought on by co-exposure to copper and silver nanoparticles. Siberian sturgeon and Atlantic salmon subjected to AgNPs showed similar branchial lesions, such as cell degeneration and epithelial necrosis (Farmen et al., 2012).

A crucial target for nanoparticles is the liver, which is the main organ for the biotransformation of harmful substances (Yao et al., 2019). According to studies, AgNPs and CuNPs cause cytotoxic changes in the liver that lead to tissue damage and the buildup of nanoparticles (Gaiser et al., 2013; Khan et al., 2024; Ostaszewska et al., 2016). The results of this investigation support previous findings that the liver is a primary location for the accumulation of AgNPs (Jarrar et al., 2014) and CuNPs (Lei et al., 2015) nanoparticles. In toxicological research, liver histopathological alterations are frequently employed as biomarkers of environmental pollution (Dabrowska et al., 2012). The current investigation found that co-exposure to nanoparticles caused dose-dependent pathological alterations in the liver. The absence of external stress was confirmed by the control group's normal hepatic architecture. In T1 group (0.2 mg/L AgNPs + 0.2 mg/L CuNPs), early signs of hepatic stress, such as pyknotic nuclei, congested sinusoids, and hyaline degeneration, were evident. These changes suggest the onset of oxidative stress and cellular dysfunction (Yin et al., 2021). These results are consistent with those of Sharma et al. (2019), who found that aquatic species experienced mild hepatic changes in response to modest nanoparticle dosages. Liver samples from the T2 group (0.8 mg/L AgNPs + 0.6 mg/L CuNPs) showed increasing inflammation, which was indicated by dilated sinusoids, hepatic damage became more pronounced, including necrosis, hemorrhages, and Kupffer cell activation. These findings indicate a heightened inflammatory response and escalating oxidative damage due to excessive ROS production (Kim et al., 2018). In comparison to the control group, hepatocyte vacuolation and hepatocyte size growth were also seen. Similar histological alterations have been reported in rainbow trout subjected to CuNPs

and AgNPs (Al-Bairuty et al., 2013; Khan et al., 2024) and carp (*Cyprinus carpio*) treated to TiO_2NPs (Hao et al., 2009). In the T3 group (1.4 mg/L AgNPs + 1.0 mg/L CuNPs), severe histological alterations such as extensive tissue necrosis, dilated sinusoids, and widespread hemorrhages were observed, reflecting irreversible damage and compromised liver function. These alterations indicate advanced hepatotoxicity and compromised metabolic processes, as do greater sinusoid widths brought on by smaller hepatocytes. Similar results have been published by (Xu et al.,2022; Khan et al., 2024), highlighting the irreversible liver damage that fish suffer from extended exposure to high nanoparticle concentrations.

This progression underscores the cumulative hepatotoxic effects of AgNPs and CuNPs at high concentrations (Sati et al., 2025). The combined analysis of oxidative stress biomarkers and histopathological findings highlights the synergistic toxicity of AgNPs and CuNPs in *Oncorhynchus mykiss*. The observed oxidative stress and tissue damage in gills and liver tissues underscore the potential risks associated with nanoparticle pollution. These findings emphasize the need for further research into the ecological impacts of nanoparticle mixtures and the development of regulatory measures to mitigate their harmful effects on aquatic ecosystems.

Chapter 7

7. SUMMARY AND CONCLUSIONS

This study provides a comprehensive evaluation of the toxicological effects of silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) on rainbow trout (*Oncorhynchus mykiss)* focusing on their individual and combined impacts on oxidative stress biomarkers and histopathological alterations. The findings underscore the ecological risks posed by nanoparticle contamination in aquatic environments and highlight the urgent need for regulatory measures to mitigate these effects. Nanoparticles, due to their unique physicochemical properties, have been widely employed across various industries. Silver nanoparticles are extensively used for their antimicrobial capabilities, while copper nanoparticles are prized for their catalytic and electrical properties. Despite their widespread applications, the release of these nanoparticles into aquatic environments has raised significant concerns regarding their toxicity to non-target organisms. This study addresses these concerns by examining the dose-dependent toxic effects of AgNPs, CuNPs, and their combined exposure on rainbow trout.

This study provides a thorough evaluation of the toxicological effects of silver nanoparticles (AgNPs) on rainbow trout *(Oncorhynchus mykiss)*, The exposure to silver nanoparticles caused a dose-dependent increase in oxidative stress, as evidenced by elevated superoxide dismutase (SOD) activity and increased lipid peroxidation (LPO) levels, reflecting the overproduction of reactive oxygen species (ROS). However, a concurrent decline in antioxidant enzymes, such as catalase (CAT) and glutathione S-transferase (GST), was observed at higher concentrations, indicating that the fish's defense systems were overwhelmed. These disruptions in enzymatic activity, coupled with oxidative damage, highlight the potential for long-term physiological harm, particularly at higher concentrations of AgNPs. Histological analysis revealed significant tissue damage in key organs. In the gills, lamellar fusion, epithelial lifting, and necrosis were prominent, compromising respiratory efficiency. In the liver, hepatocyte vacuolation, nuclear degeneration, and necrotic regions indicated severe metabolic and detoxification impairment. These results demonstrate that AgNPs exposure disrupts physiological homeostasis, leading to substantial oxidative damage and organ dysfunction.

In the case of copper nanoparticles (CuNPs), exposure also induced oxidative stress, with a significant increase in catalase (CAT) activity across all durations of exposure. Interestingly, LPO levels decreased, which might suggest the activation of compensatory mechanisms to mitigate oxidative damage. Nonetheless, histological examination highlighted severe structural damage. In the gills, hypertrophy, hyperplasia, lamellar fusion, and necrosis were observed, with damage escalating in a concentration- and time-dependent manner. The liver exhibited extensive vacuolation, cellular degeneration, and necrotic regions, reflecting disruptions in essential metabolic processes. The most pronounced effects were evident at the highest concentration of CuNPs (1.0 mg/L) and the longest exposure period (21 days), emphasizing the toxic potential of CuNPs to affect critical physiological functions.

When exposed to combined silver and copper nanoparticles (AgNPs and CuNPs) at varying concentrations, rainbow trout displayed pronounced oxidative stress and significant histological damage. The antioxidant enzyme activities of CAT, SOD, and GST initially increased, particularly in the gills and livers, indicating an active response to oxidative stress. However, prolonged exposure resulted in a decline in these enzyme activities, suggesting a depletion of the antioxidant defense system. LPO levels were markedly elevated in both gills and livers, with the highest nanoparticle concentrations causing the most severe effects. Histological analysis revealed extensive tissue damage. The gills showed lamellar fusion, hyperplasia, epithelial lifting, and necrosis, severely impairing respiratory efficiency. The liver was heavily affected, with widespread vacuolation, nuclear degeneration, and necrotic regions, indicating significant metabolic and detoxification challenges. Notably, the combined exposure to AgNPs and CuNPs resulted in enhanced toxicity compared to individual nanoparticles, suggesting synergistic interactions that exacerbate oxidative stress and tissue damage.

The findings of this study highlight the ecological risks posed by nanoparticle contamination, particularly the amplified effects of combined nanoparticles. The observed oxidative stress and histopathological damage underscore the potential for long-term physiological harm in aquatic organisms and emphasize the importance of establishing stringent regulatory frameworks to monitor and limit the release of nanoparticles into aquatic environments. Developing safe thresholds for nanoparticle concentrations is essential to protect aquatic biodiversity and maintain ecosystem stability.

In conclusion, this study demonstrates the dose-dependent toxicological effects of AgNPs, CuNPs, and their combination on rainbow trout, providing valuable insights into the mechanisms of nanoparticle-induced toxicity. The results emphasize the urgent need for further research to explore the chronic impacts of nanoparticle exposure and to assess their interactions with other environmental stressors. This knowledge will be crucial for designing effective strategies to mitigate the risks of nanoparticle pollution in aquatic ecosystems while supporting the sustainable use of nanotechnology.

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LIST OF PUBLICATIONS

S. No.	Tittle	Journal
01	Silver Nanoparticle Toxicity in Rainbow Trout: Insights into Physiological and Molecular Responses" has been accepted for publication in Toxicology Mechanisms and Methods.	Toxicology Mechanisms and Methods
02	Toxicological impact of copper nanoparticles on rainbow trout: hematological, biochemical, antioxidant, and histopathological responses with oxidative gene expression	Toxicology Mechanisms and Methods
03	Assessing the Combined Toxicity of Silver and Copper Nanoparticles in Rainbow Trout (*Oncorhynchus mykiss*) Fingerlings.	Biological Trace Element Research
04	Biogenic silver nanoparticles: Synthesis, applications and challenges in food sector with special emphasis on aquaculture	Food Chemistry: X
05	Nanotechnology in aquaculture: Transforming the future of food security	Food Chemistry: X
06	Nanotechnology in Aquaculture: Applications and Challenges In: Exploring Frontiers In Fisheries Research Emerging topics and Innovations Published by: Elite Publishing House (EPH) 2023. ISBN: 798-93-58994-57-5	Book Chapter
07	PROTEIN REQUIREMENTS IN FISH	Book chapter

LIST OF CONFERENCES AND WORKSHOPS

S. No	Conference name	Tittle	Host country
01	International conference on Current Trends in Toxicology and 43rd Annual Meeting of Society of Toxicology, India, 2024(STOX 2024): Human Health and Environmental Safety	Evaluating the Dose dependent Toxicity of silver nanoparticles in Rainbow trout: A detailed examination of physiological and molecular reactions	**India**
02	Two day inter University brainstorming Organized by SKUAST-K in collaboration with Indian Institute of Technology-Kanpur (IIT-K) and Indian National Science Academy (INSA)	Toxicological effect of copper nanoparticles on rainbow trout	**India**
03	Bioengineering and Biosciences (ICBB-2022) Society of Bioinformatics for Experimenting scientists	Impact of urbanization on the breeding biology of blue rock pigeon (*Columbia livia*) in central Kashmir, India	**India**