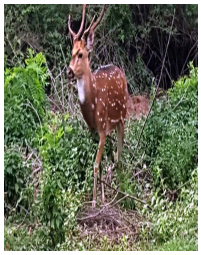

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Ethoxyquin on Haematological and Biochemical Parameters in the Fish, *Anabas testudineus* (Bloch, 1792)

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Abstract: Ethoxyquin, a quinoline fungicide, is widely used as an antioxidant in the animal feed industry, and also used as feed constituents, notably in fish meal. Determination of median lethal concentration of ethoxyquin by probit analysis showed LC_{50} value as 16.5 mg/L. Fish were exposed to one-tenth and one-fifth of LC_{50}-96 h (1.65 and 3.3 mg/L concentration, respectively) for 96 h. Ethoxyquin exposure did not alter the body weights of the animal, however, the percentage of mucous deposition showed a significant ($P<0.05$) increase at both sublethal concentrations. Fish exposed to ethoxyquin showed significant reduction in serum protein and increase in serum glucose. This could be due to lysis of RBC or catabolism of protein for the immediate release of energy in demand of toxic stress. Increase in the activities of aspartate and alanine aminotransferase may be due the toxic stress induced by ethoxyquin which altered the normal metabolism of animal. Alterations in haematological parameters may be due to haemolysing capacity of ethoxyquin and rapid oxidation of haemoglobin to methaemoglobin due to toxicant exposure. Thus, ethoxyquin at acute exposure of sublethal concentrations induced haematological and biochemical alterations in serum of fish, *Anabas testudineus*.

Keywords: Ethoxyquin, *Anabas testudineus*, haematology, alanine aminotransferase, aspartate aminotransferase

Introduction

Ethoxyquin is a widely used antioxidant in the animal feed industry, and is included in preparations of vitamins and carotenoids, in premixes and compound feed, and in feed constituents, notably fish meal (Taimr, 1994). The use of ethoxyquin in fish meal stabilises long chain polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Therefore, ethoxyquin is known to promote health in humans and animals and seems important in relation to both the quality and safety (Thorisson *et al*., 1992). International Fishmeal and Fish oil Organization (IFFO) estimates that approximately 66% of world production of fish meal is stabilized with ethoxyquin, and it has been used since 1970s. The International Maritime

Organization (IMO) regulates shipping safety and requires that fish meal should be dosed with ethoxyquin between 400 mg/ kg and 1000 mg/ kg within 12 months of shipping, ensuring that at least a level of 100 mg/ kg is present at the time of loading before shipping to mitigate the risk of combustion during transport at sea.

Apart from an antioxidant compound widely added to animal food, ethoxyquin is also used as a color preservative in paprika, chilli powder, and ground chillies as well as an anti-degradation agent used in rubber production. On the other hand, it is expected that the European Union authorities may suspend the approval for use of ethoxyquin with an immediate effect. The meeting of Standing Committee on Plants, Animals, Food and Feed (SCoPAFF) held on 27-28 April, 2016 decided and recommended to consider the alternatives for ethoxyquin due to its potential adverse impacts on fish and other animals. It was also decided to follow-up actions to suspend the use of ethoxyquin in fish meal products.

As a consequence of the use of ethoxyquin-containing feed additives, ethoxyquin is unavoidably released into the environment. Ethoxyquin as fish meal in aquaculture is more likely released directly to the broader aquatic environment around an aquaculture facility as waste feed or can be taken up by fish and then excreted again into the environment.

Fish as an aquatic vertebrate, is in direct contact with the aquatic environment and anything added to its environment may cause some impact on the hematological characteristics (Gabriel *et al*., 2007). Hence fish hematological profile was extensively used in clinical diagnosis of fish physiology in order to determine the effects of external stressors and disease conditions in fish (Fernandes and Mazon, 2003). Many investigators studied the effects of toxicants on the hematology of different fish species and reported various degrees of hematological changes and suggested that reduction in hemoglobin, RBC and PCV are related to oxygen carrying capacity of the blood (Adhikari *et al*., 2004). Biochemical and haematological parameters are the reliable biomarkers widely used to detect the adverse effects of toxicants in an organism and are extensively applied to assess environmental risk of any pollutants (Van der Oost *et al*., 2003). As a result, the present study was designed to focus on the effects of ethoxyquin on haematology and metabolic enzymes in the fish, *Anabas testudineus*.

Materials and methods

Animal:

Fresh water fish, *Anabas testudineus* (body wt 22±1.5 g; length 15±2 cm) were collected from a fish farm, Aqua fish, B.H. Road, Kottakkal, Malappuram District, Kerala, India. Prior to experiments, fishes were acclimatized to the laboratory conditions in well-aerated cemented tank (40 L capacity), provided with dechlorinated water. Preliminary test were conducted by maintaining water temperature as 28 ± 2°C, oxygen saturation of water (70 and 100 %), and pH 6.5 to 7.5 using standardized procedures as per APHA guidelines (1998).

Chemicals:

Ethoxyquin (1,2-dihydro-2,2,4-trimethyl-quinolin-6-yl ethyl ether) of 75% purity was obtained from HiMedia Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Evaluation of median lethal concentration (LC_{50}-96 h):

For determining LC_{50} value, separate circular plastic tubs of 40 L water capacity were taken and different concentrations of ethoxyquin (12-19 mg/ L) were added and 10 fishes were introduced into each tub. A control tub with 40 L of water and ten fishes were also maintained without toxicant. The lethal concentration for 50 % killing (LC_{50}) values was computed on the basis of probit analysis (Finney, 1971) for 96 h (LC_{50}-96 h).

Treatments:

Ethoxyquin was dissolved in an organic solvent, 1% Dimethyl sulfoxide (1% DMSO), and therefore it was used as a positive control in the experiment. There were two groups: one-fifth (3.3 mg/ L) and one-tenth (1.65 mg/ L) of LC_{50}-96h concentrations. Each group consists of positive (DMSO) and negative (solvent-free) controls, and the toxicant-exposed groups at different concentrations maintained for 24, 72 and 96 h, respectively.

The experiment was designed as follow:

(i) Control Groups:

Group 1 – Solvent-free

Group 2 – With solvent (1% DMSO)

Treatment Groups:

(ii) One-fifth of LC_{50}-96 h of ethoxyquin (3.3 mg/ L)

Group 3 – maintained for 24 h

Group 4 – maintained for 72 h

Group 5 – maintained for 96 h

(iii) One-tenth of LC_{50}-96 h of ethoxyquin (1.65 mg/ L)

Group 6 – maintained for 24 h

Group 7 – maintained for 72 h

Group 8 – maintained for 96 h

Collection of blood and analysis:

Each fish was held and wrapped with a clean, dry filter paper and the posterior half of its body was blotted with another clean coarse filter paper. Caudal peduncle of the fish (control and experimental groups) was severed with the single stroke from a sharp blade. After discarding the first drop of blood, the freely oozing blood was collected and centrifuged at 5000 rpm for 10 minutes to separate the blood serum, which was used for biochemical analysis. Total protein concentration in the blood serum was estimated by the method of Lowry *et al*. (1951). Quantitative estimation of glucose in blood serum was done by the method as described by Trinder (1969). The activities of alanine and aspartate aminotransferase were assayed by the method as described by Reitman and Frankel (1957).

Small quantity of blood was added to anticoagulant (1% ethylenediamine tetraacetic acid - EDTA) for haematological parameters. The blood was thoroughly mixed with the anticoagulant using a thin, blunt glass rod, during the process of collection itself. The whole blood was used for the estimation of erythrocyte, leucocyte counts (Rusia and Sood, 1992) and haemoglobin (Drabkin, 1946) in both control and experimental groups.

Statistical analysis:

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $p<0.05$ against control group (denoted as asterisks in Figures). Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

Results

Ethoxyquin exposure did not alter the body weight of the animal at both sublethal concentrations for 96 h (Fig. 1). However, the percentage of mucous deposition showed a significant ($P<0.05$) increase at both one-fifth and one-tenth of LC_{50} concentrations in time-dependent manner (Fig. 2). Mortality of the animal

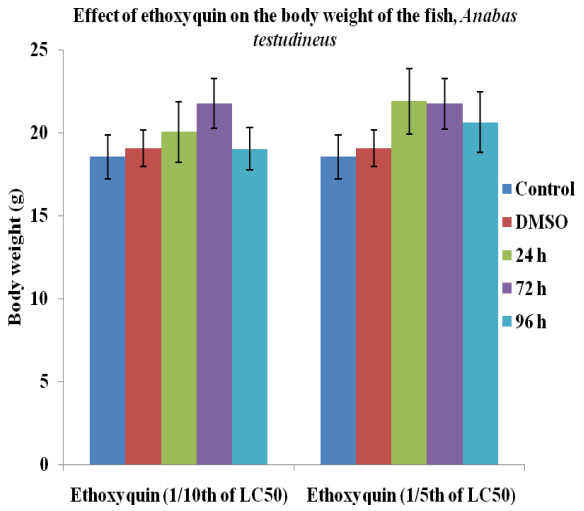

Figure 1

was continuously monitored throughout the experiment. Different concentrations of ethoxyquin showed different percentage of mortality at different time interval as shown in Table 1. Dead fishes were immediately removed and their numbers were recorded. It was observed that at the

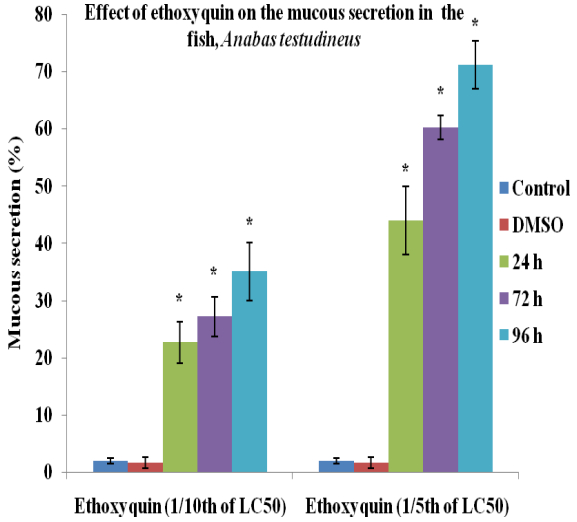

Figure 2

concentrations 12, 13 and 14 mg/ L of ethoxyquin no mortality was seen after 96 h. At 15 mg/ L of ethoxyquin 10% mortality was observed. At 16 and 17 mg/ L concentrations of ethoxyquin 40% of mortality has been recorded at 96 and 72 h, respectively. However, at 18 and 19 mg/ L of ethoxyquin all fishes were dead (100% mortality) at 72 and 48 h, respectively (Table 1). Computation of

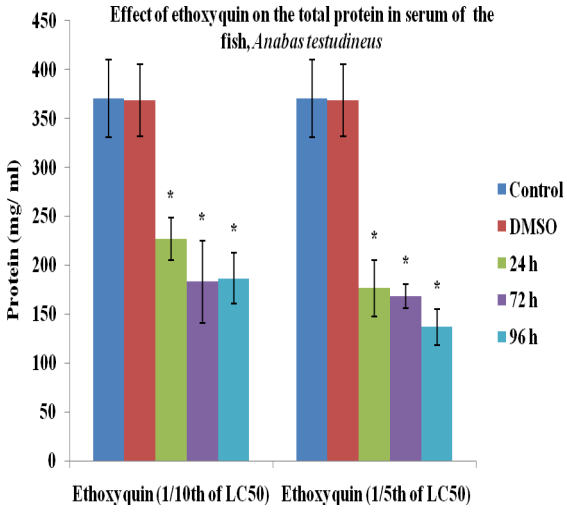

Figure 3

median lethal concentration by probit analysis showed LC_{50} value as 16.5 mg/ L (Table 2). Correlation analysis of mortality against concentrations of ethoxyquin

showed highly positive correlation (r = +0.921).

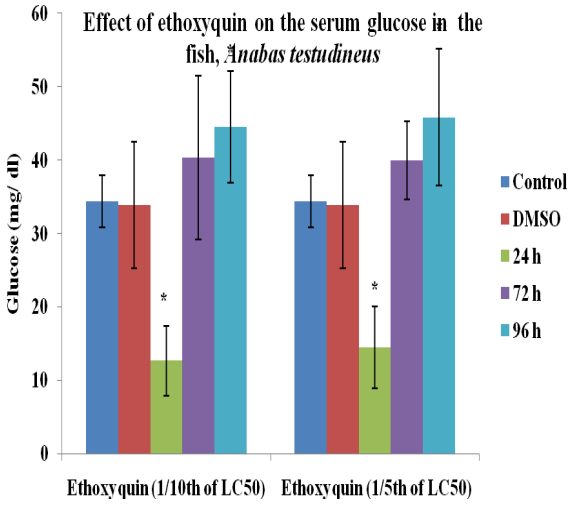

Figure 4

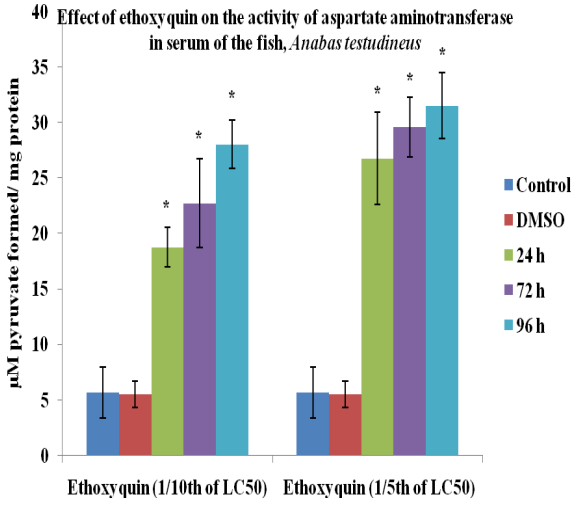

Figure 5

In ethoxyquin exposed groups the behaviour of the fish showed drastic alteration when compared to control groups. After 24 h of ethoxyquin treatment slow movement of the fish was observed along with behavioural abnormalities as frequent engulping of air, mucous secretion throughout the body, lethargic and bulging of eyes.

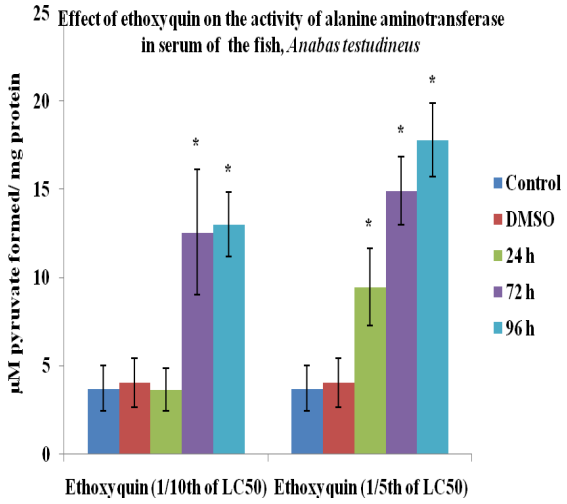

Figure 6

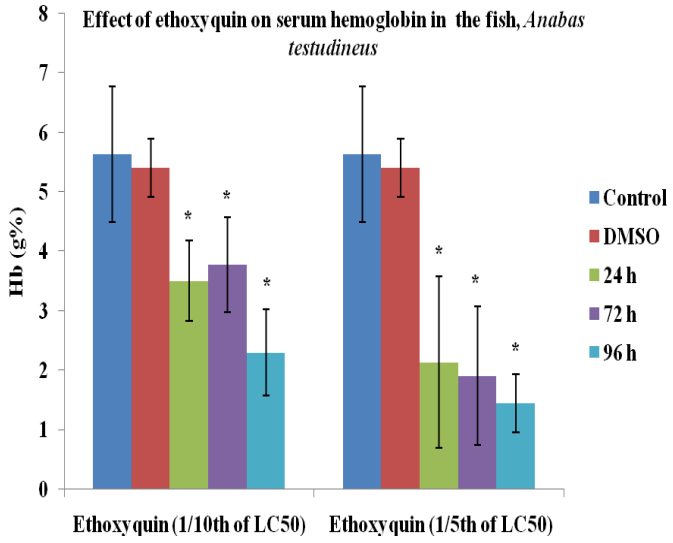

Figure 7

Fish when exposed to ethoxyquin showed significant ($p<0.05$) decrease in the serum total protein level at all durations and at both concentrations when compared to the control groups (Fig. 3). Ethoxyquin exposure showed significant ($p<0.05$) decrease in the blood glucose level in both sublethal concentrations at 24 h exposure period.

However, an increase in the blood glucose was observed after 72 h of ethoxyquin exposure. A significant ($p<0.05$) increase in the glucose level was found only after 96 h exposure in both sublethal concentrations as compared with the control groups (Fig. 4). There was a

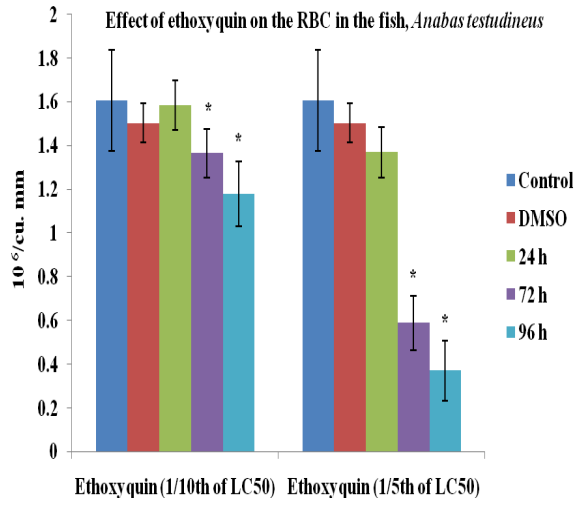

Figure 8

significant ($p<0.05$) increase in the activity of aspartate aminotransferase in the serum of fish exposed to ethoxyquin. The changes were observed in both sublethal

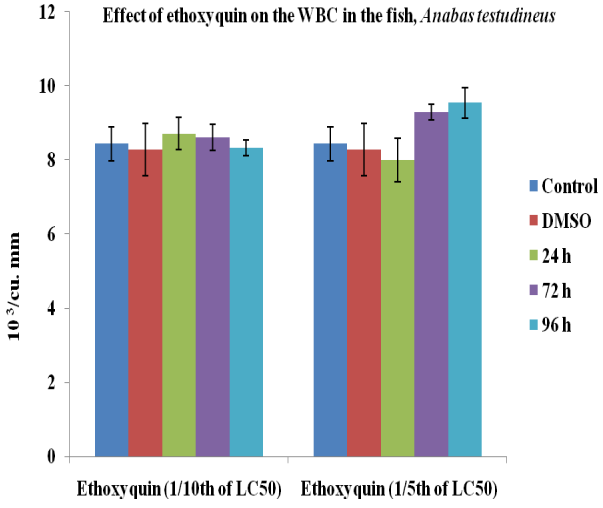

Figure 9

concentrations in time-dependent manner (Fig. 5). The activity of serum alanine aminotransferase showed significant ($P<0.05$) increase only after 72 and 96 h of ethoxyquin exposure at one-tenth of LC_{50} concentration. Whereas at one-fifth of LC_{50} concentration a significant ($P<0.05$) increase was observed in all treatment groups in time-dependent manner (Fig. 6).

Table 1: Effect of ethoxyquin on the mortality of the fish, *Anabas testudineus* for 96 h

Concentration (mg/ L)	Mortality (%)	Hour of mortality	Total (No. of animals)
12	0	96 h	10.00
13	0	96 h	10.00
14	0	96 h	10.00
15	10	96 h	10.00
16	40	96 h	10.00
17	40	72 h	10.00
18	100	72 h	10.00
19	100	48 h	10.00

Fish when exposed to ethoxyquin at both sublethal concentrations showed significant ($P<0.05$) reduction in the gram percentage of haemoglobin. However, a time-dependent decrease was noted only at one-fifth of sublethal treatment (Fig. 7). Exposure to ethoxyquin significantly ($P<0.05$) decreased the count of red blood corpuscles after 72 and 96 h at both concentrations when compared to the corresponding control groups (Fig. 8). Ethoxyquin treatment did not show significant changes in the count of white blood corpuscles at all durations of both concentrations (Fig. 9).

Discussion

Haematological parameters are widely used as a tool for assessing the health of

Table 2: Probit analysis of 95% confidence limits for effective concentrations of ethoxyquin in the fish, *Anabas testudineus*

Prob	Concentration	95% Confidence Limits	
		Lower	Upper
.01	14.22123	12.62423	14.97011
.02	14.47448	13.01074	15.16758
.03	14.63749	13.26080	15.29571
.04	14.76133	13.45121	15.39378
.05	14.86283	13.60747	15.47476
.06	14.94978	13.74137	15.54462
.07	15.02643	13.85939	15.60665
.08	15.09540	13.96551	15.66287
.09	15.15840	14.06235	15.71460
0.10	15.21662	14.15172	15.76277
0.15	15.46006	14.52334	15.96871
0.20	15.65631	14.81873	16.14182
0.25	15.82667	15.07013	16.29919
0.30	15.98123	15.29244	16.44943
0.35	16.12580	15.49384	16.59796
0.40	16.26419	15.67932	16.74876
0.45	16.39922	15.85230	16.90512
0.50	16.53320	16.01541	17.07001
0.55	16.66828	16.17095	17.24635
0.60	16.80666	16.32126	17.43732
0.65	16.95090	16.46894	17.64672
0.70	17.10424	16.61709	17.87972
0.75	17.27128	16.76977	18.14402
0.80	17.45920	16.93281	18.45231
0.85	17.68083	17.11598	18.82805
0.90	17.96370	17.33926	19.32286
0.91	18.03270	17.39233	19.44574
0.92	18.10795	17.44971	19.58065
0.93	18.19106	17.51250	19.73065
0.94	18.28433	17.58232	19.90019
0.95	18.39130	17.66163	20.09608
0.96	18.51776	17.75446	20.32961
0.97	18.67443	17.86822	20.62162
0.98	18.88474	18.01908	21.01804
0.99	19.22104	18.25668	21.66167

the organisms (Sampath *et al*., 1993). Changes in the haematological parameters are the quick responses of the animal towards environmental or physiological alterations, and provide an integrated measure of the physiological status of the organisms. Changes in haematological parameters depend upon the aquatic biotope, fish species, age, and sexual maturity and health status (Ross and Ross, 1999). Haematological and biochemical studies help in understanding the relationship of blood characteristics to the habitat and adaptability of the species to the environment. The alteration in fish haematological parameters such as RBC, WBC, Hb and packed cell volume etc., are known to be influenced by many factors including the exposure to environmental contaminants (Pandey, 1977).

In the present study ethoxyquin exposure increased the deposition of mucous throughout the body of the animal and this could be the first defense mechanism of the animal against exposure to the toxicant, ethoxyquin. Alterations in behavioural pattern was also observed after exposure to ethoxyquin in order to see if the toxic compound have severe implications for survival of the fish at short duration of 96 h. Any disturbances such as presence of toxicants in the surrounding environment could reflect on the behaviour of fishes. During the experiments, abnormal behavioural pattern was observed. Immediately after the exposure to ethoxyquin, fishes showed immediate slow movement in swimming and remained in static position for a while. After some time, fishes showed erratic swimming and jumping to avoid from the toxic environment. As they failed, then the fishes moved on the surface with wide opening of gill operculum to engulp air. Finally as a defensive mechanism the

fishes secreted mucous all over the body and bulging of eyes was also noted.

In the present study evaluation of median lethal concentration of ethoxyquin at 96 h by the method of probit analysis showed 16.5 mg/ L in the fish, *Anabas testudineus*. Correlation analysis of mortality against concentrations of ethoxyquin showed high degree of positive correlation (r = +0.921), therefore, the rate of mortality is dependent on the concentration of ethoxyquin exposed.

Exposure to ethoxyquin at both sublethal concentrations showed significant decrease in the total protein content in serum at all durations. The reduction of protein content in serum occurs due to shrinkage and lysis of RBCs causing plasma dilution and/or protein catabolism where structural protein is converted to energy (Das *et al*., 2004). The blood glucose level was decreased significantly at 24 h of ethoxyquin exposure. Later, a steady increase in the blood glucose was observed after 72 h of ethoxyquin exposure with significant increase at the end of 96 h exposure in both sublethal concentrations as compared with the control groups and this could be due to the increase in glycolysis in response to stress caused by ethoxyquin exposure (Silbergeld, 1974).

Analysis of enzyme activities is widely accepted procedure used for rapid detection to predict early warning of toxicant. Aspartate and alanine aminotransferases are liver specific enzymes. It is one of the sensitive analyses to measure hepatotoxicity of any toxicant and can be assessed within a shorter time. In the present study, ethoxyquin exposure caused significant increase in the activities of aspartate and alanine aminotransferases in the serum of fish at both sublethal concentrations. Enzyme activities could affect various chemical and biological reactions in the body of the fish. Transamination is one principal pathway for synthesis and deamination of amino acids, enabling carbohydrate and protein metabolism during fluctuating energy demands of the organism under various adaptive conditions (Gabriel and George, 2005). The changes in the enzyme activities disrupt physiological and biochemical processes and this is because the animal tries to maintain equilibrium in the presence of contaminant and therefore can be used as a relevant stress indicator.

Blood is a patho-physiological reflector of the whole body and therefore, blood parameters are important in diagnosing the structural and functional status of the animal exposed to toxicants. The significant reduction in RBC and haemoglobin contents were reported in fishes exposed at different concentrations of ethoxyquin. In the present study the decrease in RBC count during the acute treatment of ethoxyquin might have resulted from severe anemic state or haemolysing capacity of ethoxyquin. The decrease in haemoglobin content might be due to rapid oxidation of haemoglobin to methaemoglobin or the release of oxygen radical due to the stress exposure of ethoxyquin. Similar observations have been observed in fish, *Cyprinus carpio* exposed to chlorpyrifos (Ramesh and Saravanan, 2008). Ethoxyquin treatment

did not show significant changes in the count of white blood corpuscles at all duration of both concentrations. However, an insignificant increase in the count was observed after 72 h of treatment in one-fifth of sublethal concentration and this could be due to the slight tissue damage caused by ethoxyquin exposure. In fish, the white blood cells respond to various stressors including infections and chemical irritants (Christensen *et al*., 1978). The present findings conclude that acute exposure to ethoxyquin at sublethal concentrations altered haematological parameters and metabolic activities in the freshwater fish, *Anabas testudineus*. This may finally lead to threat to the survivability of the fish in its natural ecosystem.

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