Evaluation of Hematology and Biochemistry of *Cyprinus carpio* Juveniles Exposed to NeemAzal Biopesticide: Acute and Sub-chronic Toxicity Study

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ABSTRACT

The concern over detrimental effects of chemical pesticide residues on the environment has enhanced the use of bio-pesticides that may also negatively impact aquatic ecosystems. The present study determined the 96-h 50% lethal concentration (LC_{50}) of the NeemAzal (containing 10 $g \cdot L^{-1}$ azadirachtin) bio-pesticide and evaluated the sub-chronic effects of its sublethal doses (0.12, 0.24 and 0.36 $mg \cdot L^{-1}$) on blood and biochemistry of common carp (*Cyprinus carpio*) with an average weight of 14.20±2.07 g. The 96-h LC_{50} was found to be 1.19 $mg \cdot L^{-1}$ of the pesticide. As NeemAzal content increased, the leukocyte count (WBC) and the level of creatinine (Cr), urea (BUN), cholesterol, glucose (GLU), neutrophils and aspartate aminotransferase (AST) elevated ($p<0.05$). Also, total proteins (TP), triglyceride (TG), alkaline phosphatase (ALP), hemoglobin (Hb), erythrocyte count (RBC), lymphocytes and hematocrit (Hct) decreased ($p<0.05$) with increasing toxin doses. Insignificant difference was found between the mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). Levels of eosinophils, basophils, monocytes, alanine aminotransferase (ALT) and albumin (ALP) of the exposed and controlled fish were also insignificant ($p>0.05$). The present investigation suggests that long-term exposure to NeemAzal can affect the hematological and biochemical parameters and can be harmful to *C. carpio*.

Keywords: Azadirachtin, Bio-pesticide, Common carp, Environmental impact, LC_{50}

INTRODUCTION

It has been found that increased use of agricultural pesticides and inflow of their excess into the aquatic ecosystem can be highly toxic and cause acute and chronic poisoning to fish and other organisms. Pesticides can harm vital organs and cause biochemical alterations in the exposed fishes (Mishra *et al*., 2004). To conquer the effects of chemical pesticides, pesticides of plant origin, or "bio-pesticides", have come into use. It is believed that unlike synthetic pesticides, bio-pesticides are more eco-friendly since they easily degrade in the environment. However, natural products are not always safer than synthetic counterparts; so, this is the reason why we need to evaluate the toxicity of bio-pesticides.

Azadirachtin is a bio-pesticide derived from neem (*Azadirachta indica*). Almost all parts of neem trees contain bitter compounds which can reduce insect feeding and alter their growth and reproductive hormones (Ascher, 1993). Therefore, azadirachtin has been used as a pesticide in agriculture, despite its potential toxic and undesirable effects on non-target organisms in the environment. Therefore, this plant-based pesticide must also be subjected to eco-toxicological tests before being authorized for agricultural purposes. It is necessary to assess its dissemination, bioaccumulation and effects on organisms to determine the safety margin of this compound, both for human health and the environment.

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Biochemical profiles are important bio-indicators in aquatic organisms under toxic condition. Hematological indices are also important parameters for the evaluation of the physiological status of fish. Several researchers have demonstrated the adverse effects of different insecticides on common carp (*Cyprinus carpio*) (Scott and Sloman, 2004; Velisek *et al*., 2009; Saravanan *et al*., 2011; Bernardi *et al*., 2013; Reverter *et al*., 2014; Murussi *et al*., 2016). Gholami *et al*. (2015) found the 96-h LC_{50} of the active ingredient of NeemAzal to be 0.73 mg·L^{-1} for *Ctenopharyngodon idella*; the compound was shown to reduce the biochemical protein and other enzymes of the fish's blood including aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). Murussi *et al*. (2016) studied the impact of azadirachtin content in the commercial Neenmax and suggested that 60 µL·L^{-1} azadirachtin can change the behavior and hematological parameters of *C. carpio*.

Cyprinus carpio is an abundant freshwater fish found naturally in rivers draining into the Caspian Sea and rivers in the south of Iran. Easy breeding and a high growth rate have made it a good species for culture in restricted waters. Common carp are also used as an integrated species in rice fields. Bio-pesticides are usually less toxic than synthetic pesticides, but extensive use of bio-pesticides in agriculture can also destroy the surrounding biodiversity. Therefore, this study aims to determine the impact of azadirachtin on fish and warn farmers of excess use of this bio-pesticide.

MATERIALS AND METHODS

Five hundred juvenile common carp were obtained from a fish farm at Shooshtar, Ahwaz, Iran. Acclimatization in well water continued for 14 days in 500-L tanks (pH = 7.87±0.1, salinity 2.3±0.1 ppt, DO = 6.12±0.4 mg·L^{-1}) with 50% daily exchange. Feeding was done at a rate of 5% of fish biomass.

Acute toxicity test

To determine the 96-h median lethal concentration (LC_{50}) of NeemAzal (product of India, containing 10 g·L^{-1} azadirachtin) to *Cyprinus carpio*, semi-static tests were conducted. A total of 180 fish with an average weight and length of 14.20±2.07 g and 7.95±0.59 cm were used. Tests were conducted in glass aquaria (40×40×40 cm) containing 40 L water and 10 fish per aquarium. The experiment setting comprised five concentrations of NeemAzal (1, 1.18, 1.41, 1.68, and 2 mg·L^{-1}) and a control (no pesticide), each with three replicates. The tested concentrations were assigned based on the results of the range-finding test. Water temperature (27–29 °C), photoperiod (10L/14D) and pH (7.5–8) were maintained during the test. A day before the LC test, feeding was ceased. Continuous aeration was provided during the experiment. Every 24 h, water and doses of pesticide were renewed for all aquaria, and mortality in each group was recorded. Probit analysis software (SPSS version 16.0, Chicago, USA) was run to determine 96-h LC_{50}.

Sub-chronic toxicity test

Based on the acute toxicity results, juveniles of *Cyprinus carpio* were subjected to 0, 0.12, 0.24 and 0.36 mg·L^{-1} (10, 20 and 30% of the 96-h LC_{50} value) of NeemAzal for 28 days. Each concentration was replicated three times. A total of 120 fish with an average weight and length of 14.20±2.07 g and 7.95±0.59 cm were randomly distributed into 12 aquaria. To maintain the toxic concentrations, water was changed daily and the test solution was renewed every 24 h. Feeding was maintained as 5% of the biomass. Fish behaviors were observed and recorded following the methodology of Oujifard *et al*. (2015).

Sampling

At the end of the experiment, the fish from all treatments and control groups were harvested, anesthetized with 10 mg·L^{-1} clove oil, and blood samples were taken from the caudal vein. To separate the blood serum and plasma, 1 mL of collected blood sample was placed in a 1-mL vial containing EDTA (for blood serum) and heparin (for plasma). After the blood was centrifuged for 10 min at 3,000 rpm, the supernatant was collected and stored at -20 °C for the subsequent analysis.

Hematological parameters

Hematological parameters including leukocyte count (WBC), hematocrit (Hct), erythrocyte (RBC) and hemoglobin concentration (Hb) were assessed using blood serum (Oujifard *et al*., 2015). Blood smears were dried under room conditions and kept for 5 min in cold methanol before staining with Giemsa stain. Identification and counting of neutrophils, lymphocytes, basophils, eosinophils, and monocytes was done using staining characteristics (Megarani *et al*., 2020). Mean corpuscular hemoglobin (MCH), its concentration (MCHC) and volume (MCV) were measured according to the following formulas:

$$MCH = (Hb/RBC)$$

$$MCHC = (Hb \times 100)/Hct$$

$$MCV = (Hct \times 100)/RBC$$

Biochemical assays

Blood plasma was used for biochemical tests. Auto Analyzer (Hitachi 917, Japan) and Pars Azmoon kit (Co, Pars Azmoon, Tehran, Iran) were used for measuring total protein (TP), cholesterol, triglyceride (TG) and glucose (GLU). Spectrophotometric method was used to measure blood urea nitrogen (BUN) and serum creatinine (Cr). The bromocresol green method was applied to assess the albumin (Alb) level of blood serum (Fazio *et al*., 2016). Alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using Bergmeyer *et al*. (1986) and Wacker *et al*. (1956) methods.

Statistical analyses

SPSS software version 16.0 (SPSS Inc. Chicago, USA) was applied for analyzing the recorded data. One-way analysis of variance (ANOVA) followed by Duncan test were performed to identify significant differences among treatments. The tests were considered significant at $p<0.05$.

RESULTS

Acute toxicity

Table 1 shows the toxic values for different time durations and their 95 and 99% lower and upper confidence limits. The 96-h LC_{50} and LC_{99} values of NeemAzal for common carp were found to be 1.19 and 2.82 $mg \cdot L^{-1}$, respectively.

Sub-chronic toxicity

During the 28-day experimental period, the experimental fish were closely observed and any abnormal behavior recorded (Table 2). As the number of experimental days increased, overactive response to external stimuli, irregular swimming, anxiety and anorexia were more evident at higher doses (0.24 and 0.36 $mg \cdot L^{-1}$).

Hematological results

Table 3 presents the hematological parameters of blood of the common carp subjected to different levels of NeemAzal for 28 days. Contrary to WBC levels, the amounts of Hct, Hb and RBC were significantly decreased ($p<0.05$) after exposure to the bio-pesticide. There was no significant

Table 1. Acute toxicity test of NeemAzal on *Cyprinus carpio* during 96-h exposure.

LC	Conc. ($mg \cdot L^{-1}$)	Confidence limits			
		LCL 0.05	UCL 0.05	LCL 0.01	UCL 0.01
25	0.75	0.59	0.89	0.54	0.94
50	1.19	1.00	1.40	0.95	0.07
75	1.89	1.56	2.38	1.49	1.27
99	2.82	2.24	4.01	2.11	0.45

Note: LCL = Lower confidence limits; ULC = Upper confidence limits

Table 2. Behavioral changes of *Cyprinus carpio* larvae exposed to NeemAzal at the end of the experiment.

Parameter	Control	Conc. (mg·L^{-1})		
		0.12	0.24	0.36
Irregular swimming	-	+	++	++
Nervous response to external stimuli	-	+	++	+++
Loss of balance	-	+	++	++
Anorexia	-	+	++	++

Note: − = None (0%); + = weak (10%); ++ = mild (10–50%); +++ = moderate (50–70%); ++++ = severe (>70%)

Table 3. Hematological parameters of *Cyprinus carpio* subjected to different concentrations of NeemAzal for 28 days.

Index	Sub-lethal concentration of NeemAzal (mg·L^{-1})			
	Control	0.12	0.24	0.36
WBC (10–3·mm^{-3})	27.16±0.76^{c}	27.60±0.85^{c}	29.66±0.57^{b}	32.66±0.57^{a}
RBC (10–6·mm^{-3})	1.60±0.0^{ab}	1.66±0.15^{a}	1.45±0.05^{bc}	1.35±0.05^{c}
Hb (g·dL^{-1})	5.70±0.10^{a}	5.73±0.25^{a}	5.03±0.65^{ab}	4.93±0.15^{b}
Hct (%)	26.30±0.50^{a}	26.33±1.52^{a}	22.83±0.76^{b}	21.33±1.52^{b}
MCV (fl)	164.38±3.13^{a}	158.39±7.29^{a}	157.48±1.02^{a}	157.90±5.60^{a}
MCH (pg)	35.63±0.63^{a}	34.51±1.87^{a}	34.80±5.34^{a}	36.56±1.07^{a}
MCHC (%)	21.67±0.32^{a}	21.78±0.31^{a}	22.11±3.46^{a}	23.18±1.20^{a}
BAS (%WBC)	1±0^{a}	1±0^{a}	1±0^{a}	1.5±0.5^{a}
EOS (% of WBC)	3±1^{a}	2±1^{a}	3.5±0.5^{a}	2±0^{a}
MON (% of WBC)	6±2^{a}	5±1^{a}	5.5±0.5^{a}	4±0^{a}
Lym (% of WBC)	73.33±4.5^{a}	73±2^{ab}	68.33±0.57^{bc}	64.33±0.57^{c}
Neut (% of WBC)	17±1^{d}	19.5±0.5^{c}	21.66 ±0.57^{b}	27±1^{a}

Note: Means±SD in the same row superscripted with different lowercase letters are significantly ($p<0.05$) different.

difference ($p>0.05$) in the level of MCV, MCH and MCHC between the exposed and control groups. For white blood cells, significant difference ($p<0.05$) was observed in the number of lymphocytes, which decreased from the control group (73.33) to the 0.36 treatment (64.33), and in neutrophils, which increased from the control group (17) to the 0.36 treatment (27). No effects of the bio-pesticide were observed in terms of monocytes, basophils or eosinophils.

Biochemical results

Biochemical results are presented in Table 4 and Figure 1. As the NeemAzal content increased, increases in BUN, GLU, Cr, cholesterol and AST content were observed. Concentrations of Alb and ALT did not vary among the exposed and control groups. Results also showed that sub-chronic exposure to the bio-pesticide decreased the levels of TG, TP and ALP ($p<0.05$).

Table 4. Biochemical parameters of *Cyprinus carpio* exposed to sub-lethal concentrations of NeemAzal for 28 days.

Index	Sub-lethal concentration of NeemAzal (mg·L^{-1})			
	Control	0.12	0.24	0.36
BUN (mg·dL^{-1})	6.00±0.50^{b}	6.60±1.35^{b}	7.46±0.55^{ab}	8.73±0.25^{a}
GLU (mg·dL^{-1})	55.83±2.77^{c}	57.40±1.15^{c}	63.63±1.40^{b}	68.93±2.51^{a}
TG (mg·dL^{-1})	136.67±4.50^{a}	134.67±10.50^{ab}	134.00±3.46^{ab}	124.00±4.0^{b}
Cholesterol (mg·dL^{-1})	129.00±9.0^{b}	139.00±5.0^{ab}	140.00±10.0^{ab}	144.67±5.50^{a}
TP (g·dL^{-1})	3.53±0.05^{a}	3.40±0.10^{ab}	3.20±0.10^{bc}	3.03±0.15^{c}
Alb (g·dL^{-1})	1.60±0.20^{a}	1.46±0.05^{a}	1.40±0.20^{a}	1.38±0.02^{a}
Cr (mg·dL^{-1})	0.23±0.03^{b}	0.30±0.06^{a}	0.30±0.0^{a}	0.29±0.01^{ab}

Note: Different letters in each row indicate statistical difference ($p\leq0.05$) between means.

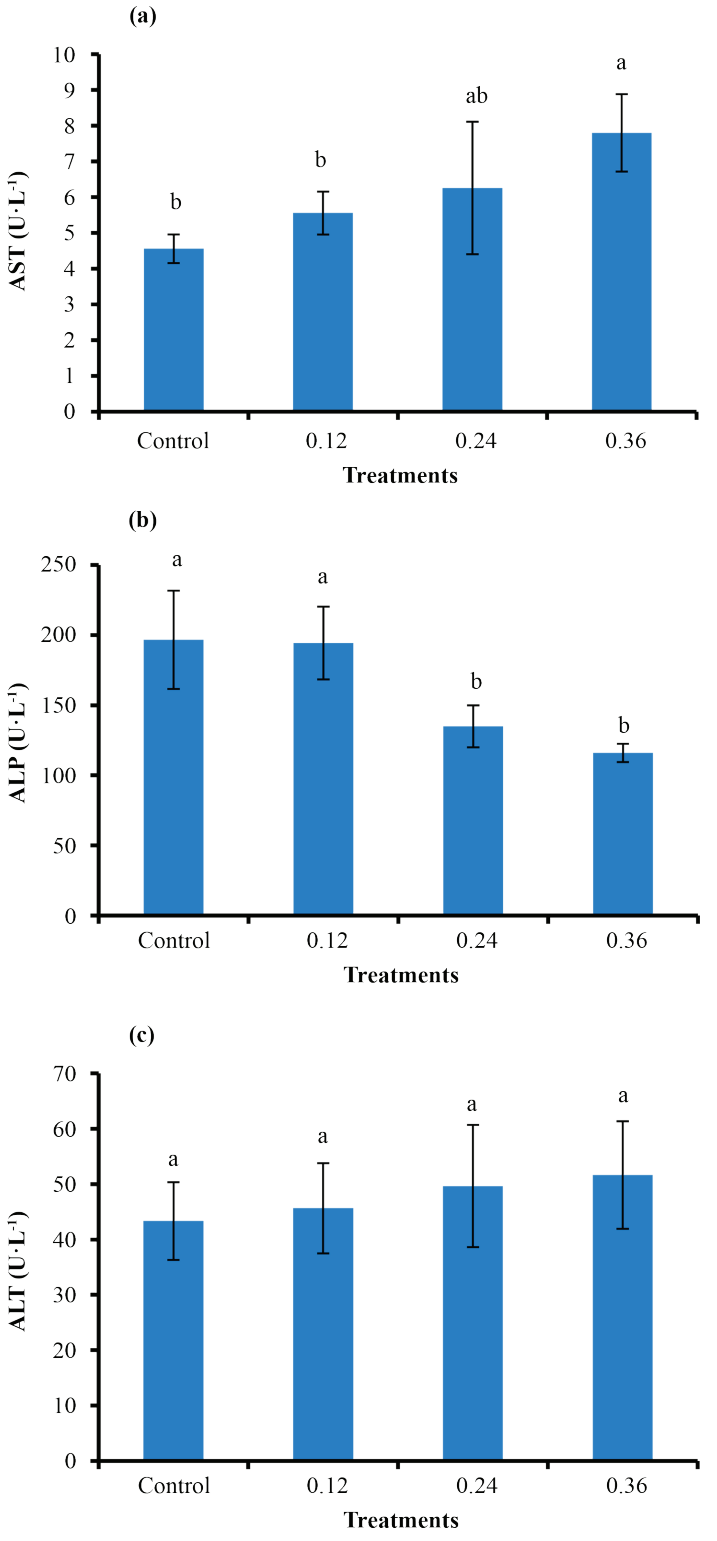

Figure 1. AST (a), ALP (b) and ALT (c) activities in the serum of *Cyprinus carpio* subjected to sub-lethal concentrations of Neem Azal (mg·L^{-1}).

DISCUSSION

Although pest management agents derived from natural sources provide an alternative to synthetic chemicals, excess accumulation and non-selective destruction of some of these bio-pesticides can cause serious impairments to the ecological environment. Generally, different levels of LC_{50} can be seen in different studies, which could be due to different experimental conditions and materials. Other studies have also shown the 96-h LC_{50} values of neem bio-pesticide for *Catla catla* (2.04 $mg \cdot L^{-1}$), *Labeo rohita* (2.36 $mg \cdot L^{-1}$), *Cirrhinus mrigala* (2.78 $mg \cdot L^{-1}$) (Das *et al*., 2002) and *Cyprinus carpio* (80 $\mu L \cdot L^{-1}$) (Murussi *et al*., 2016). In this study, the 96-h LC_{50} value of NeemAzal for common carp was found to be 1.19 $mg \cdot L^{-1}$, which is lower than the previously mentioned studies. This difference might be due to the type and the concentration of the active ingredient in the tested pesticide and how the extract was obtained. For example, Murussi *et al*. (2016) used Neenmax containing a maximum of 1,200 $mL \cdot L^{-1}$ of Aza A and B as active ingredients. Winkaler *et al*. (2007) used the aqueous extract of the dried leaves in well water and found the 24-h LC_{50} of 4.8 $g \cdot L^{-1}$ for *Prochilodus lineatus*. It is also important to know whether the LC value is based on the active ingredient present in the pesticide or the pesticide itself. The 96-h LC_{50} value of NeemAzal active ingredient for *C. idella* was 0.73 $mg \cdot L^{-1}$ (Gholami *et al*., 2015), which shows no difference with our study. Different products of neem pesticide have different LC values for specific fish species. For example, the LC_{50} values of Neem Gold and Nimbecidine on L*epidocephalichthys guntea* fingerlings were 0.525 and 0.135 $mg \cdot L^{-1}$ (Mondal *et al*., 2007), but the same value for Neemgold to *Danio rerio* was 2.980 $\mu g \cdot L^{-1}$ (Ansari and Ahmad, 2010), while LC_{50} of Neemsheild on *L. rohita* was 44.61 $mg \cdot L^{-1}$ (Maitra *et al*., 2014). Differences in the sensitivity of fish species to neem can be due to the variation in the amount of active ingredients extracted from different parts of the plant and even the plant source (Luo *et al*., 1999). Toxic effects of neem leaf extracts on three life stages of *Oreochromis mossambicus* were observed at 1.67 $g \cdot L^{-1}$, 2.27 $g \cdot L^{-1}$ and 5.83 $g \cdot L^{-1}$ for fry, juveniles and adults, respectively (Dhara *et al*., 2016). Therefore, such variation in the lethal toxicity may also be attributed to variation in fish species, age and size, test methods and water quality.

Toxic substances can affect the behavior of fish. In our study, NeemAzal clearly changed the behavior of the specimens (Table 2). This type of change can be found in other studies on fish exposed to pesticides. Rao *et al*. (2005) stated that the interruption between the nervous and muscular junctions due to poison accumulation causes the changes in fish behavior. Murussi *et al*. (2016) showed significant changes in the traveling distance, turn angle, time spent immobile and the number of immobile episodes of fishes exposed to 60 $\mu L \cdot L^{-1}$ azadirachtin.

Stressors can also change the hematological parameters of fish. Exposure to toxin can damage the hematopoietic tissues of fish and cause negative effects on blood cells. Decreases in RBC, Hct and Hb are recognized as a symptom of anemia (Banaee *et al*., 2011). In the present study, the reduction in Hb, RBC and Hct levels of the experimental fish (Table 3) was evident. These reductions might be due to damage made to the gills, release of oxygen radicals, and reduction of Hb in erythrocytes due to the toxicant. Haemodilution due to gill damage also can reduce the erythrocyte count. The kidney is an important hematopoietic organ in fish that receives a large flow of blood from the gills and, consequently, can be a target organ of aquatic contamination (Mariano *et al*., 2021). Therefore, long-term exposure of fish to NeemAzal could have affected the hematopoietic organs and reduced the hematological parameters of the fish. Similarly, decrease in RBC count, Hb and Hct values in fish exposed to toxicants have been reported (Ahmadi *et al*., 2014). Murussi *et al*. (2016) exposed *C. carpio* to 60 $\mu L \cdot L^{-1}$ azadirachtin and found lower hematimetric index, hemoglobin and red cell distribution. In contrast, Carvalho and Fernandes (2006) showed an increase in the above parameters of fish exposed to toxic agents.

White blood cells were significantly increased in the fish exposed to NeemAzal. Our results are in line with other studies that reported an increased leucocyte count in fishes exposed to pesticides (Reddy and Bashamohideen, 1989).

Venkata Ramudu *et al*. (2009) marked WBC increase as a defense mechanism against the pesticide that has entered the body of a fish. The significant increase of WBC during sub-lethal treatment might be due to the defense reaction of the fish immune system against NeemAzal action in damaging the gill tissue. Wedemeyer and McLeay (1981) also suggested that an increase of WBC count could be due to an increase in granulocytes and monocytes, which function as phagocytes to salvage debris from injured tissues. Decline in total lymphocyte count and increase in the number of neutrophils, eosinophils and monocytes in carps poisoned by different pesticides were also reported previously (Sopinska and Guz, 1998). Increases in WBC count have been reported effects of chemical pollution in a number of fish populations (David *et al*., 2015). In contrast to the present findings, decreased WBC counts have been reported in fish after exposure to concentrations of poison (Shamoushaki *et al*., 2012). In the present study, exposure to NeemAzal led to a decrease in lymphocytes, whereas the neutrophil level increased. Decrease in the percentage of lymphocytes (Svoboda *et al*., 2001) and an increased percentage of neutrophil cells (Banaee *et al*., 2008) was reported in *C. carpio* exposed to diazinon.

The decrease in TP and Alb was in accordance with increasing toxin dose (Table 4). This result was in agreement with that of Gholami *et al*. (2015), who reported lower protein content with increased dose of azadirachtin in grass carp *C. idella*. Banaee *et al*. (2011) indicated the reduction in the levels of TP and Alb due to hepatic impairment from pesticide exposure. In this study the decrease in TP and Alb levels could be explained by the destruction of liver cells, which are important sites of protein synthesis. It has been suggested that, in response to a stressor, physiological activity of the body increases; to sustain physiological activity, more energy is utilized and the amounts of TP and Alb decrease (Martinez *et al*., 2004). This could be the reason for reduced levels of TP and Alb in fish exposed to NeemAzal pesticide.

Any disorder in the kidney and gills elevates the BUN level of fish serum. In this investigation, the amount of Cr and BUN in the serum rose as the toxin level increased (Table 4), which indicates that the gills and kidneys were damaged by the toxic compound. Cholesterol and TG have been used to evaluate nutritional status of fish. Bernet *et al*. (2001) indicated that any damage to the liver can increase the concentration of TG and cholesterol of the blood. In this investigation, the amount of TG and cholesterol also increased in the serum of exposed fish (Table 4), and may have been due to liver damage. Seyit *et al*. (2000) also suggested that increased serum cholesterol could be a result of liver damage or nephrotic syndrome. Similar results were obtained by Yang (2003), who reported higher levels of BUN, CR, cholesterol and TG concentrations in the serum of common carp (*C. carpio*) exposed to gallium. Bernet *et al*. (2001) also suggested that high observed concentrations of TG were a result of nephritic syndrome or glycogen storage disease.

Blood glucose is the main source of an animal's energy. An increased level of glucose in plasma also is a response to stressors. In this experiment, there was no significant difference between a treatment of 0.12 $mg \cdot L^{-1}$ NeemAzal and the control groups; however, GLU was significantly higher in plasma of fish exposed to higher concentrations of the pesticide (Table 4). Hastuti *et al*. (2003) suggested that an increase in blood glucose level was the result of an increase in plasma catecholamine hormones. He also stated that catecholamine hormone activates the enzymes involved in the catabolism of liver and muscle glycogen stores, and suppresses the secretion of the hormone insulin, so that blood glucose increases. Previous studies showed an increase of blood GLU of animals in response to the toxin (Winkaler *et al*., 2007). Higher GLU was also noted by other researchers in fish exposed to pollutants (Ahmad, 2011; Banaee *et al*., 2011).

Increase in ALT and AST activities in the serum of fish serves as an indicator of tissue damage or liver disfunction (Mariano *et al*., 2019; Li *et al*., 2020). In this experiment, increased levels of AST and ALT were recorded, supporting earlier findings (Agrahari *et al*., 2006; Banaee *et al*., 2011). Our results indicate that exposure to NeemAzal had adverse effects on the liver physiology of the fish and increased their protein

catabolism. ALP activity was also significantly reduced in this study (Figure 1b). Inhibition of ALP activity in tissues (Gill *et al*., 1990) and serum (Newcomb, 1974) of fishes exposed to toxicants was previously reported. Khoshbavar-Rostami *et al*. (2004) associated ALP reduction to liver damage in fish due to poisoning. Anderson *et al*. (2002) also stated that decreases in ALP in various organs may be due to the decline in the rate of synthesis caused by lowered metabolic demands and electrolytic imbalance caused by tissue over-hydration.

CONCLUSION

In the present study, considerable alteration occurred in different blood parameters including biochemical and hematological profiles of *Cyprinus carpio* treated with sub-lethal concentrations of NeemAzal. These findings indicate that NeemAzal can be toxic to fish, and therefore people applying this product must be aware of its potential toxicity in natural ecosystems.

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