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Original Article

Effects of intraperitoneal injection of safflower (*Carthamus tinctorius*) extract in Caspian roach (*Rutilus caspicus*) broodstocks in exposure to ammonia stress

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Abstract: The objective of the present study was to investigate the effects of safflower extract on some serological parameters and the resistance to ammonia stress in the Caspian roach (*Rutilus caspicus*). For this purpose, 150 fish with an average weight of 61.73±0.32 g were allocated into 15 aquaria. The fish were divided into one control group and four treatment groups, with three replications for each group. Fish of the treatment groups were intraperitoneally injected with different levels of safflower extract, including 50 mg/kg (treatment 1), 100 mg/kg (treatment 2), 200 mg/kg (treatment 3), and 300 mg/kg (treatment 4), respectively. The fish in the control group just received normal saline via IP injection. After 2 weeks, blood samples were taken to evaluate some haematological and biochemical parameters. According to the results, serum glucose levels were significantly lower in all treatment groups compared to control 2 weeks post-injection. Total protein levels were measured higher in treatments 1 and 2 compared to control, but treatments 3 and 4 were recorded lower than control one. The levels of ALP and AST enzymes also decreased in fish that received safflower extract. The results of ammonia stress showed that injection of different levels of safflower extract led to an increase in the survival rate of Caspian roach in exposure to ammonia stress.

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Introduction

The Caspian roach, *Rutilus caspicus*, a member of the cyprinid family, is a freshwater fish inhabiting the Caspian Sea basin. This fish is a part of the diet of some valuable Caspian Sea fish, such as perch and sturgeons (Hasanpour et al., 2015, 2016; Eagderi et al., 2022). In recent years, the population of this species has significantly decreased due to several reasons, such as overfishing, sea pollution, and destruction of natural restoration environments. As a result, it has been included in the list of endangered fish species (IUCN Red List) (Kiabi et al., 1999). Every year, fingerlings of this species are released into the sea to restore for their restocking. However, despite these efforts, the downward trend of the catch of this fish has not decreased much (Statistical Yearbook of Iran Fisheries Organization, 2019). Some research has investigated the cultivation of this valuable species up to market size, but the economic results have not been encouraging for farmers (Piri et al., 2014). To restore fish stocks, it is important to provide the broodstock with the highest efficiency and production. This can be achieved by reducing management stress, improving rearing conditions, and providing proper diet and supplements that increase the immune system function.

In recent years, several studies have been conducted concerning using plant extracts to stimulate the immune system in fish (Citarasu, 2010). In this regard, the safflower, *Carthamus tinctorius* L. (Asteraceae), is one of the traditional medicinal plants. Several medicinal properties have been reported in humans (Li et al., 2013). In some studies, this plant has been noted for its diabetes control effects and anticancer properties in lab mice (Jun et al., 2011). Despite extensive studies on this plant in medical science and laboratory animals, there have been limited studies on the effects of this plant in aquatic

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animals, and most of these investigations are also related to the effects of safflower seed oil in the diet of fish (Altundag et al., 2014; Dernrkbasi et al., 2015). Few studies have been done on the effects of safflower extracts on fish e.g., Dadras et al. (2016) on great sturgeon (*Huso huso*), Mazandarani et al. (2019) on common carp (*Cyprinus carpio*) and Zaigari et al. (2019) on rainbow trout (*Oncorhynchus mykiss*). For the use of any herbal extract and drug in animals, its medicinal effects and side effects must first be investigated in the pilot study. Considering the importance of using immune stimulants in the Caspian roach to increase stress tolerance during artificial reproduction, the present study investigated the effects of safflower extract via injection method on some immune parameters and ammonia stress tolerance in broodstock fish.

Materials and Methods

Fish preparation and maintenance: A total of 150 Caspian roach broodstocks were obtained from the Sijwal bony fish reproduction and rearing center (Golestan Province, Iran). The fish with an average weight of 61.7±3.1 g were transported to the lab in plastic bags and were divided into two fiberglass tanks (1.5 x 1.5 meters with 40 cm of water) and reared for one week. Then, they were divided into 15 fiberglass tanks containing 150 L of water (10 fish per tank) and acclimatized to the experimental conditions for 14 days. During the study period, fish were fed commercial pellet (Faradaneh Co, Iran) twice a day (3% biomass), and water temperature, pH, and total ammonia nitrogen were 25.3±2.1°C, 7.7±0.43 and 0.13±0.07 mg N /L, respectively.

Treatments and sampling procedure: To prepare the injectable ethanolic extract, safflower was washed three times (with sterile water), dried, and ground. Ethanol (96%) was added as a solvent to soak the powder at a ratio of 1:10, and then this mixture was transferred to a dark glass container and mixed well on a shaker for 48 hours at room temperature. The solution was passed through a filter paper, the solvent was evaporated at 40°C in a rotary evaporator, and finally, the remaining material was packed as an ethanolic extract in sterile containers (Harikrishnan, 2009). In this study, five experimental groups were considered, including 4 treatments and one control group (each with three replicates). The fish of the treatment group received 0.3 ml of extract via intraperitoneal injection at four dosages of 50, 100, 200, and 300 mg/kg bw. 0.3 ml of sterile normal saline (0.9% sodium chloride) was injected into the control group using a similar method. In this regard, safflower extract was dissolved in 0.9% sodium chloride at 40°C to prepare the injectable solution. The fish were anesthetized using 100 ppm eugenol, and the injection was done using an insulin syringe. All treatments and control groups were reared two weeks post-injection.

Serological and hematological findings: Eight fish from each treatment were randomly selected for blood sampling after two weeks of injection. The fish were anesthetized with 100 ppm eugenol, and blood sampling was done via caudal vasculature with a 21-gauge syringe. Part of the blood samples were placed in different microtubes for serological tests. Blood serum was separated by a centrifuge (15 minutes, 8000 rpm), and biochemical parameters, including glucose, protein, ALP, AST, and ALT, were measured by laboratory kits (Pars Azmoun Co, Iran). The remaining part of the blood was prepared for hematological tests. Neubauer hemocytometer was used to measure the red blood cell (RBC) counts and white blood cell (WBC) counts. The samples were diluted 100 times with Dice solution (Dacie and Lewis, 2001). Blood hematocrit was measured by the standard microhematocrit method, and the hemoglobin values were determined by the cyanomet hemoglobin method using lab kites (Pars Azmoun Co, Iran) (Blaxhall and Daisley, 1973). The main components of the RBC indices include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated based on the routine formula (Dacie and Lewis, 2001).

Ammonia stress: Ten fish from each group were exposed to a concentration of 0.6 mg/l of molecular ammonia (NH_3). For this purpose, ammonium chloride solution (NH_4Cl) (Merck company,

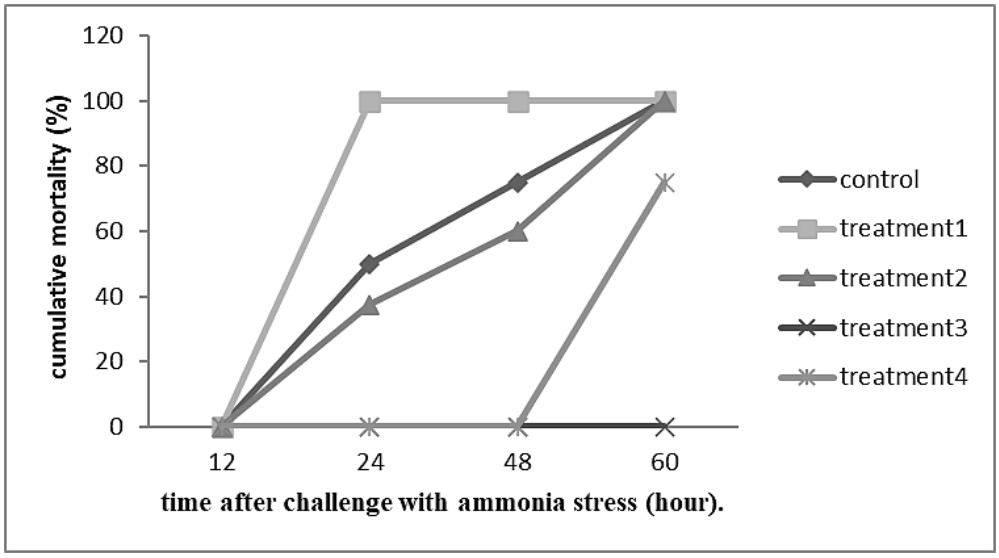

Figure 1. The mortality rate in the Caspian roach, *Rutilus caspicus*, broodstocks injected with different levels of safflower extract when exposed to lethal concentrations of unionized ammonia (Treatment 1: injected with 50 mg/liter of safflower extract, Treatment 2: injected with 100 mg/liter of safflower extract, Treatment 3: injected with 200 mg/liter of safflower extract, and Treatment 4: injected with 300 mg/liter of safflower extract).

Germany) was prepared from the laboratory store. The water temperature was kept at 25°C with a heater, and KOH solution was used to control the pH = 8.2. The amount of ionized ammonia (NH_4) and molecular ammonia (NH_3) was calculated and determined based on Emerson et al. (1975) after measuring total ammonia, considering temperature and pH. The ammonia solution was prepared for each treatment and replaced daily.

Statistical analysis: Data analysis and determination of significance levels were performed using SPSS 18 software. Data were subjected to a one-way analysis of variance (one-way ANOVA) followed by Duncan's posthoc test with a 95% confidence level. All data were expressed as mean ± standard deviation (SD).

Results

According to the results, the mortality rate 24 hours post-challenge in the control group, treatments 1 and 2, were 50, 100, and 37.5%, respectively. 75% of fish in the control group, 100% of fish in treatment 1, and 60% of fish in treatment 2 group died 48 hours after exposure, at the same time, up to 48 hours after exposure. No mortality was recorded in the treatments 3 and 4. Finally, 60 hours after exposure, 100% mortality was recorded for all control groups, treatment 1 and treatment 2, and 60% for treatment 4. Mortality was not observed in the treatment 3 to 60 hours after exposure (Fig. 1).

The serum total protein levels two weeks after receiving safflower extract via intraperitoneal injection are shown in Figure 2. In fish injected with safflower extract, there was a significant difference in total protein levels compared to the control group ($P<0.05$). Based on the results, total protein levels in fish that received 50 and 100 mg/kg bw of extract were significantly higher than the control group, i.e., these values were significantly lower in fish that received 200 and 300 mg/kg bw of extract compared to the control group (Fig. 3).

The serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) enzymes in the treatments and control one are shown in Figures 4, 5 and 6, respectively. In the fish groups that received 100, 200, and 300 mg/kg bw extract, the levels of AST and ALP were significantly lower than the control group and treatment 1 ($P<0.05$). ALT levels were significantly higher in fish receiving 300 mg/kg bw than in other groups (Fig. 6).

Based on the results, intraperitoneal injection of safflower extract did not cause significant changes in the red blood cell count (RBC), hematocrit, hemoglobin, MCV, MCH, and MCHC levels. The

Table 1. Hematological indices of Caspian roach injected with different levels of safflower extract.

Parameters	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
RBC (× 10^6 cell/µl)	1.45±0.3	1.40±0.5	1.31±0.3	1.45±0.4	1.22±0.6
WBC (10^3 cell/µl)	31.1±4.4^a	28.6±5.3^a	20.2±3.4^b	11.1±2.4^c	12.1±2.3^c
Hemoglobin (mg/dl)	7.32±0.63	7.11±0.83	6.93±0.62	6.44±0.72	6.64±0.77
Hematocrit (%)	32.12±1.21^a	29.27±2.11^{ab}	27.32±1.92^b	27.33±2.33^b	26.18±2.41^b
MCV (fl)	221.5±13.46	209.1±17.6	208.5±10.1	188.6±18.8	214.6±15.9
MCH (pg)	50.5±6.4	50.8±4.5	52.9±3.8	46.4±8.4	54.3±10.4
MCHC (g/dl)	22.8±4.4	24.3±5.5	25.4±3.7	25.3±4.8	25.4±1.6

Control: injected with normal saline; treatment 1: injected with 50 mg/kg of safflower extract; treatment 2: injected with 100 mg/kg of safflower extract; treatment 3: injected with 200 mg/kg of safflower extract; treatment 4: injected with 300 mg/kg of safflower extract. Means with different letters (a–c) in the same row indicate significant differences (P<0.05).

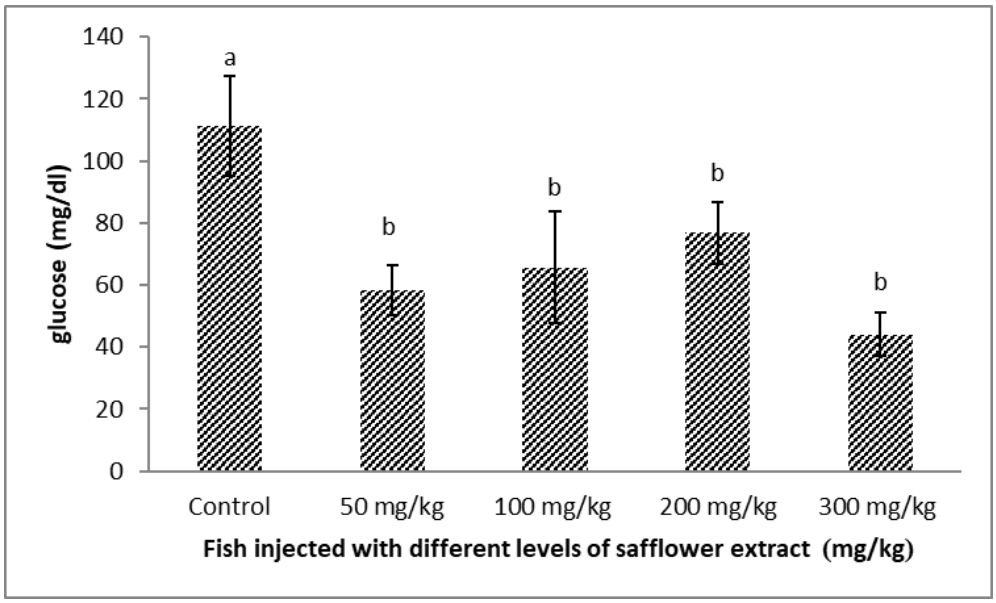

Figure 2. Glucose levels in the serum of Caspian roach injected intraperitoneally with different levels of safflower extract.

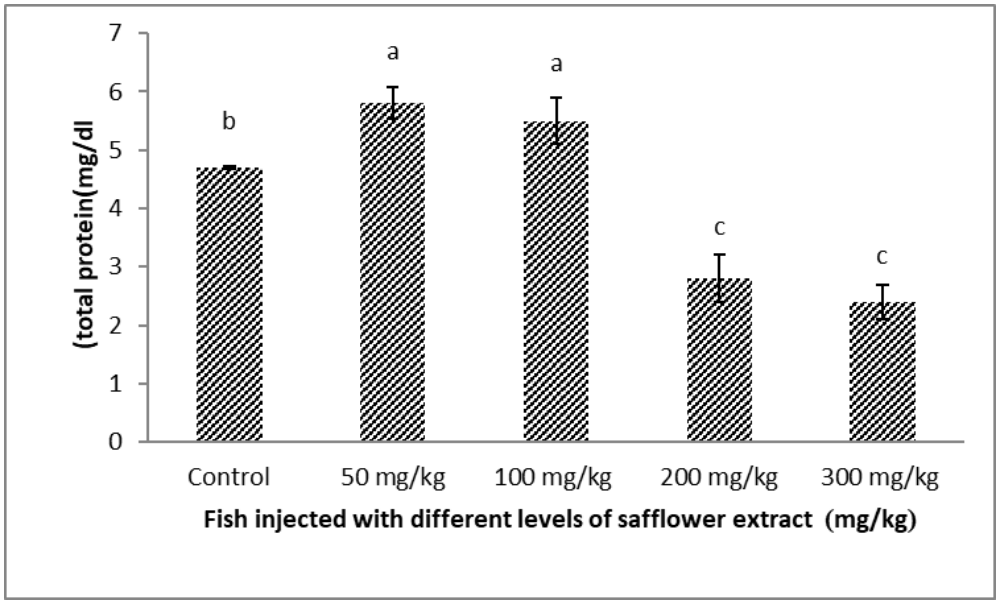

Figure 3. Total protein levels in the serum of Caspian roach injected intraperitoneally with different levels of safflower extract.

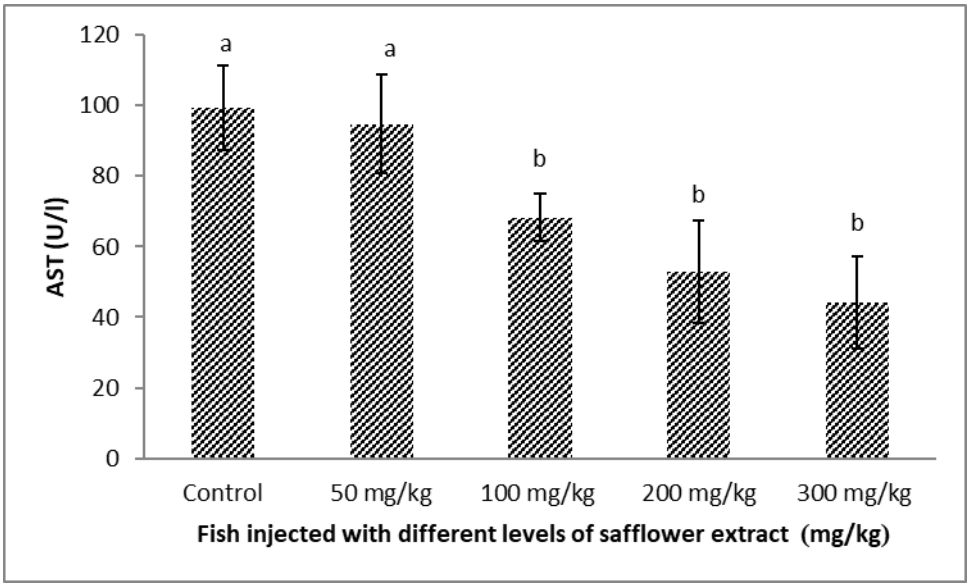

Figure 4. Aspartate aminotransferase (AST) levels in the serum of Caspian roach injected intraperitoneally with different levels of safflower extract.

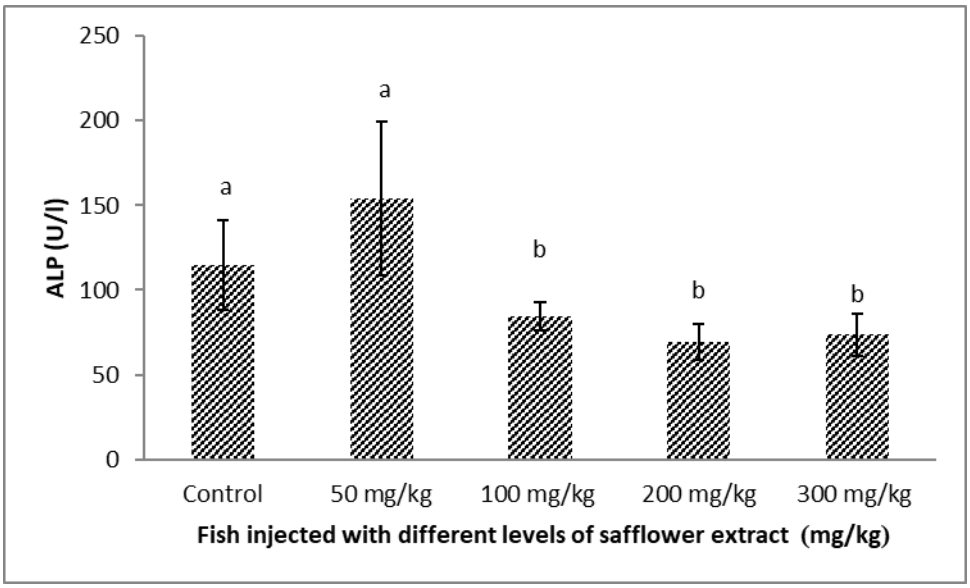

Figure 5. Alkaline phosphatase (ALP) levels in the serum of Caspian roach injected intraperitoneally with different levels of safflower extract.

number of white blood cells (WBC) in fishes injected with 100 to 300 mg/kg bw extract was significantly decreased compared to the control group and fish that received 50 mg/kg extract. Increasing the extract dose led to a significant decrease in the number of white blood cells of the Caspian roach (Table 1).

Discussions

In fish stock management and restocking programs, it is crucial to provide optimal breeding conditions and proper maintenance for the broodstocks to achieve their highest performance. In many cases, broodstocks caught from the sea are not ready to spawn. Keeping them in closed environments causes stress and weakens their immune system (Stoskopf, 1993). According to reports, dietary and herbal supplements can be beneficial in boosting the immune system (Awad and Awaad, 2017). The Caspian roach inhabits both freshwater conditions and the brackish waters of the Caspian Sea. This fish is not exposed to high ammonia levels in its natural habitats. However, the possibility of increased ammonia is a concern in closed-rearing environments with high density and less water exchange. In this study, injecting Caspian

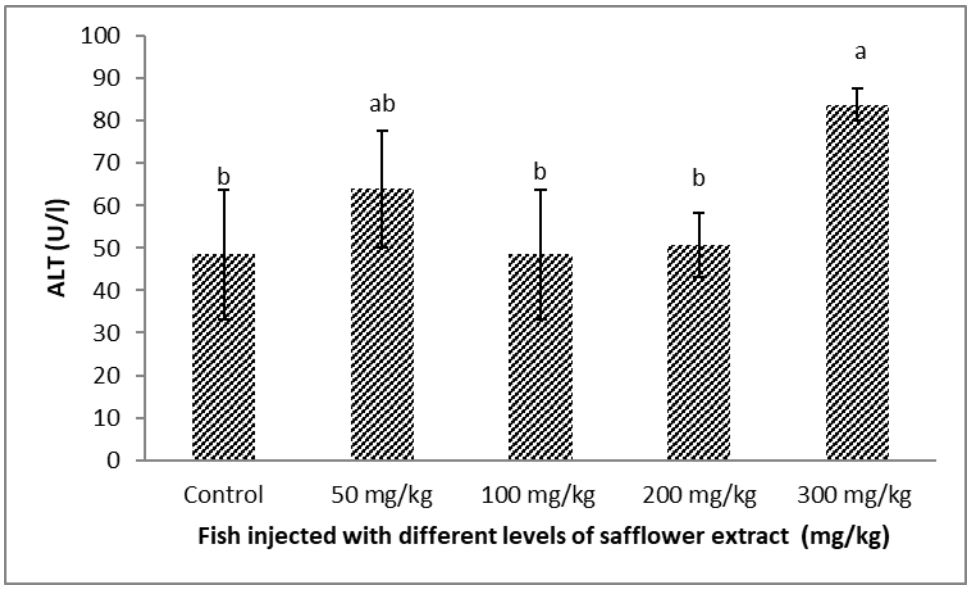

Figure 6. Alanine aminotransferase (ALT) levels in the serum of Caspian roach injected intraperitoneally with different levels of safflower extract.

roach broodstocks with 200 and 300 mg/kg of safflower extract improved their resistance to ammonia stress. The fish that received these injections did not experience any mortality during the 48-hour post-challenge period. The control group and the fish receiving 50 and 100 mg/kg of extract had mortality rates between 60 and 100% after exposure to 0.6 mg/liter of unionized ammonia for 48 hours.

In a study, Mazandarani et al. (2016) determined that the LC50 of unionized ammonia for young Caspian roaches was 1.68 mg/liter during 96 hours of exposure. The high mortality rates observed in broodstocks during the first 48 hours at a concentration of 0.6 mg/liter suggest that broodstocks may be more susceptible to stress than young-reared fish. However, it is important to note that these experiments were conducted at different times and under different conditions, so this comparison cannot be made with certainty.

Some studies have been conducted to determine the LC50 value of molecular ammonia for different fish species. However, the results vary depending on the species and size of the fish. For instance, Person-Le Royet et al. (1995) reported the LC50 for sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), and turbot (*Scophthalmus maximus*) to be 1.97, 2.73, and 3.32 mg/l, respectively, during 96 hours. Wajsbrot et al. (1991) calculated the LC50 value for rainbow trout larvae as 0.32 mg/l; Ruffier et al. (1981) reported the LC50 for catfish (*Ichtalarus punctatus*) as 1.3 mg/l, and Guan et al. (2010) found the LC50 value for common carp between 2.16 and 2.32 mg/l. These studies suggest smaller fish are more sensitive to molecular ammonia than larger ones.

In the current work, the levels of liver enzymes AST and ALP decreased in fish injected with safflower extract compared to the control group. However, in fish that received 300 mg/kg of safflower extract, the levels of ALT were significantly higher. These enzymes are typically used to evaluate liver damage. Usually, the levels of AST and ALP enzymes increase in liver disorders (Banaei et al., 2011). However, in cases of long-term intoxication, there have been reports of a decrease in ALP values, too. Therefore, these enzyme levels' decrease and increase have been recorded. For example, ALP values were reduced in the Caspian roach due to manganese poisoning (Hoeini et al., 2014) and in common carp due to copper poisoning (Hoseini et al., 2016).

Based on the enzyme test results, it can be concluded that injectable safflower extract does not harm liver cells. It might even improve the fish's ability to tolerate ammonia stress, especially in those fish that were given different concentrations of safflower extract during the study. However, this is just a hypothesis; further investigation is needed to

confirm it.

In the present study, the total protein levels increased in fish treated with safflower extract at concentrations of 50 and 100 mg/kg. However, the treatments injected with 200 and 300 mg/kg of the extract showed a decrease in total protein levels compared to the control group. Dadras et al. (2016) found that adding safflower extract to the diet of great sturgeon led to a decrease in serum ALP, AST, and ALT enzyme levels and an increase in lysozyme, total protein, and total immunoglobulin. Although there are few studies on reducing blood glucose in fish treated with safflower extract, this effect was proven in laboratory mice through numerous studies using the intraperitoneal injection method. (Asgari et al., 2012).

According to hematological results, intraperitoneal injection of safflower extract significantly reduced WBC counts in fish that received 100 mg/kg or more. Furthermore, there was a significant decrease in WBC counts when the concentration of the extract was increased to 200 and 300 mg/kg. In a study conducted by Mazandarani et al. (2019), it was found that feeding common carp with safflower extract did not affect their blood parameters and led to an increase in their survival rate when exposed to salinity stress (Mazandarani et al., 2019). However, in the present study, the treated fish with safflower extract had lower serum protein levels and a significant decrease in white blood cell counts, which could indicate a partial weakening of their immune system. This means that increasing stress tolerance in fish does not necessarily mean an increase in their immunity. For instance, corticosteroid drugs can reduce inflammation and stress but ultimately lead to a weakened immune system (Stoskopf, 1993). Therefore, before introducing safflower extract as a supplement for fish, further investigations should be conducted for each species of fish, considering their sex, age, and physicochemical conditions.

Conclusion

This study suggests that injecting safflower extract into the peritoneal cavity of the Caspian roach can reduce liver damage and improve its tolerance to high levels of unionized ammonia. To help the Caspian roach broodstock withstand ammonia stress, a dose of 200 mg/kg is recommended through intraperitoneal injection. However, it is worth noting that using this extract may weaken the fish's immune system.

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