

Assessment of the Mint-Copper Nanoparticles Treatment to Control the Infection of Saprolegniasis in *Cyprinus carpio* L.

Sarab W. Alwash¹, J.K. Al-faragi¹, Tagreed M. Al-Saadi²

¹ Department of Pathology- College of veterinary Medicine- University of Baghdad/ Iraq

² Collage of Education for Pure Science-Ibn Al-Haitham/ University of Baghdad/ Iraq

Correspondence:sarabwahab90@gmail.com

Abstract

The goal of this approach is to produce a copper nanoparticle-based alternative method for *saprolegniasis* control in *Cyprinus carpio* L. For this purpose, six different transactions of 120 fish (20 fish/therapy) were used. Which were treated with copper nanoparticles at concentrations of 10mg/l, 20mg/l, CuSO₄ 0.5mg/l, and mint 100mg/l after being contaminated with *Saprolegnia* spp. (2×10^4 zoospores/l). one as a positive control (infected with *Saprolegnia* spp. without antiseptics) and the other as a negative control (no infection no disinfectants). Blood samples were taken from fish after 14 days of therapy for hematological and biochemical analysis. The average values of blood levels have improved significantly. RBC count and hemoglobin content were found to be marginally higher (p0.05) in all NPs treatments as compared to the C- group. Total protein and globulin were significantly increased (p0.05) in both treatments, in addition to the positive and negative groups (C- and C+). When compared to the C+ and C- groups, both treatments saw a significant decrease in albumin (p0.05). CuNPs were tested for potency using chemical processes (green synthesis), and then diagnosed using FTIR, X-ray, EDX, and SEM. The nanometer scale ranged from 11 to 34. According to this report, copper nanoparticles have antifungal properties.

Keywords: Copper, Nanoparticles, Chemical reduction, Characterization, *C. carpio*

1- Introduction

A nanoparticle (NP) is a microscopic material that behaves as a single entity in terms of distribution and effects, and varies significantly from bulk materials of similar composition. The scale, form, and chemical atmosphere of NPs affect their properties (Murray *et al.*, 2000) and despite their widespread use, the toxicity of NPs has become increasingly important for the last decade (De-Jong and Borm, 2008). Because of their special characteristics such as specific surface area, durability, inertness, optical properties, and size dependent tunable surface chemistry, many nanomaterials (NMs) have been widely used in electronics, supercapacitors, environmental, and therapeutic applications (Nel *et al.* 2006, Nair and Chung 2015). Functional NM research and development has exploded in popularity as a result of their improved physicochemical properties, which make them suitable candidates for use in real-world applications. Because of their release into the atmosphere, NMs' physicochemical properties and physiological structure are closely linked to their environmental toxicology. However, the relevance of these physicochemical properties to the availability of NMs in the atmosphere is still unknown. The rapidly expanding industrial applications of NMs have sparked significant questions about their potential eco-toxic effects when released into

the atmosphere. (Fairbrother and Fairbrother 2009; Jacques *et al.*, 2017). CuNPs are also commonly used in fungicides, algacide, and herbicides, but they can cause genotoxicity and oxidative DNA damage at the cellular level. (Song *et al.*, 2012). Aquaculture developed to adopt more productive means to feed increasing human populations (Ajeel & Al-faragi, 2013). The increase in aquaculture is said to be paralleled with a corresponding increase in the occurrence of infectious diseases, resulting often from high stocking densities and stress conditions that favor the occurrence and spread of pathogens (Ajeel & Al-faragi, 2013). Cultured fish suffer a wide variety of bacterial, viral, parasitic and fungal diseases (Ajeel & Al-faragi, 2013).

2- Material and methods

2-1- Experimental Design of Artificial Infection

A commercial fish farm in Babel, Iraq, provided approximately 120 stable *C. carpio* weighing 805g. Prior to the start of the experiment, the fish were conditioned in a laboratory environment for two weeks. The fish were grouped into 6 treatment classes in duplicate (2 tanks/treatment) and uniformly stocked into 12 rubber gaskets (10 fish/tank). The fungi isolates were then added to the fish ecosystem (2×10^4 zoospores per liter) and kept for 14 days in the aquarium tanks. Water quality parameters were monitored every day in each tank when signs of cotton wool like fungal were visible on the fish: [Dissolved O₂ (mg /l) 6.15 ± 0.5 ; Temperature (°C) 20 ± 2 ; pH 7.11 ± 0.05]. Blood samples were collected from fish 14 days after infection with *saprolegnia* and treatment to for hematological and biochemical analyses.

2-2- Preparation of Copper Nanoparticles

Using copper (II) sulfate pentahydrate as a precursor salt and starch as a capping agent, Cu nanoparticles were created through a chemical reduction process. The process begins with intense stirring of 0.1 M copper (II) sulfate pentahydrate solution into 120 mL of starch (1.2 percent) solution for 30 minutes. Under continuous rapid stirring, 50 mL of 0.2 M ascorbic acid solution is applied to the synthesis solution in the second step. After that, 30 mL of 1 M sodium hydroxide solution was slowly applied to the prepared solution when it was heated at 80 °C for 2 hours with continuous stirring. The solution's color changed from yellow to ocher. After the reaction was completed, the solvent was removed from the heat and allowed to settle overnight until the supernatant solution was carefully discarded. The precipitates were filtered out of the solution and washed three times with deionized water and ethanol to remove the residual starch bound to the nanoparticles. The resulting ocher-colored precipitates are dried at room temperature. Nanoparticles were dried and placed in a glass vial for further examination. (Khan *et al.*, 2016).

2-3- Green Synthesis of mint-Copper Nanoparticles

CuNPs powder in the amount of 0.2g was prepared and deposited in brown bottles. With continuous stirring, 100 mL of plant leaf extract was slowly mixed with CUNPs solution (1:6). The mixture was incubated for 24 hours at room temperature. The color shift was monitored on a regular basis (after 30 minutes and 90 minutes). The colour changed from light brown to dark brownish, indicating the formation of M-Cu NPs. To clear any impurities, the M-Cu NPs is washed in deionized water. Thereafter, the NPs were allowed to dry and so as to be used.

2-4- Characterization of Nanoparticles

2-4-1- X-ray Diffraction analysis

The prepared nanoparticles were XRD analyzed on a PANlytical X'Pert PRO diffractometer with Cu Ka radiation (1.54 Å) as a source, which was run at 40 kV and 30 mA. (Khan *et al.*, 2016)

2-4-2- Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, JEOL JSM-6490A) fitted with an energy-dispersive X-ray spectrometer (EDX) (6490 LA) was used to analyze the morphology and chemical structure of the synthesized nanoparticles (Khan *et al.*, 2016).

2-4-3- Fourier Transform Infrared (FTIR) spectroscopy

The NicoletMagna-IR Spectrometer model 550 was used to capture FTIR spectra of copper NPs in KBr pellets.

2-4-4- Energydispersive X-ray spectroscopy (EDX)

The EDX was performed at a 20.0 kV acceleration voltage. Sprinkling condensed nanoparticles onto double-sided adhesive carbon conductive tape that was placed on a microscopic stub of copper was used to make the sample. The sample is then gold-sputter-coated using an ion sputtering system (JFC 1500).(Khan *et al.*, 2016)

2-5- Blood and Serum Collection

Blood was taken from the caudal vein. According to the procedure defined by Hrubc and Smith(2000), blood was transferred to heparin anticoagulant tubes for RBCs ($10^6/l$) and WBCs ($10^3/l$) counts. A hemoglobin test kit was used to determine the amount of hemoglobin in the blood (g/dl).

2-6- Analytical Statistics

SPSS V. 16 software was used for statistical analysis. To evaluate the relevant variations between variables, one method was used: study of variance (ANOVA). At a chance value of 5%, the variations in means were examined.

3- Result and Discussion

3-1- Scanning electron microscopy analysis

The surface morphology of synthesized nanoparticles was studied using SEM analysis. Cu nanoparticles' surface morphology is depicted in (Figure 1). The spherical or semi shape spherical of the Cu nanoparticles as prepared was revealed by SEM image. Stabilizers are important in determining particle size distribution and preventing clustering and flocculation. (Qiuli *et al.*, 2010)

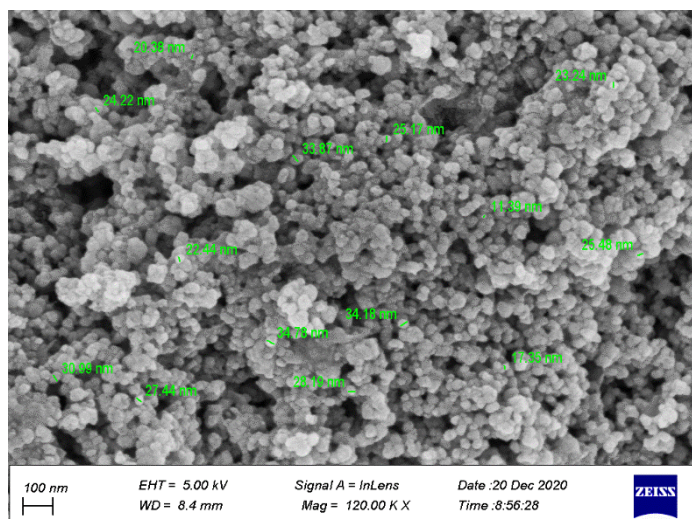


Figure 1 :- Scanning electron micrographs (SEM) of Cu nanoparticles

3-2- Fourier transformation infrared spectroscopy (FTIR):

Photochemical study of aqueous leaf extract *M. spicata* was shown by FTIR spectroscopy (Figure 2).

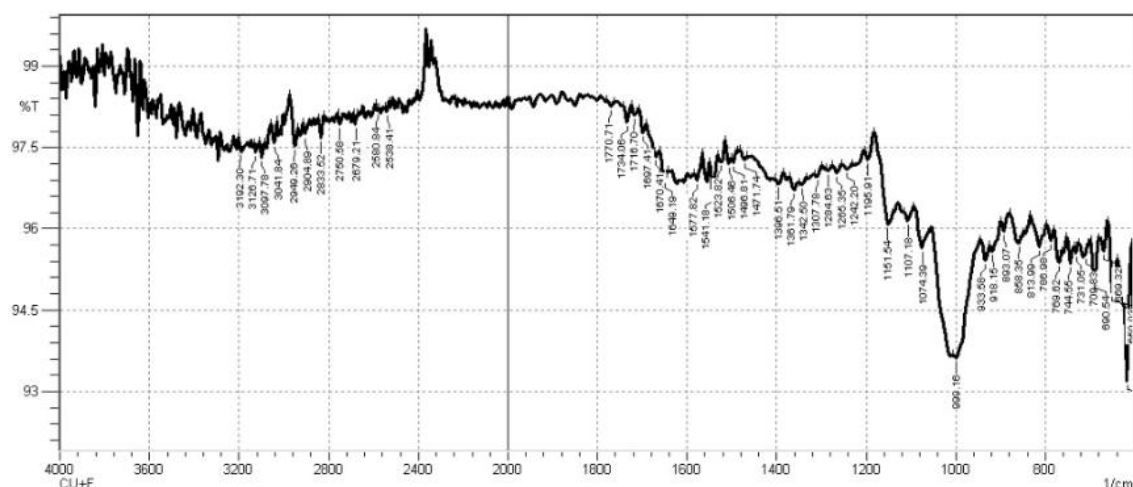


Figure 2 :- FTIR spectrum of copper nanoparticles synthesized by aqueous leaf extract of *M. spicata*.

3-3- Analysis x-rays (XRD):

The structure of the copper sample was determined via X-ray diffraction (XRD). (Figure 3) shows an XRD pattern of copper nanoparticles. The patterns of Cu-NPs exhibited strong diffraction peaks at 2θ (43.303° , 50.425° , 74.093° , 89.902° , and 95.08°). All of the diffraction peaks could be indexed to those of face-centered cubic copper according to this method agrees with the JCPDS card no. (JCPDS Card No.: 85-1326), with the emergence of one peak returning to CuO-NPs at 2θ (36.4212°) without other peaks appearing, indicating the purity of phase and the absence of impurities. The coexistence of two crystalline phases, metallic Cu and Cu₂O, was shown by the XRD diffraction pattern. This clearly shows that the zero-valent copper nanoparticles produced during the chemical reduction stage decompose due to Cu's reduced stability (Aslam *et al.*, 2000), and Cu₂O can be formed by oxidation (Feng *et al.*, 2012).

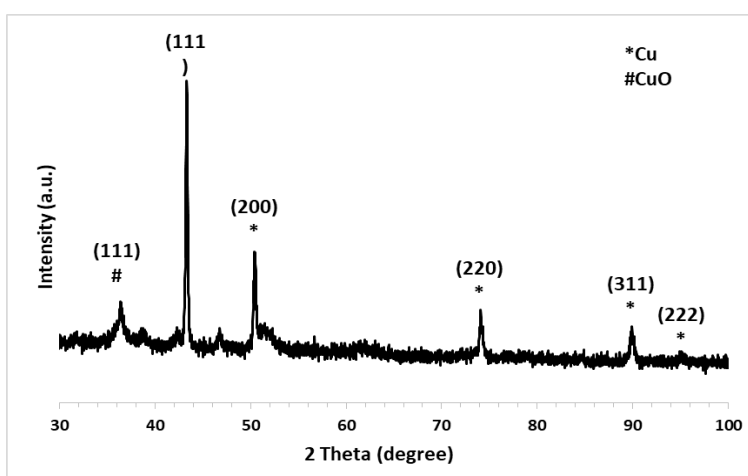


Figure 3:- XRD pattern of Cu and CuO nanoparticles

3-4- Energy-dispersive X-ray spectroscopy

The elemental composition of the synthesized nanoparticles was determined using EDX spectroscopy. The Cu nanoparticles' EDX spectrum (Fig. 4) verified the presence of Cu, O, and C. The binding energy of oxygen (OK) has a plateau around 0.57 keV, while CuL, CuK, and CuKb have peaks at binding energies of 0.94, 8.04, and 8.94 keV, respectively (Khan *et al.*, 2011). In addition, a peak corresponding to carbon (CK) has emerged at 0.27 keV. Except for a significantly improved peak of carbon, the EDX spectrum of Cu nanoparticles obtained is close to that stated earlier by (Kooti and Matouri, 2010). The existence of carbon-based stabilizers was confirmed by the carbon and oxygen peaks in the samples (Singh and Raykar, 2008). Carbon peaks in the spectrum may be due to the carbon tape that was used to install the sample on the stub. The elemental analysis of the prepared Cu nanoparticles is shown in Table (1).

Table 1: 1 Energy-dispersive X-ray (EDX) spectroscopy of Cu and CuO nanoparticles

Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	22.54	0.37	48.23

O	K series	17.01	0.21	27.32
Cu	L series	60.45	0.34	24.45
Total		100.00		100.100

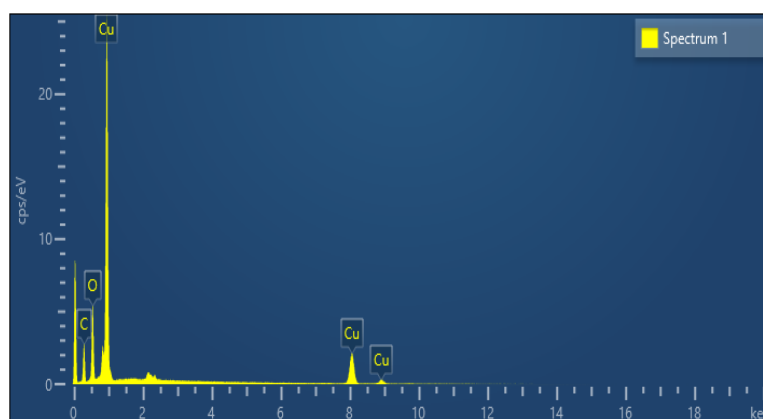


Figure 4 :- EDX spectrum of Cu and CuO nanoparticles

3-5- Haematological Parameters

After infection with *Saprolegnia spp.*, WBC is found to increase in (C+) relative to (C-). When compared to group C-, the number was slightly higher ($p < 0.05$) (C-). In contrast to the (C-) group, WBC counts increased significantly in all care classes. T1 had the highest value, which was slightly higher than T3 and T4. The number and quality of white blood cells (WBCs) are often used as indicators of immune response and disease (Cagiran, 1990). Changes in WBC and differential counts have been stated to be significant in determining a fish's health status. Leukocytes are usually lower in healthy fish and higher in sick fish, which may be seen as a significant indicator for infectious diseases (Gabriel *et al.*, 2004).

The current analysis found a substantial improvement ($P < 0.01$) in *C. carpio* WBC count in C+ relative to C-. These findings are consistent with Jamalzadeh *et al.*, (2009) findings that WBC levels are higher in fungal-infected fish than in stable *Caspian Salmon (Salmo ciscaucasicus)*. In a similar trend to the current research, Shah *et al.*, (2015) recorded improvements in rainbow trout.

RBCs count ($\text{Cell} \times 10^6/\text{l}$) observed a substantial decrease ($p < 0.05$) in C+ group after 14 days from infection by *Saprolegnia spp.* relative to C- group after 14 days from infection by *Saprolegnia spp.* T1 has the largest value, with a major ($p < 0.05$) boost as compared to all other therapy groups (T2, T3, T4) and control groups (C- and C+). PCV (percent) findings after infection with *Saprolegnia spp.* indicate major variations ($p < 0.05$) in the (C+) group relative to the (C-) group (C-). The PCV (percentage) in the C+ group was slightly higher than in the C- group. PCV (%) was significantly higher in T1 (treated with 20 mg M-CuNPs) than in T2, T3, T4, and C+ classes. After 14 days of infection with *Saprolegnia spp.*, Hb levels in the (C+) population indicate a substantial decrease ($p < 0.05$) as comparison to the (C-) group. Furthermore, as opposed to the C- and C+ levels, both care groups (T1, T2, T3, and T4) demonstrated significant

variations ($p < 0.05$). The number of RBCs, PCV value, and Hb content in infected groups, C+, were significantly lower than in the control group. This may be due to anemia caused by hyphae that enter infected fish's blood vessels and induce hemorrhage, hematopoietic tissue injury, haemodilution and osmotic imbalance, and mucus release lethargy. These findings are consistent with McLeay (1973).

Table 1:- Hematological parameters of *C. carpio* after 14 days from infection with *Saprolegnia spp.* and treated with M-CuNPs, CuSO₄ and mint.

Treatment	WBC $\times 10^3/\mu\text{l}$	RBC $\times 10^6/\mu\text{l}$	PCV (%)	Hb (g/dl)
Control -ve Non infected	19.35 $\pm$ 0.12 c	2.007 $\pm$ 0.034 a	25.23 $\pm$ 0.19 ab	8.41 $\pm$ 0.67 a
Control +ve Infected	21.68 $\pm$ 0.02 bc	1.620 $\pm$ 0.017 b	15.95 $\pm$ 0.23 c	5.31 $\pm$ 0.09 c
T1	25.73 $\pm$ 0.02 a	2.083 $\pm$ 0.009 a	26.46 $\pm$ 0.15 a	8.82 $\pm$ 0.06 a
T2	23.13 $\pm$ 0.01 ab	1.987 $\pm$ 0.009 a	21.13 $\pm$ 0.15 ab	7.04 $\pm$ 0.06 ab
T3	22.88 $\pm$ 0.16 b	1.677 $\pm$ 0.024 b	18.36 $\pm$ 0.18 bc	6.12 $\pm$ 0.06 bc
T4	22.76 $\pm$ 0.01 b	1.730 $\pm$ 0.012 b	20.43 $\pm$ 0.12 ab	6.80 $\pm$ 0.05 ab

3-6- Biochemical Test

Total Protein, Albumin and Globulin and A/G ratio

Total protein (TP), albumin (AL), and globulin (GL) concentrations in *C. carpio* serum contaminated with *Saprolegnia spp.* and treated with M-CuNPs, CuSO₄, and mint (Table 2). After 14 days, the C- group's TP and AL levels were significantly higher ($p < 0.05$) than the C+ group's. T1 and T2 TP levels were found to be significantly higher in the C- and C+ classes, respectively. T3 and T4 TP levels, on the other hand, showed no major differences ($p < 0.05$) as compared to the C- group. As applied to all treated groups, the AL level in the C+ category showed a substantial improvement ($p < 0.05$). (T1, T2, T3 and T4). GL levels, on the other hand, increased significantly in the C+ group after 14 days relative to the C- group. In addition, GL in both treated groups showed substantial differences as compared to the C- group. GL levels have differed significantly ($p < 0.05$) between treatment classes (T1, T2, T3 and T4). The A/G ratio shows a substantial reduction in T1, T2, T3, and T4 treatments as compared to C - and a non-significant difference as compared to C + in T1, T2, T3, and T4 treatments.

C+ showed a substantial decline ($P < 0.01$) in total protein and albumin levels during the experimental duration as compared to the control group C-. These findings are consistent with those of Shah *et al.* (2015), who discovered that total serum protein and albumin content in *Saprolegnia spp.* infected rainbow trout decreased dramatically as

compared to the normal and control groups. This may be attributed to changes in aerogram values in infected fish, which indicate the effect of dermato/systemic mycosis. As a result, it can be inferred that fungal infection causes haemostatic imbalances in fish, which are mirrored in their haemato-biochemical profile. The latest observations are supported by Mastan *et al.*, (2009). According to Yang and Chen (2003), the reduction of total serum protein and albumin in infected fish is due to blood vessel injury, which causes a large amount of blood portion to be lost, resulting in hypoproteinaemia.

The A/G ratio of *C. carpio* was found to be slightly lower ($P > 0.05$) in T1, T2, T3, and T4 after the experiment ended in comparison to Control(-) and no noticeable change ($P > 0.05$) in comparison to Control(+), with the lowest ratio observed in T1. In response to infection, the A/G ratio decreases, indicating a transition from albumin to globulin formation (Foott *et al.*, 1996). which may be a sign of inflammation (Osman *et al.*, 2009).

Table 2:- Total Protein, albumin, globulin level and albumin/globulin ratio of *C. carpio* treated with M-CuNPs, CuSO₄ and mint.

Group	Total protein	Albumin	Glubulin	A\G
C-	4.25 ± 0.09 b	1.93 ± 0.15 a	2.32 ± 0.06 b	0.83 ± 0.09 a
C+	3.15 ± 0.01 c	1.22 ± 0.09 d	1.93 ± 0.06 c	0.63 ± 0.03 b
T1	5.17 ± 0.01 a	1.74 ± 0.01 a	3.43 ± 0.03 a	0.50 ± 0.03 c
T2	4.59 ± 0.01 ab	1.65 ± 0.18 ab	2.93 ± 0.02 ab	0.56 ± 0.09 c
T3	4.05 ± 0.00 b	1.52 ± 0.09 c	2.52 ± 0.03 ab	0.60 ± 0.03 bc
T4	4.32 ± 0.01 b	1.58 ± 0.09 c	2.74 ± 0.04 ab	0.58 ± 0.00 c

4- Conclusion

The research indicate that M-CuNPs are selective as an antifungal in the treatment of *Saprolegniasis*. M-CuNPs may thus be used as a promising alternative to chemotherapeutic compounds in aquaculture to achieve viable, cost-effective, cleaner, and environmentally friendly fish processing.

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