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Antibacterial Effect of Silver Nanoparticles on Hematological and Biochemical Parameters in Common Carp Infected with *Aeromonas hydrophila*

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Abstract

This study aimed to investigate the antibacterial effect of Nanosilver on haematological and biochemical parameters in common carp, *Cyprinus carpio* infected with *Aeromonas hydrophila*. Silver nanoparticles (AgNPs) were prepared chemically from silver nitrate and biologically from lemon extract. The characterization of silver nanoparticle was studied using a UV spectrophotometer, TEM, SEM, and FTIR, which showed that the AgNPs was spherical shape, size 30-50 nm. Experimentally, 150 fish (40 ±5g) were divided into eight groups, all treatment groups except control negative group were infected experimentally with *A. hydrophila* by intraperitoneal injection (0.1 ml; 1. 43×10^{-7} CFU/ml) and were treated with different concentrations of AgNPs as follows; [chemical method:T1 0.125 g/ l, T2 0.25g /l], [biological method: T3 0.045 g/l, T4 0.075 g/l and T6 0.15g/l] and T5 treated with oxyteteracycline (OTC) (0.4 g/l). All groups were treated by bath treatment for 10 days for 30 min per day for 10 days. Results indicated that WBC count was significantly decreased in all treatment groups compared to C+ group. Lymphocyte percentages registered significant decreased in T4, T5, T6 groups compared to T1 group. Albumin showed a significant ($P<0.05$) in all of treated groups compared to C+ group. The reduction of nitro blue tetrazolium (NBT) by radical oxygen produce from neutrophils cell showed a significantly decreased ($P\leq0.01$) in C+ and C - groups compared to all treated groups. nanoparticle based treatment technologies could overcome threats of multi-drug resistance syndrome either as an alternative or as supplementary to oxytetracycline therapy and it reduces the use of antibiotics and the potential for environmental effects.

Keywords: *Cyprinus carpio*, Nanosilver treatment, Hematology, differential leucocyte count, Nitrobluetetrazlium

Introduction

With the rapid growing of aquaculture has resulted in the rapid increase of infectious diseases. Bacterial diseases are the most common diseases in intensive fish rearing facilities which could hinder the aquaculture industry (Ibrahim *et al.*, 2013) *A. hydrophila* is a major bacterial pathogen, which causes dermal ulceration, haemorrhagic septicaemia and high mortality rate up to 80% in acute cases in many fish species (Cipriano *et al.*, 1984; Janda and Abbott, 2010 ; Alsaphar, 2012). The routine use of antibiotics has led to severe biological and ecological problems, especially the development of antibiotic resistance (Waiho *et al.*, 2021). There is a need to search alternative method to control pathogens. Recently, Nanoparticles have been attracted the attention of researchers toward their potential biomedical applications (Zhang *et al.*, 2019). AgNPs have special interest, especially in biomedicine, these carried a great revolution in the biological and medicinal fields in the modern area as compared to other materials (Yaqoob *et al.*, 2020) Therefore, the aim of the present study is to investigate the efficiency of silver

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nanoparticles as antibacterial agent against *A. hydrophila* in common carp through studying the haematological parameters (RBC and WBC count, Hb content, PCV value and differential leucocyte count), biochemical profile (albumin, globulin and total protein) and Respiratory Burst Activity [(using Nitrobluetetrazolium assay (NBT)].

MATERIALS AND METHODS

Experimental design

The study was done in the University of Baghdad, College of Veterinary Medicine, and Ichthyology Laboratory. The isolation of *A. hydrophila* was identified by vitek2 kit rapid identification system. Healthy fish of *C. carpio* weighted 40±5g were acclimatized for two weeks prior to the experiment and maintained on control basal diet. Fish were stocked in a two glass tank through (70 × 40 × 40 cm). Water temperature, pH and dissolved oxygen concentrations (DO) were measured daily (22.17±0.27 °C, 7.5±0.03 pH and 6.47±1.99 mg/L DO). Then, a total of 150 fish were randomly selected and distributed in to 15 bath (two replications for each treatment except control positive one bath) filled with chlorine-free tap water at a rate of 10 fish per trough and two replicates were retained for each of (Except control positive only one bath) (control negative, control positive, T11.25g/10L, T2 2.5g/10L, T3 0.5g/10L, T4 0.75g/10L,T5 40g/10L and T61.5g/10L). All treated groups were injected intra peritoneal with the bacterial suspension (0.1ml, 1.43×10^{-7} CFU/ml). All groups were treated by bath treatment for 10 days for 30 min per day. The clinical signs were appeared after 48-72 hr and start treated. After 10 days from treatment period, blood samples were collected from caudal vessel for determination of haematological and biochemical parameters.

PREPARATION OF SLIVER NANOPARTICLES ($AgNO_3$NPS)

1. Biochemical preparation of AgNO3NPs

This is done according to the method of Dadosh, (2009) by dissolving 2.5 g silver nitrate ($AgNO_3$) in 800 ml of distilled water, and heating the solution to 95-100 ° C for boiling afterwards, start adding (0.32 g + 125 ml) ml of sodium citrate (C6H5O7Na3). By Moliere (0.01mu). Diagnosis was carried out with FTIR and TEM.

2. Biosynthesis preparation of AgNPs

Biosynthesis preparation of AgNO3NPswas carried out using locally fresh lemon (citrus lemon) following the procedure of Swain et al. (2014) with slight modification. characterized by UV , and indicated a prominent peak at 450nm the range of AgNPs and TEM,SEM The nanoparticles have a nearly spherical shape, smooth surface and size ranged between 30-50nm.

Blood Collection and Samples preparation

The blood samples were collected from two fish which randomly selected per tank from caudal vein puncture using plastic syringe 3 ml (gage 1. 5x21). The first part of blood was transferred to tub coated with lithium heparin that work as anticoagulant and used for determination the haematological parameters and NBT test. The second part was divided in to two portions, transferred to tubes contain gelatine and permitted to clot for two hour. Serum was separated by centrifugation and stored at freeze (-20◦C) and were used for biochemical parameters (total protein, Albumin and Globulin content).

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Haematological Parameters

The haemoglobin content, packed cell volume, total erythrocytes and leukocytes count of the blood was estimated. RBC and WBC were diluted with appropriated diluting fluids (deices fluid) and the total content was calculated using improved Neubauer haemocytometer Blaxhall and Daisley (1973). Cyan-methaemoglobin method was used (Mustafa, 2012). The Hb concentration was determined as g dl^{-1} of blood from a standard graph.

Differential leukocytes count determination:

Differential leukocytes count was performed in duplicate for each sample according to Stoskoph (1993).

Biochemical Profile

Total protein (TP) was assayed through colorimetric test kit (Bio (France)) by biuret method, separately as described by Siwicki and Anderson (1993). Albumin was determined in serum samples by a BCG method at wave length of 550 nm according Kit from Bio (France) was used. The total globulin fraction (g/dl) was generally determined by subtracting the albumin from the total protein.

Determination of respiratory burst activity using Nitrobluetetrazolium (NBT)

Reactive oxygen radical production by neutrophils during respiratory burst activity was evaluated by the reduction of NBT to formazan blood samples were mixed with (20mg/10ml) NBT in equal proportion (200ul/200ul) and incubated for 30 min at 25◦C taken 50µl of this mixture and 1 ml of dimethyl formamide (SRL, India) was added to solubilize the reduced formazan product in tube. Then, centrifuged at 2000rpm for 5 min and the solution was taken. The reduced extent of NBT was measured at an optical density of 540 nm with dimethyl formamide as the blank by using UV- Spectrophotometer (Anderson *et al.*, 1992).

Statistical Analysis

The Statistical Analysis System- SAS (2012). Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare among means in this study. P values less or equal to 0.05 were considered significantly different.

Results and Discussion

Clinical Sings Pre-Treatment with AgNPs

The infected fish became moribund in 2-3 days while the mortality was started from the third day post injection with A. hydrophila. The external signs were included: loss of scales, eye abnormalities, rot in the pectoral fin, haemorrhage and darkling of the skin, ulceration, degenerated to opacity exophthalmia and bursting that dislodged the eyeball out of the socket when both eye were affected (Fig.1). Also, the sings included abnormal swimming, the abdomen became distended and scales bristle out from the skin with focal haemorrhage. Internally, there was enlargement of the liver and the cooler was pale, congestion in internal organs and haemorrhagic and necrotic spots in kidney. These results are in line with Badawi and Bawazir (2019) who found enteritis and haemorrhage in the stomach, liver, gill epithelia, renal

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hematopoietic tissue and necrosis of the splenic sheath and kidneys showed necrotic foci This could be due to toxins produced by *A. hydrophila*. In this study, it clearly indicated that *A. hydrophila* was the causative agent of the mortality of *C. carpio*. Numbers of virulence factors derived from *A. hydrophila*. A known generally that *A. hydrophila* produce α –haemolysis and β- haemolysis (Howard et al., 1987; Hirono and Aoki 1991).These findings are in agreement with Salman (2014).

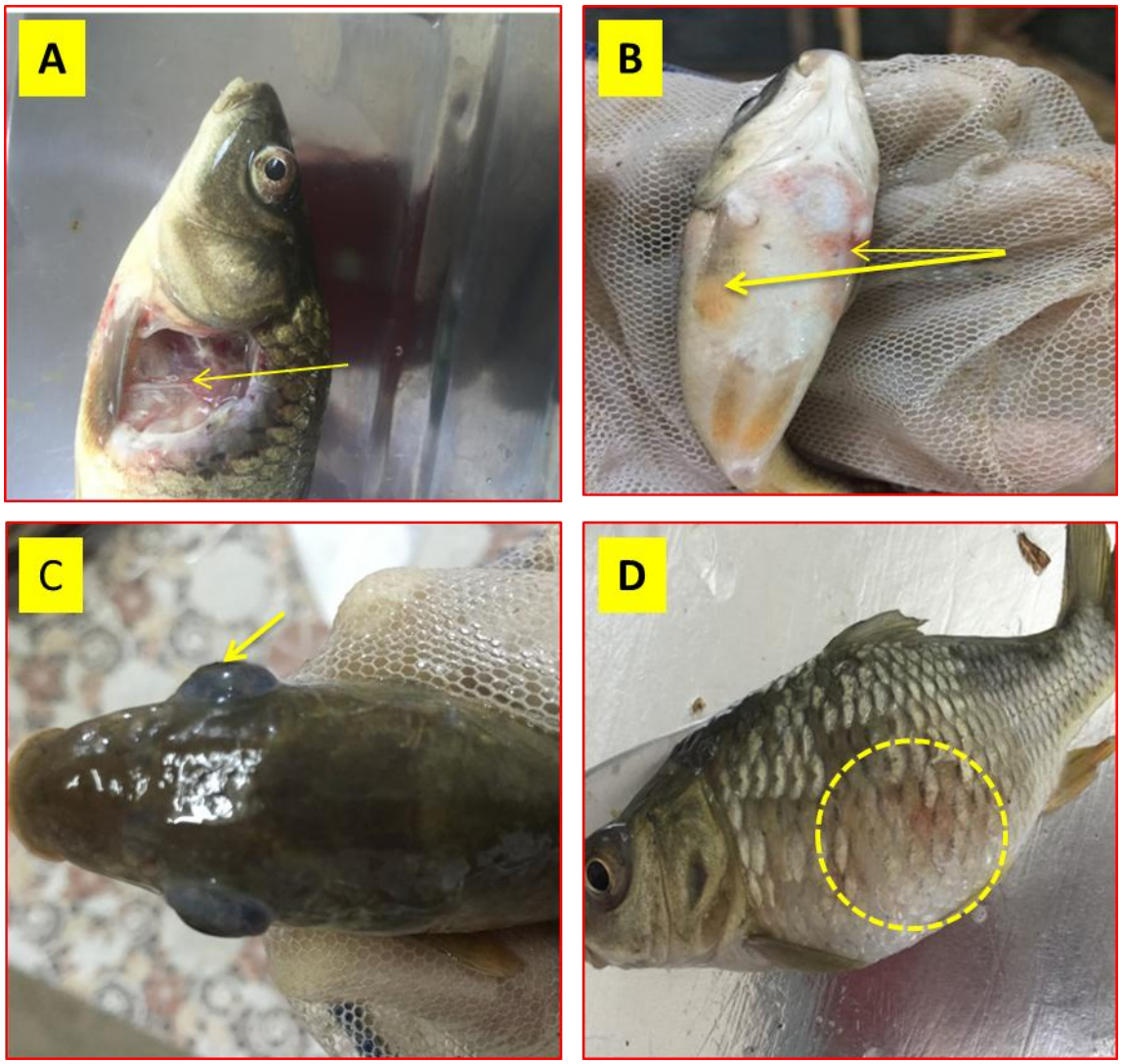

Fig. 1: External clinical sings A: showing the ulceration of skin B: demonstrating the haemorrhagic of skin C: exophthalmia of eyes D: showing loss of scales and distended of abdomen.

Haematological Parameters

The results of haematological parameters are presented in Table 1. RBC count of *C. carpio* post infection by *A. hydrophila* and treatment with AgNPs for 10 day revealed that T1, T4 and T5 showed slightly increased in RBC count compared to control groups (C+ and C-) but there were no significant differences ($P>0.05$). Also, there were no significant differences ($P \geq 0.05$) between the treatments (T1, T2, T3, T4, T5 and T6) groups and the control groups. Bhuvaneswari et al. (2018) indicated that RBC is being destroyed by the leucocytosis activity

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with subsequent erythroblastosis in erythrocytic anaemia infected by *A.hydrophila* of *C. carpio.* This result study was within normal range of RBCs count according to Glomski et al. (1992) who registered that the number of erythrocytes in circulating blood in common carp was most often within 0.5-3.0× 10^6/mm^3. However, the reduction number of RBC in T6 and T3 could be indicated that RBC is being destroyed by the leucocytosis activity with subsequent erythroblastosis in erythrocytes anaemia (Steinhagen et al., 1990). Also, the reduced number of erythrocytes and haemoglobin concentration was reported in cichlid, *Etroplus suratensis* infected by ulcerative epizootic syndrome (Pathiratne and Rajapakshe, 1998). This study is in agreement with Haney et al. (1992), who recorded a decrease in the RBC number and haemoglobin concentration may be caused by bacterial agent could be attributed to increased destruction, loss, or suppression of RBCs. This is could be associated with hem dilution brought about by loss of body fluids from advanced haemorrhagic–necrotic lesions. No significant differences (P≥0.05) were observed for Hb content and PCV% in all treatments (T1, T2, T3, T4, T5, T6) groups. Chau (1976) observed reduction in Hb content and haematocrit value in Golden shiner infected with a tail rot disease. This data indicated that *A. hydrophila* haemolysis and decrease the Hb and PCV value. Results of WBC count recorded significantly (P ≤0.05) decreased in all treatment groups (T1, T2, T3, T4, T5 and T6) compared to C+ group. But, there were no significant differences (P>0.05) among the treated groups (T1, T2, T3, T4, T5 and T6). Also, all the treatment groups showed significant increase (P≤0.05) compared to C- group. WBCs counts are generally used as indicator for immune response and diseases (Cagirgan1990). Changes in WBCs count has been reported to play an important role in assessment of health status of fish, and the increased in all of treatment gropes T1,T2,T3,T4and T6 compared with C- WBCs are usually an immunity reaction to foreign agents such as pathogens.The increase of WBC count in infected group could be due to response of immunity system to bacterial infection . These findings are in agreement with Harikrishnan *et al.* (2003), whom found that the WBC count of goldfish, *Carassius auratus* infected with *A. hydrophila* increased significantly as reported in this study for common carp infected with *A. hydrophila*. This increase according to Pathiratne and Rajapakshe (1998) may have a positive role in increasing goldfish immunity against *A. hydrophila*. In contrary, Al- Hilial (2021) registered significant increase in WBC count in *C. carpio* at high dose of Curcumin-AgNPs (12mg/l) (which was used as antifungal against *Saprolegnia spp.)* compared to infected fish (C+). This study is agreed with Imani et al. (2015) who found that elevated levels of WBC after treatment for AgNPs may be due to their immunomodulatory effect on the immune system of rainbow trout. Also, the result of this study is similar to Soltanian, and Fereidouni (2016).

Table 1: Effect of different treatments of AgNPs on haematological parameters (RBC count, PCV%, Hb content and WBC count) of *C. carpio*.

Treatment groups	Mean ± SE			
	RBCx10^6/µl	Hb g/dl	PCV%	WBCx10^3/µl

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C -	2.42 ±0.12	7.00 ±1.00	21.00 ±3.00	19.40 ±2.88b
C+	2.00 ±0.42	4.65 ±0.65	13.50 ±2.50	28.00±1.02 a
T1chem AgNPs 0.125g/l	2.52 ±0.16	6.00 ±0.00	18.50 ±0.50	21.00 ±9.73 b
T2chemically 0.25g/l	2.30 ±0.40	5.30 ±0.70	15.50 ±1.50	22.10 ±1.09b
T3bio AgNPs 0.045g/l	2.03 ±0.13	6.55 ±0.95	20.00 ±3.00	23.50 ±1.56b
T4bio AgNPs 0.075g/l	2.63 ±0.57	7.15 ±1.85	21.50 ±5.50	21.50 ±8.70b
T5 OTC 0.4g/l	2.43 ±0.06	7.15 ±1.16	21.50 ±3.50	22.70 ±9.66b
T6 bio AgNPs 0.15g/l	2.09 ±0.09	6.30 ±0.70	19.50 ±2.50	23.10 ±1567 b.
LSD value	0.990 NS	3.271 NS	10.01 NS	14213.0 *

Means having with the different letters in same column differed significantly. * (P≤0.05), NS: Non-Significant. n=4 samples

Differential leucocytes count

Results of differential leukocyte counts for *C. carpio* are summarized in Table 2. The percentages of neutrophils recorded a significant increase in T3 compared to T1. While, the percentage of lymphocytes recorded a significant decrease (P≤0.05) in T4, T5 and T6 groups compared to the T1 group, but these groups did not show any significant differences compared to the other groups and the C- groups. The percentage of monocytes showed a significant increase (P<0.05) in the T4, T5 and T6 treated groups respectively, compared to the control treatment C + and T1 groups. According to Nemati et al. (2019) who found increase of lymphocytes, the number of monocytes and granulocytes when treated Henna, *Lawsonia inermis* in common carp infected with *A.hydrophila* by enhanced the non-specific humeral and cellular responses and disease resistance against *A. hydrophila*. Also, Serezli et al. (2005); Sahu et al. (2007); Harikrishnan and Balasundaram (2003) who showed that the herbal treatment may have a positive role in increasing fish immunity and immune system depending on the species, water temperature and drug used. While, an increase in the number of lymphocytes may indicates a specific immune stimulation. Therefore, differential leukocyte count clarifies the role of therapy in nonspecific defensive activities in fish.

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Table2: Effect of different treatments of AgNPs and OTC in differential leukocytes count of *C. carpio* infected with *A. hydrophila.*

Treatment groups	Mean ± SE (%)			
	Neutrophils	Lymphocytes	Eosinophils	Monocytes
C-	10.00 ±0.35ab	82.00 ±0.31 ab	1.00 ±0.02	7.00 ±1.06 abc
C+	13.00 ±0.5 ab	80.00 ±0. 5 ab	1.00 ±0.01	6.00 ±0.50 bc
T1CHI 0.125g/l	9.00 ±0.5 b	86.00 ±0.5 a	1.00 ±0.01	4.00 ±0.55 c
T2CHI 0.25g/l	10.00 ±0.35 ab	83.00 ±0.5 ab	1.00 ±0.02	6.00 ±1.05 bc
T3BIO 0.045g/l	14.00 ±0.94 a	80.00 ±02 ab	1.00 ±0.01	5.00 ±0.75 c
T4BIO 0.075g/l	11.00 ±0.5 ab	78.00 ±0.1 b	1.00 ±0.01	10.00 ±0.80 a
T5OTC 0.4g/l	12.00 ±0.32ab	78.00 ±0.20 b	1.00 ±0.02	9.00 ±0.95 ab
T6BIO0 .15 g/l	13.00 ±0.36 ab	79.00 ±0.22 b	1.00 ±01	7.00 ±1.00 abc
LSD value	4.501 *	6.027 *	0.50 NS	3.50 *
Means having with the different letters in same column differed significantly.* (P≤0.05), NS: Non-Significant.				

Biochemical Profile

Results of total protein, globulin and albumin globulin ratio are presented in Table 3. The result of TP showed no significant differences in T1, T2, T3,T4, T5 and T6 compared to C + and C- groups. Albumin content showed significantly (P<0.05) decreased in all treated groups in comparison with C+ group. The globulin content showed no significant in all treatments T1, T2, T3, T4, T5 and T6 compared with control C+ and C – groups. While, A/G ratio recorded significant decreased (p≤0.05) in T1 and T2 compared to C+ group. Result of this study, the maximum total protein in C- (3.65g/dl) and minimum result in C+ (3.45g/dl) these values agreed with total protein level observed as a maximum in healthy fish (3.8± 0.3 mg/dl) and minimum in diseased fish (2.13 ± 0.25 g/dl), this indicated that due to infection protein contents automatically became low. Same as albumin and globulin level was also high in healthy fish and low in diseased fish. These findings are agreed with Kumar et al. (2005) and kumar et al. (2013). Changes in total protein content, in comparison to basic range, may be used as a clinical indicator in assessment of the health status, stress and body condition in aquatic species (Riche, 2007). The increase in the serum protein and globulin levels could be indicate a strong innate immune response in fish (Wiegertjes et al.,1996) and this mean in the present study the affected total protein and globulin levels were restored to near normal levels in T1,T2 and T4 in the treated groups which indicating that the treatment with act as a immunostimulatory.

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Table 3: Effect of different treatments of AgNPs on biochemical profile (total protein, albumin, globulin content g/dl and albumin globulin ratio) of *C. carpio*.

Treatment groups	Mean ± SE			
	Total protein g/dl	Albumin g/dl	Globulin g/dl	A/G
C-	3.65 ±0.15	1.78 ±0.02 bc	1.87 ±0.12	0.95 ±0.08 ab
C+	3.45 ±0.11	1.97 ±0.07 a	1.48 ±0.12	1.33 ±0.13 a
T1CHI0.125g/l	3.57 ±0.07	1.67 ±0.02 c	1.90 ±0.10	0.88 ±0.06 b
T2CHI 0.25g/l	3.6 ±0.05	1.75 ±0.00 bc	1.85 ±0.05	0.94 ±0.02 b
T3BIO0.045g/l	3.50 ±0.00	1.75 ±0.10 bc	1.75 ±0.10	1.01 ±0.11 ab
T4BIO0.075g/l	3.57 ±0.17	1.70 ±0.05 ab	1.85 ±0.10	0.91 ±0.03 ab
OTC0.4g/l	3.50 ±0.10	1.80 ±0.05 bc	1.72 ±0.12	1.05 ±0.10 ab
T6BIO0.15g/l	3.45 ±0.00	1.70 ±0.00 bc	1.75 ±0.00	0.97 ±0.00 ab
LSD value	0.379 NS	0.170 *	0.324 NS	0.265 *
Means having with the different letters in same column differed significantly. * ($P \leq 0.05$), NS: Non-Significant.				

Nitroblue tetrazolium (NBT) reduction assay

The reduction of Nitro Blue Tetrazolium (NBT) by radical oxygen produce from neutrophils cell of *C.* showed a high significantly increased ($P \leq 0.01$) in all treatment groups (T1, T2, T3, T4, T5 and T6) compared to C- and C+ groups. The highest values were reported in T4 and T6 which showed significant increased relative to other treatment groups. As well as, no significant differences were found among T1, T2, T3 and T5 (Table 4). These results established the stimulation of nonspecific immunity in infected and treated fish which could be due to improves the immune response to overcome the stress caused by infection, These results are in line with Hamad and Mustaf (2018), and also are in agreement with Shah et al. (2015) whom demonstrated that the increases in NBT reduction in infected samples were accepted as a response of cellular immune system to bacterial infection and with an increase in antibody production, which helps in recovery of the fishes, exposed to the infections agent (Seth and Saxena, 2003). This study also agreed with Ramazan et al. (2005) who stated that the values of NBT in the OTC treated fish were considerably higher than those in the control fish in the first sampling, but during the second sampling there was a slight decrease in the treated group, the decrease of OTC-treated group level of NBT reduction relative to control group this result prove that OTC diminutive phagocytic activation of phagocytic cells and it was removed from the body after 20 days of treatment. Also, these result are in accordance with Aziz and Mustafa (2019) whom observed significant increase in NBT value and survival rate in C. carpio in all treated groups infected with *Flavobacterium columnare* and treated by ozonized water. However, this study are in disagreement with Taffaella et al. (1999) whom didn't find any effect on respiratory burst activity or phagocytosis of kidney macrophages in turbot, *Scophthalmus maximus* after oral administration of oxytetracycline.

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Table 4: Effect of different treatments of AgNPs NBT reduction assay of *C. carpio*.

Groups	Mean ± SE of OD
C-	1.23 ±0.02 d
C+	1.30 ±0.03 d
T1CHI 0.125mg/ml	1.54 ±0.01 c
T2 CHI.0.25mg/ml	1.46 ±0.03 c
T3BIO 0.05mg/ml	1.53 ±0.07 c
T4 BIO 0.075mg/ml	1.76 ±0.04 a
T5 OTC 0.4 mg/ml	1.56 ±0.04 bc
T6BIO 0.15mg /ml	1.65 ±0.02 ab
LSD value	0. 11 **
P-value	0.0001
Means having with the different letters in same column differed significantly. ** (P≤0.01).	

Conclusions

With further evaluation, nanoparticle based treatment technologies could overcome threats of multi-drug resistance syndrome either as an alternative or as supplementary to oxytetracycline therapy and it reduces the use of antibiotics and the potential for environmental effects. Further investigations are needed using AgNPs program in the form of a water bath or with feed against a wide range of pathogens in different species of fish.

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