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Impact of Butylated Hydroxyanisole on serum anti-oxidant status and histological analysis in Zebrafish *Danio rerio*

Petchiammal Kanagaraj[1], Sangeetha Soundararajan[1*], Shalini Elumalai[1], Deepa Rani Sadhasivam[2*]
[1]PG & Research Department of Zoology, Pachaiyappa's College, Chennai, Tamil Nadu, India
[2]PG& Research Department of Zoology, Ethiraj College for Women, Chennai, Tamil Nadu India.
***Corresponding authorsMail id:** drdeepaarivan@gmail.com

Abstract
The synthetic anti-oxidant Butylated Hydroxyanisole (BHA) was tested for its chronic toxicity against zebrafish*Danio rerio*. Toxicity assays initiated with finding the LD_{50} value of BHA against zebrafish. It was calculated as 54.119±3.015µM, hence the different concentration of BHA set within the LD_{50} value. The concentrations 1µM, 5µM, 10µM and 25µM of BHA were made to expose to zebrafish for 21 days continuously. The chronic toxicityof BHA was evaluated by the biochemical level in the serum, differential count of leukocytes, count of erythrocytes in the blood and histological analysis of organs live, muscle and gills. The toxicity of BHA revealed by biochemical alterations in the serum, abnormal count of leucocytes and disorganization of tissues in organs at higher concentration of BHA to fishes. The present study clearly demonstrates the chronic toxicity of BHA with zebrafish model.

Key words:
Butylated Hydroxyanisole; Chronic Toxicity; *Danio rerio*; anti-oxidants; Histology

1. Introduction

Synthetic anti-oxidants have been extensively used in different kind of products such as pharmaceuticals, food products cosmetics, etc., (Liu et al., 2015; Wang et al., 2016). Among variety of synthetic anti-oxidants, Butylated hydroxyanisole (BHA) is synthetic phenolic anti-oxidant which is one of the major compound used in commercial products such as beverages and food products with the maximum permissible limit of 200mg/kg in food products regulated by the U.S. Food and Drug Administration (FDA), the European Union (EU) and CODEX STANDARD (Freitas and Fatibello-Filho, 2010; Zhou et al., 2015).In order to identify the functions of clinical importance of compounds, the evaluation of toxicity in animal model is more important. Before, human clinical trials it is also essential to evaluate the dosage concentration and to assess the side effects on animals. The screening of hazardous effects on various animal models including chick, mice, rat, guinea pig, artemia and zebra fish models was evaluated and reported in several studies. Among other model organisms, zebra fish gained much attention in developmental biology, molecular genetics and drug discovery (Spitsbergen and Kent, 2003; Hill et al., 2005). Most importantly, zebrafish (*Danio rerio*) have been chosen for their size, quick development, transparent and easy of farming (MacRae and Peterson, 2015). It is also important to notice that 80% genes of Zebrafish was matched with human gene (De Esch et al., 2012). Researchers have also utilized the zebrafish as a model organisms for metabolic diseases. It is not only used as a model for biomedical research but also used to evaluate the toxicity nature of organic and inorganic pollutants. There are numerous research reports available majorly on impact of environmental contaminants and pharmaceutical compounds on zebra fish model (Zon et al., 2005; Garcia et al., 2016). Zebrafish is a powerful animal model to evaluate accumulation of test compounds, toxicity mechanism and to identify toxicity end points (Cassar et al., 2019). In addition, organic pollutants have been identified regularly in the environment, raising concerns on the hazardous pollutants (Ma et al., 2012). The synthetic anti-oxidants has been reported as endocrine disruptors causing the weak estrogenic activity, perturbation in steroidogenesis and dysfunction of reproductive system in fish (Jeong et al., 2005; Kang et al., 2005; Soto et al., 1995). The impact of synthetic anti-oxidants on development stages of zebrafish embryo has been reported by Yang et al., 2018. All the synthetic anti-oxidants causing the negative influence in the growth of zebrafish.

In the present study, the effect of BHA on biochemical changes in serum biomarkers in the serum after exposure of BHA to fish. Histology analysis have been done to study the impact of BHA on tissue level changes in fish.

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2. Materials and Methods

2.1. Acclimation and Breeding of Zebra Fishes

Commercially procured Zebra Fishes (6-8 months old) were acclimated to laboratory condition by maintaining them at 28°C in a 14:10 hours light and dark photoperiod for around a week (Sulukan et al., 2017). During acclimation the animals were fed with *Artemia salina ad libitum.* The excess ammonia load was removed by siphoning. After proper acclimation 20 males and 10 females were chosen and allowed to spawn in a tank over night at optimum conditions for successful breeding.

2.2. Chronic Toxicity Studies

Chronic toxicity studies were carried out for 21 days as per the OECD guidelines using bath immersion method (OECD, 2006).Butylated Hydroxyanisole (BHA) was purchased from Sigma, India for this study. Different concentrations of BHA were exposed to fish to measure LD_{50}value. According to the LD50 value the experiment concentrations fixed and the selected animals were subjected to concentrations of BHA 5µM, 10µM and 25µM prepared using fresh water and control animals maintained without BHA treatment. All the tanks were maintained at optimum conditions viz. temperature - 25±1 °C, total hardness of water (dgH) - 13 N°, oxygen concentration >60%, pH = 8.3–8.6. However, variation in pH was observed in CaO solutions (in 100 mg/L - pH 10.5, in 400 mg/L - pH 11.8, in 800 mg/L - pH 12.4), in MgOH solutions (in the concentration 1600 mg/L - pH 10.2), and in MgO solutions (from 100 to 800 mg/L - pH 10.4 to 11.1). The entire experiment was conducted in 14:10 hrs light and dark photoperiod in triplicates and repeated thrice.

2.3. Collection of blood and preparation of serum

Blood was collected from the zebrafish by made transverse incision near to the dorsal aorta. Initially a small puncture was made on the fish skin and with help of scissors the puncture was made 0.3-0.5 cm. By gentle pressing the fish, the blood was welling up from incision and immediately collected using a micro syringe. A 10-12 fishes was sacrificed to collect 100 µl blood and it was allowed to clot then spun the clot for 10 min at 2500 rpm; then the serum was collected on the top of centrifuge.

2.4. Serum Bio-Chemical Assay

The anti-oxidant enzymesCatalase (CAT) activity(Aebi, 1984),Lactate dehydrogenase (LDH) activity(Domingues et al. 2010)Glutathione S-transferase (GST) activity(Domingues et al., 2010) and reduced glutathione (GSH) activity (Saldak and Lindsay, 1968) were measured in fish serum after exposure.

2.5. Hematological analysis

For hematology studies, the blood was collected and smeared on glass slides. The blood cells stained with Wright-Giemsa. The leucocyte differentials were counted under microscope (Feldman et al., 2000).

2.6. Histopathology

Method described by Humason (1962) was adopted for histopathological analysis. 72 hpf one gram tissue from each animal exposed to different BHA concentrations (0, 25 and 50µm) were soaked in fixative overnight. After fixation the tissues were washed thoroughly in running tap water and dehydrated using increasing percentage of ethyl alcohol. After complete dehydration the tissues were treated with methyl benzoate and embedded in paraffin wax. The wax blocks were then sectioned (5µm Thickness) using rotary microtome. The sections were fixed on a slide cleaned using xylene and stained using hematoxylin followed by counter staining using Eosin. The slides were air dried and covered using coverslips and photomicrographs were taken for analysis.

2.7. Statistical Analysis

The experiment procedures were replicated three times for statistical analysis. The results were expressed as mean±standard deviation. Analysis of Variance (ANOVA) was used to measure the significance. Differences were considered significant at $P<0.05$.

3. Result and Discussion

The study ofthe toxic effect of BHA on zebrafish initiated with measure the LD50 value of BHA against zebrafish. Among the different concentrations of BHA used in this study, the higher mortality rate was observed above 50µM upon chronic exposure for 21 days. The LD50 value is calculated as 54.119±3.015µM using Graphpad Prism 9.2. The experimental dosages of BHA as 1µM, 5µM, 10µM and 25µM set by based on the LD50 value and the without BHA treatment considered as experiment control group. Previous reports were made on the acute toxicity of BHA against zebrafish embryo, Yang et al., (2018) have reported that LD50 value of BHA as 99µM for 92 hrs BHA exposure to zebrafish larvae. In the present study revealed the chronic toxicity of BHA on adult zebrafish and the adult fishes were tolerates the higher concentrations of BHA for 21 days. In the case of anti-oxidant activity in the serum, all the bio-marker level tested in this study were increased after 21 days with higher concentrations. Catalase activity at the concentration of 25µM was increased moderately

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(3.56U/g of protein) when compared to control (0.63 U/g of protein) (Fig. 1). Similarly, the LDH activity was also increased upto threefold as 3300U/ g of protein (Fig. 2). LDH is involves in anaerobic path wayand it is a main indicator enzyme which is synthesized against oxidative stress, so that the enzyme could be used as biomarker to study the toxicity of compounds (Li et al., 2011). Similar results were also reported by Li et al., 2018 on exposure of zebrafishes to gabapentin. Li and his co-workers reported increasing activity of CAT with respect to increasing concentration of gabapentin however unlike the present study LDH activity was similar to control in lower concentrations of gabapentin and elevated in higher concentrations of gabapentin. In the case of activity of GST and GSH was not significant but increased at higher concentration upon chronic toxicity (44 U/g of Protein and 28.66 U/g of protein respetively) (Fig. 3 & 4). GST level was increased when the organism exposed oxidant stress and it could coordinate with GSH to detoxify the compounds. Both the enzymes reduce the oxidative stress (Stephensen et al., 2002; Jakoby and Ziegler, 1990). Li et al., (2018) showed the higher level of these biomarkers in zebrafish against given toxicity compound.

The toxicity effect of BHA on hematology parameters in zebrafish also observed in the present study. Hematology is preliminary diagnostics used in human and veterinary medicine. The hemtalogic alterations indicates the metabolic pathway of disease occurs. In the case of white blood cells, lymphocytes, eosinophils and basophils were counted as reduced level in the blood after chronic exposure of BHA. The count of lymphocytes at 25µM dose decreased upto 64% while the control fishes showed 82% of lymphocytes. The toxic compound might induce the apoptosis of lymphoid cells in the fish (Schwartzman and Cidlowski, 1994). Previous studies have reported that chronic exposure of toxic compound causes the lymph-toxicity, lymphoid hypoplasia and decreased lymphopoesis (Mendelsohn et al., 1977). Similarly, eosinophils and basophils were reduced the level at the higher concentration of BHA (0.5% and 0.17%) (Table. 1).

Interestingly, the monocytes were significantly increased in the blood after 21 days of exposure of BHA (21%). Neutrophils were moderately increased in blood at higher concentration of BHA exposure. The upregulation of mRNA level of glucocorticoid receptors or alteration the gene expression might be the reason for the leukocyte differentiations. The red blood cells were counted after the experimental studies. BHA exposure dropped the level of erythrocytes significantly at higher concentration of BHA (Relative erythrocyte number is 37). Low level of RBC can lead to anemic condition in fish.

In the present study, tissue level changes were recorded after BHA exposure in fishes. The organs liver, muscle and gills were analyzed by histology technique. Similarly, muscle epithelial disorganization, epithelial movement, odema, lamellar fusion and curling of secondary lamellae were observed at higher concentration exposure of BHA (Fig. 5a & b). In the case of gill tissues, congestion of blood vessels, fusion of lamellae and hyperplasia of the branchial arch were noticed at the concentration 25µM of BHA in chronic exposure (Fig. 5c & d).The liver tissue had degeneration or necrosis, inflammation and pynkosis at higher concentration of BHA in chronic toxicity studies. Moreover, swelling of the cells, vacuolar degeneration, irregular shape of cells and bi-nucleated cells were observed (Fig. 5e & f). Metal nanoparticles and toxic compound exert the similar effect on hepatocytes, muscle and gills (Diniz et al., 2013; Govindasamy and Rahuman, 2012).

4. Conclusion

The present research study clearly demonstrates the toxic effect of BHA on biochemical alterations and tissue level changes in zebrafish as animal model. Even-though the synthetic anti-oxidant has more advantageous in food and beverages industry, above permissible limit pave the way to dysfunction metabolic pathway. This is preliminary study to illustrate the negative effect of industrial chemical. In future, the study could elaborate to molecular level changes and marker gene expression studies.

Conflicts of interest

The authors declared no conflict of interest.

References

Aebi, H., 1984. Catalase. Methods Enzymol. 2, 673–684.

Cassar, S., Adatto, I., Freeman, J.L., Gamse, J.T., Iturria, I., Lawrence, C., Muriana, A., Peterson, R.T., Van Cruchten, S. and Zon, L.I., 2019. Use of zebrafish in drug discovery toxicology. Chemical research in toxicology, 33(1), pp.95-118.

De Esch, C., Slieker, R., Wolterbeek, A., Woutersen, R. and de Groot, D., 2012. Zebrafish as potential model for developmental neurotoxicity testing: a mini review. Neurotoxicology and teratology, 34(6), pp.545-553.

Diniz, M.S., De Matos, A.P.A., Lourenço, J., Castro, L., Peres, I., Mendonça, E. and Picado, A., 2013. Liver alterations in two freshwater fish species (*Carassius auratus* and *Danio rerio*) following exposure to different TiO2 nanoparticle concentrations. Microscopy and Microanalysis, 19(5), pp.1131-1140.

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Domingues, I., Oliveira, R., Lourenço, J., Grisolia, C.K., Mendo, S. and Soares, A.M.V.M., 2010. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 152(3), pp.338-345.

Feldman, B.F., Zinkl, J.G. and Jain, N.C., 2000. Schalm\U+ 00b4\s veterinary hematology.

Freitas, K.H.G. and Fatibello-Filho, O., 2010. Simultaneous determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food samples using a carbon composite electrode modified with Cu3 (PO4) 2 immobilized in polyester resin. Talanta, 81(3), pp.1102-1108.

Garcia, G.R., Noyes, P.D. and Tanguay, R.L., 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacology & therapeutics, 161, pp.11-21.

Govindasamy, R. and Rahuman, A.A., 2012. Histopathological studies and oxidative stress of synthesized silver nanoparticles in Mozambique tilapia (*Oreochromis mossambicus*). Journal of Environmental Sciences, 24(6), pp.1091-1098.

Hill, A.J., Teraoka, H., Heideman, W. and Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicological sciences, 86(1), pp.6-19.

Humason, G.L., 1962. Animal tissue techniques. Animal tissue techniques.

Jakoby, W.B. and Ziegler, D.M., 1990. The enzymes of detoxication. The Journal of biological chemistry (Print), 265(34), pp.20715-20718.

Jeong, S.H., Kim, B.Y., Kang, H.G., Ku, H.O. and Cho, J.H., 2005. Effects of butylated hydroxyanisole on the development and functions of reproductive system in rats. Toxicology, 208(1), pp.49-62.

Kang, H.G., Jeong, S.H., Cho, J.H., Kim, D.G., Park, J.M. and Cho, M.H., 2005. Evaluation of estrogenic and androgenic activity of butylated hydroxyanisole in immature female and castrated rats. Toxicology, 213(1-2), pp.147-156.

Li, X., Zhou, S., Qian, Y., Xu, Z., Yu, Y., Xu, Y., He, Y. and Zhang, Y., 2018. The assessment of the eco-toxicological effect of gabapentin on early development of zebrafish and its antioxidant system. RSC advances, 8(40), pp.22777-22784.

Li, Z.H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Kolarova, J., Li, P. and Randak, T., 2011. Acute toxicity of carbamazepine to juvenile rainbow trout (Oncorhynchus mykiss): effects on antioxidant responses, hematological parameters and hepatic EROD. Ecotoxicology and environmental safety, 74(3), pp.319-327.

Liu, R., Song, S., Lin, Y., Ruan, T. and Jiang, G., 2015. Occurrence of synthetic phenolic antioxidants and major metabolites in municipal sewage sludge in China. Environmental science & technology, 49(4), pp.2073-2080.

Ma, Y., Han, J., Guo, Y., Lam, P.K., Wu, R.S., Giesy, J.P., Zhang, X. and Zhou, B., 2012. Disruption of endocrine function in in vitro H295R cell-based and in in vivo assay in zebrafish by 2, 4-dichlorophenol. Aquatic toxicology, 106, pp.173-181.

MacRae, C.A. and Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nature reviews Drug discovery, 14(10), pp.721-731.

Mendelsohn, J., Multer, M.M. and Bernheim, J.L., 1977. Inhibition of human lymphocyte stimulation by steroid hormones: cytokinetic mechanisms. Clinical and experimental immunology, 27(1), p.127.

OECD, 2006. Fish Embryo Toxicity Assays. No. 203 85 422. German federal environmentagency.

Schwartzman, R.A. and Cidlowski, J.A., 1994. Glucocorticoid-induced apoptosis of lymphoid cells. International archives of allergy and immunology, 105(4), pp.347-354.

Sedlak, J. and Lindsay, R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical biochemistry, 25, pp.192-205.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N. and Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental health perspectives, 103(suppl 7), pp.113-122.

Spitsbergen, J. M., and Kent, M. L. (2003). The state of the art of the zebrafish model for toxicology and toxicologic pathology research–advantages and current limitations. Toxicol Pathol 31 (Suppl.), 62–87.

Stephensen, E., Sturve, J. and Förlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 133(3), pp.435-442.

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Sulukan, E., Köktürk, M., Ceylan, H., Beydemir, Ş., Işik, M., Atamanalp, M. and Ceyhun, S.B., 2017. An approach to clarify the effect mechanism of glyphosate on body malformations during embryonic development of zebrafish (*Daino rerio*). Chemosphere, 180, pp.77-85.

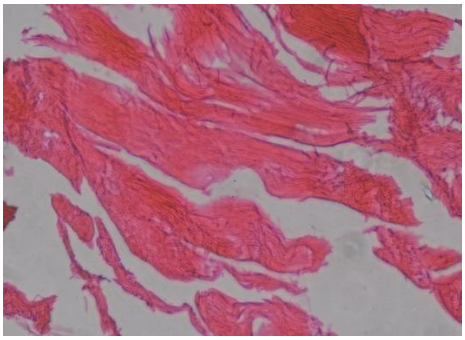
Wang, W., Asimakopoulos, A.G., Abualnaja, K.O., Covaci, A., Gevao, B., Johnson-Restrepo, B., Kumosani, T.A., Malarvannan, G., Minh, T.B., Moon, H.B. and Nakata, H., 2016. Synthetic phenolic antioxidants and their metabolites in indoor dust from homes and microenvironments. Environmental science & technology, 50(1), pp.428-434.

Yang, X., Sun, Z., Wang, W., Zhou, Q., Shi, G., Wei, F. and Jiang, G., 2018. Developmental toxicity of synthetic phenolic antioxidants to the early life stage of zebrafish. Science of the Total Environment, 643, pp.559-568.

Zhou, S., Li, Z., Lv, X., Hu, B. and Jia, Q., 2015. Preconcentration of synthetic phenolic antioxidants by using magnetic zeolites derived with carboxylatocalix [4] arenes combined with high performance liquid chromatography. Analyst, 140(17), pp.5944-5952.

Zon, L.I. and Peterson, R.T., 2005. In vivo drug discovery in the zebrafish. Nature reviews Drug discovery, 4(1), pp.35-44.

Figures and Table



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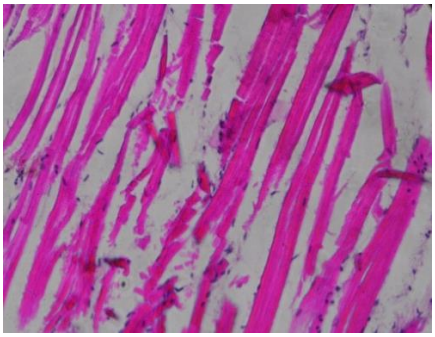

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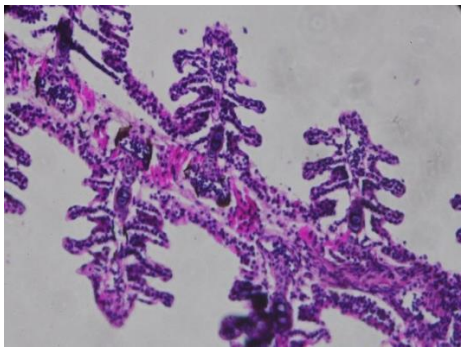

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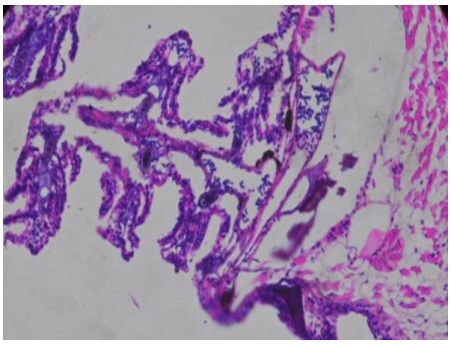

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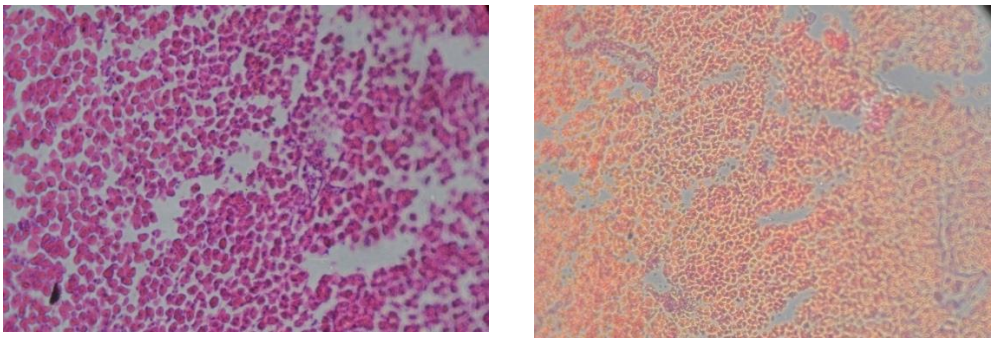

(e) **(f)**

Fig. 5: Histology analysis of muscle, gills and liver after chronic exposure of BHA to zebrafish. The muscle tissues (b) broken compared to control muscle (a); degenerative gills were observed (d) and control gill (c); disintegration of liver tissues (f) control liver (e)

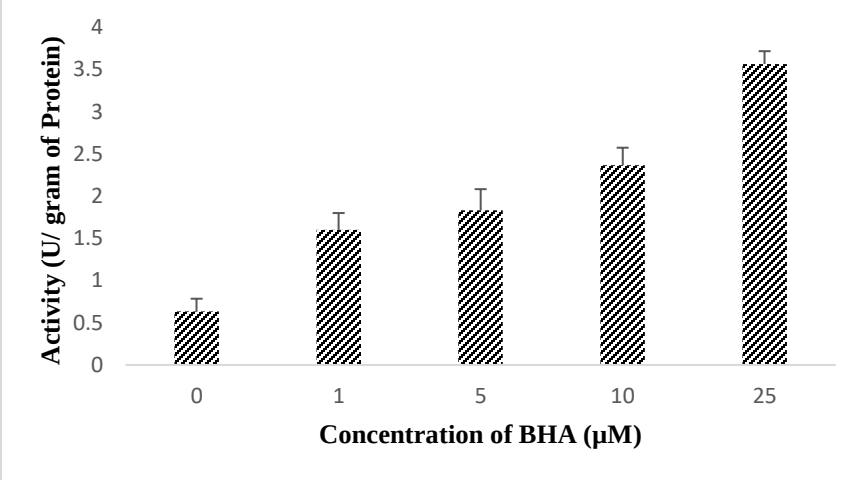

Fig. 1: The activity of catalase in the serum of fish after BHA exposure.

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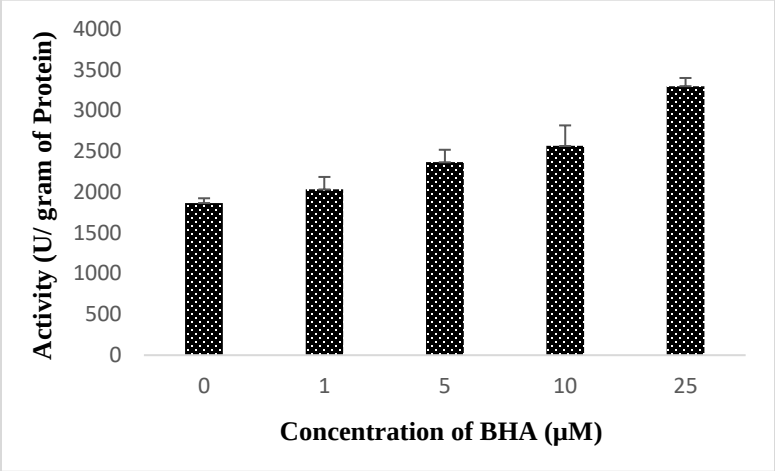

Fig. 2: LDH activity increased in the serum after chronic exposure of BHA to fish

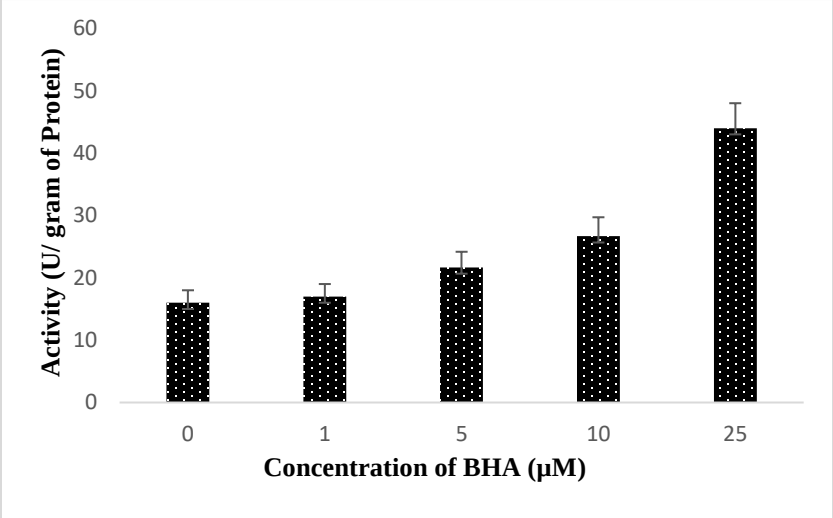

Fig. 3: Glutathione S-transferase activity in the serum against BHA treatment on fish

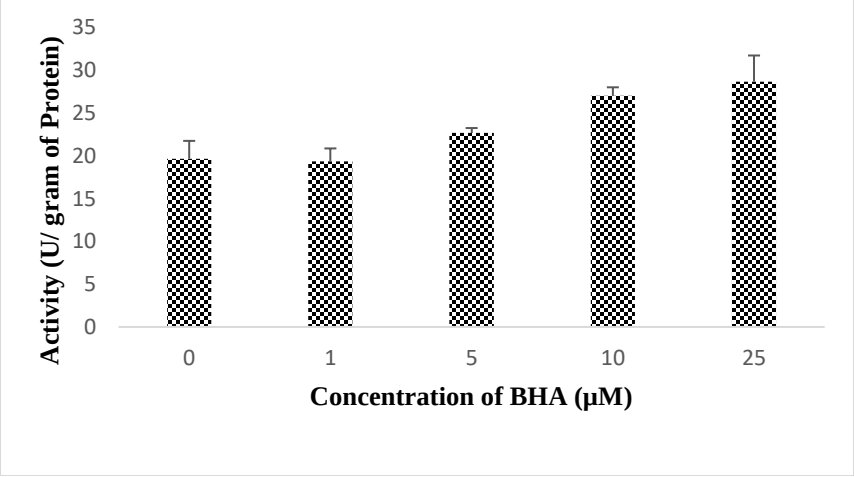

Fig. 4: The activity of GSH in the serum of fish after chronic exposure of BHA

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Table. 1: Effect of BHA on differential count of blood cells in zebrafish

Blood Cells (%)	Concentration of BHA (µM)				
	0	**1**	**5**	**10**	**25**
Lymphocytes	82±3	82.66±2.516	80.33±2.08	78.33±1.527	64±3.605*
Monocytes	9.3±1.52	11.66±1.527	12.87±2.516	13.33±2.08	21±3.605*
Neutrophils	12.33±2.5	14±1.73	15.66±1.05	16.6±1.527	23±4.358*
Eosinophils	3.66±0.577	1.8±0.3	1.6±0.5	1.46±0.321	0.5±0.2*
Basophils	2.3±0.556	0.43±0.28	0.321±0.21	0.146±0.07	0.17±0.112
Erythrocytes (REN)	55±5.567	53.3±3.05	50.3±2.912	45±4	37±2.5

Values are mean±SD

*Significantly different from control; $p > 0.05$.