EFFECIENCY OF COPPER NANOPARTICLES COATED MINT AS ANTI-FUNGAL AGAINST SAPROLEGNIASIS DISEASE IN COMMON CARP

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ABSTRACT

The present study was aimed to find out the efficacy of M-CuNPs, mint, and CuSO4 in controlling saprolegniasis in common carp. The efficacy of Cu-NP which prepared by using chemical method was examined. 120 fish of weight 80±10g were groups divided into six replications (10 fish/replicate) and the groups were treated as follows: (C-) health without any infection and without treatment, (C+) infected and without treated, (T1 and T2) The fish were infected with the fungus and treated with M-CuNPs at concentrations 20, and 10 mg/l respectivly.T3: fish infected with fungus and treated with copper sulfate at concentration 0.5mg/l. T4: Infected fish were treated at a concentration 100 mg/L of mint. After 14 days of treatment, samples were collected from fish for study the histopathological changes. The copper nanoparticles treatment, 20 mg/l showed the highest survival rate (85%). The survival rate of the control group (C+)(50%). Histopathological studies revealed a significantly increased ($p<0.05$) percentage of gill epithelial proliferation and epithelial lifting in fish from nanoparticles groups relative to C(+) and C(-). Copper nanoparticles appear to be a good disinfectant against *Saprolegnia* infection at the dose of 20 mg/l.

Key words: aquaculture, *cyprinus carpio*, copper sulphate, fungi

كفاءة جسيمات النحاس النانوية المطلية بالنعناع كمضاد للفطريات ضد مرض عفن الماء في أسماك الكارب الشائع

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المستخلص

يهدف البحث إلى معرفة فاعلية M–CuNPs والنعناع وكبريتات النحاس في السيطرة على عفن الماء في الكارب الشائع. تم فحص فاعلية جسيمات النحاس النانوية المحضرة بالطريقة الكيميائية. تم توزيع 120 سمكة بوزن 80±10 غم عشوائياً على ست مكررات (10 سمكة/ مكرر) وزعت على النحو التالي: (C–) صحية بدون إصابة وبدون علاج ، (C+) مصابة بدون علاج، (T1 وT2) أصيبت الأسماك بالفطر وتم علاجها بـ M–CuNPs بتراكيز 20ملغم/لتر و10ملغم/لتر على التوالي. T3: أصيبت الأسماك بالفطر وتم علاجها بكبريتات النحاس بتركيز 0.5ملغم /لتر. T4: عولجت الأسماك بتركيز 100ملغم/لتر. بعد 14 يومًا من العلاج، تم جمع عينات من الأسماك لدراسة التغييرات النسيجية المرضية. أظهرت معالجة الجسيمات النانوية النحاسية 20 ملغم/لتر أعلى معدل بقاء (85%). كان معدل البقاء على قيد الحياة للمجموعة الضابطة (C+)(50%).أظهرت الدراسات النسيجية المرضية زيادة معنوية ($P<0.05$) في نسبة تكاثر الظهارة الخيشومية ورفع الظهارة في الأسماك من مجموعات الجسيمات النانوية بالنسبة إلى مجموعات(C+)و(C–).أظهرت جزيئات النحاس النانوية على أنها مطهر جيد ضد عدوى عفن الماء بجرعة 20ملغم/لتر.

الكلمات المفتاحية: الأحياء المائية، الكارب الشائع، الجزيئات النانوية، كبريتات النحاس، فطريات.

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INTRODUCTION

Aquaculture is one of the most rapidly expanding food-producing industries. Aquaculture set a new world record for production in 2012, accounting for half of all fish intended for human consumption. By 2030, this percentage is projected to rise to 62 percent (13). Aquaculture has become a significant source of income in areas where natural fishery productivity has declined due to population growth and biodiversity loss (12). Aquaculture contributes to meet the increasing needs of the population of the aquatic organism, including fish, because the commercial fishing from open water suffers from a decrease in its contribution to filling this deficit to 50% of the consumer needs of seafood in all parts of the world(17).Common carp *Cyprinus carpio* L. Considers the far more common form of fish cultured in Iraq; this fish species has many characteristics that make it ideal for breeding in Iraq's aquatic climate, including weight gain in a short period of time and tolerance to many pathogens (2). Elevated fish production in Iraq has associated with an increase in diseases in culture ponds. These diseases can be caused by pond management mistakes (such as feed or water quality issues) or infectious agents such as viruses, bacteria, fungi and parasites (2). The elimination of fish pathogens is unquestionably critical for the future of the aquaculture industry; infectious diseases, especially fungal diseases, are unquestionably the leading cause of economic losses in the industry (1). Saprolegniasis in teleost fish is a major problem affecting both wild and farmed freshwater fish production, but this disease is more abundant in the cultured fish, which adversely affects the fish industry(7).

Saprolegniasis is caused by oomycetes, including the *Saprolegnia* and *Achlya* species, which infect fish in fish farms and aquaculture(22).Saprolegniasis in fish usually begins as a cotton wool-like white to gray or brownish growth on the head, dorsal fin, and then spreads to other parts (27). Bronopol, hydrogen peroxide, sodium chloride, and copper sulphate are often used. As a result, studies are being performed to find more friendly, stable, and cost-effective *anti-Saprolegnia* compounds that are eco-friendly and have little or very low environmental impact (16). Nanomaterials are being used in very novel ways in scientific research, and nanotechnologies and nanosciences are rapidly developing disciplines with enormous potential in industrial and innovation. research, nanomaterials display various multidisciplinary activities in both the aquaculture and the agriculture sector (4). A nanoparticle is a microscopic particle that acts as a single entity in terms of transport and properties, differing significantly from bulk materials of similar composition. The scale, form, and chemical atmosphere of NPs affect their properties (19). Cu-NPs can also be used in fungicides, algaecide and herbicides (26). Therefore, the aim of this study is to assess anti-fungal activity of mint-copper nanoparticles against saprolegniasis dieases.

MATERIALS AND METHODS

Isolation and recognition of *Saprolegnia spp.* from water media: Baiting technique was used to isolate aquatic fungi from water samples obtained from the Tigris River in Baghdad, Iraq.15- 20 ml of river water was pumped into a sterile petri dish containing Chloramphenicol to extract pure cultures from the environment. After that, sesame seeds (5-7 seeds per petri dish) were added (3), they incubated in 20 ^{o}C for 7 days and examined every day to observe the hyphae(fig. 1 B,C)

Experimental design of artificial infection

A fish farm in Babel, Iraq, provided 120 healthy *C. carpio* weighing 80±10g.Fishes were acclimatized for two weeks in laboratory conditions before the beginning of the study. The fish were randomly stocked into 6 replicated groups in glass aquaria (10 fish/tank). The fish were kept in a natural photoperiod of 12 h. light/12 h. dark. The fungi isolates were introduced to the fish environment (2 X 10^4 zoospores per liter). When there were signs of cotton wool on the fish, different concentrations of treatment were added to the tanks, and the fish were treated as follows: C (-) control health without treatment, C (+) control infected with fungi without treatment; T1 and T2:-fish were infected and treated with M-CuNPs 10 and 20 mg/l per hour respectively; T3:-fish were infected and treated with copper sulphate 0.5 ml/l for 30 min for 6 successive days and T4:-fish were

infected and treated with mint 100mg/l for 7 successive days. Water quality parameters were monitored every day in each tank as follows: {Temperature (^{o}C) 22 ± 1, Dissolved O2 (mg l-l) 6.10± 0.5, and pH 7.10 ± 0.05}.

Preparation of copper nanoparticles

Cu nanoparticles were created through a chemical reduction process that employed copper (II) sulfate pentahydrate, a precursor salt and starch as a capping agent. The process begins with the addition of 0.1 M copper (II) sulfate pentahydrate solution into 120 mL of starch (1.2 %) solution, followed by intense stirring for 30 minutes. In the second step, 50 mL of 0.2 M ascorbic acid solution is applied to the synthesis solution while stirring continuously. Following that, 30 mL of 1 M sodium hydroxide solution was steadily applied to the prepared solution while stirring constantly and heating at 80 ^{o}C for 2 hours. The color of the solution changed from yellow to ocher. After the reaction was completed, the solvent was removed from the heat and allowed to settle overnight before discarding the supernatant solution with caution. The precipitates were removed from the solution through filtration and washed three times with deionized water and ethanol to remove the excess starch bound with the nanoparticles. The collected ocher color precipitates are dried at room temperature. After drying, the nanoparticles were placed in a glass vial.

Clinical examination and survival rate

Amlacker outlined the process after observing about 120 fish with irregular behaviors and external lesions on the skin and gills (6). Percentage survival was calculated using the following equation:

Survival rate (%) =final number of fish survivor/initial number of fish stocked×100

Histopathological studies

The histopathological studies were carried out in the manner mentioned by Myers *et al*.,(21). Selected tissues (skin with muscles and gills) were fixed in a 10% formaldehyde solution for 48-72 hours. Following that, the tissues were systematically washed and prepared into paraffin blocks. To reveal the fungal hyphae, tissue blocks were sliced (5-7 µm thickness) and stained with Haematoxylin and Eosin (H&E), and skin parts were stained with Periodic Acid Schiff (PAS). Light microscopy was used to view the slides, and photographs were taken using an Optika Vision Microscopy Digital UBS camera. According to Bernet *et al*.,(8) detailed anatomy descriptions were created for the experiments. Histological characteristics were determined, assessed as necessary, and graded according to the number of lamellae in the gill parts. According to Mustafa (20) quantitative analysis was performed on secondary lamellae that were complete from tip to the root.

Statistical analysis

Statistical processing was performed using SPSS V. 16 Software. One was a measurement of variance (ANOVA) was used to evaluate the major variations between variables. The variations in ways is analysed at 5 percent likelihood point.

RESULTS AND DISCUSSION

Isolation and identification of *Saprolegnia spp*. : Macroscopically and Microscopically:- The morphological qualities of the contagious development states on SDA(fig.1 A) show up as a round mass of fibers, whitish in shading and earthy in the center, and described by an inescapable and thick mycelium, following 24-72 hours of incubation at 20 ^{o} C. The presence of stretched non-septate hyphae in growth disengages was utilized to recognize them along with masses, different long and width, straightforward and has cell film. Such sporangia were loaded up with huge number of spores what isolated from the basal substantial hyphae by measure called Saprolegnoid (Fig.1 C). In light of morphological highlights like the improvement of agamic stages (zoosporangium, zoospores, and pimple), coenocytic. Hyphae, and the shortfall of oogonia, the strain disengaged in our example was affirmed as *Saprolegnia spp.* (15). Microscopical analysis revealed that *Saprolegnia spp.* hyphae were present. They had a branched non-septet appearance, were translucent, and had a cell membrane. This trait is shared by all members of the *Saproleginiaceae* family (11), whereas the presence of zoosporangia is cylindrical or spherical in form, with a large number of spores, and this characteristic is renewed by Saproleginoid. This is similar with Seymour results (24).

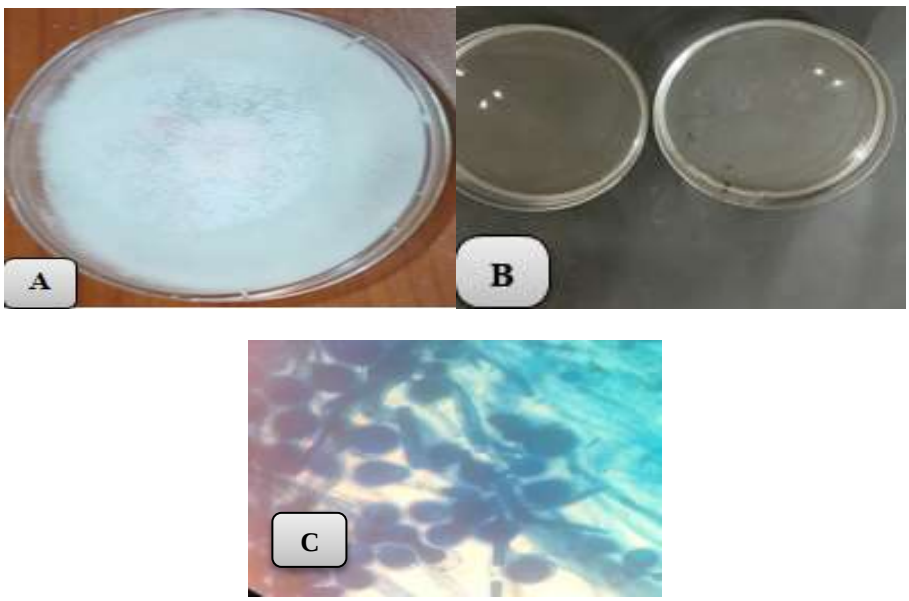

Figure 1. A- *Saprolegnia spp.* Long hairs with a whitish cottony color appeared after 3-4 days of culture on SDA at 20°C.B- On sesame seeds, wet culture of developing *Saprolegnia spp.* C- Lacto-phenol cotton blue staining revealed that the hyphae morphological characteristics were typical of *Saprolegnia spp*. x40

Clinical signs and survival rate: The results of the clinical signs for all groups and survival rate are summarized in Table 1. The presence of filamentous strands called hyphae was the most common clinical symptom in the C (+) group that became infected after 3-5 days of fungal exposure (Fig. 2). It begins with spherical spots on the fish body that grow larger and extend all over the fish body, covering 40-60% of the body surface with the fungus, resulting in whitish patches and gill infection(fig.2 A,B, and C). Some cases were ulcerated after 4-7 days of infection, the hyphae penetrating through the skin, and the fish were totally coated in a dense layer of fungi. At the end of the trial, the mortality rate reached up to 50%, as shown in Table 1. The M-CUNPs gave good results in the treatment, infected fish showed no evidence of fungal growth or disease, particularly at concentrations of 20 mg/l (T1) and 10 mg/l (T2) respectively as a bath for 3 days and the rate of survival was 85% and 70%. For T1,the growth of hyphae disappeared after 8 days of therapy, the whitish patching stopped after 10 days, and the skin color returned to normal after 12 days. The development of hyphae in T2 disappeared (fig. 2 D and E) after the 9th day of the experimental period. The whitish patching disappeared after 12 days of the trial, and 14 days after infection, the skin returned to normal. The chance of survival rate in T3 was 95%. The hyphae disappeared after 9 days of therapy, the whitish patching disappeared after 10 days, and the skin returned to normal after 11 days from the age of experimentation. The hyphae disappeared after 10 days of therapy in T4, the whitish patching disappeared after 10 days of the experimental period, the color of the skin returned to normal after 11 days of the experimental period, and there was a 60% survival rate. The onset of Saprolegniasis symptoms on infected positive controls after 7 days of fungal infection occurred as filamentous threads called hyphae; these findings are consistent with Muhsin (18). Furthermore, the findings are consistent with Robert et al., (23) hypothesis that hyphae can cover up to 40 or 50 percent of the body surface and gills, with mortalities ranging from 10 to 50 percent Saprolegniasis is distinguished by brownish patches of cottony fungal growth on the skin, including the gills, according to Bruno and Wood (23). In most cases, fish die from respiratory failure caused by widespread gill infection, organ failure in rarer cases, and reduced osmoregulation caused by wounds covering a wide surface area (10). When to the infected fish (C+), the treatment groups (T1 and T2) that treated against Saprolegniasis showed signs of recovery. This indicated that M-CuNPs was very active disinfectant can kill and depressing the fungal by rupturing the cell membrane of

fungal (5) As a result, this research, as well as Soltani et al., (25), showed that nanoparticles have antifungal properties in *Saprolegnia sp*

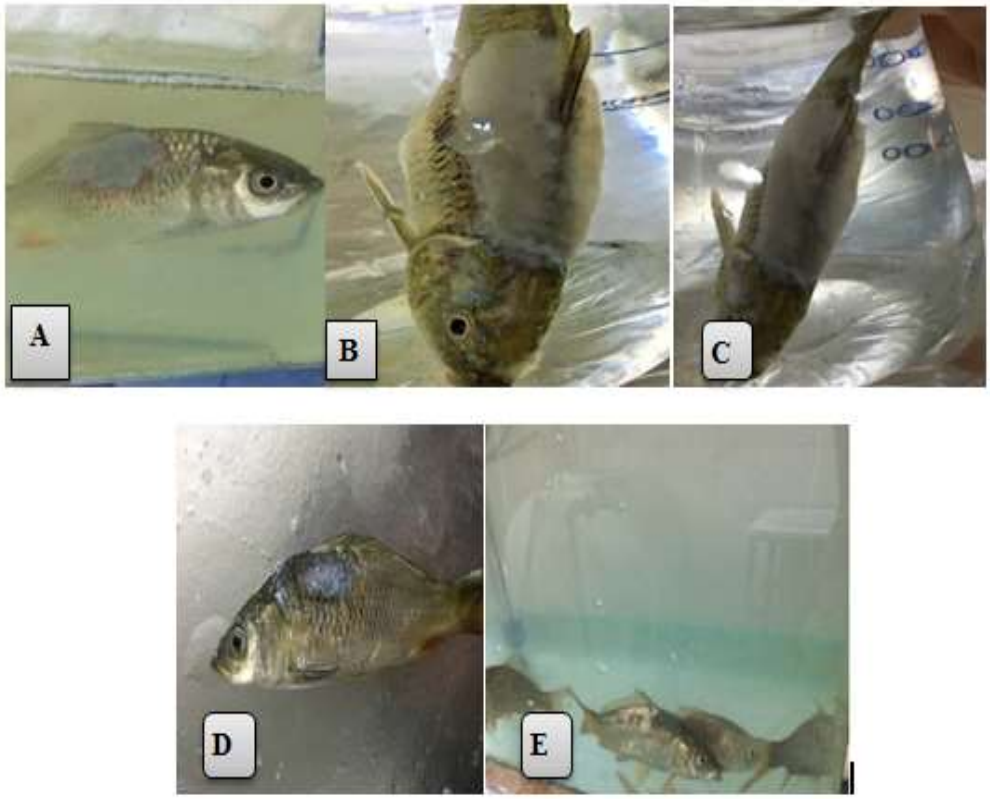

Figure 2. A,B,C: Cotton wool-like hyphal mats and ulcerations on the body explain the gross lesion in common carp. D,E: Fish-healing.

Table1. Clinical signs and endurance rate for all treatments gatherings of *C. carpio* contaminated with *Saprolegnia spp*.

Group	Fungal growth and clinical signs	No. of fish	Dead fish	Survive fish	Survival Rate %
C-ve	- - -	20	0	0	100
C+ve	+++	20	10	10	50
T1 (20mg/l)	- - -	20	3	17	85
T2 (10mg/l)	- - -	20	6	14	70
T3 (0.5mg/l)	- - -	20	1	19	95
T4 (100mg/l)	- - /+	20	8	12	60

+: signs persist / - : signs vanish

Histopathological studies

Gill : The gill morphology in the control group highlighted normal structures which showed typical appearance of primary lamellae and secondary lamellae (Fig.3 A). Gills from *Saprolegniasis* infected non treated group showed epithelial lifting, destruction of lamellar epithelium with massive haemorrhage and various cellular accumulate between primary lamellae and shorting of the secondary lamellae (Fig.3 B). In the treatment groups (T1, T2, T3, and T4), these changes were less pronounced (Fig.3 C - F). Fish from processed water (T1, T2, T3, and T4) had higher levels of gill epithelial proliferation and epithelial lifting, as well as fusion of secondary and main lamellae, than C+ and C- groups for the majority of these observed lesion forms.

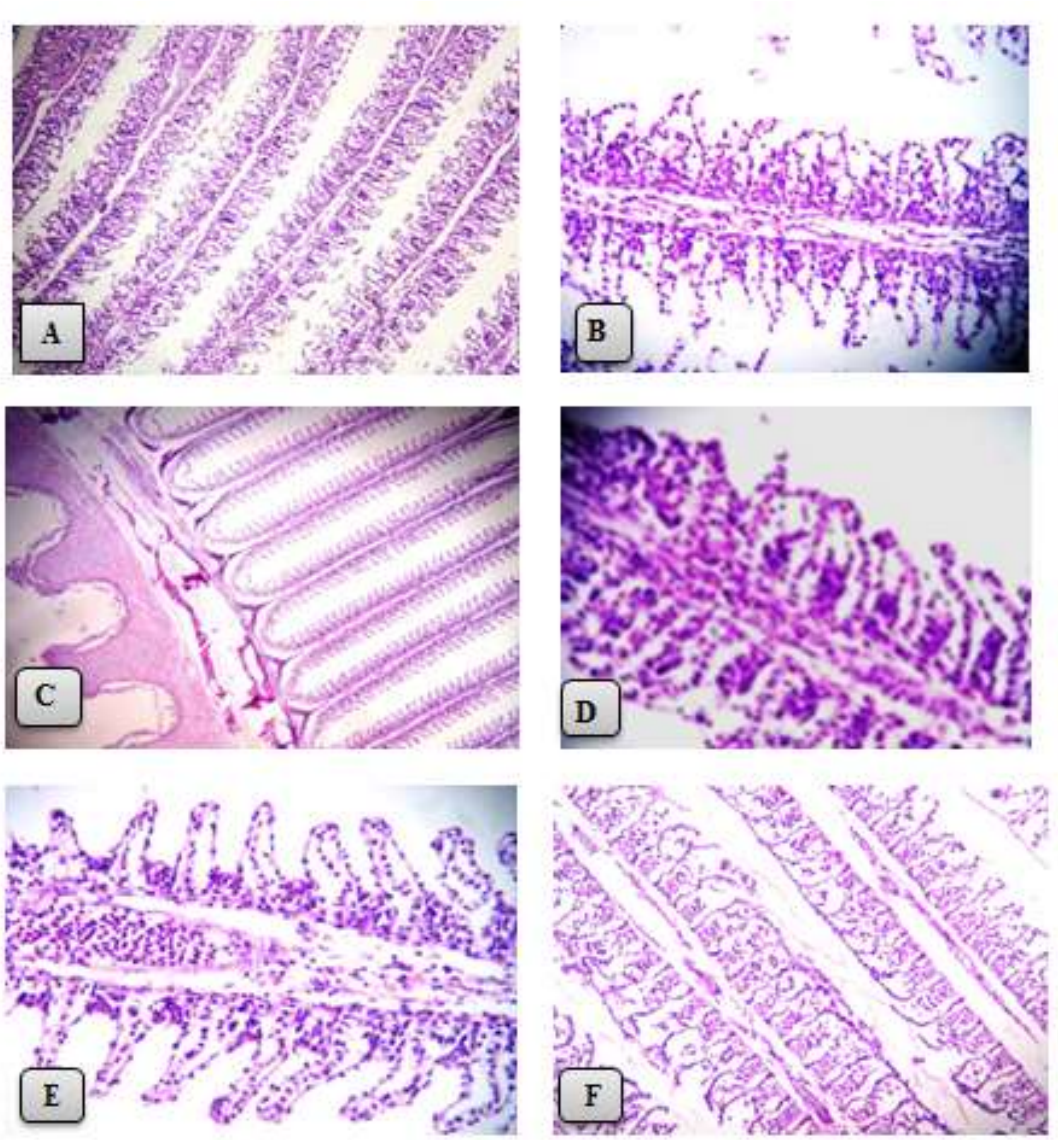

Figure 3. Histological sections through gills of *C. carpio* A: Negative control group showing normal appearance of primary lamellae and secondary lamellae. B: Positive control group infected gills showing epithelial lifting, destruction of lamellar epithelium with massive haemorrhage and various cellular accumulate between primary lamellae and shorting of the secondary lamellae and epithelial sloughing of gill rakers.(C-F): treated groups with copper nanoparticles, copper sulphate and Mint. H and E stain; x 10

Skin: The control group showed normal histological structure of skin layers, epidermal layer, basal layer and stratum compactum (fig.4 A). Skin infected non treated group showed complete and partial loss of epidermis associated with intense vaculation of survival skin cell (fig.4 B). Other findings showed massive dermal and hypodermal necrosis that covered with thin epidermal cells, also evidence of dermal edema (fig.4 C-D). With MNC penetration, the skin of groups (T1 and T2) showed an improvement in the number of mucous secreting cells (Fig. 5 A and B). T3, on the other hand, showed extreme dermal necrosis as well as intramuscular edema. (Fig.5 C). T4 show slight epidermal hyperplasia with numerous of mucous cells and slight cellular infiltration(Fig.5 D). In the present study, *C. carpio* from the control group shows the normal structural organization of gills while an infected and treated group shows histopathological alterations namely (epithelial lifting, hyperplasia, fusion of lamellae, hemorrhage, and telangiectasis). The epithelial lifting could possibly be examples of general defense mechanisms since the gap through the bloodstream is increased by separating the epithelium of the lamellae One of the main histological changes found in fish exposed to

stress is cell proliferation, which results in hyperplasia and contributes to lamellar fusion, as seen in the current research. The current study revealed histopathological changes in infected injured area and gills of *C. carpio* infected with *Saprolegnia spp.* including narcotization of dermis and hypodermis. Penetrating fungal hyphae was clearly observed in the muscular layer. While the skin of *C. Carpio* treated with M-CUNPs showed an increase of mononuclear cells (monocytes/macrophages and lymphocytes) (MNCs) and slight sloughing of a most superficial layer of the epidermis which indicating the disappearance of the causative agent . The histopathological changes in skin and gills of *C. carpio* could be attributed to the enzymatic activity of the *Saprolegnia spp.* which fed the tissue. Similar types of changes in the skin of *Saprolegnia spp.* infected fish have been reported by Hatai and Hoshiai (14).

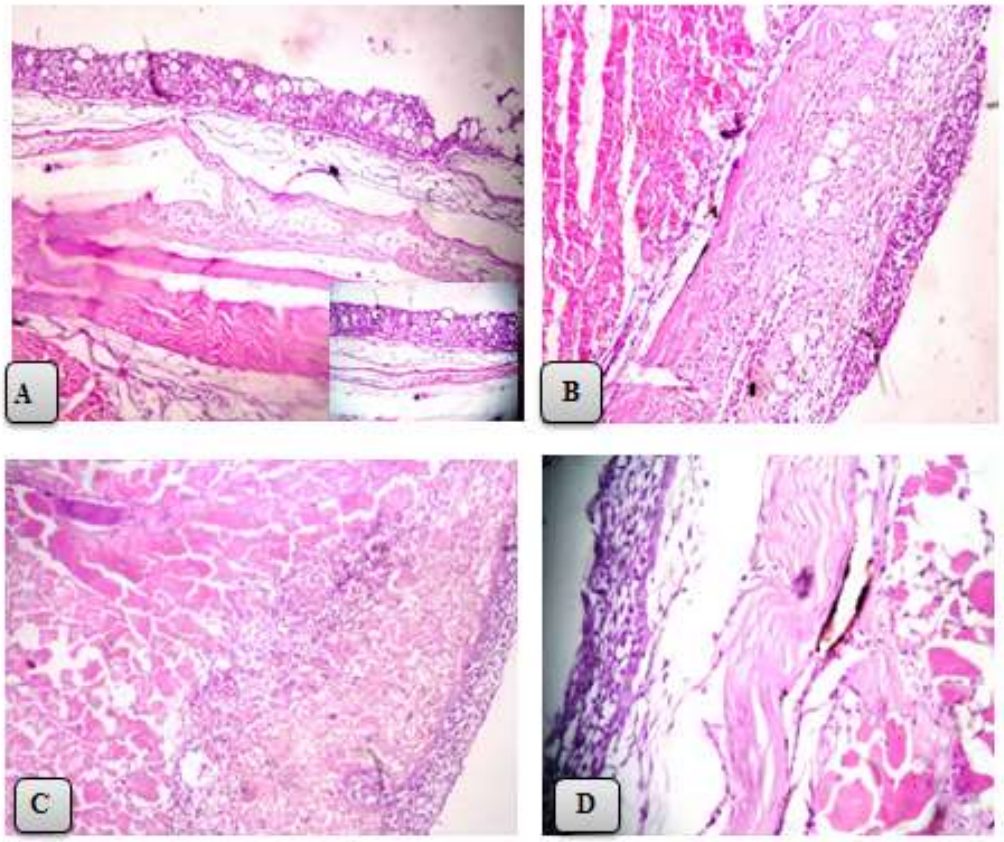

Figure 4. Histological structures seen through the skin of a *C. carpio* infected with *Saprolegnia spp.* and treated with M-CUNPs,$CuSO_4$ and mint (A): The epidermal layer (EP), the basal layer (BL), and the stratum compactum are both visible in the control skin. (B-D) positive regulation of full epidermis erosion and ulceration, as well as penetration of mononuclear cells (MNCs); (C) Increase in the number of warning cells, fibrous material deposition with sloughing, and epidermal tissue destruction (D) cellular debris mixed with fungal hyphae with extreme vacuolation packed with fungal material indicating total loss of epidermal layer with cellular debris mixed with fungal hyphae ; Thickness 5-7µm. x10

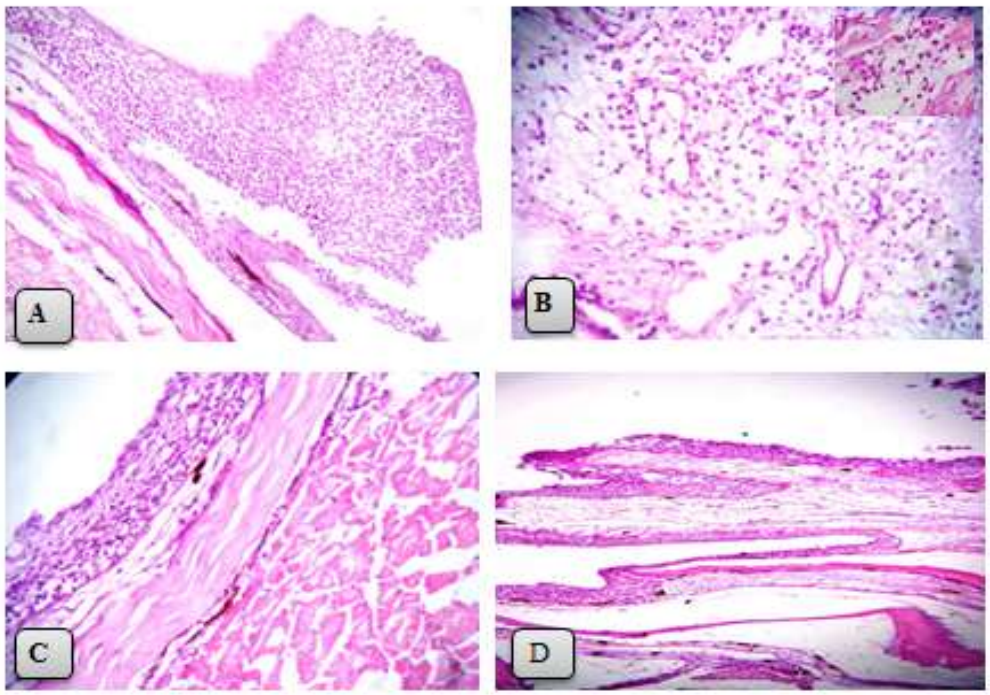

Figure 5. Histological structures seen through the skin of a *C. carpio* infected with *Saprolegnia spp.* and treated with M-CUNPs (A) T1 MNC penetration increased the amount of mucous secreting cells; (B)T2 showing a rise in the amount of mucous-secreting cells; (C) T3 displaying a well-organized system with a higher number of mucous cells; (D) T4 including mild epidermal hyperplasia; x40

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Conclusion

The findings showed 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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