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Research Paper

HAEMATOLOGICAL RESPONSES IN THE FRESHWATER FISH, *Anabas testudineus* (BLOCH, 1792) EXPOSED TO SUBLETHAL CONCENTRATION OF ACID ORANGE 7

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Abstract

Acid orange 7, one of the most popular synthetic azo dyes, widely used in textile, cosmetics, plastics and leather industries was exposed at sublethal concentration (0.27 g/L) to the freshwater fish, *Anabas testudineus* for 24, 72 and 96 h maintaining ten animals per group along with the control. Acid orange 7 exposure decreased the erythrocyte count in all treatment group in time-dependent manner, and the decrease in number of red cells in the blood is often associated with development of anemia, hypoxic condition or damage in the haematopoietic tissues. Acid orange 7 increased the number of leukocytes after 72 and 96 h exposure groups, which indicate the immune response of the fish against the exposed toxicant. The reduction in the concentration of haemoglobin and haematocrit after acid orange exposure could be due to blood cell destruction thereby leading to anemia in fish. Acid orange 7 decreased mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in the treatment groups thereby indicating abnormal haemoglobin synthesis, failure of blood osmoregulation and plasma osmolarity. The decrease in serum total protein concentration suggests the stress response of fish to the exposure of acid orange, and also reflects the increase in demand for energy. The present study showed an increase in the activities of alanine and aspartate aminotranferases after acid orange exposure demonstrating tissue damage in the fish. The present study concluded that acid orange 7, an azo dye caused sublethal toxicity in the freshwater fish, *Anabas testudineus* as evidenced by haematological changes. Therefore, it was suggested to restrict the use of synthetic dyes and release of untreated

effluents into the water bodies, if otherwise, may consequently affect the fish population.

Key words: *Acid orange 7, Haematology, Anabas testudineus, Sublethal toxicity, Alanine aminotranferases, Aspartate aminotranferases*.

INTRODUCTION

The textile industry consumes a substantial amount of water in its manufacturing processes used mainly in the dyeing and finishing operations of the plants. The wastewater from textile plants is considered as the most polluting of all the industrial sectors based on the volume generated as well as the composition of effluent (O'Neill et al., 1999). The increased demand for textile products proportionally raised the production rate, and the use of synthetic dyes together contributed to severe pollution problems in current times. Dyes are the major compound or pollutant discharged from textile industry, which evaporate into the air and are absorbed through skin showing allergic reactions that even harm children before the birth (Kant, 2012). It is estimated that more than 10,000 different dyes and pigments are used industrially and above 7 X 10^5 tons of synthetic dyes are annually produced worldwide (Robinson et al., 2001). In the textile industry, due to the inefficiency of dyeing process upto 2,00,000 tons of dyes are discharged into the environment during the dyeing and finishing operations in the form of effluents every year (Perkins, 1991). It has been reported that azo dyes are the largest group of colorants constituting 60-70% of all organic dyes produced in the world with respect to the number and production volumes (Bafana et al., 2011). Azo dyes have a wide range of applications in the textile, pharmaceutical and cosmetic industries, and are also used in food, paper, leather and paints.

Acid orange 7, an azo dye, is widely used in textile industry and to some extent in cosmetics, plastics, leather, ink and paints. Acid orange 7 is a non-reactive dye used as a coloring agent in all cosmetic products and also used in oxidative hair dye formulation (Roy and Saha, 1981). In textile industry, acid orange is primarily used as a dye for silk and wool, polyamide fibre fabric of direct printing, and also as indicator and biological shading on leather, and paper. Polluted water bodies as a result of dye exposure from various sources are considered as the source of acute or chronic toxicity for aquatic organisms. As a result, textile azo dyes have certainly gained attention in the field of toxicology, environmental monitoring and assessment. In toxicological research, biomarkers are widely used as early diagnostic tools to detect the adverse effects caused by chemical pollutants (Parkin and Shelton, 1994). Several studies make use of haematology as a biomarker of pollutant exposure in order to monitor the interaction between a toxicant and biological system (DaCuna et al., 2011).

A major part of the world's food is being supplied from fish source, so it is essential to secure the health of fishes. Fish live in very intimate contact with their environment and are therefore very susceptible to physical and chemical changes which may be reflected in their blood components (Saravanan et al*.,* 2010). Blood is a liquid connective tissue which truly reflects physical and chemical changes occurring in organism. Hence, detailed information can be obtained on general metabolism and physiological status of fish in different groups of age and habitat (Parma et al*.,* 2007; Ololade and Oginni, 2010). Blood provides an ideal medium for toxicity studies where blood and tissue of living organisms are very sensitive to changes and are extensively used in Ichthyology research.

Hematological indices are valuable tool for the evaluation of physiological changes taking place in fish. Blood cell responses are important indicators of changes in

the internal and external environment of animals. In fish, exposure to chemical pollutants can either increase or decrease their haematological levels. Thus blood offers important profile to study the toxicological impact on animal tissues. Different blood parameters are often subjected to change depending upon stress condition and various other environmental factors (Golovina, 1996). The changes in blood profile depend on fish species, age, cycle of the sexual maturity of spawners or due to the disease condition (Luskova, 1997). Generally, a decrease in nonspecific immunity of the fish due to exposure of pollutants has been shown to alter the haematological parameters (Svoboda et al., 2001). Besides monitoring health status and stress responses, haematological parameters have been used to predict the systematic relationship and physiological adaptations of animals. Thus hematological indices are the toxicologic endpoints that quickly reflect the poorer condition of fish than other commonly measured parameters.

The main objective of the present study was to evaluate the sublethal toxic effect of one of the azo dyes, acid orange 7, on haematological response in the fish, *Anabas testudineus*. Use of haematological parameters as indicators of stress can provide valuable information concerning the physiological status of fish in a changing environment, and it provides an ideal tool for the present ecotoxicological study.

MATERIALS AND METHODS

Collection and maintenance of animal

Freshwater fish, *Anabas testudineus* weighing 22 ± 2.2 g and length 11 ± 1.6 cm were collected from the fish farm, Sea world aquarium, Kozhikode District, Kerala. Fishes were acclimatized to the laboratory conditions for four weeks with constant supply of water and good lighting system. They were maintained in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water maintaining light and dark at 12: 12 h. The study was conducted in accordance with the animal ethical guidelines of the country.

Preliminary tests

The physico-chemical features of the tap water were estimated as per American Public Health Association guidelines (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures.

Selection of test concentration

The median lethal concentration or LC_{50} value of acid orange 7 determined by Probit analysis, with a confident limit of 5 % level (Finney, 1971) for 96 h was 2.7 g/ L. In the experiment, one-tenth of median lethal concentration of acid orange 7 i.e., 0.27 g/ L was selected as sublethal concentration.

Chemicals

Acid orange 7 (CAS-633-96-5) of 97% purity was purchased from Tokyo Chemicals Industry Private Limited, India. Drabkin reagent, Grumwald-Giemsa stain, bromcresol green, 2-oxoglutarate, DL-α-alanine and 2,4-dinitrophenyl hydrazine were obtained from Himedia Research Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Experimental design

The selected test concentration i.e., 0.27 g/ L was maintained for four durations i.e., 24, 72 and 96 h, respectively along with control group. Single dose with different durations were used in present study and ten fish specimens were used for every test and also in control groups. The first group of fishes was maintained in toxicant-free water and was used as control and the remaining group was treatment groups exposed to acid orange 7 at 0.27 g/ L concentration for 24, 72 and 96 h, respectively.

Killing of animals and collection of blood samples

The fish was caught very gently using a small dip net, one at a time with least disturbance. At the end of each exposure time, fishes were weighed and decapitated. After each exposure period, blood from the control and treatment groups were collected by cardiac puncture using 2 ml glass syringe and transferred into a vial containing 1% ethylenediaminetetraacetic acid as anticoagulant. The whole blood was then immediately used for hematological studies. Remaining blood samples were centrifuged at 1000-2000*g* for 10 min at 4°C to obtain serum for biochemical estimation.

Haematological parameters

Erythrocyte count

Erythrocytes were counted using improved Neubauer counting chamber as described by Rusia and Sood (1992). The collected blood was drawn up to 0.5 marks in red blood cell (RBC) pipette and Hayem's diluting fluid is drawn up to the mark 101. It makes the dilution as 1: 200 and the solution was mixed thoroughly by gently rotating the pipette several times in the form of '8'. It was allowed to stand for 2 or 3 min. Meanwhile the counting chamber and cover glass was cleaned and the cover glass was placed over the ruled area. The solution was again mixed gently and the stemful of solution was expelled and a deep of fluid was allowed to flow under the coverslip holding the pipette at 40º angles. It was then allowed to stand for 2 or 3 min for RBC's to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of RBC's was counted in five small squares of the RBC column under high power. The number of RBC's per cubic mm was calculated as: Number of cells counted X Dilution factor/ Depth factor X Volume of sample. Finally the total erythrocyte count was expressed as number of red blood cells/mm^3 of blood.

Leukocyte count

Leukocytes were counted by the method of Russia and Sood (1992) using Neubauer type haemocytometer. The collected blood was diluted to 1:20 with Turck's diluting fluid, mixed well and allowed to stand at room temperature for 2 min. The cells were counted under the microscope by using a counting chamber from all 4 "W" marked corner squares. The number of leukocytes per cubic millimeter was calculated as:

Number of cells x Dilution factor x Depth factor / Area counted

Haemoglobin content

Haemoglobin content was estimated by cyanmethaemoglobin method (Dacie and Lewis, 1984). Briefly, 20 µl of blood was diluted by adding 5 ml of Drabkin's solution, and mixed well. The solution was allowed to stand at room temperature for 5 min and the absorbance was measured at 540 nm using Ultraviolet-Visible Spectrophotometer.

Erythrocyte indices

Erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using the values of haematocrit (ct), haemoglobin (Hb) and total erythrocytes as per the standard formulas described by Dacie and Lewis (1984).

MCV (pg) = Haematocrit (%)/ RBC count in millions/ mm^3 × 10.

MCH (pg) = Haemoglobin (g/ dl)/ RBC count in millions/ mm^3 × 10.

MCHC (g/ dL) = Hb (g/ dL)/ Hct (%) × 100.

Haematocrit (packed cell volume) was also calculated using standard formula:

Haematocrit (%) = RBC count (in millions/mm3)/ total blood sample volume × 100

Biochemical parameters

After collecting the blood it was kept undisturbed for clotting in room temperature for 30 min to 1 h. Then it was centrifuged at 1,000–2,000 *g* for 10 min in a cold centrifuge so as to obtain the blood serum, which was then used for biochemical studies.

Estimation of serum total protein

Total protein concentration in the tissue was estimated by the method of Lowry et al. (1951). An aliquant of the test sample was mixed with alkaline copper reagent and vortexed, they were allowed to stand for 10 minutes at room temperature. Folin-Ciocalteau reagent (1 N) was added to each of the tubes, vortexed and allowed to stand for 20 minutes at room temperature. The optical density was read at 650 nm on a Ultraviolet-Visible Spectrophotometer. A standard calibration was prepared using different concentrations of bovine serum albumin.

Serum albumin

Serum albumin was estimated according to the method as described by Bishop et al. (2000). Briefly, 5 µl of sample was diluted to 200 µl of bromcresol green reagent, and after the incubation for 5 min at room temperature, the absorbance was measured at 670 nm.

Serum globulin

The concentration of serum globulin was calculated from the known values of serum total protein and serum albumin.

Serum globulin = Serum total protein – serum albumin.

Aspartate aminotransferase

The activity of aspartate aminotransferase was assayed by the method as described by Reitman and Frankel (1957). The reaction mixture containing aspartate (0.1 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37ºC for 1 h. After incubation, 250 µl of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, to stop the reaction sodium hydroxide (0.1 N) was added, mixed and incubated at room temperature for 10 min. The absorbance was read at 510 nm against the blank. A standard calibration was prepared by using different concentrations of sodium pyruvate. The results were expressed as µM pyruvate formed per mg protein.

Alanine aminotransferase

The activity of alanine aminotransferase was assayed by the method as described by Reitman and Frankel (1957). The reaction mixture containing DL-α-alanine (0.2 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37ºC for 1 h. After incubation, 250 µl of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, to stop the reaction sodium hydroxide (0.4 N) was added, mixed and incubated at room temperature for 10 min. The absorbance was read at 510 nm against the blank. A standard calibration was prepared by using different concentrations of sodium pyruvate. The results were expressed as µM pyruvate formed per mg protein.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against the control group. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

RESULTS AND DISCUSSION

Effect of acid orange 7 on haematological parameters in the fish, *Anabas testudineus*

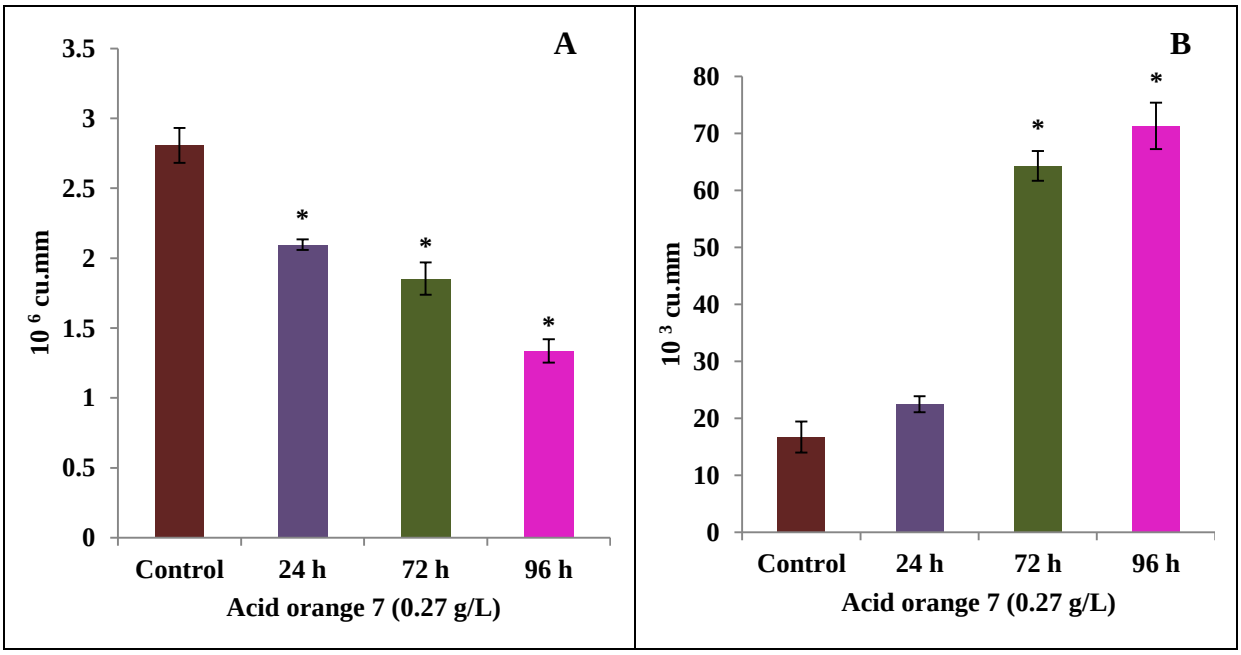
Several investigators have established normal ranges for various blood parameters in fish to detect fish physiology and pathology, and the analysis of blood indices provide reliable information on metabolic disorders, deficiencies and chronic stress status of the toxicant-exposed animal. Thus the adverse effects of toxicants on the blood constituents of an organism can be assessed by haematological parameters. Blood consists of a liquid part termed plasma, which leads to the formation and function of three different cells namely erythrocytes, leukocytes and thrombocytes. Erythrocytes are red blood corpuscles (RBC) that are in greater amounts in the blood stream, and fish erythrocytes are nucleated where they carry oxygen and carbonic gas. Leukocytes are white blood corpuscles (WBC), which are the defensive cells used widely to assess the immune system whereas thrombocytes are related to blood clotting with the organic defense and have phagocytic function (Hrubec et al., 2000). Exposure to acid orange 7 at sublethal concentration (0.27 g/L) showed significant ($P<0.05$) and time-dependent decrease in the erythrocyte count when compared to the control group (Fig. 1a). The decrease in number of red cells in the blood is often associated with development of anemia, hypoxic condition or damage in the haematopoietic tissues (Junqueira et al., 2006). This could be also due to the stimulation of lipid peroxidative system by acid orange 7 resulting in the production of lipid peroxides which hemolyses the RBCs as evident by the study conducted in our laboratory. The major pathological consequences of free radical induced membrane lipid peroxidation include increased membrane rigidity, attenuation or inhibition of anti-inflammatory cytokine production like the adiponectin, increased cellular deformation, reduced erythrocyte survival through increased necrosis, and increased lipid fluidity which starts as a chain of inflammatory reactions causing endothelial dysfunction (Arika et al., 2016).

Sublethal exposure of acid orange 7 increased the number of leukocytes, where significant ($P<0.05$) rise were noticed after 72 and 96 h treatment groups (Fig. 1b). White blood cells count play a vital role in immune function by protecting the body

against antigen invasion and are formed from pluripotent hematopoietic stem cells found in the red bone marrow. Sublethal exposure of acid orange 7 increased the number of leukocytes after 72 and 96 h group which indicate the immune response of the fish against the exposed toxicant.

Haemoglobin is a conjugated protein containing haeme as prosthetic group and globin as the protein part apoprotein (Zhiteneva et al., 1989). Haemoglobin is the primary intracellular protein of the RBCs that transport oxygen and carbon dioxide to tissues during respiration. Acid orange 7 when exposed to fish at sublethal concentration significantly ($P<0.05$) decreased the concentration of haemoglobin in all treatment groups in time-dependent manner when compared to the corresponding control group (Fig. 1c). The reduction in the concentration of haemoglobin after acid orange exposure could be due to blood cell destruction thereby leading to anemia in fish.

Haematocrit represents the percentage of red blood cell volume of whole blood volume, which is also referred as packed cell volume (PCV) used extensively to access anemia. Thus any factors that influence RBCs have been shown to affect the haematocrit because RBCs comprise 99% of the total cells of the blood (Wintrobe and Greer, 2009). Fish exposed to acid orange 7 showed significant ($P<0.05$) decrease in the packed cell volume in the blood of the fish in a time-dependent manner when compared with the corresponding group of control animal (Fig. 1d). The decrease in packed cell volume after acid orange 7 exposure represents anemic condition in the exposed fish.



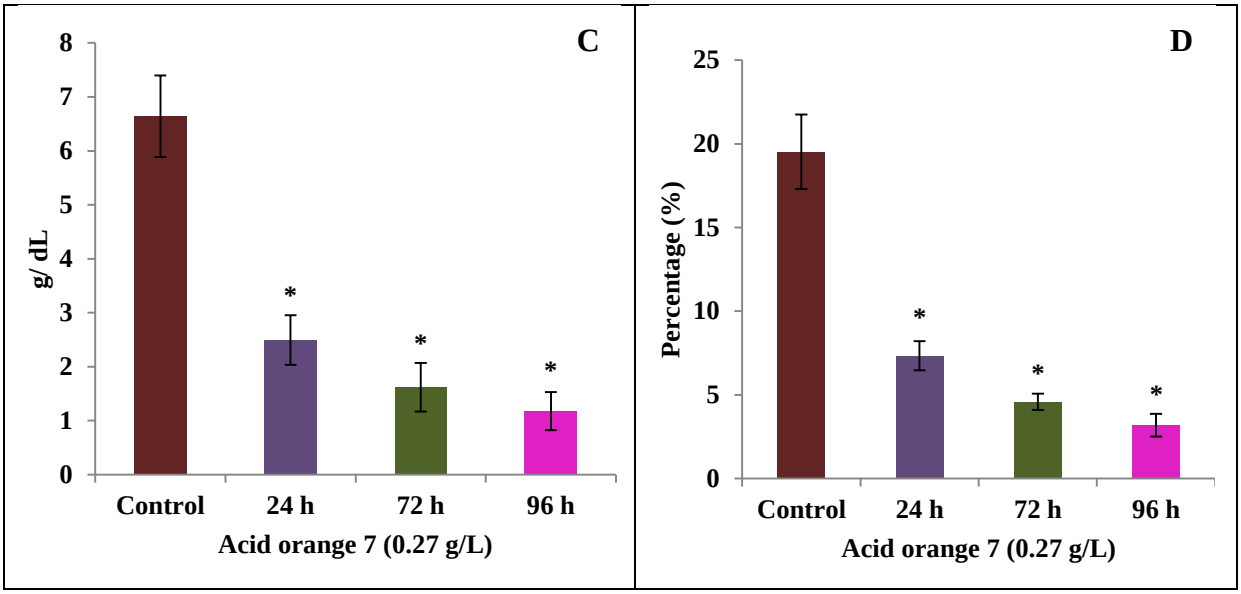


Fig. 1 Effects of acid orange 7 on (a) erythrocyte count, (b) leukocyte count, (c) haemoglobin concentration and (d) packed cell volume in the blood of the fish, *Anabas testudineus*. Data are presented as Mean ± standard deviation for 10 animals per group. Asterisks (*) denotes significant at P<0.05 against the control group.

Mean corpuscular volume (MCV) is a measure of the average volume or size of a red blood cell. Accordingly, low MCV indicates microcytic, the size is smaller than the average size, normal MCV indicates normocytic with normal average size of RBC and high MCV indicates macrocytic representing larger than average size of RBC (Aslinia et al., 2006). The mean corpuscular volume in the blood of the acid orange 7 exposed fish showed significant (P<0.05) decrease in a time-dependent manner when compared with the corresponding control group (Fig. 2a). Low MCV in the blood of the acid orange 7 exposed fish was consistent with microcytic anemia.

Mean corpuscular haemoglobin (MCH) is a calculation of the average amount of haemoglobin inside a single red blood cell. Acid orange 7 exposure significantly (P<0.05) decreased the mean corpuscular haemoglobin in all treatment groups (Fig. 2b) thereby indicating abnormal haemoglobin synthesis, failure of blood osmoregulation and plasma osmolarity (Stookey et al., 2007).

Mean corpuscular haemoglobin concentration (MCHC) is a calculation of the average concentration of haemoglobin inside a single red blood cell. Exposure to acid orange 7 at sublethal concentration decreased the mean corpuscular haemoglobin concentration after 72 and 96 h of treatment than that of the control group (Fig. 2c). The results suggest that exposure to sublethal concentration of acid orange 7 drastically affected the total population of red cells in *Anabas testudineus* in a time-dependent manner. MCV reflects the size of red blood cells while MCH and MCHC are used mathematically to define the concentration of hemoglobin and to suggest the restoration of oxygen carrying capacity of the blood (Groff and Zinkl, 1999). The reduction in the levels of MCH and MCHC in acid orange-exposed groups indicates the diminished oxygenation, and insufficient production of red cells with insufficient haemoglobin.

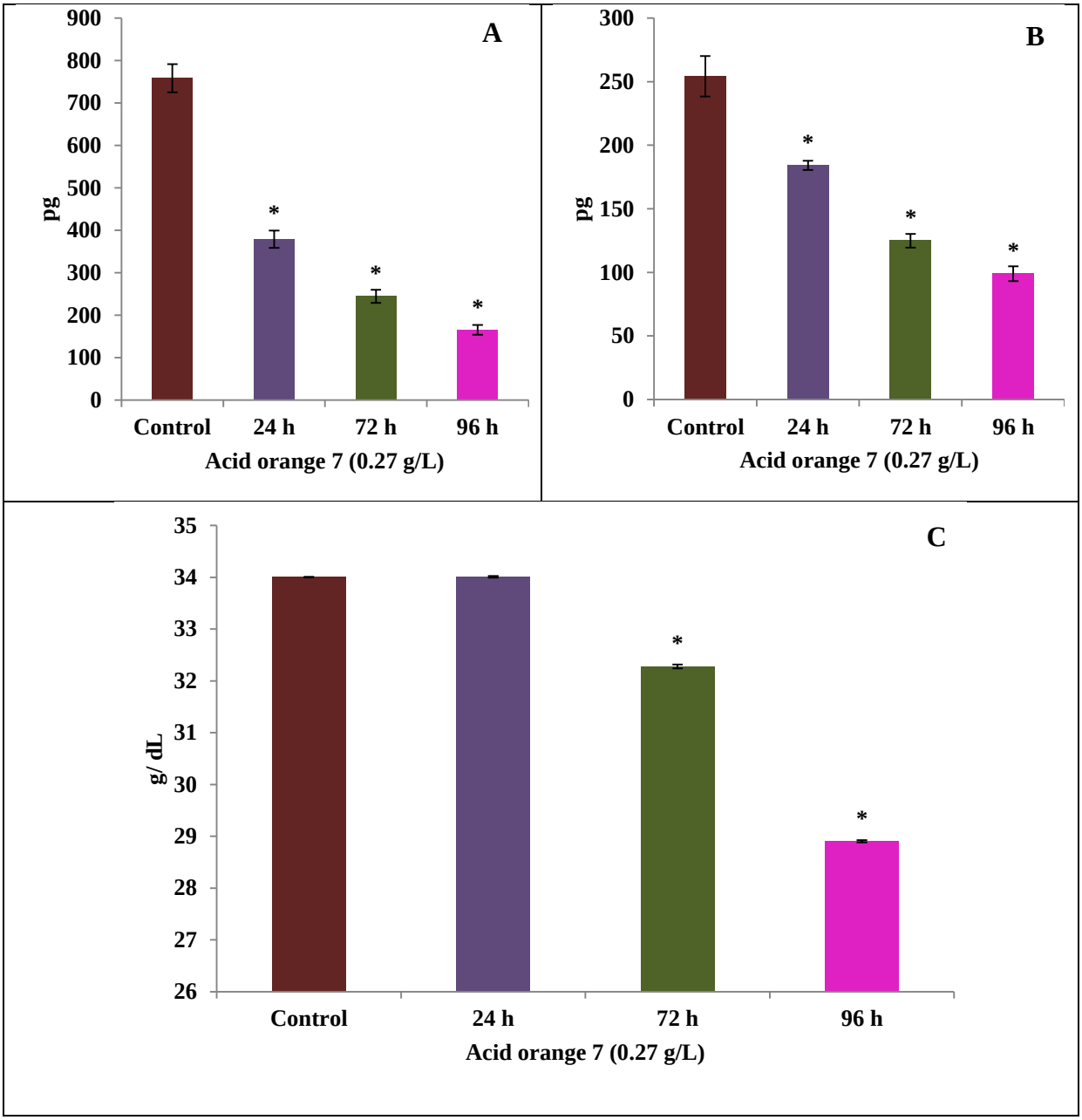


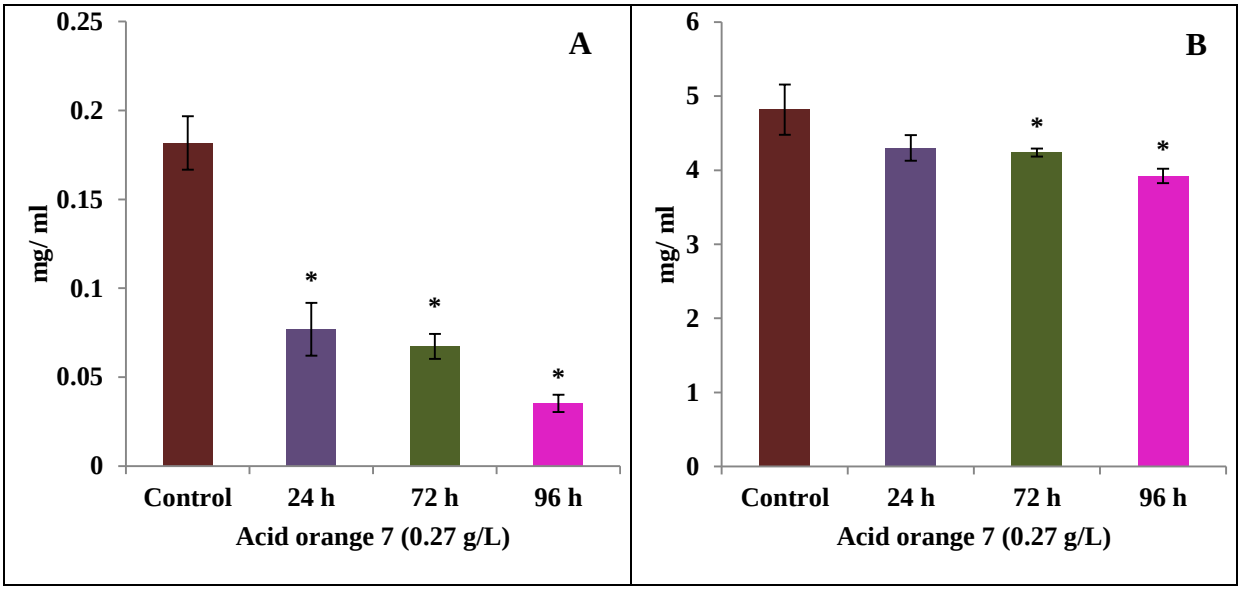
Fig. 2 Effects of acid orange 7 on the (a) mean corpuscular volume (MCV), (b) mean corpuscular haemoglobin (MCH), (c) mean corpuscular haemoglobin concentration (MCHC) in the blood of the fish, *Anabas testudineus*. Data are presented as Mean ± standard deviation for 10 animals per group. Asterisks (*) denotes significant at P<0.05 against the control group.

Effect of acid orange 7 on the biochemical parameters in the serum of the fish, *Anabas testudineus*:

The vital function of blood circulation in carrying materials from one part of the fish to another can be detected by the level of plasma proteins, which include globulins, fibrinogens and albumins. Plasma proteins provide transporting, buffering, nutritive, protective, and energetic functions in the fish reflect the health status (Kaur and Saxena, 2018). Blood serum total protein is a fairly labile biochemical system, precisely reflecting the condition of the organism, and the alteration in the total serum protein may be influenced by several internal or external factors. Albumin and globulin, the

major constituent of the total protein, was widely used to examine disorders of the immune system, renal and hepatic dysfunctions (Giron-Pizer et al., 2007). In the present observation, the levels of serum albumin, globulin and total protein decreased significantly ($p<0.05$) in a time-dependent manner after orange 7 treatment when compared with the corresponding control group (Figs. 3a, b and c). This could be due to the increase in demand for energy as a result of impaired liver function or kidney damage (Gray and Doolittle, 1992). The results was in agreement with another study when acid orange 7 exposed to the fingerlings of *Labeo rohita* reported similar alterations in the haematological and biochemical parameters (Barot and Bahadur, 2015).

Measurement of enzyme activity in blood serum is considered as a sensitive indicator of cellular damage. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are group of enzymes responsible for the conversion of amino acids and α-ketoacids to release necessary energy required for the synthesis of new protein (Sreedevi et al., 1992). Alanine and aspartate aminotranferases not only function as link enzymes between the protein and carbohydrate metabolism, but also serve as indicators of altered physiological or stress condition (Mauro et al., 2006). During tissue necrosis, transaminases pass into the circulation from the damaged organs so that the activities of the enzymes increase in the blood (Borges and Wassermann, 2007). Therefore, the quantification of enzyme activity can serve as a valuable biomarker of pollutant exposure. The present study showed significant ($P<0.05$) increase in the activities of alanine and aspartate aminotransferases in the serum after acid orange 7 exposure (Figs. 4a and 4b) thereby demonstrating tissue damage in the fish. Similar observations have been observed when cypermethrin was exposed to the fish, *Cyprinus carpio* (Al-Ghanim, 2014).



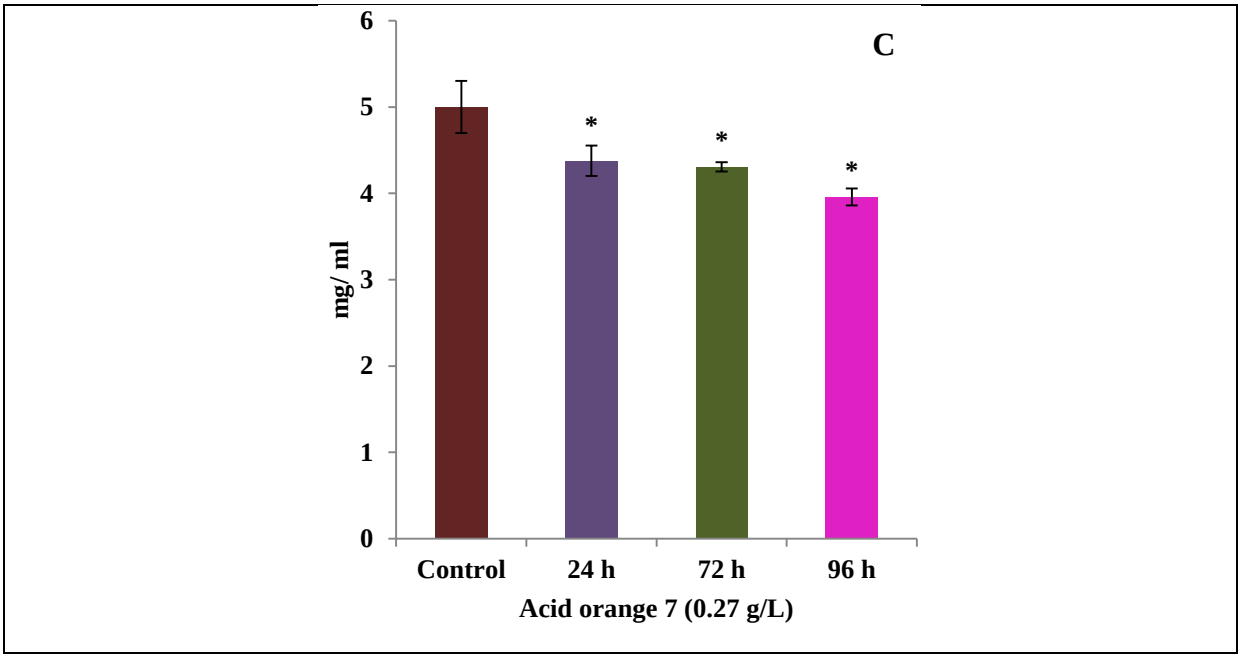


Fig. 3 Effects of acid orange 7 on the (a) level of serum albumin, (b) level of serum globulin, and (c) serum total protein in the fish, *Anabas testudineus*. Data are presented as Mean ± standard deviation for 10 animals per group. Asterisks (*) denotes significant at P<0.05 against the control group.

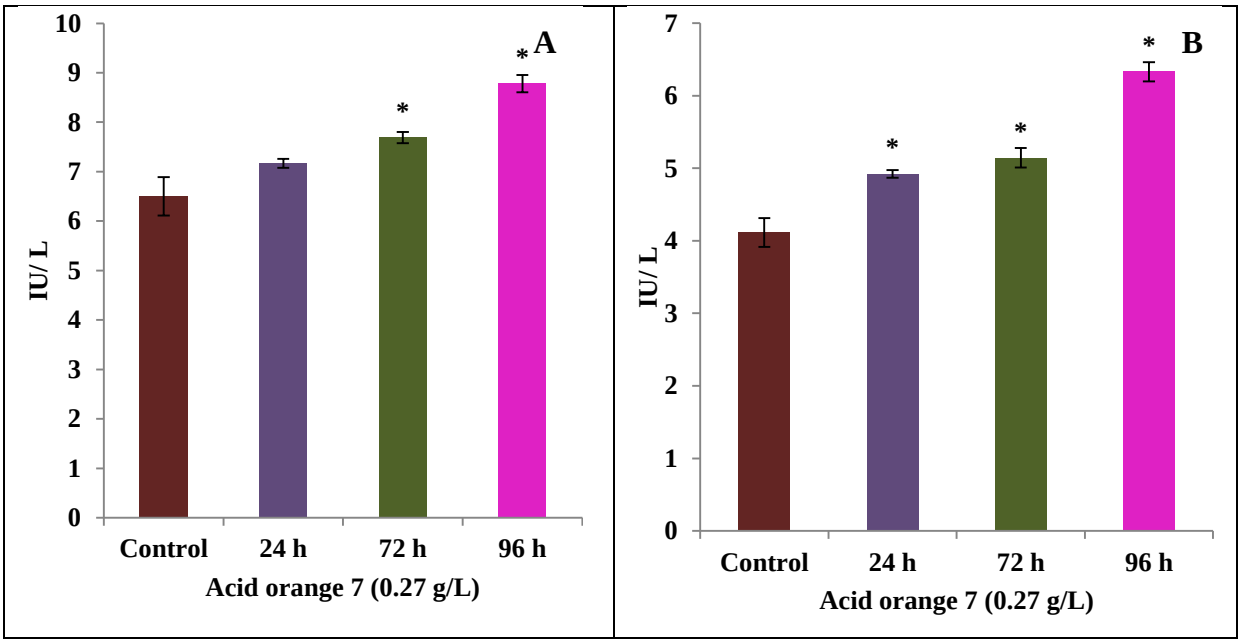


Fig. 4 Effects of acid orange 7 on the activity of (a) alanine aminotransferase, and (b) aspartate aminotransferase in blood serum of the fish, *Anabas testudineus*. Data are presented as Mean ± standard deviation for 10 animals per group. Asterisks (*) denotes significant at P<0.05 against the control group.

CONCLUSIONS

Haematological parameters are the most sensitive indices in monitoring the toxicity of any chemicals exposed at sublethal concentrations. The present study concluded that acid orange 7, an azo dye caused sublethal toxicity in the freshwater fish,

Anabas testudineus as evidenced by haematological changes. High degree of such alterations could lead to severe anemia and finally results in the death of fish. Therefore, it was suggested to restrict the use of synthetic dyes and release of untreated effluents into the water bodies, if otherwise, may consequently affect the fish population.

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