EFFICACY OF SILVER ZEOLITE ON HEMATOLOGY AND SURVIVAL RATE IN *CYPRINUS CARPIO* INFECTED WITH *SAPROLEGNIA SPP.*

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ABSTRACT

This study was undertaken to evaluate the effect of silver zeolite (SZ) on hematological parameters and survival rate in common carp, *Cyprinus carpio* infected with *Saprolegnia spp.* A total of 120 fish (weight 100 g) were divided into six treated groups in duplicate (10 fish/tank) as follows: T1, T2, T3 and T4 that infected with *Saprolegnia spp*. (2×10^4 zoospore/ml) and treated with SZ at (100,200 and 300 mg/l) while T4 infected with *Saprolegnia spp.* and treated with copper sulfate (5g/100 L); C+ group, fish were infected with Saprolegnia without treatment and C- served as control negative group. Following 14 days from infection with *Saprolegnia spp.* and treatment with SZ, considerable changes were observed in blood indices. In pre-treatment, results showed a significant increased (P≤0.05) in WBC count in all infected fish (C+, T1, T2,T3 and T4) in comparison to C- group. RBC, Hb content and PCV% registered a significantly decreased (P≤0.05) in all infected fish (C+,T1, T2,T3 and T4) relative to the control group (C-). Post treatment, WBC registered significantly decreased in treated groups (T1,T2,T3 and T4) compared to C+ in WBC count. The best survival was recorded in T3 (85%) followed by T2 and T1 (65%, and 60%) respectively. In conclusion Silver zeolite seems to be a valuable disinfectant agent against *Saprolegnia spp.;* at the dose of 300 mg/l.

Keywords: carp- clinoptilolite- copper sulphate-fungus

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فعالية الزيولايت الفضي على المعايير الدمية ومعدل البقاء في اسماك *Cyprinus carpio* المصابة تجريبيا بعفن الماء

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المستخلص

هدفت هذه الدراسة إلى تقييم تأثير الزيولايت الفضي على الصفات الدمية ومعدل البقاء في اسماك الكارب الشائع المصابة تجريبيا بفطر *Saprolegnia spp*. وزعت 120 (معدل الوزن 100 عم) عشوائياً الى ستة معاملات مكررة (10 سمكة/حوض) على النحو التالي: T1 و T2 و T3 المصابة بـ*Saprolegnia spp* والمعالجة بالزيولايت الفضي بمعدل (200و100 و 300 ملغم/ لتر).) بينما كان T4 مصابًا *Saprolegnia spp* والمعالجة بكبريتات النحاس (5 غم/ 10 لتر) وكذلك (C+) المصابة بـ *Saprolegnia spp* و معاملة السيطرة السلبية(C−). بعد 14 يومًا من الإصابة بالفطر والمعالجة بالزيولايت أظهرت النتائج قبل العلاج زيادة معنوية (P≤0.05) في وعدد كريات الدم البيضاء في جميع الأسماك المصابة (C + و T1و T2و T3و T4) مقارنة بمعاملة C− وسجل عدد كريات الدم الحمراء والهيموكلوبين والنسبة المئوية لحجم كريات المضغوطة انخفاضًا معنويًا (P≤0.05) في جميع الأسماك المصابة (C + وT1 وT2 وT3 و T4) مقارنة بمعاملة C−. بعد العلاج ، أظهرت النتائج أن هناك انخفاضًا معنويًا في المجموعات المعالجة (T1وT2 وT3 وT4) مقارنة بـ C+ في عدد كريات الدم البيضاء. مقارنة بمعاملة السيطرة(C+). تم تسجيل أفضل معدل بقاء في T3 (85%) و 65% و 60% في T2وT1 على التوالي. بناء على هذه النتائج، أثبتت الدراسة فاعلية الزيولايت في معدل البقاء والصفات الدمية ضد مرض عفن الماء في اسماك الكارب الشائع بمعدل 300ملغم/لتر.

الكلمات المفتاحية: الكارب – كلينوبتيلولايت– كبريتات النحاس– فطريات

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INTRODUCTION

Around the world, aquaculture now produces 80 million tons of fish (7). In this fast growth, fish are exposed to a wide variety of diseases, notably bacteria, viruses, and fungi (17, 19). In recent years, severe fungal infections have significantly contributed to the increasing morbidity and mortality. Without a doubt, infectious diseases, especially fungi infections, are the biggest contributor to economic losses in aquaculture (14, 29,). Diseases in fish farms and aquaculture are caused by oomycetes like Saprolegniales, which include the Saprolegnia and Achlya species (21). Saprolegniasis in teleost fish is a major problem affecting both wild and farmed freshwater fish productions (2, 6)**.** *Saprolegnia spp.* are secondary infections, but under certain situations, they may also operate as main pathogens, inflicting severe economic losses to aquaculture (27). Infected fish with *Saprolegnia spp.* often have lumps on their skin and gills that range in color from cottony white to gray, brown, red, or greenish (29)**.** Zeolites are microporous crystalline hydrated aluminosilicates that have many applications due to their biocompatibility, such as ion exchange and adsorption- desorption properties (1). Silver is recognized to have a broad antibacterial range and to be generally harmless compared to other antibacterial metals. Among various antibacterial metals, silver is known to have a wide antibacterial spectrum and is relatively safe (5). SZ may impart antibacterial and antifungal characteristics to resins or synthetic polymers when combined with zeolite containing silver ions (28). The antifungal activity of SZ has received only marginal attention and only a few studies have been published on this topic (20). Hence, this study was aimed to evaluate the efficiency of SZ on hematology and survival rate in common carp infected with *Saprolegnia spp*.

MATERIALS AND METHODS

Experimental design

The current study was carried out in the Fish Diseases Laboratory at the College of Veterinary Medicine/University of Baghdad for one month. A total of 120 of healthy *C. carpio* (average weight 100 g) were obtained from a commercial farm from Al-Mahawil. Initially, the health status of the experimental fish was inspected, after that the fish were dipped in formalin (37%: (15ml/100L) for 30 min. or until the appearance of the stress on fish, after two weeks of acclimation for the fish were stocked in two bath with dimension of 150 × 20 ×40 cm. Then, fish were randomly distributed into 12 tanks at rate of 10 fish per tank (two replicates/treatment) as follows: T1, T2, T3 and T4 that infected with *Saprolegnia spp*. (2×10^4 zoospore/ml) and treated with SZ at (100,200 and 300 mg/l) while T4 infected with *Saprolegnia spp.* and treated with copper sulfate at concentration of 5g/100 L; C+ group, fish were infected with Saprolegnia without treatment and C- served as control negative group. Each of the six treatment groups was fed the formulated diets at a daily rate of 2% body mass during the experimental study. The experimental fish were preserved under a 12 h light/dark cycle. The chemo-physical tests of the water were measured daily during the experimental period as follows: [Temperature: 20.2±0.8 °C, Dissolved oxygen: 6.1±0.3 mg/l, pH: 7.5±0.2] as the optimize condition of carp fish.

Silver- modified- clinoptilolite (zeolite) preparation

A silver modification of clinoptilolite was achieved using the ion exchange method according to the procedure described by Nikawa *et al.* (20). Breifly, 100 g of NZ was placed in 250 ml of 3% (w/v) $AgNO_3$ liquid at pH 50.2 (to avoid metal precipitation) and then stirred at 300 rpm for 24 hours in a dark environment (due to silver's light sensitivity) to obtain maximal silver exchange onto the zeolites. Filtration was used to extract the zeolites from the liquid, which was then rinsed with deionized water, left to dry at 60 °C and saved ready for use.

Isolation and identification of *Saprolegnia spp.:* For the isolation of *Saprolegnia spp.*, water samples were collected from the Tigris River/Baghdad, Iraq, and the biting method was used to isolate the aquatic fungus (4, 18)**.** In order to get pure colonies from the environment, 15–20ml of stream water was placed into a sterile petri plate with chloramphenicol. At that time, 5-7 sesame seeds, *Sesamum indicum* were placed in each petri dish (3). The plates were kept at 20 °C for 7 days, and every 24 hours, hyphae were

looked. The macroscopic appearance included color, shape and other characteristic features of colonies of *Saprolegnia spp.*, which were determined on Sabroud dextrose agar (SDA) and on sesame seeds. Direct microscopic examination included the removal of infected areas of samples and washed 2-3 times with sterile dH_2O and then transmitted to another clean slide and adding 1-2 drops of lactophenol Cotton Blue stain. Then, it was covered with a cover slip and left for 2 min. The examination was conducted using a light microscope under high magnification (x100) to determine the shape of hyphae. The spore suspension was scored in 1 ml of solution by using a "Newbaurhemocytometer chamber."

Hematological parameters

Blood samples were drawn from caudal peduncle (n=6). Then, samples were transferred to heparin anticoagulant tubes for determination of RBCs (10^6/µl) and WBCs (10^3/µl) count. Hemoglobin content (g/dl) was detected using Cyan-methaemoglobin method (16).

Statistical analysis

Statistical Analysis System (22) was applied to determine the impact of various study parameters. Least Significant Difference (LSD) test (Analysis of Variance-ANOVA) were applied to compare means significantly. A P value of less than 0.05 was detected as significantly different**.**

RESULTS AND DISCUSSION

Isolation and identification of *Saprolegnia spp.:* All isolates were classified as *Saprolegnia spp.* depend on the morphological features of fungal colonies were appeared after 24-48 h from incubation on SDA as rounded mass of filaments, white in color and brown in the center. Also, these fungal colonies characterized by an extensive and dense mycelium (Figure 1 A). While, the fungal growth colonies in water media appeared after 3-5 days from culturing as small growth, white in color and diameter ranged 1-1.5cm (Figure 1 B). asexual reproduction of fungus isolate was distinguished by the appearance of branched non-septate hyphae as well as masses varying in width and length that are translucent and have a cell membrane. These zoosporangia were densely packed with spores (Figure 1 C and D).

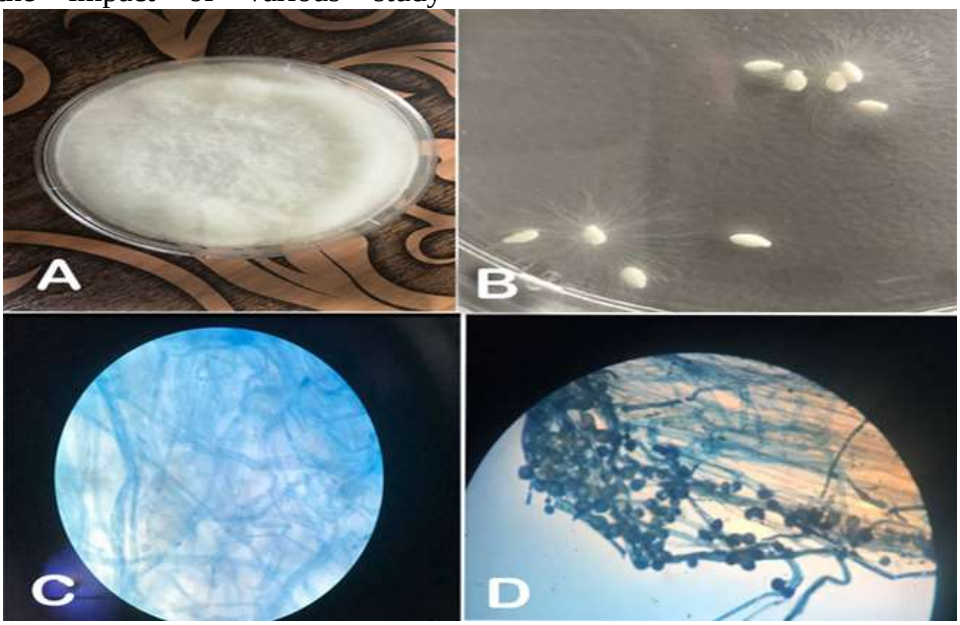

Figure 1. A- *Saprolegnia spp.* colonies on SDA at 20ºC for 3-4 days started with cysts of long hairs with white cottony color. B- Wet culture of developing *Saprolegnia spp.* growth on sesame seeds. C- The wet smear showing non-septate hyphae. D- Part of sesame seed colony stained with lactophenol cotton blue showing zoospores.

Hematology: Results of WBCs count showed that at 1 and 3 days pre and post treatment with SZ , WBCs count reported significantly increased ($P \leq 0.05$) in C+ and in all treated groups (T1, T2, T3 and T4) relative to C- group. While, all treated groups (T1, T2, T3 and T4) registered significantly decrease ($P \leq 0.05$) post treatment with SZ pre- treatment with SZ (Table 1).

Table 1. Results of WBCs×10^3/µl count of *C. carpio* at 1 and 3 days pre and post treatment with silver zeolite

Groups	Mean ± SE of WBCs count×10^3/µl			LSD value
	Day 1 pre treatment	Day 3 pre treatment	post treatment	
C- : healthy fish	24.56 ±0.08 B b	24.46 ±0.02 B c	24.81 ±0.01 E a	0.043 *
C+ infected with *Saprolegnia spp.*	30.42 ±0.57 A a	31.12 ±0.58 A a	31.08 ±0.01 A a	1.635 NS
T1: 100 mg\L SZ	28.84 ±0.63 A ab	31.13 ±1.46 A a	26.19 ±0.02 B b	3.182 *
T2: 200mg/L SZ	30.21 ±0.88 A a	30.94 ±0.59 A a	25.74 ±0.01 C b	2.116 *
T3: 300mg/L SZ	30.04 ±0.87 A b	32.26 ±0.57 A a	24.91 ±0.01 D c	2.092 *
T4: $CuSO_4$ 5g/100L	30.18 ±0.56 A a	31.12 ±0.58 A a	24.32 ±0.02 F b	1.631 *
LSD value	2.025 *	2.353 *	0.035 *	---

Means with different capital letters in the same column and small letters in the same row are indicated significantly different. * (P≤0.05).

Results of RBCs count reported that at pre-treatment (1 and 3 days) and post treatment with SZ, RBCs count stated significantly decreased (P<0.05) in C+ and in all treated groups (T1, T2, T3 and T4) in comparison to C- group. However, all treated groups (T1, T2, T3 and T4) reported significantly increased (P<0.05) post treatment compared to pre-treatment with SZ (Table 2)

Table 2. Results of RBCs×10^6/µl count of *C. carpio* at 1 and 3 days pre and post treatment with SZ

Groups	Mean ± SE of RBCs×10^6/µl count			LSD value
	Day 1 pre treatment	Day 3 pre treatment	Post treatment	
C- : healthy fish	2.87 ±0.02 A a	2.88 ±0.01 A a	2.86 ±0.01 A a	0.046 NS
C+ infected with *Saprolegnia spp.* without treatment	1.78 ±0.01 C a	1.67 ±0.01 D b	1.57 ±0.01 F c	0.032 *
T1: 100 mg\L SZ	1.91 ±0.02 B b	1.73 ±0.01 C c	2.18 ±0.01 E a	0.048 *
T2: 200mg/L SZ	1.70 ±0.01 D c	1.87 ±0.02 B b	2.72 ±0.01 D a	0.034 *
T3: 300mg/L SZ	1.50 ±0.01 F c	1.67 ±0.01 D b	2.76 ±0.01 C a	0.034 *
T4: $CuSO_4$ 5g/100L	1.59 ±0.01 E b	1.61 ±0.01 E b	2.82 ±0.01 B a	0.035 *
LSD value	0.041 *	0.031 *	0.031 *	---

Means with different capital letters in the same column and small letters in the same row are indicated significantly different. * (P≤0.05).

Hb content pre-treatment (1 and 3 days) and post treatment with SZ recorded that there were significant differences (P<0.05) in C+ and all treated groups (T1,T2, T3 and T4) compared to C- group. But, all treated groups (T1, T2, T3 and T4) recorded significantly increased in Hb content post treatment with SZ relative to these groups in pre-treatment with SZ (Table 3).

Table 3. Results of Hb content (g/dl) of *C. carpio* at 1 and 3 days pre and post treatment with SZ

Groups	Mean ± SE of Hb content (g/dl)			LSD value
	Day 1 pre treatment	Day 3 pre treatment	After treatment	
C- : healthy fish	8.73 ±0.02 A a	8.73 ±0.02 A a	8.73 ±0.01 A a	0.046 NS
C+ infected with *Saprolegnia spp.*	4.87 ±0.01 B b	4.96 ±0.01 B a	4.98 ±0.01 C a	0.034 *
T1: 100 mg\L SZ	4.54 ±0.02 BC b	4.79 ±0.17 BC b	7.44 ±0.33 B a	0.753 *
T2: 200mg/L SZ	4.36 ±0.33 BC b	4.60 ±0.01 BC b	8.56 ±0.64 A a	1.449 *
T3: 300mg/L SZ	4.03 ±0.22 C b	4.44 ±0.32 C b	8.43 ±0.01 A a	0.791 *
T4: $CuSO_4$ 5g/100L	4.28 ±0.33 BC b	4.58 ±0.01 BC b	8.81 ±0.14 A a	0.726 *
LSD value	0.653 *	0.465 *	0.934 *	---

Means with different capital letters in the same column and small letters in the same row are indicated significantly different. * (P≤0.05).

On the other hand, results revealed that the PCV (%) pre-treatment (1 and 3 days) and post treatment with SZ there were significantly decreased (P<0.05) in C+ and all treated groups (T1,T2, T3 and T4) compared to C-group. However, all treated groups (T1, T2, T3 and T4) verified significantly increased in PCV (%) post treatment with SZ compared to these groups in pre-treatment with SZ (Table 4).

Table 4. Results of Packed Cell Volume (%) of *C. carpio* at 1 and 3 days pre and post treatment with SZ

Groups	Mean ± SE of PCV %			LSD value
	Day 1 pre treatment	Day 3 pre treatment	After treatment with SZ	
C- : healthy fish	32.70 ±0.35 A a	31.38 ±0.36 A a	32.73 ±0.01 A a	2.169 NS
C+ : infected with *Saprolegnia spp.* without treatment	20.46 ±0.47 B a	21.47 ±0.86 B a	21.78 ±0.86 D a	2.621 NS
T1: 100 mg\L SZ	20.70 ±0.95 B b	21.12 ±1.01 B b	27.85 ±0.63 C a	3.042 *
T2: 200mg/L SZ	21.35 ±0.49 B b	20.56 ±0.99 B b	29.75 ±0.19 BC a	2.252 *
T3: 300mg/L SZ	21.94 ±0.89 B b	20.67 ±0.95 B b	30.86 ±0.27 AB a	2.66 *
T4: $CuSO_4$ 5g/100L	20.77 ±0.96 B b	21.81 ±0.32 B b	32.38 ±0.34 A a	2.142 *
LSD value	2.257 *	2.480 *	1.910 *	---

Means with different capital letters in the same column and small letters in the same row are indicated significantly different. * (P≤0.05).

WBCs are involved in the control of immune activities, and as a protective reaction to stress, their quantity increases in fish. High WBCs counts suggest tissue damage caused by infection, extreme physical stress, and leukemia. Thus, in the current investigation, an increase in WBCs may have been produced by tissue damage-induced immune system activation (23). Results of the current study in pretreatment (1 and 3 days) showed a significantly increased (P≤0.05) in the WBC count of *C. carpio* in C+ compared to C-. These results are in agreement with Jamalzadeh *et al*. (11) who observed that the WBC was higher values in fungal infected fishes than healthy Caspian Salmon, *Salmo ciscaucasicus*. In the present work, the increases in WBC count in infected *C. carpio* (C+) and treated groups (T1, T2, T3, and T4) may be a result of the cellular immune system's reaction to fungal infection. Similar observation in rainbow trout were also

documented as in the current research (24). The rise in WBCs is connected with a stimulation of the immune response and an increase in antibody production, which aids in the survival and recovery of infected fish 26). Following treatment, there was a significantly decreased of WBCs count in treated groups (T1, T2, T3, and T4) when compared to C+. These results are correlated with the results on culture media, which showed inhibition for fungal growth on SDA, and with the clinical signs that appeared after treatment in all treatment groups which showed the fungal hyphae disappeared from the surface of skin, healing all injuries from the infected area, the color of skin returning to normal, stress reduction, and the fish returning to normal behaviors (data not shown). Infected groups, C+ and treated groups (T1, T2,T3, and T4) exhibited substantial reductions in RBC count, PCV value, and Hb content compared to un-infected group (C-) prior to treatment. This decline may be related to anemia induced by hyphae that invade the blood vessels of infected fish and cause bleeding, damage to hematopoietic tissues, haemodilution with osmotic imbalance, and lethargy owing to mucus secretion, these outcomes are consistent with Shah and Altindag (25). Accelerated erythroclasis (i.e., red blood cell fragmentation) as a result of altered membrane permeability and/or enhanced mechanical fragility. These results are consistent with Suhail e*t al.* (26) and Gill and Epple (8). Other explanation, This could be because Saprolegnia mycelia penetrate deeply, causing wounds that result in blood loss (13). Our findings are also in agreement with Zaki (30) whom reported that *Tilapia nilotica* infected with *Saprolegnia parasitica* resulted significantly decreased in RBCs count, Hb content and PCV. Post-treatment, results of the current study showed significant increases in RBC count, PCV and Hb in all treatment groups compared to the infected group C+. These results are correlated with the results on culture media, which showed inhibition for fungal growth on SDA, and with the clinical signs that appeared after treatment in all treatment groups T1, T2, T3 and T4, which showed the fungal hyphae disappeared from the surface of skin, healing all injuries from the infected area, the color of skin returning to normal, stress reduction, and the fish returning to normal behaviors. Two antifungal mechanisms are proposed to explain the behavior of AgNP-coated zeolite: first, the fungi are directly killed by the silver ions released from the filters (fungicidal effect); second, by passing through the AgNP-coated zeolite, the fungi are contaminated with silver ions, but still survive; however, they cannot form colonies on the surface of the fish, as the silver ions inhibit their replication and growth ability (fungistatic effect) (12, 15).

Survival rate

Survival registered highest rate in T4 (90%) followed by T3 (85%) then T1 and T2 (65 and 60%) respectively and the survival percentage in C+ was 50%.The highest rate of survival particularly T3 compared to infected group (C+) suggests that SZ was very efficient disinfectant, can inhibit their replication and growth ability of Saprolegnia (fungistatic effect) (12). Similar results reported by Hamad *et al*. (9) who reported that the ability of ozone to reduce Saprolegnia infection in common carp. Our results are also in consistent with Hlial et al. (10) whom demonstrated the best antifungal activity of curcumin–silver nanoparticles against Saprolegniasis in common carp.

CONCLUSIONS

In conclusion Silver zeolite seems to be a valuable disinfectant agent against *Saprolegnia spp.;* at the dose of 300 mg/l. The results of this study demonstrate the first success using SZ for the treatment of *Saprolegnia spp.* in common carp.

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