Study the Antimicrobial Activity of Metallic Nanoparticles and their Effect on Animal Model

Zahra Ghulam Haider[1], Farzana Rashid[1]*, Iqra Noshair[1], Abdullah Etezaz[2], Sadia Iqbal[1], Sadia Akhtar[1], Adila Sana[1]

[1]Department of Zoology, Lahore College for Women University, Jail Road, Lahore 54000, Pakistan
[2]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

Abstract- Green synthesis of metallic nanoparticles is a promising way of preparing nanoparticles using plant extracts that have lesser toxicity in any organisms. In the present work, we analyzed the antibacterial activity of *Calotropis gigantea* (ZnO) and (Cu) mediated nanoparticles in an in vitro assay against *S. aureus*, *E. faecalis*, *K. pneumoniae* and *E. coli*. Where, against *Aeromonas hydrophila* (*A. hydrophila*) in an in vivo assay employing *Labeo rohita* (*L. rohita*). *L. rohita* were divided into seven groups for both (ZnO) and (Cu) nanoparticles respectively: Control, *A. hydrophila* infected, Cu NPs (75 µg/L), ZnO NPs (75 µg/L), Cu NPs+I (75 µg/L) and ZnO NPs+I (75 µg/L) fish treated groups. Change in different bio-indicators such as hematological, histological and enzyme analysis were observed. Interestingly, the infected fish treated with both types of ZnO NPs and Cu NPs exhibited a higher survival rate than the untreated infected fish and demonstrated signs of recovery from infection, providing a compelling indication of the positive impact of photosynthesized ZnO NPs and Cu NPs. Disruptions in hematological and histological parameters were found in the infected fish. Both ZnO NPs and Cu NPs showed recovery on the hematological and histological parameters. Analysis of oxidative stress also revealed provoking evidence of the positive impact of ZnO and Cu NPs treatment against disease progression in the infected fish. The major finding of the study was that the higher concentrations of the nanoparticles (75 µg/L in the case of ZnO NPs and 75 µg/L in the case of Cu NPs) were more effective in fighting against disease. In conclusion, our work presents novel insights for the use of green-synthesized ZnO and Cu NPs as economic and innocuous antibacterial candidates in aquaculture.
Mention the abstract for the article. An abstract is a brief summary of a research article, thesis, review, conference proceeding or any in-depth analysis of a particular subject or discipline, and is often used to help the reader quickly ascertain the paper's purpose. When used, an abstract always appears at the beginning of a manuscript, acting as the point-of-entry for any given scientific paper or patent application.

Index Terms- Znic Oxide, Nanoparticles, *Calotropis gigantean*, *Labeo rohita*, *Aeromonas*

I. INTRODUCTION

Nanotechnology is a perspective of advance synthetic chemistry mainly concerned with the development of nanoparticles. Synthesis of nanoparticles with different shapes, sizes, structure which find its significance and applications in different fields such as medicine that benefit the human being [1-3]. Biological methods for nanoparticles (NPs) synthesis using microorganisms, enzymes, and plants or plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods. Thus, there is a requirement for a new simple and eco-friendly green synthesis processes, those restricted the use of toxic chemicals and higher energy inputs [4].

The plant *Calotropis gigantean* (*C. gigentea*) belonging to the family Asclepiadanceae, also called as Alarka, Shwetarka, Mandara, Vasuka is distributed through out India, dry wast land [5]. *Calotropis gigantea* is a species of *Calotropis* native to Cambodia, Indonesia, Malaysia, Philippines, Thailand, Sri Lanka, India and China [6-8]. The different parts of the plant are used in Indian traditional medicine for the treatment of painful muscular spasm, dysentery, fever, rheumatism, asthma and as an expectorant and purgative [9]. To the best of our knowledge, biological approach using leaf extract of *C. gigantea* has been used for the first time as a reducing material as well as surface stabilizing agent for the synthesis of spherical shaped ZnO-NPs. The structure, phase, and morphology of synthesized product were investigated by the standard characterization techniques.

Zinc oxide (ZnO) is a widely known semiconductor photocatalyst due to its affordability and large band gap energy resulting in a maximum rate of oxidation. However, it has a higher efficiency in the degradation of dyes. Furthermore, ZnO has a higher photocatalytic nature in NPs due to an increase in surface area compared to bulk materials. This increase in the surface area of the ZnO nanoparticles allows a higher rate of degradation of textile dyes (crystal violet, basic blue, and methylene red) [10]. Copper oxide (CuO) NPs have attracted due to its strong catalysts, electrophoresis, photonics, and antioxidant bacteria, depending on size, shape, and surrounding environment. CuO nanoparticles have been used as excellent disinfectants in water treatment plants, because of effective antibacterial properties and resistance mutations in addition to the characteristics of antimicrobials [11]. This study focuses on synthesizing the ZnO and Cu NPs using a solution precipitation method from *C. gigantea* leaf through green synthesis.

The increasing tendency of microbial infections, rapid emergence of drug-resistance to recent antibiotics and quick evolution through mutation necessitate development or modification of antimicrobial compounds and alternative treatments [12]. Advanced research in nanotechnology recently comes with the development of nano-scale objects with prominent antimicrobial actions against multidrug resistant pathogens suggesting a platform to fight against bacterial mutation arch [13-14]. Among the most advanced nanotechnological applications, metal nanoparticles provide the most effective results with their unique mode of actions [15].

Diseases are the major stumbling block towards sustainable growth of the aquaculture. Fish are threatened by many pathogens, especially viruses and bacteria, often with serious consequences [16-17]. Due to the increase in disease outbreak and the development of microbial resistance, there is an urgent need to prevent infectious agents and other harmful microorganisms without propagating antibiotic resistance pattern [18-19]. One of the possibilities is to use nanoparticles as antimicrobial drug in aquaculture but their potential use for disease control is not fully explored yet. Rohu (*Labeo rohita)* is the most important freshwater fish species having high consumer preference and market demand. It is considered to be the leading aqua-crop species in freshwater aquaculture system. With this contextual information, the present study was undertaken to assess the effects of ZnO and Cu nanoparticles in combination on hematological, histological enzymatic activities and disease resistance in rohu [20].

II. MATERIAL METHODS

2.1. Preparation of *C. gigantea* Leaves Extract

The *C. gigantea* (Milky weed) leaves were collected from the Government College Lahore. All reagents used were of analytical grades takes from Sigma Aldrich and all experiments were performed using distilled water. Fresh leaves of *C. gigantea* were collected, dry under the shadow place and crushed into small pieces. About 50g of dried finally chopped leaves and 100 mL of sterilized distilled water yielded the extract. The mixture was boiled for 60 minutes. The extract was filtered through Whatman No.1 filter paper, the filtrate was collected in 250 cm^3 Erlenmeyer flask and used thereafter [21]. The extract was divided into two parts (Figure 1).

2.2. Synthesis of Zinc oxide (ZnO) NPs

The green synthesis of of ZnO NPs was prepared following the method reported in the literature [22] by using one part of 50 mL of *C. gigentea* leaves extract was heated to 60-80ºC using a magnetic stirrer heated for the production of ZnO NPs. As the temperature reached above 60 °C added 5 g of Zn $(NO_3)_2$ to the plant extract. Heat the mixture until it changes into a paste of a deep yellow color. This paste was collected in a ceramic crucible and heated at 400 °C for 2 hours in an air heated furnace. A light-yellow powder was acquired. This was carefully collected and grinded in a pestle mortar to get a fine powder shown in figure 2.

2.3. Synthesis of Copper Nanoparticles (Cu) NPs

The resultant leaves extract other one part was filtered. Then a separate beaker were taken for the mixing of 1g of $CuSO_4$ into distilled water and add this solution into the leaves extract and boiled until it change into the paste of black color. This paste was collected in a ceramic crucible and heated at 400 °C for 2 hours in an air heated furnace. A dark black color powder was acquired. This was carefully collected and grinded in a pestle mortar to get a fine powder (Figure 3).

2.4. Characterization of *C. gigentea* Cu and ZnO NPs

The synthesized ZnO and Cu NPs were characterized by using a combination of spectroscopic techniques such as UV-visible spectroscopy (UV-Vis; AE-S70-1U; AELAB GUANGZHOUCo.,Ltd., Guangzhou, China, A & E Lab Instruments (Guangzhou) Co., Ltd., Guangzhou, China), X-ray diffraction (XRD D8 discover, Bruker, Germany), Fourier transform infrared spectroscopy [FTIR; (P/N 206-72010, Tracer SHIMADZU, Kyoto, Japan)] and Scanning Electron Microscopy (SEM; Evo LS10 Zeiss, Jena, Germany) to analyze their crystallinity, structure, composition, and size. ImageJ (https://imagej.net/ij/, accessed on 21 August 2022) was used to determine the size of synthesized NPs.

2.5. Bacterial Strains

Non-repetitive thirty (30) clinical isolates from skin exudates from pus and wounds etc., were collected from microbiology laboratory of hospital and general hospital of Lahore and characterized by morphological and biochemical tests, i.e., citrate and catalase test using 16S rRNA sequencing [23]. The selected strains were *Pseudomonas aeruginosa* (*P. aeruginosa*; MN900691). *Streptococcus* sp., *Escherichia coli* (*E. coli*; MT448673) and *Staphylococcus aureus* (*S. aureus*; MT448672). These selected bacterial strains were sensitive to amikacin and resistant to ciprofloxacin.

2.6. Antibacterial Activity of ZnO and Cu NPs

Antibacterial activity of *C. gigentea* leaf extract, and ZnO and Cu NPs, was determined against Gram negative and Gram positive bacteria using various concentrations (5, 10, 15 and 20 mg/mL) of green synthesized ZnO and Cu NPs and using plant extract, following Kirby-Bauer disc diffusion method [23]. The antibacterial tests were performed in the light, including UV. Isolated strains were sub cultured in nutrient broth at 30 ºC for 24 h. Sterilized standard filter paper discs were soaked in different concentrations of ZnO and Cu NPs. Blank disc saturated with distilled water was used as negative control and antibiotics [amikacin and ciprofloxacin] were used as positive controls. The experiment was performed in triplicate and ZOI was measured.

2.7. Cytotoxicity Effect of ZnO and Cu NPs by Brine Shrimp Lethality Assay

The brine shrimp lethality bioassay is a simple bioactive chemical cytotoxicity test. It is based on test substance capacity to kill a simple zoological organism brine shrimp [24]. To perform this test dried eggs of brine shrimp (0.5 g) were added to a 500 ml glass beaker filled with 400 ml of artificial seawater. The suspension was aerated by bubbling air into the funnel and kept for 24 to 48 hours at room temperature. After aeration had been removed the suspension was kept undisturbed for 1 hour whereby the remaining egg settled down.

In order to use the only active larvae one side of the separating funnel was covered with aluminum foil and the other illuminated with a lamp whereby the phototropic larvae were gathering at the illuminated side. With a pipette, 30 to 40 brine shrimp larvae were collected and transferred to a deep-well microtiter plate filled with 0.2 ml of salt water. The dead larvae counted (N), a solution of 20 µg of ZnO and Cu NPs in 5,10 µL of DMSO (Dimethyl sulfoxide) was added and the plate was kept at room temperature in the dark. After 24 hours the number A of the dead larvae in each well was counted again under microscope.

The surviving larvae were killed by addition of 0.5 mL methanol so that subsequently the total number G of the animals could be determined. Each test row was accompanied by blind sample containing pure DMSO instead of a test solution. As a positive control with 100 % mortality, actimycin D (10 µ/mL) was used.

The mortality rate M was calculated using the following formula:

$M = (\frac{(A-B-N)}{(G-N)}) \times 100$

Where

M = Percentage of the dead larvae after 24 hours

A = Number of the dead larvae after 24 hours

B = Average number of the dead larvae in the blind samples after 24 hours

N= Number of the dead larvae before starting the test

2.8. Experimental Specimen and Toxicity Effect of Green Synthesized ZnO and Cu NPs

The toxic effects of green synthesized ZnO and Cu NPs were studied in vivo. For this purpose, ninety (90) *Labeo rohita* (*L. rohita)* with 25 g in weight and 6-7 inches long were used as the experimental specimen they were purchased from the Himalaya Fish Hatchery Murediky G. T road Lahore. Fish were acclimatized for two weeks before initiation of experiment. Any sick fish were excluded. During acclimatization fish were fed to the basal diet. For the experiment the healthy specimens were then arbitrarily transferred to six (6) glass aquaria that were T1 (Control group), T2 (*A. hydrophila* challenged group), T3 (High concentration of Cu NPs 75µg/L), T4 (High concentration of ZnO NPs 75µg/L), T5 (High concentration of Cu NPs 75µg/L + Infected) and T6 (High concentration of ZnO NPs 75µg/L + Infected) were given orally in the fish water aquarium for 15 days.

2.9. Hematological Observations

Blood was collected directly from gills of fish of each group using sterilized insulin syringes (1 ml). Each blood sample was poured into the labelled purple tubes according to the relative group. Blood samples in purple tubes that contained EDTA an anticoagulant were used for hematology.

2.10. Histological Examination

For histological studies, the gills and liver of *L. rohita* were taken and fixed into 10% formalin. Next, slides were prepared and stained with hematoxylin and eosin (H and E). The slides were examined under the camera fitted microscope at 10X. There was no alteration of histology of control group fish but infected group fish showed the histological alterations in gills and liver of *L. rohita*.

2.11. Statistical Analysis

All the experiments including antimicrobial, cytotoxicity activity was done in triplicate and results were calculated as a mean ± standard deviation (SD) by using Microsoft Excel (2013).

III. RESULTS

The change in color of ZnO (yellow) and Cu solution (black) was observed and taken as an confirmation of reduction of zinc nitrate dehydrate and copper sulfate salt in ZnO and CuO NPs respectively.

3.1 Characterizations of ZnO and Cu NPs

3.1.1 UV Visible Spectroscopy

The Systronics UV-visible spectrometer was used to investigate the optical characteristics of the green synthesized. ZnO and Cu NPs. Absorption spectrum of the ZnO and Cu NPs is represented in Figure 4A ,B that range from 300-800 nm. The ZnO and Cu NPs showed its absorption band at range of 460nm and 385nm respectively. The purity of the ZnO and Cu NPs was determined by the absence of any other peak in the range of absorption spectrum.

3.1.2. X- ray Diffraction

X-ray diffraction (XRD) is an innovative technique used to determine the structure and crystalline size of nanoparticles (NPs). The XRD patterns for ZnO and Cu NPs fall within the diffraction angle range of 20° to 80° as shown in Figures 3 and 4 ZnO nanoparticles exhibit a prominent peak at 2θ = 36.27° along with additional peaks at 31.67°, 34.43°, 47.53°, 62.97°, and 73.42° with corresponding diffraction indices.

In contrast, Figure 4 shows the peaks for Cu at angles of 27.52°, 37.36°, 48°, 54.75°, 62.19°, and 74°, corresponding to the (110), (004), (200), (211), (213), and (301) planes, respectively, which align well with the JCPDS card (21-1272). The purity of both ZnO and Cu NPs is confirmed by the specific peak positions with no extraneous peaks observed in the diffraction patterns. Additionally, the size of the NPs is inversely correlated with peak width as the peak width increases the size of the NPs decreases (5A,B).

3.13. FT-IR Spectroscopic Analysis of the ZnO and Cu NPs

FT-IR spectra is powerful analytical technique used for the identification of the functional group and chemicals bond in materials. ZnO and Cu NPs were synthesized by using the leaves of *C. gigentea* and their FT- IR spectra in 100% transmittance mode ranged from 4000-5000 cm^{-1} as shows in figure 6 A,B.

According to FT-IR spectrum of ZnO NPs the lowest region peak formed was 500–700 cm^{-1} which represented the formation of ZnO NPs. The highest peak 1533, 2362 and 3648 cm^{-1} indicated the formation of O-H bond. Where, the formation of the Cu NPs was determined by peak appearing at the range 3600-3400 cm^{-1} (N-H stretch), 3400-2400 cm^{-1} (O-H stretch), 988-830 cm^{-1} (C-H bend) and peak at 1612 cm^{-1} (C=C stretch), and 1271 cm^{-1} (C-O bend).

3.1.4. Scanning Electron Microscopy

The ZnO and Cu NPs size and morphology was determinate by using SEM as shown in figure 7A and B respectively. SEM micrograph indicated the single and number of aggregates. The data showed that spherical-shape ZnO NPs and Cu NPs showed amorphous spherical-shape with diameter ranges of 10-35nm. The figure 6A and B showed that smaller size ZnO and Cu NPs were formed with an average size 15nm and 18nm respectively.

3.2. Antibacterial Effect of Green Synthesized ZnO and Cu NPs

3.2.1. Antibacterial Activity of ZnO NPs against Bacterial Strains

The antimicrobial activity of green synthesized ZnO NPs were examined by disc diffusion against the different Gram positive and negative bacterial strains. When different concentration (5, 10, 15 and 20 mg/ mL) of ZnO NPs were examined the zone of inhibition (ZOI) were formed alone and along with the antibiotics as showed in Figure 8 and Table 1.

3.2.2. Antibacterial Activity of Cu NPs against Bacterial Strains

The antimicrobial activity of green synthesized Cu NPs were examined by disc diffusion against the different Gram positive and negative bacterial strains. When different concentration (5, 10, 15 and 20 mg/ mL) of Cu NPs were examined the ZOI were formed alone and along with the antibiotics as showed in Figure 9 and Table 2.

The results represented that among the bacterial strains *S. aureus* were indicated the highest ZOI of 22 mm and *E.coli* indicated the

minimum ZOI of 18 mm at the concentration of 20 mg / mL of green synthesized ZnO NPs while, in case of Cu NPs the bacterial strains *S. aureus* was (Figure 9 and Table 1) showed the lowest ZOI of 16 mm and *E.coli* showed the highest ZOI of 17 mm at the concentration of 20 mg / mL of green synthesized Cu NPs (Figure 10 and Table 2).

3.3. Cytotoxicity Assay of Green Synthesized ZnO and CuNPs

The brine shrimp lethality assay was used to determine the cytotoxicity of zinc oxide and copper oxide NPs. Different concentration of both ZnO and Cu NPs (5mg/mL, 10mg/mL, 15mg/mL and 20mg/mL). The suspension of the NPs did not exhibit any significant acute toxicity within 24h. There were four or five larvae found to be dead larvae was calculated to be 6.13% (15mg/mL) and 6.53% at highest (20mg/mL) concentration of ZnO NPs while, Cu NPs has 15.3% (20mg/mL) so, significant toxicity was observed on the shrimp larvae. The results presented in table 3 showed that the ZnO and Cu NPs were virtually toxic on the shrimp larvae as the concentration of the nanoparticles increased.

3.4. Hematological Analysis

Hematological parameters were assessed after 15 dpi. Significant differences in Hb (Figure 8a), RBCs (Figure 8b), MCH (Figure 8c), MCHC (Figure 8d), Hct (Figure 8e), WBCs (Figure 8f), PLT (Figure 8g), MCV (Figure 8h), monocytes (Figure 8i), eosinophils (Figure 8j), neutrophils (Figure 8k) and lymphocytes (Figure 8l) were found in the C, I, Cu NPs, Z NPs, Cu NPs+I and Z NPs+I groups. However, WBC was significantly high in the I and C NPs + I Hematological parameters were assessed after 15 dpi.

3.5. Histopathological Examination

For histological observations, fish organs gills and liver were analyzed. *L. rohita* were examined for histological changes on 15^{th} day.

3.5.1. Histopathological Observation of Gills

The gills of fish of control group are composed of four gill arches and each arch consists of several gill filaments. In fish gills there are two types of lamella. The secondary lamella protrudes from the primary lamella and sometimes referred to as the gill filament which extends from the gill arch. Examination of gill sections was performed after 15 days of bacterial infection (*A. hydrophila*) with the treatment of ZnO NPs that were synthesized by the *C. gigantea* leaves. In case of T1 (control group) histology of gills of *L. rohita*, normal structure of epithelium, primary lamella, secondary lamella, pillar cells and erythrocytes were observed with some minor alterations. Fish of T2 (infected group) showed excessive damage with fusion of primary lamella, fusion of secondary lamella, lamellar disorganization, hyperplasia, dilated club tips and lamellar aneurysm. In T3 (antibiotic treated group) there was inflammation in the gills structure and dilated club tips, hyperplasia and fusion of lamella were also seen. In case of T4 (High concentration of ZnO NPs treated group) blood congestion, hyperplasia, hemorrhage, disorganization of Lamella and lamellar aneurysm were observed.

The Fish of T5 (higher concentration of Cu NPs+ infected) exposed epithelium lifting, hypertrophy of the secondary lamella, fusion of primary lamella, dilated club tips and vacuolation. The infected group T6 that was treated with high concentration of ZnO NPs showed some defects in the gills of *L. rohita* that were curling of lamella, epithelium lifting, fusion of secondary lamella. Hypertrophy in the secondary lamella and disorganization of lamella were also observed.

3.5.2. Histopathological Observation of Liver

The liver is a major organ which performs metabolic functions and has detoxical role of endogenous waste products. In case of control fish, regular structure and systematic arrangement of hepatocytes (cells of liver) were observed. Liver of control group had normal structure with no conservative pathological changes. Normal hepatocytes with polygonal shape and normal hepatic portal veins were seen. On the other hand, liver of T2 group infected with fungus showed a large number of deformities such as focal area of necrosis, condensed blood vessels, karyolysed cells, degenerated hepatocytes, interstitial space, infection and necrotic portal vein.

In T3 green synthesize Cu NPs group with higher concentration necrosis, focal area of necrosis, swollen hepatocytes, degenerated hepatocytes hemorrhage and infection were observed. In liver of group T4 exposed to high concentration of zinc oxide Nanoparticles there were blood congestion, hemorrhage, swollen hepatocytes and condensed blood vessels. Liver of T5 (High concentration of Cu NPs +infected) showed infiltration and necrosis. Swollen hepatocytes, interstitial space and hemorrhage were also seen. Group T6 that were exposed to the high concentration of ZnO NPs and infected as well were examined and showed necrotic portal vein, condensed blood vessels and interstitial space with polygonal hepatocytes.

IV- DISCUSSION

Nanoparticles have significant potential in various biological, physical, and chemical applications because of their small size and large surface area. In the biological field, including aquaculture, their applications are particularly promising. Currently, the trend in aquaculture development is focused on intensifying and commercializing fish production, with a primary emphasis on enhancing fish immunity through dietary supplementation. Green synthesis methods often use natural, non-toxic materials, which reduces the environmental impact and potential toxicity to aquatic ecosystems. Chemically synthesized nanoparticles can involve hazardous substances and produce harmful byproducts. Nanoparticles produced through green synthesis are typically more biocompatible with living organisms including fish. This is because they are often made from natural or less toxic materials which minimize adverse effects on fish health.

Green synthesis methods generally use plant extracts, microorganisms, or natural polymers which are less likely to introduce harmful chemicals into the environment. Chemically synthesized nanoparticles may contain residual toxic chemicals or stabilizers that can be harmful to fish. Green synthesis can be more cost-effective because it often uses readily available and inexpensive natural materials. The green synthesis approach aligns with sustainable practices by minimizing waste and utilizing renewable resources. It often requires milder conditions and avoids the use of hazardous chemicals making it a more environmentally friendly option. Overall, the use of green-synthesized nanoparticles in fish treatment supports safer, more environmentally friendly, and sustainable practices compared to their chemically synthesized counterparts.

The present study was designed to green synthesize of NPs by *C. gigantea* leaves (Zinc nitrate salt) and Cu NPs (Copper sulphate salt). The antibacterial effects of these NPs were examined against gram positive *E. faecalis*, *S. aureus* and gram negative bacteria i.e. *E. coli*, *K. pneumoniae*, *A. hydrophila*, and *P. aeruginase*. Bacterial susceptibility to these nanoparticles was checked by disc method in which different concentrations of nanoparticles (5, 10, 15 and 20 mg/mL), *C.gigantea* leaves extract and antibiotic disc were also used. In our results nanoparticles showed antibacterial effects. The size of inhibitory zone differed depending upon the specie of bacteria as well as the concentration of nanoparticles.

In our study *S. aureus* displayed inhibitory zones of 14, 15, 19 and 22 mm for ZnO NPs disc at doses of 5, 10, 15 and 20 mg/mL and in Cu NPs *E.coli* showed 10 nm 12 nm 11 nm and 17 nm. ZnO NPs as Zn-nano discs were evaluated for their antibacterial effectiveness against the Gram positive bacterium *S. aureus* by [25]. The bacterium *S. aureus* was resistant to all ZnO NPs concentrations, according to the results of the antibacterial activity for the discs soaked with 6, 7, 8 and 9 ppm of ZnO NPs with the average size (mm) of the regions of inhibition surrounding the discs ranged from 2.41 to 6.19 mm.

The toxicity assay of green-synthesized nanoparticles (NPs) using brine shrimp is an inexpensive, easily accessible, simple, and reliable method. In the present study, the cytotoxic effects of green-synthesized ZnO and Cu NPs were assessed using the brine shrimp lethality assay. Brine shrimp larvae were exposed to different concentrations (5, 10, 15, and 20 mg/mL) of NPs and *C. gigantea* leaf extract to evaluate their toxicity in marine aquatic ecosystems. It was observed that the percentage of dead larvae increased over time.

The brine shrimp assay is a widely used preliminary screening tool due to its simplicity and sensitivity. This assay involves exposing brine shrimp larvae to different cool due to its simplicity and its mortality rates. Studies have shown that Cu NPs at concentrations ranging from 5 mg/mL to 20 mg/mL exhibit minimal acute toxicity, with mortality rates comparable to or lower than controls treated with vehicle alone (DMSO) [24].

It was observed that the plant extract did not exhibit any significant acute toxic activity within 24 hours. The death of the larvae was attributed to the accumulation of nanoparticles (NPs) in their guts, as the brine shrimp were unable to eliminate the ingested particles. This accumulation was thought to be the primary cause of mortality. The findings indicated that the percentage of brine shrimp mortality increased with the concentration of NPs. Therefore, it was concluded that the mortality rate is both dose- and time-dependent. Similar results have also been reported by other researchers [25,26].

For fisheries experts, the analysis of hematological parameters has played a significant role to detect change in fish anatomy and it cause change in behavior of fish. To assess the patho-physiological status of different living things exposed to any toxins, blood parameters are considered as the best biomarker [26,27]. In the present research, remarkable alterations in components of blood were noticed in challenged fish as fish of control and NPs treated groups showed normal hematology. Hemoglobin level was recorded that was high in treated groups then infected group where the Hb level in ZnO NPs treated was high as compared to Cu NPs treated group. The WBC level of infected group was increased then NPs treated groups. The platelets level of infected group was high then NPs treated groups where platelets level in Cu NPs treated group was high as compared to the ZnO NPs treated group. The MCH percentage of the control group is high as compared to the infected group where the percentage of MCH was high in ZnO NPs as compared to the Cu NPs treated group. The MCHC percentage was decreased in infected group while, MCHC percentage in Cu NPs were increased as compared to the ZnO NPs treated group. The RBCs count was low in the infected group as compared to the ZnO NPs and Cu NPs treated groups. The Hct percentage in the present study was low in infected group but after the treatment the Hct percentage was high in treated ZnO NPs and Cu NPs groups as compared to infected group. The present study showed the decreased level of MCV in ZnO NPs and Cu NPs treated groups and the MCV level in ZnO NPs decreased then Cu NPs whereas the level of MCV was remarkably increased in infected group.

The number of platelets after the day 15 sampling platelets showed a significant increase. Likewise, [28] reported a study in which *Grass carp* exposed to endo sulfan that exhibit acute toxicity and it showed a significant increase in platelet number. The WBCs counts were high in this analysis that could be due to stress conditions. The majority of the blood is made up of white blood cells that guard the organism during the time of infection, damage, bleeding and it prevents the foreign agents to enter in the body [30]. The WBCs level rises remarkably during the stress to combat under the stress conditions and defend organism from any damage [31]. As stated by [32] acute Endosulfan exposure in *Clarias gariepinus* showed WBCs and Thrombocyte elevated whereas hemoglobin, red blood cells, MCV and MCHC dramatically decreased.

In histopathological analysis, gills and liver were used to analyze the health of *L rohita*. In Gills, normal structure of epithelium, primary lamella, secondary lamella, pillar cells and erythrocytes were observed with some minor alterations in untreated group. The treatment with ZnO+Cu NPs showed several forms of histopathological changes as lamellar fusion, hyperplasia, lamellar disorganization, hyperplasia, dilated club tips, lamellar aneurysm, blood congestion, hyperplasia, hemorrhage, disorganization of lamella, hypertrophy of the secondary lamella, necrosis and vacuolation. Likewise, [33] reported a study in which few chosen *L. rohita* tissues treated to phenol were analyzed for histopathological changes and the observed changes in gills were hyperplasia, lamellar fusion, necrosis and lamellar disorganization. The majority of the fish gill histological responses were linked to circulation issues as well as backward and forward changes [34].

The histology of fish liver of control group exhibited typical structure and there were no pathological abnormalities whereas infected fish indicated infiltration, vacuolization, hemorrhage, necrotic portal vein, necrosis, hepatocyte degeneration, hyper trophy and blood congestion. The pathological changes observed in the current research were same to the [35] in which *Catla catla* were exposed to phenol. Similarly, [36] exposed phenol in *Oreochromis aureus* juveniles and same histopathological changes were observed as in the present study. Hypertrophy is due to increase in cellular size and hepatic enzyme activities can

be increased or decreased by the metals and can lead to histopathological hepatic change [37]. A variation between the rate of a substance's production in parenchymal cells and the rate at which it is released into the bloodstream indicate vacuolization.

Similarly, [38] observed *Tilapia zillii*, a freshwater fish that was exposed to aluminum and histopathological abnormalities were seen in the gills and liver that result the same changes as of present study. Likewise [39] showed respiratory epithelium necrosis, primary gill lamellae destruction and deterioration of secondary gill lamellae *Rarbora daniconius* exposed to pesticide endosulfan. [40] noticed cell membrane breakdown, blood clotting and congestion, pyknosis, necrosis, and hyperplasia and vacuolation of liver cells after exposure to Cypermethrin in *L. rohita*. An attempt has been made to study the possible changes in the histopathology of liver and gills in the present study.

the present study.

V- CONCLUSION

Cu and ZnO NPs were synthesized by using *C. gigantea* leaf extract. it is safe, eco-friendly and cheapest method. Antibacterial activity of Cu and ZnO NPs was checked against the multidrug resistant bacterial strains by the disc and well diffusion method. Nanoparticles showed strong antibacterial potential against gram negative and gram positive bacteria. The antimicrobial activity is dose dependent. As the dose is increased, the antibacterial activity increases. Moreover, well diffusion assay is more effective as compared to disc diffusion assay for antibacterial activity. ZnO NPs was showed best results then Cu NPs The concentration of Cu and ZnO NPs increases in cytotoxic analysis showed that they have toxic effect on brine shrimp larvae. Both Cu and ZnO NPs demonstrated effectiveness in improving hematology, histology and in bacterial challenged fish.

ACKNOWLEDGMENT

The preferred spelling of the word "acknowledgment" in American English is without an "e" after the "g." Use the singular heading even if you have many acknowledgments.

REFERENCES

[1] Barbara, K., & Nora, S. (2005). United States environmental protection agency (US EPA) Proceedings: Nanotechnology and the Environment: Applications and Implications. In *Progress Review Workshop III. Arlington, VA* (pp. 1-8).

[2] Prasad, T. N. V. K. V., & Elumalai, E. (2011). Biofabrication of Ag nanoparticles using Moringa oleifera leaf extract and their antimicrobial activity. *Asian Pacific Journal of Tropical Biomedicine*, *1*(6), 439-442.

[3] Hoag, E. G., Collins, J. B., Holcomb, J. L., Hoag, J. R., Nadagouda, M. N., & Varma, R. S. (2009). Degradation of bromothymol blue using nano iron synthesized through greener method. *J. Mater. Chem*, *19*, 8671-8677.

[4] Govender, Y., Riddin, T. L., Gericke, M., & Whiteley, C. G. (2010). On the enzymatic formation of platinum nanoparticles. *Journal of Nanoparticle Research*, *12*, 261-271.

[5] Sharma, P. C., & Jain, S. (2008). Synthesis and in vitro antibacterial activity of some novel N-nicotinoyl-1-ethyl-6-fluoro-1, 4-dihydro-7-piperazin-1-yl-4-oxoquinoline-3-carboxylates. *Acta Pol Pharm*, *65*, 551-556.

[6] Ahmad, N., & Sharma, S. (2012). Green synthesis of silver nanoparticles using extracts of Ananas comosus.

[7] Alagumuthu, G., & Kirubha, R. (2012). Synthesis and characterisation of silver nanoparticles in different medium. *Open Journal of Synthesis Theory and Applications*, *1*(2), 13-17.

[8] Singh, R. P., Shukla, V. K., Yadav, R. S., Sharma, P. K., Singh, P. K., & Pandey, A. C. (2011). Biological approach of zinc oxide nanoparticles formation and its characterization. *Adv. Mater. Lett*, *2*(4), 313-317.

[9] Yashni, G., Al-Gheethi, A., Mohamed, R., Hossain, M. S., Kamil, A. F., & Abirama Shanmugan, V. (2021). Photocatalysis of xenobiotic organic compounds in greywater using zinc oxide nanoparticles: a critical review. *Water and Environment Journal*, *35*(1), 190-217.

[10] Bouafia, A., Laouini, S. E., Ahmed, A. S., Soldatov, A. V., Algarni, H., Feng Chong, K., & Ali, G. A. (2021). The recent progress on silver nanoparticles: synthesis and electronic applications. *Nanomaterials*, *11*(9), 2318.

[11] Roy, A., Gauri, S. S., Bhattacharya, M., & Bhattacharya, J. (2013). Antimicrobial activity of CaO nanoparticles. *Journal of biomedical nanotechnology*, *9*(9), 1570-1578.

[12] Mandal, S. M., Roy, A., Ghosh, A. K., Hazra, T. K., Basak, A., & Franco, O. L. (2014). Challenges and future prospects of antibiotic therapy: from peptides to phages utilization. *Frontiers in pharmacology*, *5*, 105.

[13] Sen, A., & Alikamanoglu, S. (2014). Characterization of drought-tolerant sugar beet mutants induced with gamma radiation using biochemical analysis and isozyme variations. *Journal of the Science of Food and Agriculture*, *94*(2), 367-372.

[14] Woodford, N., Turton, J. F., & Livermore, D. M. (2011). Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS microbiology reviews*, *35*(5), 736-755.

[15] Rai, M. K., Deshmukh, S. D., Ingle, A. P., & Gade, A. K. (2012). Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. *Journal of applied microbiology*, *112*(5), 841-852.

[16] Smith, H. L., & Thieme, H. R. (2012). Persistence of bacteria and phages in a chemostat. *Journal of mat* Romero, J., Feijoó, C. G., & Navarrete, P. (2012). Antibiotics in aquaculture–use, abuse and alternatives. *Health and environment in aquaculture*, *159*(1), 159-198.*hematical biology*, *64*(6), 951-979.

[17] Leung, T. L., & Bates, A. E. (2013). More rapid and severe disease outbreaks for aquaculture at the tropics: implications for food security. *Journal of applied ecology*, 215-222.

[18] Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological indicators*, *8*(1), 1-13.

[19] Swain, P., Das, R., Das, A., Padhi, S. K., Das, K. C., & Mishra, S. S. (2019). Effects of dietary zinc oxide and selenium nanoparticles on growth performance, immune responses and enzyme activity in rohu, Labeo rohita (Hamilton). *Aquaculture nutrition*, *25*(2), 486-494.

[20] Vidya, C.; Hiremath, S.; Chandraprabha, M.N.; Antonyraj, M.L.; Gopal, I.V.; Jain, A.; Bansal, K. Green synthesis of ZnO nanoparticles by Calotropis gigantea. Int. J. Curr. Eng. Technol. 2013, 1, 118–120

[21] Liaqat, I.; Liaqat, M.; Tahir, H.M.; Ali, N.M.; Arshad, M.; Arshad, N. Motility effects biofilm formation in Pseudomonas aeruginosa and Enterobacter cloacae. Pak. J. Pharm. Sci. 2019, 32, 927–932.

[22] Sarah, Q. S., Anny, F. C., & Misbahuddin, M. (2017). Brine shrimp lethality assay. *Bangladesh Journal of pharmacology*, *12*(2), Online-Jun.

[23] Ydollahi, M., Ahari, H., & Anvar, A. A. (2016). Antibacterial activity of silver-nanoparticles against Staphylococcus aureus. *African Journal of Microbiology Research*, *10*(23), 850-855.

[24] Mustafa, G., Tahir, H., Sultan, M., & Akhtar, N. (2013). Synthesis and characterization of cupric oxide (CuO) nanoparticles and their application for the removal of dyes. *African Journal of Biotechnology*, *12*(47), 6650-6660.

[25] Maurer-Jones, M. A., Gunsolus, I. L., Murphy, C. J., & Haynes, C. L. (2013). Toxicity of engineered nanoparticles in the environment. *Analytical chemistry*, *85*(6), 3036-3049.

[26] Mapar, M. (2018). Waveguide evanescent-field microscopy for label-free monitoring of biological nanoparticles: fabrication, characterization and application. Chalmers Tekniska Hogskola (Sweden).

[27] Sumithra, K., Lakshmi, B. V., & Jothiramalingam, S. (2018). Antioxidant and Larvicidal Studies of Copper Oxide and Zinc Oxide Nanoparticles by Musa acuminata Leaves as Reducing Agent.

[28] Maheswaran, R., Devapaul, A., Muralidharan, S., Velmurugan, B., & Ignacimuthu, S. (2008). Haematological studies of fresh water fish, Clarias batrachus (L.) exposed to mercuric chloride. *International Journal of Integrative Biology*, *2*(1), 49-54.

[29] Hussain, R., Javed, M. T., & Khan, A. (2012). Changes in some biochemical parameters and somatic cell counts in the milk of buffalo and cattle suffering from mastitis. *Pak. Vet. J*, *32*(3), 418-421.

[30] Hasan, Z., Ghayyur, S., Hassan, Z. U., & Rafique, S. (2015). Histomorphometric and Hematological Profile of Grass Carp (Ctenopharyngodon idella) during Acute Endosulfan Toxicity. *Pakistan Veterinary Journal*, *35*(1).

[31] Babu Velmurugan, E. I. C., Senthilkumaar, P., Uysal, E., & Satar, A. (2016). Hematological parameters of freshwater fish Anabas testudineus after sublethal exposure to cypermethrin. *Environ. Pollut. Prot*, *1*, 32-39.

[32] Deshmukh, D. (2016). Hamatological response in a freshwater fish Channa Striatus exposed to endosulfan pesticide. *Bioscience Discovery*, *7*(1), 67-69.

[33] Ndimele, P. E., Jenyo-Oni, A., Kumolu-Johnson, C. A., Chukwuka, K. S., & Onuoha, S. (2015). Effects of acute exposure to endosulfan (organochlorine pesticides) on hematology of African Mud Catfish, Clarias gariepinus (Burchell, 1822). *Bulletin of environmental contamination and toxicology*, *95*, 164-170.

[34] Butchiram, M. S., Kumar, M. V., & Tilak, K. S. (2013). Studies on the histopathological changes in selected tissues of fish Labeo rohita exposed to phenol. *J Environ Biol*, *34*(2), 247-251.

[35] Van Dyk, J. C., Marchand, M. J., Pieterse, G. M., Barnhoorn, I. E., & Bornman, M. S. (2009). Histological changes in the gills of Clarias gariepinus (Teleostei: Clariidae) from a polluted South African urban aquatic system. *African Journal of Aquatic Science*, *34*(3), 283-291.

[36] Tilak, K. V. B. R., Ranganayaki, N., & Manoharachari, C. (2006). Synergistic effects of plant-growth promoting rhizobacteria and Rhizobium on nodulation and nitrogen fixation by pigeonpea (Cajanus cajan). *European Journal of Soil Science*, *57*(1), 67-71.

[37] Abdel-Hameid, N. A. H. (2007). Physiological and histopathological alterations induced by phenol exposure in Oreochromis aureus juveniles. *Turkish Journal of Fisheries and Aquatic Sciences*, *7*(2).

[38] Paris-Palacios, S., Biagianti-Risbourg, S., & Vernet, G. (2000). Biochemical and (ultra) structural hepatic perturbations of Brachydanio rerio (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulfate. *Aquatic Toxicology*, *50*(1-2), 109-124.

[39] Hadi, A. A., & Alwan, S. F. (2012). Histopathological changes in gills, liver and kidney of fresh water fish, Tilapia zillii, exposed to aluminum. *International Journal of Pharmacy & Life Sciences*, *3*(11).

[40] Ghanbahadur, A. G., & Ghanbahadur, G. R. (2012). Study of gonadosomatic index of fresh water fish Cyprinus carpio. *DAMA International*, *1*(1), 32-33.

[41] Murussi, C. R., Costa, M. D., Leitemperger, J. W., Flores-Lopes, F., Menezes, C. C., Loebens, L., ... & Loro, V. L. (2016). Acute exposure to the biopesticide azadirachtin affects parameters in the gills of common carp (Cyprinus carpio). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *180*, 49-55

AUTHORS

First Author – Zahra Ghulam Haider, MS scholar, Department of Zoology, Lahore College for Women University, Lahore;

Second Author – Prof. Dr. Farzana Rashid, Department of Zoology, Lahore College for Women University, Lahore

Third Author – Iqra Noshair, Ph.D scholar, Department of Zoology, Lahore College for Women University, Lahore; ORCID-0009-0006-8174-8652

Forth Author – Abdullah Etezaz, Ph.D scholar, Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan;

Fifth Author – Sadia Iqbal, Ph.D scholar, Department of Zoology, Lahore College for Women University, Lahore;

Sixth Author – Sadia Akhtar, Ph.D scholar, Department of Zoology, Lahore College for Women University, Lahore;

Seventh Author – Adila Sana, MS scholar, Department of Zoology, Lahore College for Women University, Lahore;

Correspondence Author – Prof. Dr. Farzana Rashid +92307-7777730

Figure 1. Preparation of leaves extract (A): *C. gigantea* Plant (B): Dry leaves of *C. gigantea* (C): Weight the dry leaves (D): Boiled crushed dry leaves on hot plate

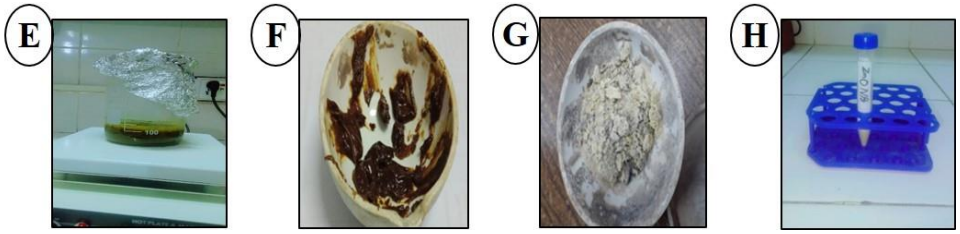


Figure 2. Synthesis of ZnO NPs (E): Addition of Zinc nitrate into leaves extract (F): Paste of leaves extract (G): Calcining (H): ZnO NPs

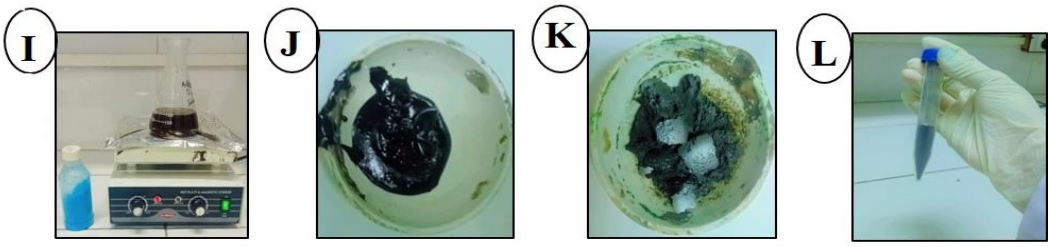


Figure 3. Synthesis of Cu NPs (I): Addition of copper sulphate into leaves extract (J): Paste of leaves extract (K): Calcining (L): Cu NPs

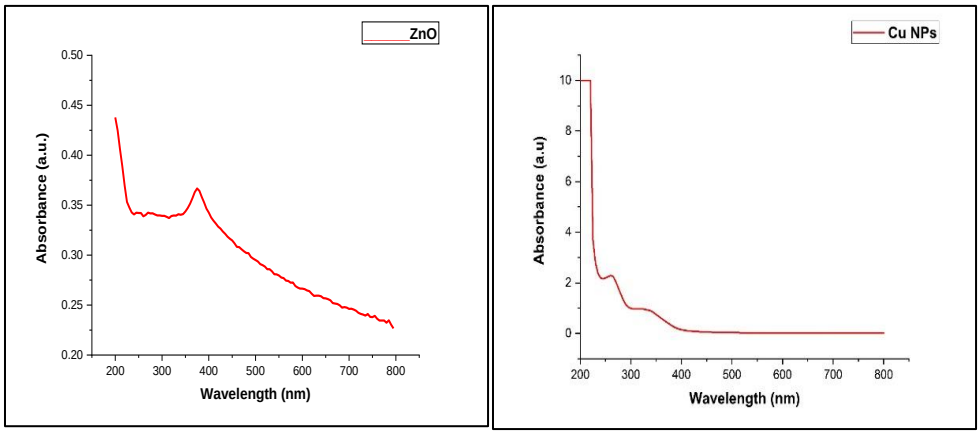


Figure 4. UV Visible Spectra of Green Synthesized ZnO and Cu NPs

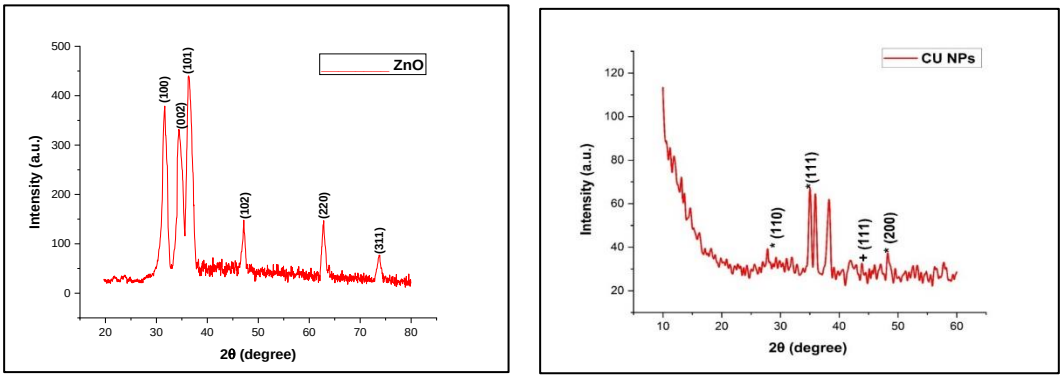


Figure 5. X-ray Diffraction of Green synthesized ZnO and Cu NPs

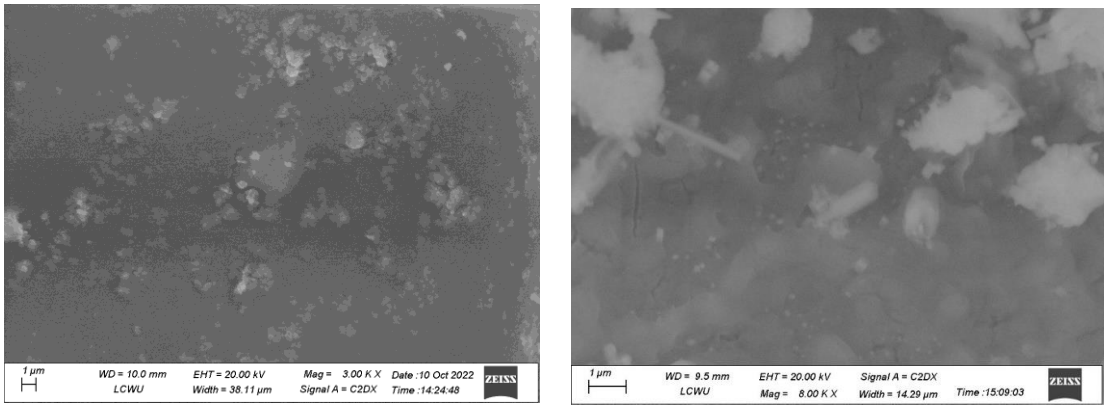
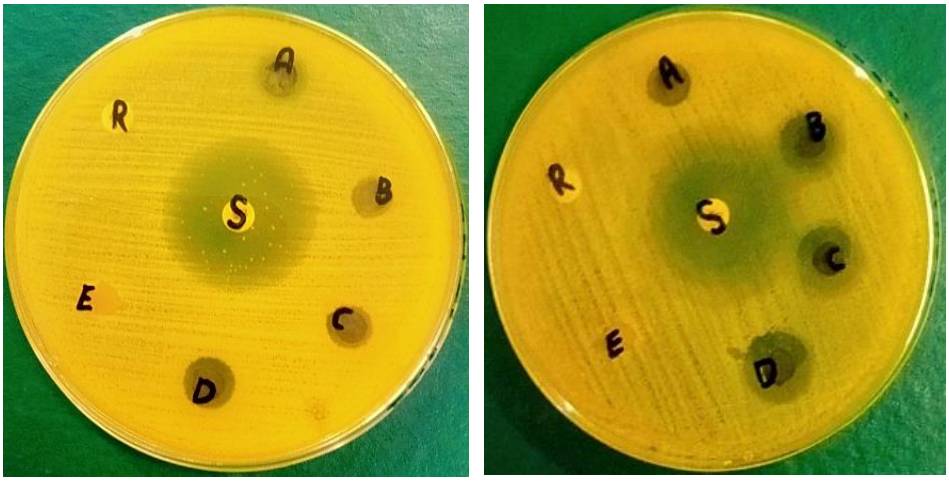


Figure 7. SEM micrograph of green synthesized ZnO and Cu NPs

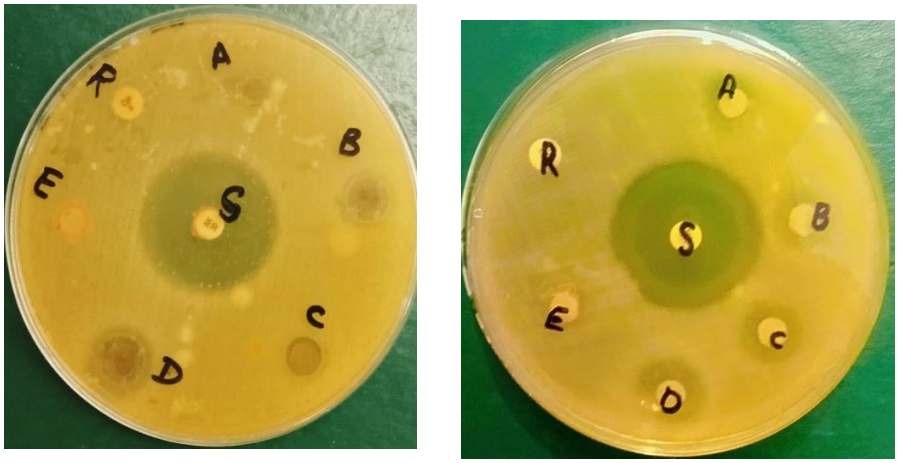


E. coli ***S. aureus***

Figure 8: Antimicrobial activity of ZnO NPs against bacterial strains by disc diffusion assay. Where A: 5 mg / ml, B: 10 mg / ml, C: 15 mg / ml, D: 20 mg / ml of ZnO NPs E: plant extract, S: bacteria sensitivity to antibiotics (amikacin) and R: bacteria resistant to antibiotics (ciprofloxin).

Table 1. Antimicrobial activity of *C. gigantea* synthesized ZnO NPs by disc diffusion method.

Bacterial Strains	**Zone of Inhibition (mm)**						
	Concentration of ZnO NPs (mg/mL)					**Antibiotics**	
	Plant Extract	**5**	**10**	**15**	**20**	**CIP**	**Amikacin**
K. pneumoniae	15±0.5	14±0.1	16±0.2	14±0.1	19±0.1	R	26±0.1
S. aureus	13±0.1	14±0.5	15±0.1	19±0.2	22±0.2	R	29±0.2
E. coli	14±0.3	15±0.1	14±0.2	15±0.1	18±0.5	R	30±0.5
E. faecalis	12±0.2	14±0.2	11±0.5	15±0.3	19±0.5	R	29±0.2



E. coli ***S. aureus***

Figure 9. Antimicrobial activity of Cu NPs against bacterial strains by disc diffusion assay. Where A: 5 mg / ml, B: 10 mg / ml, C: 15 mg / ml, D: 20 mg / ml of Cu NPs E: plant extract, S: bacteria sensitivity to antibiotics (amikacin) and R: bacteria resistant to antibiotics (ciprofloxin).

Table 2. Antimicrobial activity of *C. gigantean* synthesized Cu NPs by disc diffusion method.

Bacterial Strains	**Zone of Inhibition (mm)**						
	Concentration of Cu NPs (mg/mL)					**Antibiotics**	
	Plant Extract	**5**	**10**	**15**	**20**	**CIP**	**Amikacin**
K. pneumoniae	14±0.5	14±0.1	16±0.2	14±0.1	15±0.1	R	27±0.1
S. aureus	11±0.1	10±0.5	14±0.1	17±0.2	16±0.2	R	25±0.2
E. faecalis	13±0.3	15±0.1	14±0.2	13±0.1	15±0.5	R	28±0.5
E. coli	10±0.2	12±0.2	13±0.5	11±0.3	17±0.5	R	30±0.2

Table 3. Cytotoxic potential of ZnO and Cu NPs by Brine shrimp lethality Assay

Samples	**Concentration of samples (mg/mL)**	**Initial no of dead larvae "N"**	**No. of dead larvae after 24 hours "A"**	**Average no. of dead larvae in blind samples after 24 hours "B"**	**Total no. of larvae "G"**	**% age Mortality rate "M" (A-B-N)/Gx 100**
ZnO NPs	**5**	0	0	0	15.6	0%
	10	1	1	0	17.6	0%
	15	2	1	0	16.3	6.13%
	20	0	1	0	15	6.53%
	Plant Extract	0	0	0	12	0%
Cu NPs	**5**	1	1	0	20	0%
	10	1	1	0	19.3	0%
	15	1	0	0	11.3	0%
	20	0	2	0	13	15.3%
	Plant Extract	0	0	0	11	0%

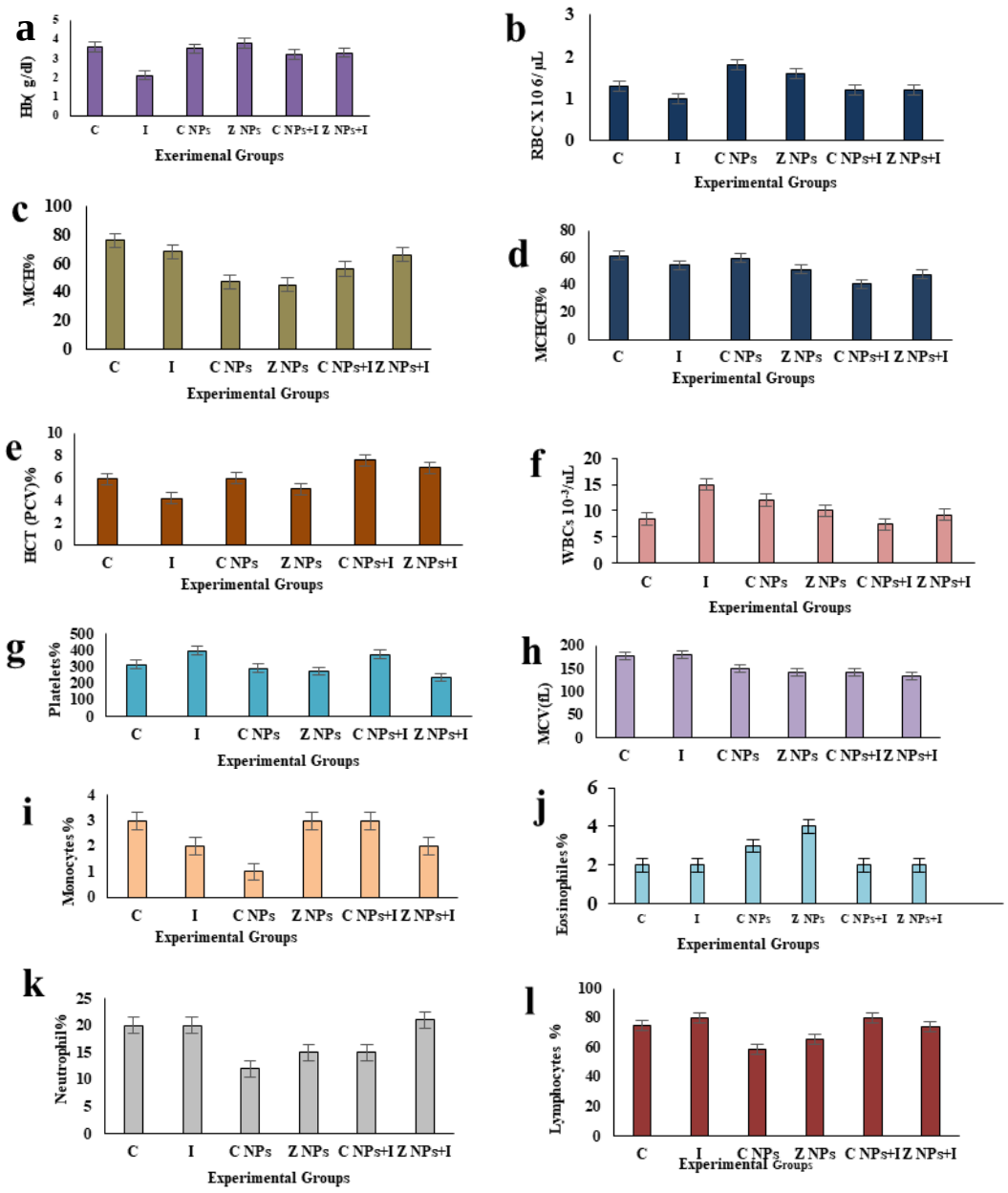


Figure 10: Hematological indices in control (Con), infected (I), copper NPs treated (C NPs), zinc NPs treated (Z NPs), copper NP-infected(Cu NPs + I) and zinc oxide nanoparticles- infected group (ZnO +I) at 15 dpi; (a) hemoglobin (Hb), (b) red blood cell count (RBCs), (c) mean corpuscular hemoglobin (MCH), (d) mean corpuscular hemoglobin concentration (MCHC), (e) hematocrit (Hct%), (f) white blood cell (WBCs), (g) platelets (PLT), (h) mean corpuscular volume (MCV), (i) monocytes, (j), eosinophils, (k) neutrophiles and (l) lymphocytes Data are presented as mean tested using two-way ANOVA with post hoc Tukey's test

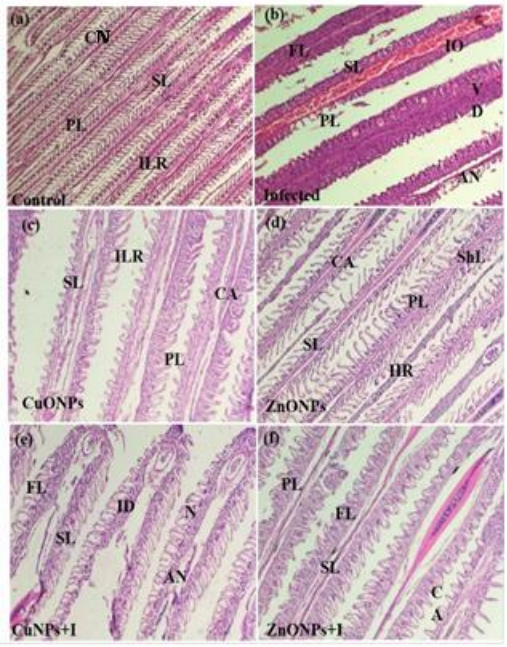


Figure 11: Gill tissues of control (Con), infected (I), Cu NPs treated ZnO NPs treated fish Cu NPs+I, and ZnO NPs+I treated fish(a), (b), (c), (d), (e) and (f) respectively; cellular necrosis (CN), fusion of lamellae (FL), primary lamellae (PL), secondary lamellae (SL), aneurism (AN), interstitial and shortening of lamellae (ShL). 10X magnification

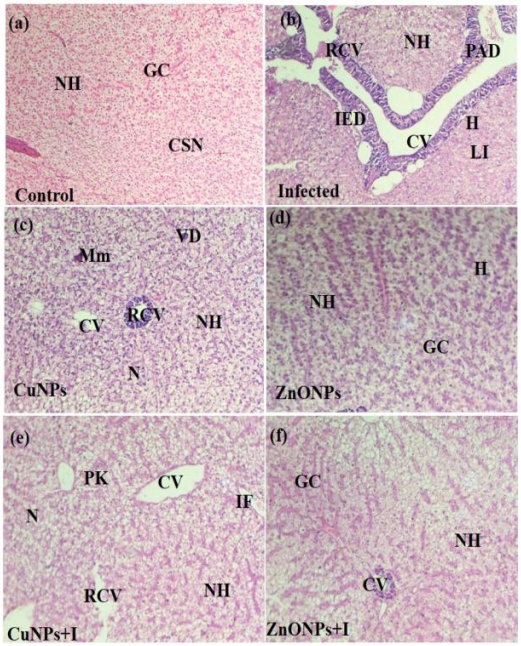


Figure 12: Liver tissues of control (Con), , infected (I), CuNPs treated, ZnO NPs treated , Cu NPs+I, ZnO NPs+I treated fish, (a), (b), (c), (d), (e) and (f) respectively; normal hepatocytes (NH), granular cytoplasm (GC), vacuolization of hepatocytes (V), central spheroidal hepatocyte nucleus (CSN), cell necrosis (N), degeneration of pancreatic area (DPA), pyknotic nuclei (PN), cytoplasmic degeneration (CD), leukocytes infiltration (LI), rupturing of the central vein (rCV) and hyperplasia (HP). 10X magnification