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Toxicity Effects of Green Synthesized Zinc Oxide Nanoparticles on Hematology and Biochemical Parameters in *Cirrhinus mrigala*

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KJI and HM conceptualized the experimental design and did project administration. MK conducted the research trial and drafted the manuscript. NK, TK, AS, KJI, UA, MF, MK, MBAK, US and RH wrote, reviewed and edited the manuscript.

Key words
ZnO-NPs, Hematology, Serum biochemistry, *A. indica, C. mrigala*

ABSTRACT

Current research was aimed to analyse the toxic effect of ZnO-NPs on hematology and serum biochemistry parameters in *Cirrhinus mrigala* with a post-exposure period of 96 h and 15 days. The experimental fish were divided into four groups (10 fish per group) i.e. control group (without ZnO-NPs supplementation), T1, T2, and T3 groups were fed on ZnO-NPs at the dose rates of 40, 80, and 120 mg/L in the aquaria. *Azadirachta indica* leaves were used to synthesize ZnO-NPs. Fish came into contact with ZnO-NPs through aqueous exposure. Blood sampling was carried out after 96 h and 15 days of exposure by puncturing the caudal vein of fish. Hematological analysis of blood showed a significant rise in the values of WBCs, MCHC, and PLTs, but a significant reduction in the counts of RBCs, Hb, and Hct in all treatments, while MCH and MCV showed non-significant change as compared to control. Serum biochemistry, such as ALP and ALT levels, significantly decreased in all experimental groups after 96 h of exposure and significantly increased after 15 days. Total protein (TP) levels did not change significantly, but albumin (ALB) levels decreased after 96 h and 15 days of exposure, and globulin (GLO) levels decreased significantly after 15 days of exposure. Bilirubin levels decreased significantly on a 40 mg/L dose. Cholesterol levels significantly increased after 96 h but showed non-significant change after 15 days. Triglyceride and HDL-C levels also showed significant changes. In conclusion, ZnO-NPs induced varying degrees of nano-toxicity in *C. mrigala* as compared to the control group. So, *C. mrigala* can be used as a good bio-indicator to track the toxic potential of nanoparticles, which could help to protect aquatic life and deal with toxic waste in aquatic bodies and also ZnO-NPs showed varying degree of toxicity with respect to time.

INTRODUCTION

In aquaculture, fish face several harsh effects; improper handling, high intensity and have to fed on artificial diets (Kumar *et al*., 2017). Artificial fish feed containing essential elements like zinc is important to maintain proper growth, normal physiological status as well as to maximize fish resistance against abiotic and biotic stressors (Kumar *et al*., 2017; Tawfik *et al*., 2017). Zn is second microelement in fish and metabolize many enzymatic processes (Tawfik

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et al., 2017; Uniyala *et al*., 2017) but can't be stored, so regular intake of Zn is necessity for fish body (Zalewski *et al*., 2005). In freshwater, fish have the ability to uptake Zn from food (Glover and Hogstrand, 2002) and from water (Hogstrand *et al*., 1998).

Zinc-oxide nano-particles (ZnO-NPs) have antifungal, antibacterial, chemical stability, conductivity, and catalytic properties (Liu *et al*., 2014). There are two NP synthesis methods (Priyadarshana *et al*., 2015). One strategy uses sputtering, etching, mechanical milling, and electro-explosion, while the second uses physical, chemical, and biological approaches to synthesis NPs (Hasnidawani *et al*., 2016). Conventionally manufactured NPs are pure but not cost-efficient and can lead to hazardous byproducts that have harmful consequences. These procedures need capping and stabilizing agents (Suntako, 2015). In green synthesis, plant extracts are the reducing medium while phytochemicals are the capping agent, biocatalyst, and natural stabilizer for NPs (Senthilkumar and Sivakumar, 2014). It doesn't need high energy, temperature, pressure, expensive apparatus, or toxic chemicals (Nagar and Nagar, 2015). Thus, green synthesis of NPs is cheaper, non-toxic, and eco-friendly than costly and harmful approaches (Sundrarajan *et al*., 2015; Lingaraju *et al*., 2016).

ZnO-NPs are used in cosmetics, hair, skin, sunshades, food additives, vitamins, wound treatments, anti-infection medicinal goods, and disinfectants. The rising usage of ZnO-NPs in consumables and food has raised concerns about their harmful impacts on human and aquatic health (Osmond and Mccall, 2010). As with other nanoparticles, the pros and downsides must be evaluated. World-wide, 3700 tonnes of ZnO-NPs are released annually (Haizhou *et al*., 2012). Uncontrolled usage and release of NPs prevent an accurate estimate. Since aquatic habitats are thought to be the final recipient of these engineered materials, it is crucial to investigate NPs effects on fish and other aquatic creatures (Zeumer *et al*., 2020; Haghighat *et al*., 2021).

Recent studies showed found that chemically manufactured nanoparticles are 10 times more hazardous than green ones (Mashjoor *et al*., 2019). Another study suggests using green-produced ZnO-NPs as growth promoters to boost viral resistance (Abdelkhalek and Al-Askar, 2020). ZnO-NPs exhibit antibacterial and cytotoxic properties (Aldalbahi *et al*., 2020). The toxicity of green manufactured NPs to fish is very little understood. A previous study reported that common carp reacts to waterborne NP exposure (Hao and Chen, 2012). Dietary ZnO-NPs (Connolly *et al*., 2016; Chupani *et al*., 2018) and lower dosages produce cytotoxicity, oxidative stress, blood biochemical parameter alterations, and tissue damage. Also, ZnO-NPs disrupt Nile tilapia's antioxidant defence mechanism (Abdelazim *et al*., 2018).

Azadirachta indica is used for the green synthesis of ZnO-NPs in current research and is generally known as neem. *A. indica* has been reported to possess anti-ulcer, anti-tumor, anti-bacterial, analgesic, anti-yeast, anti-inflammatory, anti-fungal, anti-hyperglycemic, anthelminthic, and anti-malarial activities. Its leaves have been used for treatment of neuromuscular pains, malarial fever, and chickenpox (Hashmat *et al*., 2012).

One of the most potential biomarkers to evaluate the toxicity effects of nanoparticles in fish is alterations in biochemical and hematological parameters (Adhikari *et al*., 2004). The *Cirrhinus mrigala* (mori) used in the current study is an Indian major carp having high fecundity and is very sensitive to environmental pollution (Chauhan *et al*., 2007). There is no data available on the toxicity effect of green synthesized ZnO-NPs from neem leaves on fish. Current research was aimed to study the water-born toxicological impacts of graded levels of green synthesized ZnO-NPs on the hematological and biochemical profiles of *C. marigala*.

MATERIALS AND METHODS

Preparation of leaf extract

The fresh leaves of *A. indica* were collected from the Cholistan area at the Baghdad campus of the Islamia University of Bahawalpur, Pakistan. After drying in the shade for one week at room temperature (25°C), the leaves were chopped and ground with a mechanical grinder into a fine powder. Plant powder of about 10g was soaked in 100 ml of double distilled water. The mixture was then heated on a hot plate at 75 °C for 60 min. After that, it was cooled and filtered through filter paper and stored at 4°C until carried out for further analysis (Lakshmi *et al*., 2017).

Biosynthesis of zinc-oxide nanoparticle (ZnO-NPs)

The synthesis of ZnO-NPs was carried out by soaking 0.016g of zinc nitrate into 100 ml of distilled water. The mixture was heated on the hotplate at 90°C, stirred continuously for 1 h using a magnetic stirrer, and then mixed in 10ml of plant extract. The final mixture was centrifuged in a centrifugation machine for 15 min at a rate of 6000 rpm, and 20 ml of sodium hydroxide solution (NaOH) was added at a drop rate of 3 ml per minute. The whole process was repeated three times to obtain the required amount of ZnO-NPs. The first sign of ZnO-NPs synthesis was a colour change in the mixture. After 1 day in an oven, ZnO-NPs were obtained in crystal form and characterized using a UV-visible spectrophotometer (Lakshmi *et al*., 2017; Bhuyan *et al*., 2015). LC50 was determined during preliminary experiment by following,

100 individuals of fish were placed in 10 glass aquaria (10 fish each) to determine 96Hr LC50 of *Cirrhinus mrigala*. Stock solution of ZnO-NPs was prepared and a total of ten concentrations were set in geometric series until the onset of LC50. Mortality of fish was noted and subject to probate analysis for final value of LC50. Then, green synthesized ZnO-NPs were administered orally and intravenously in *C. mrigala.*

Experimental design

Experimental fish, *C. mrigala*, of an average weight of 15±7 g were collected from the Hasilpur Government Fisheries Centre and transported safely to the fish research laboratory at the Department of Zoology, Islamia University Bahawalpur. The fish were acclimatized in well aerated glass-aquaria (92×46×46 cm, L×W×H). Fish were satiated with an artificial diet for 15 days, two times a day, at a rate of 3% of their body weight.

After acclimatization, fish were randomly allocated into four groups labelled as T0 (control, ZnO-NPs, 0.00 mg/L), T1 (ZnO-NPs, 40 mg/L), T2 (ZnO-NPs, 80 mg/L), and T3 (ZnO-NPs, 120 mg/L) (10 fish in each group, each having 3 replicates). ZnO-NPs sonication was carried out in a bath-type sonicator at room temperature for about 30 min. After sonication, nanoparticles were daily mixed in the water of each experimental group at 0, 40, 80, and 120 mg/L/treatment. The experimental period was divided into two phases, 96 h and 15 days. Water quality parameters were the same throughout the experiment (temperature, 27.52±0.23 °C; DO, 7.19±0.19 mg/L; pH, 6.4±0.28).

Blood sampling and serum separation

After a ZnO-NPs contact period of 96 h (1st blood sampling) and 15 days (2nd blood sampling), blood samples were taken from 3fishes/replicas, the fish were starved for 24 h. Anesthesia was administered to fish in each group at random using buffered tricaine methane sulphonate (MS-222) (100 gmL^{-1}) as a method of anesthesia. Blood was collected through the caudal vein using a plastic syringe and then added to heparinized tubes for hematological examination. Plain tubes without anticoagulants were used to collect blood serum from 5 fishes/replica, which was subsequently centrifuged at 300 rpm for 15 min at 4°C. The supernatant serum was taken out and kept at -20°C until it was used in plastic Eppendorf tubes (Ghazi *et al*., 2022).

Hematological studies

All hematology parameters like white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLTs) were estimated by an automated hematological analyzer (Celltac, α, MEK-6550 Ltd., Japan).

Serum biochemistry

Alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), bilirubin (TBIL), cholesterol (CHO), triglycerides (Trg), high-density lipoprotein (HDL-C) and globulin (GLB) biochemical parameters in serum samples were evaluated using an automated chemistry analyzer (Hitachi 7600-110 Ltd., Japan).

Statistical analysis

One-way ANOVA using SPSS was applied on collected data to determine significant variations between the experimental and control groups, and values were tabulated as a mean ± SEM (standard error of mean). Significant differences between the mean values were observed at $p \leq 0.05$ using Tukey's multiple-comparison test.

RESULTS

Hematological analysis

Hematological analysis of *C. marigala* exposed to ZnO-NPs after 96 h and 15 days is presented in Table I. Fish exposed to different doses of ZnO-NPs exhibited a significant increase in the levels of WBCs, MCHC and PLTs, except on 120mg/L nanoparticles, where the level of WBCs was the same as the control group. RBCs, Hb, and Hct levels decreased significantly in comparison to the control group. However, immunity response was found to be lowered at 120mg/L ZnO-NP in relation to control and other treatment groups, indicating high toxicity induced by nanoparticles. MCV showed a non-significant difference among all treatments with respect to control. PLTs, counts fall at a dose level of 40mg/L ZnO-NP as compared to the control group. Moreover, when the comparison of these indices was carried out among experimental groups, the highest levels of RBCs, Hb, and Hct were recorded in control, while those of MCH, MCHC, and PLTs at a dose level of 120 mg/L ZnO-NP and WBCs at a dose level of 80 mg/L ZnO-NP after 96 h and 15 days of nanoparticle exposure, respectively.

Serum biochemistry analysis

The effect of ZnO-NPs on serum biochemistry is exhibited in Table II. A significant difference in the level of ALT, ALP, bilirubin, triglyceride, and HDL-C was recorded in all experimental groups after 96 h and 15 days of nanoparticle exposure as compared to control.

Table I. Effect of ZnO-NPs on hematological profile of *C. marigala* after 96 h and 15 days.

Parameters	Control (0 mg/L)	ZnO-NPs (40mg/L)	ZnO-NPs (80mg/L)	ZnO-NPs (120mg/L)	p-value
After 4 days					
WBCs ($\times 10^3$ µl)	182.07±7.80^c	233.03±3.450^b	276.90±3.64^a	193.77±3.20^c	0.001
RBCs ($\times 10^6$ µl)	1.72±0.03^a	1.65±0.02^b	1.40±0.01^c	1.11±0.05^d	0.001
Hb (g/dl)	8.63±0.51^a	7.86±0.21^b	7.47±0.15^b	6.24±0.25^c	0.022
Hct (%)	36.13±2.03^a	31.50±1.00^b	28.13±2.58^b	21.07±1.7^c	0.023
MCV (fL)	210.20±14.90	190.87±3.75	230.86±60.16	189.83±22.45	-------
MCH (pg)	51.15±2.28	48.97±0.73	52.00±0.71	53.97±0.42	------
MCHC (g/dl)	23.95±2.34^b	24.97±0.21^{ab}	26.63±1.99^{ab}	29.80±3.57^a	0.039
PLTs (10^3/µl)	77.00±2.00^c	67.00±5.29^d	104.00±5.57^b	121.33±3.51^a	0.001
After 15 days					
WBCs ($\times 10^3$ µl)	192.07±8.90^c	229.03±3.450^b	278.90±3.74^a	199.86±3.19^c	0.001
RBCs ($\times 10^6$ µl)	1.73±0.02^a	1.51±0.04^b	1.29±0.02^c	0.99±0.02^d	0.001
Hb (g/dl)	8.73±0.35^a	7.23±0.07^b	6.93±0.15^b	5.77±0.30^c	0.021
Hct (%)	36.63±1.70^a	30.67±1.05^b	27.47±2.68^b	19.47±2.55^c	0.022
MCV (fL)	211.0±11.60	202.30±10.84	212.73±17.91	196.90±28.50	-------
MCH (pg)	50.27±1.61	49.70±1.41^c	53.73±0.40	54.23±1.92	-------
MCHC (g/dl)	23.90±1.87^a	23.57±0.61^a	25.53±2.13^{ab}	30.10±5.72^b	0.042
PLTs (10^3/µl)	81.00±4.00^c	69.67±3.05^d	107.00±2.00^b	138±4.58^a	0.001

Values are means ± SD of the triplicate samples. Means with similar superscripts in a row show statistically non-significant (P>0.05) difference.

Table II. Effect of ZnO-NPs on serum biochemistry of *C. marigala* after 96 h and 15 days.

Parameters	Control (0 mg/L)	ZnO-NPs (40mg/L)	ZnO-NPs (80mg/L)	ZnO-NPs (120mg/L)	p value
After 96 h					
ALT (U/ml)	35.00±2.00^a	30.50±1.00^b	24.50±1.20^c	33.10±0.45^a	0.002
ALP (U/ml)	57.17±4.75^a	50.37±1.11^b	39.77±2.05^c	23.27±1.59^d	0.001
Total protein (g/dl)	2.50±0.20	2.60±0.10	2.70±0.10	2.52±0.10	--
Albumin (g/dl)	1.08±0.10^{ab}	1.17±0.06^{ab}	1.23±0.12^a	0.71 ±0.20^b	0.029
Globulin (g/dl)	1.42±0.10	1.43±0.15	1.47±0.06	1.37±0.06	--
Bilirubin (g/dl)	0.58±0.12^a	0.42±0.11^b	0.52±0.09^{ab}	0.55±0.07^{ab}	0.027
Cholesterol (mg/dl)	178.33±4.16^d	187.60±2.35^c	211.33±1.53^b	233.33±4.04^a	0.001
Triglyceride (mg/dl)	119.67±5.03^b	70.33±2.08^d	86.33±3.06^c	136.33±3.06^a	0.001
HDL-C (mg/dl)	72.00±2.65^a	43.00±2.00^c	72.33±1.53^a	58.67±1.53^b	0.001
After 15 days					
ALT (U/ml)	39.17±1.53^d	49.20±2.13^c	58.13±1.35^b	63.73±1.16^a	0.001
ALP (U/ml)	56.10±1.47^c	63.17±1.52^b	68.50±1.00^a	54.10±2.06^c	0.001
Total protein (g/dl)	2.60±0.10	2.47±0.05	2.43±0.05	2.57±0.25	--
Albumin (g/dl)	1.16±0.05^a	1.10±0.10^{ab}	1.06±0.06^{bc}	0.83±0.14^c	0.027
Globulin (g/dl)	1.43±0.12^a	1.37±0.05^a	1.43±0.05^a	1.09±0.10^b	0.038
Bilirubin (g/dl)	0.60±0.10^a	0.40±0.10^b	0.50±0.10^{ab}	0.53±0.05^{ab}	0.025
Cholesterol (mg/dl)	165.00±92.67	170.67±2.08	173.67±2.08	169.33±3.05	--
Triglyceride (mg/dl)	120.33±3.05^b	78.67±2.08^d	91.00±2.00^c	142.33±3.51^a	0.001
HDL-C (mg/dl)	70.00±2.64^a	39.33±1.52^c	67.67±1.53^a	61.33±2.08^b	0.021

Values are means ± SD of the triplicate samples. Means with similar superscripts in a row show statistically non-significant (P>0.05) difference.

ALP and ALT levels were reduced significantly in all experimental groups after 96 h and increased after 15 days of ZnO-NPs exposure in comparison to control. The level of total protein was non-significantly changed, albumin and globulin level was same with respect to control except on provision of high dose 120 mg/L nanoparticles. Albumin level reduced after 96 h and 15 days, and globulin level reduced after 15 days significantly as compared to control. The bilirubin level decreased significantly on 40 mg/L nanoparticles as compared to control. Cholesterol levels increased significantly as the dose level of ZnO-NPs increased after 96 h but showed a non-significant difference in all experimental groups as compared to control after 15 days of nanoparticle exposure. The level of triglyceride decreased on 40mg/L nanoparticles exposure as compared to control, but as the dose level increased, the triglyceride level also increased significantly. There was also a significant difference in HDL-C levels after 40mg/L and 120mg/L nanoparticle exposure compared to control and 80mg/L nanoparticle exposure. Triglyceride and HDL-C levels can be listed in relation to dose levels as follows: triglyceride; 40mg/L<80mg/L<control<120mg/L and HDL-C; 40mg/L<120mg/L<control<80mg /L.

DISCUSSION

The toxic influences of various nanoparticles in living organisms relies upon several factors including; aggregation status, chemical composition, morphology, size, structural and surface properties of these nanoparticles. These features significantly affect the physiological interactions between organism's tissues and nanoparticles. The extensive applications of these nanomaterials in various fields of medical and industries prompted a comprehensive exploration of their harmful reactions in living organisms (Xiang *et al*., 2018).

Haemato-immunological reactions are commonly used to indicate physiological stress in fish in response to some toxicants (Ciji *et al*., 2012). In current study, fish fed on different doses of supplemented ZnO-NPs exhibited a significant rise in the values of WBCs, MCHC and PLTs, but significant reduction in the counts of RBCs, Hb, and Hct in comparison to fish fed on basal diet while MCH and MCV showed no significant differences with respect to control. Previous studies also reported dose dependent increase in WBCs, MCHC and PLTs but decrease in the number of RBCs in grass carp nourished by ZnO and ZnSO4 supplemented diets, in rainbow trout and Heteroclarias (Oti and Avoaja, 2005; Kori-Siakpere *et al*., 2008) fed with ZnO-NPs supplementations. Alkaladi *et al*. (2015) studied percentage decrease in the values of Hb and Hct in *Oreochromis niloticus* upon administration to sub-lethal concentrations of ZnO-NPs while non-significant difference on MCH and MCV. Further, reduction in the level of Hb and Hct has been correspondingly observed in Fe2O3-NPs treated fish and Ag-NPs treated silver carp (Shaluei *et al*., 2013).

Heavy metal nanoparticles destroy the membrane integrity inducing ROS (reactive oxygen species) pathway (Wise *et al*., 2010). Upon exposure to some toxicant, RBCs swell up leading to hemolysis and anemic condition in suspected organisms (Kori-Siakpere *et al*., 2008). Reduction in the counts of RBCs, Hct and Hb adversely affect the values of other hematological indices; MCHC, MCH, MCV and consequently lead to anemic condition (Kori-Siakpere *et al*., 2008).

In the present study WBCs level decreased at high dose 120mg/L nanoparticles, similar to current results, previous studies indicate a significant fall in the counts of WBCs in *Ctenopharyngodon idella* (Faiz *et al*., 2015), in Clarias (Oti and Avoaja, 2005) and Heteroclarias species (Kori-Siakpere *et al*., 2008) when fed with ZnO-NPs. WBCs reduction (leucopaenia) might be a result of ZnO-NPs bioaccumulation's in tissues which exert toxic effect on cells manufacturing from spleen (Firat, 2007) due to high amount of corticosteroid hormones which prevent and heal the inflammation areas (Celik *et al*., 2013). The values of WBCs may increase (lead to elevation in immunity response) or decrease (indicate unhealthy actions of blood forming tissues) in response to the entry of some toxic particles or stressors like chemical pollutant and infections in the body of all vertebrates including fish (Kori-Siakpere *et al*., 2008; Moharram *et al*., 2011; Olurin *et al*., 2012).

Biomolecules, mainly proteins face oxidative stress leads to variations in these molecules due to transition in metallic ions and are potential biomarkers for the indication of physiological disturbances (Kanwal *et al*., 2019). In present study, the amount of ALP and ALT was found to be reduced significantly in all experimental groups after 96 h and increased after 15 days compared to control. The level of Total protein not affected by ZnO-NPs. The albumin and bilirubin level decrease consistently in experimental groups after exposure period of 96 h and 15 days, while globulin showed non-significant difference after 96 h, but their level rise after 15 days relative to control. Cholesterol level was significantly high after 96 h but showed non-significant change after 15 days. The level of triglyceride and HDL-C were decreased in all experimental groups than the control one but at 120mg/L triglyceride level increased significantly as compared to other groups.

Kanwal *et al*. (2019) describe a significant decrease in albumin, and globulin in ZnO-NPs treated fish while no effect on total protein. The high immunity in response

to protein elevation indicated the stressful and weakened situation in fish, making it defenseless to others diseases. Further, previous researches have studied the low levels of albumin and globulin and unchanged total protein in *Channa punctatus* exposed to Cr, Ni and Co polluted water (Javed and Usmani, 2015), plasma protein in *Oreochromis niloticus* fed with Cd and Cu nanoparticles (El-Serafy *et al*., 2013) and in contrast to our study, total protein rise in common carp after transportation stress of 7 h (Dobsikova *et al*., 2006) to compete immune-toxic challenge and high energy requirement induced by nanoparticles mediated strain. In our study these parameters were not significantly different between the groups, indicating that NP exposure did not alter the overall immunological response of fish, similar to the study of Farsani *et al*. (2017), who studied the effect of ZnO-NPs on *oreochromis niloticus*.

Cholesterol level increased significantly after 96 h but showed a non-significant difference after 15 days. The level of triglyceride decreased on 40mg/L nanoparticles but as the dose level increased, the triglyceride level also increased significantly. There was also a significant difference in HDL-C levels compared to control. Similar to the study of Hajirezaee *et al*. (2020) who documented the same trend in cholesterol and triglyceride level when applied TiO2-NPs to *Cyprinus carpio*. Also Harsij *et al*. (2020) reported high level of triglyceride on high dose of Se-NPs on rainbow trout.

CONCLUSION

It can be inferred from the results of variations in different parameters that green synthesized ZnO-NPs from neem induced varying degrees of nano-toxicity in *C. mrigala* as compared to control group. The toxicity results of current study revealed that green nano-particles are less toxic *C. mrigala* can serve a suitable bio-indicator to monitor toxic potential of nanoparticles. Moreover, elevation or reduction in hematological or biochemical parameters can be used to judge the physiological status and fish health. Therefore, nano-toxicity should be investigated comprehensively before carrying out their further applications in different fields.

DECLARATIONS

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IRB approval

The experiment was conducted after the approval of the Ethical Review Committee of the Islamia University of Bahawalpur, Multan.

Ethical statement

The fish were cultured and harvested according to the ethical guideline, i.e. the fish were anesthetized with MS-222 and samples were collected for different analysis.

Statement of conflict of interest

The authors have declared no conflict of interest.

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