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RESEARCH ARTICLE

Effect of Bisphenol-A on Serum Biochemistry and Liver Function in the Freshwater Fish, *Catla catla*

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A B S T R A C T

Bisphenol-A (BPA) is an anthropogenic chemical mimicking 17β estradiol actions. It is used as a monomer for plastic production. In the present study, adult female (age around 18 months) *Catla catla*, a widespread cyprinid fish in the Pakistani rivers, were exposed to increasing concentrations (10, 100, 1000 µg/l) of BPA for 2-weeks. Classical toxicological endpoints like liver enzymes and alterations in liver histology were investigated. In addition, the effect of BPA on hepatosomatic index (HSI) and *vitellogenin (vtg)* mRNA expression was also examined. A concentration-dependent increase was recorded in serum liver enzymes like aspartic aminotransferase (AST) and alanine aminotransferase (ALT) compared to control. The biomarkers for kidney function like serum creatinine level and uric acid also significantly increased in fish exposed to BPA. HSI did not change in groups exposed to low concentrations of BPA (10 and 100 µg/l); however, a significant increase as compared to control was observed in female fish exposed to 1000 µg/l BPA. Hepatic *vtg* mRNA increased with increase in exposure concentration but only the highest concentration (1000µg/l) was capable of inducing a significant *vtg* expression. Present study showed that bisphenol-A has far reaching detrimental effects on fish health by altering metabolic profile of liver and kidney and induced significant histopathological changes in the liver. Moreover, it also exerted environmental estrogenic effects by inducing vitellogenin, similar to natural estrogens. The results presented here are similar to earlier studies reporting estrogenic actions of BPA.

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INTRODUCTION

Aquatic ecosystem receives a large number of pollutants that pose major threats to the aquatic fauna during their developmental and adult life stages. Such anthropogenic chemicals are major problem around the globe and toxicity of anthropogenic chemicals is usually dependent on their concentrations in the environment, persistent nature and bioaccumulation/bioavailability (Sayed *et al.,* 2011).

Bisphenol-A (BPA) is a xenobiotic and solely manmade chemical. BPA is a plastic monomer used for the synthesis of polycarbonate plastics, epoxy resins and thermal papers. It is also used as a precursor of flame retardant (Corrales *et al.,* 2015) and as a constituent of dental sealant (Suzuki *et al.,* 2000). To cope the rising demand of plastic and plastic products, large volume of BPA is produced worldwide (Corrales *et al.,* 2015). Production of BPA in large quantity resulted in subsequent release in the environment especially aquatic environment.

BPA enters into the water bodies through manufacturing plants and effluent discharge. BPA is also released during transport and processing, degradation of plastic and PVC pipes also result in BPA release (Flint *et al.,* 2012). BPA gained much attention in the recent years because it has the affinity for estrogen receptors (ERα and β) present on cell and nuclear membrane and with peroxisome proliferator receptors (PPRs) (Corrales *et al.,* 2015). BPA also interact with thyroid and androgen receptor and alter the titer of androgens and thyroid hormones in the body (Faheem *et al.,* 2017b). In aquatic environment, BPA is usually degraded in 0.5-6 days (Mihaich *et al.,* 2012). Because of its unremitting release

in the aquatic environment many aquatic organisms, especially fish, are continuously exposed to BPA.

Role of BPA an as endocrine disruptor have been studied extensively. Various reports suggested that BPA caused reproductive impairments and oxidative stress in different fish species (Mandich *et al.,* 2007; Maradonna *et al.,* 2014; Faheem and Lone, 2017; Faheem *et al.,* 2017a, 2017b, 2018b). Relationship between toxicant exposure and fish health is an important area of research. Study of physiological and histopathological biomarkers are important to detect response of a fish species to a toxicant and vitellogenin has been an important biomarker of endocrine disruption. Therefore, in the current work, estrogenic and non-estrogenic effects of bisphenol-A were studies by estimating liver vitellogenin expression, liver and kidney function, and liver histology.

Catla catla is an edible and commercially important aquaculture species of Indo-Pak subcontinent. Determination of responses of *Catla catla* will be vital for environmental monitoring considering its importance as edible species and its consumption related effects on human physiology.

MATERIALS AND METHODS

Healthy adult female *Catla catla* (length, 39.9±1.10 cm; weight, 1023.53±99.97 g), were obtained from Himalaya Fish Hatchery, Lahore, Pakistan. Before start of experiment, fish were acclimatized for 14 days in cement tanks at ambient temperature and photoperiod. During this period, commercially available carp pellet diet was given to fish twice a day. Water temperature, dissolve oxygen, and pH were recorded every other day.

After acclimatization, fish were divided into four groups. Three groups were exposed to increasing concentration (10, 100, 1000 µg/l) of BPA for 14 days, and the fourth group was vehicle control. BPA stock solution was made by dissolving BPA in ethanol, and required dilutions were made by adding appropriate amount of stock in the aquaria water. Approximately, 75% water was changed on every second day and fresh BPA solution was added after water change. No food was provided during the tenure of the experiment. Experiments and fish handling was performed according to OECD guidelines for fish toxicity (OECD, 1992).

Sample collection: After 14 days, fish was anesthetized. Clove oil was used as an anesthetic. After recording length and weight of every fish, blood was drawn from caudal peduncle and serum was separated by centrifuging blood at 1000g for 10 min. Serum was aliquoted and kept at -80°C until analysis. Fish were humanely sacrificed, liver tissue was removed, cleaned and weighed to the nearest milligram. A portion of liver was preserved in 10% buffered formalin for histological examination while remaining tissue was quick frozen by placing in liquid nitrogen for few minutes and then stored at -80°C.

Calculation of hepatosomatic index: Hepatosomatic index (HSI) was calculated using formula.

HSI = (liver weight (g)/body weight (g))*100

Biochemical analysis: Serum biochemical parameters *e.g.* aspartic amino- transferase (AST), alanine amino-transferase (ALT), creatinine (Cr) and uric acid were determined by commercially available kits (Randox reagents).

Histological examinations: The tissue samples preserved in formalin were dehydrated by passing through ascending concentrations of alcohol. After clearing with xylene, the liver tissues were impregnated with wax. Tissue sections were cut (5µ thick). Hematoxylin and eosin (H & E) stains were used to stain the tissue. After staining, slides were examined carefully and photographs were taken with high resolution camera fitted on a microscope (Leica, Japan).

Real time qPCR: Vitellogenin expression in liver was studied by real time qPCR. One microgram of total RNA from liver tissue was reverse transcribed using oligo dT primers. Real-time qPCR was performed using CFX 96 (Bio-Rad) with Syber Green fluorescent label. Geometric mean of three most stable reference genes (*18s, gapdh, tbp*) was used as reference control following Faheem *et al.* (2018a). Primers used in gene expression study are listed in Table 1. Melt curve analysis was performed after amplification to ensure amplification specificity. Each sample was run in duplicate and cycle threshold value generated by Software (CFX Manager, Version 3.1). $2^{-\Delta\Delta Ct}$ method was used to calculate change in transcript abundance (Livak and Schmittgen, 2001).

Statistical analysis: All data of biochemical and gene expression studies are expressed as means ± S.E.M. One way analysis of variance (ANOVA) and Tukey's post hoc test was used to determine the statistical difference among means. All the statistical analysis was performed on SPSS (IBM, Version 20). P value less than 0.05 was considered as significant.

RESULTS

No significant change in hepato-somatic index (HSI) was observed in female *Catla catla* exposed to 10 and 100 µg/l BPA. However, fish exposed to highest concentration (1000 µg/l) of BPA had significantly higher HSI as compared to control group (Fig. 1).

Histological examination of adult female fish liver exposed to increasing concentrations of BPA is given in Fig. 2. Hepatic tissue of fish from control group showed normal architecture. Central vein was lined with epithelial cells and hepatocytes were arranged in cords. Control group hepatocytes had centrally located nucleus with no vacuoles. Similar picture of hepatocytes was observed in the livers of fish exposed to low concentration (10 µg/l) of BPA. Few vacuoles were seen and shrinkage of pancreatic cells (the fish has heptopancreas) were observed in fish liver exposed to 100 µg/l BPA. Major histopathological alterations were only observed in hepatocytes of fish exposed to 1000 µg/l BPA. These alterations involved loss of normal architecture of hepatocytes, shrinkage of pancreatic cells, lipid vacuolization, hemorrhage and necrosis.

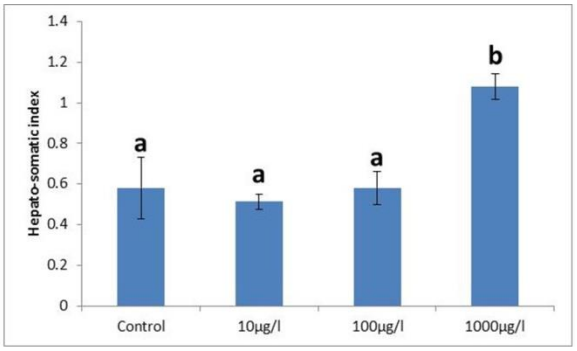

Fig. 1: Hepato-somatic index (HSI) of adult female *Catla catla* exposed to 10, 100 and 1000 µg/1 BPA for 14 days. Data are expressed as mean ± SEM. n=3. Different letters indicate significant differences among groups, P<0.05.

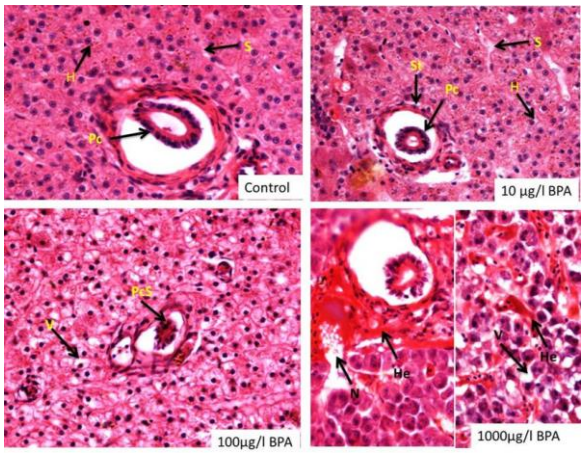

Fig. 2: Liver tissue from adult female *Catla catla* exposed to graded concentration of BPA (10, 100 and 1000 µg/1) for 14 days. Panereatic cells (Pc); Hepatocytes with central nucleus (H); Sinusoids (S); Supporting tissue (St); Hemolysis (He); Shrinkage of pancreatic cells (PcS); Vacuolization (V); Necrosis (N). H & E stain, 400X.

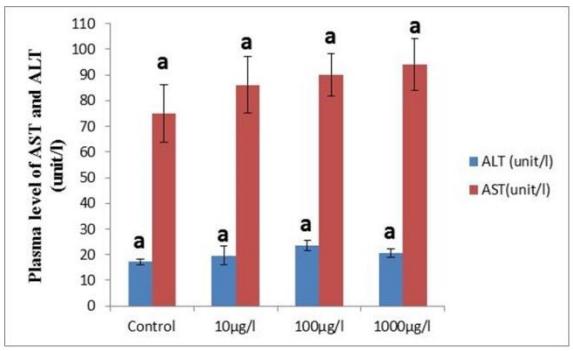

Fig. 3: Serum level of alanine amino transferase (ALT) and aspartic amino transferase (AST) in female *Catle catla* exposed to 10, 1000 and 1000 µg/1 BPA for 14 days. Data are expressed as mean ± SEM. n=3. Different letters indicate significant differences among groups. P<0.05.

Table 1: Primer sequences, amplicon lengths and annealing temperature of selected genes

Genes	Primer sequence 5'to 3'	Amplicon size	Annealing temperature (°C)
gapdh	ATCA-CAGCCACGCAGAAGACC CAGGAATGACTTTGCCCACAGC	126	60
18S	CGGTGAACCTTGGTGACTCT CTTGGATGTGGTAGCCGTTT	189	60
tbp	AACAGCTTGTCCCTCCTGGA TCCAGGAGGGACAAGCTGTT	213	60
vtg	GTTGCTCTCCAGACCTTTGC GCAGAGCCTCCACCTTGTAG	180	60

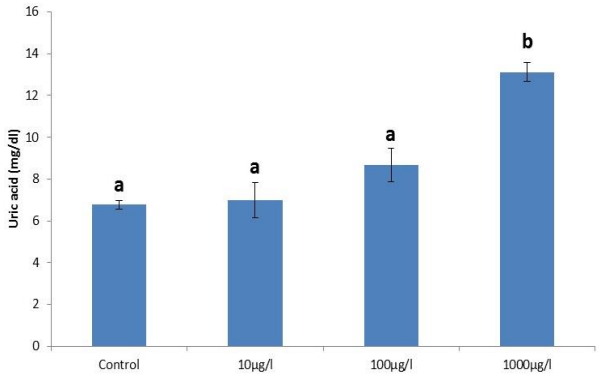

Fig. 4: Serum level of uric acid in female *Catla calta* exposed to 10, 100 and 1000 µg/1 BPA for 14 days. Data are expressed as the mean ± SEM. n=3. Different letters indicate significant difference among groups. P<0.05.

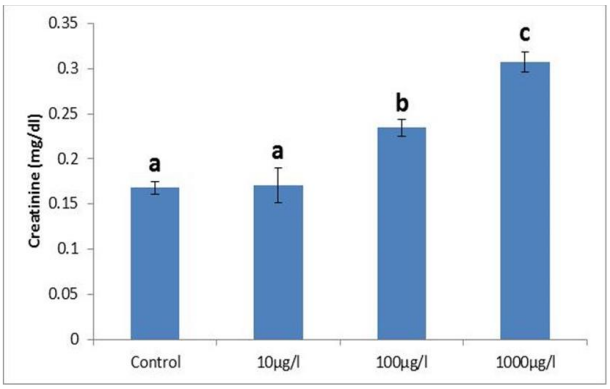

Fig. 5: Serum level of creatinine in female *Catla calta* exposed to 10, 100 and 1000 µg/1 BPA for 14 days. Data are expressed as the mean ± SEM. n=3. Different letters indicate significant difference among groups. P<0.05.

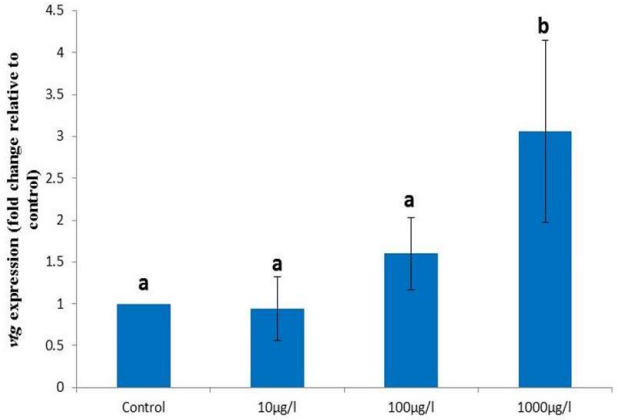

Fig. 6: Relative gene expression of the biomarker gene vitellogenin (*vtg*) normalized to different reference genes and mean of three selected genes (*gapdh, 18s, tbp*), in liver of adult female *Catla catla* after 14 days of exposure to graded concentration s of BPA. Data are expressed as the mean fold change ± SEM. n=3. Different letters indicate significant differences among groups. P<0.05.

An increase in serum ALT and AST (Fig. 3) was recorded but the increase was not significant (P>0.05). A statistically significant increase (P<0.05) in kidney function parameters was recorded after exposure to increasing concentrations of BPA for 14 days. Level of serum creatinine increased significantly in 100 and 1000 µg/l BPA groups (Fig. 4) while uric acid increased only in 1000 µg/l BPA group (Fig. 5).

Vitellogenin mRNA expression did not change in group exposed to 10µg/l but with an increase in concentration, *vtg* mRNA increased. A significant ($P<0.05$) induction of *vtg* mRNA was only observed in group exposed to 1000µg/l /BPA (Fig. 6).

DISCUSSION

Vital organs of fish *e.g.* liver, kidney, gills and brain are important target of any toxicant. Various studies showed that alteration in physiological and blood biochemical parameters are caused by contaminants in the surrounding environment (Ololade and Oginni, 2010; Sayed and Hamed 2017). Study of serum biochemistry provides a valid indication of damage by pollutant and are mirror image of the environmental pollution and are useful for detection of tissue pathophysiological status (Sayed and Hamed 2017; Abdel-Tawwab and Hamed, 2018)

Increased level of serum ALT and AST indicate liver damage (Bhattacharya *et al.,* 2008) and have been used as toxicopathological biomarker (Coppo *et al.,* 2016). Similar increase in level of serum AST and ALT was reported in heavy metal exposed fish (Mekkawy *et al.,* 2011) and other endocrine disrupting chemicals (Bhattacharya *et al.,* 2008). In the present study, exposure of increasing concentrations of BPA to adult female *Catla catla* caused non-significant increase in activity of both enzymes. Similar non-significant increase in AST and ALT was reported in *C. gariepinus* exposed to diazinon (Adedeji *et al.,* 2009). Significant increase in hepatic enzymes (ALT, ALP, AST) was recorded in *Oreochromis niloticus* exposed to 1.64 µg/l BPA for 6 weeks (Abdel-Tawwab and Hamed, 2018). This difference in hepatic enzyme level may be due to different concentrations and exposure time. In our study acute exposure of BPA was used while Abdel-Tawwab and Hamed (2018) used chronic exposure in their study.

Serum creatinine and uric acid are important indicators of renal health and kidney function and biomarkers for muscle and purine metabolism (Hamed and Tawwab, 2017). Glomerulus damage, impair metabolism of carbohydrates and increased muscle tissue catabolism therefore may cause increased creatinine level in blood (Hadi *et al.,* 2009). A significant increase was recorded in serum creatinine level of fish exposed to 100 and 1000µg/l BPA while uric acid level significantly increased only in fish exposed to 1000 µg/l BPA. Significant increase in creatinine and uric acid may indicate that BPA affect muscle and purine (nucleic acid) metabolism. This increase may also be due to the damage of renal tubules. Degeneration and necrosis of glomerulus and decrease in hematopoietic tissue in the same fish species after BPA exposure was reported by Faheem *et al.* (2016). This decrease in hematopoietic tissue may be a cause of increase in serum uric acid.

Histopathological evaluation of key organs is an important tool to evaluate the effect of a toxicant on fish health. Liver is the main organ of detoxification, any alteration in the liver tissue may lead to compromised functions in fish. Exposure of BPA to female *Catla catla* resulted in degenerative changes in hepatocytes. Similar histopathological alteration was recorded in *Clarias gariepinus* liver exposed to nonylphenol for 15 days (Sayed *et al.,* 2012). Alteration in liver histology includes change in normal structure, vacuolation of hepatocytes and dilation of sinusoids. Structural protein degeneration may be the reason of sinusoid dilations after BPA exposure. Vacuolation of hepatocytes is non-specific response of fish after exposure to a toxicant (Roberts, 1978), hepatocyte vacuolation observed here may be due to lipid accumulation. Juvenile *Sparus aurata* exposed to BPA had severe lipid accumulation and degeneration of liver paranchyma and hepatocytes (Maradonna *et al.,* 2014). Exposure of 1000µg/l BPA to *Oreochromis mossambicus* for 10 and 20 days resulted in large amount of hepatocyte vacuolization (Chitra and Maiby, 2014).

Vitellogenin (vtg) is the precursor of egg yolk and used as a biomarker for estrogenic endocrine disruption (Matozzo *et al.,* 2008). Vitellogenin is synthesized in female fish under the influence of endogenous estrogenic hormones. Increased mRNA level of *vitellogenin* or presence of vitellogenin protein in serum of male and juvenile fish or abnormal level of vitellogenin in female fish, out of reproductive season, is the sign of endocrine disruption. Increased level of hepatic *vtg* mRNA in female fish suggests BPA has estrogenic activity in *Catla catla*. Results reported here are in accordance with the recent study where administration of 1-100µg/g BPA to immature yellowfin seabream resulted in increased hepatosomatoc index and serum vitellogenin (Negintaji *et al.,* 2018). Increased vitellogenin level was also reported in *Cyprinus carpio* exposed to similar chemical and experimental conditions (Mandich *et al.,* 2007). Significant induction of vitellogenin after BPA exposure was earlier reported in different species of fish (Sohoni *et al*., 2001; Ishibashi *et al.,* 2005; Lee *et al.,* 2007; Mandich *et al.,* 2007; Faheem *et al.,* 2017c; Negintaji *et al.,* 2018). All these studies reported that BPA is capable of vtg induction; however difference among studies can be due to difference in fish species used as model, species-specific estrogen receptor binding, water temperature and exposure time (Crain *et al.,* 2007; Faheem *et al.,* 2017c).

Present study indicate that bisphenol-A at environmentally relevant concentration is capable of inducing *vtg* mRNA and damage vital organs of fish leading to altered level of enzymes that can cause potential harm to fish health and reproduction. If such fish with heavy load of BPA are consumed regularly by humans also can create similar problems to their health.

Authors contribution: The study was supervised by KPL. All experiments were performed by MF and real time was performed by MF and SK. MF prepared the manuscript.

REFERENCES

Abdel-Tawwab M and Hamed HS, 2018. Effect of bisphenol A toxicity on growth performance, biochemical variables and oxidative stress biomarkers of Nile tilapia, *Oreochromis niloticus* (L.). J Appl Ichthyol pp:1-9.

Adedeji OB, Adeyemo OK and Agbede SA, 2009. Effects of diazinon on blood parameters in the African catfish (*Clarias gariepinus*). Afr J Biotechnol 8:3940-6.

Bhattacharya H, Xiao Q and Lun L, 2008. Toxicity studies of nonylphenol on rosy barb (*Puntius conchonious*): A biochemical and histopathological evaluation. Tissue Cell 40:243-9.

Chitra KC and Maiby S, 2014. Oxidative stress of Bisphenol-A and its adverse effects on liver of fresh water fish *Oreochromis Mossambicus.* Int J Sci Res 3:221-4.

Coppo JA, Mussart NB and Fioranelli SA, 2016. Physiological variation of enzymatic activities in blood of bullfrog, *Rana catesbeiana* (Shaw, 1802). Revista Vet 12:22-7.

Corrales J, Kristofco LA, Baylor SW, *et al.,* 2015. Global assessment of bisphenol A in the environment: Review and analysis of its occurrence and bioaccumulation. Dose Response 13:1-29.

Crain DA, Eriksen M, Iguchi T, *et al.,* 2007. An ecological assessment of bisphenol- A: evidence from comparative biology. Reprod Toxicol 24: 225-39.

Faheem M and Lone KP, 2017. Oxidative stress and histopathologic biomarkers of exposure to bisphenol-A in the freshwater fish, *Ctenopharyngodon idella.* Braz J Pharm Sci 53:e17003.

Faheem M, Jahan N and Lone KP, 2016. Histopathological effects of bisphenol-A on liver, kidneys and gills of Indian major carp, *Catla catla* (hamilton, 1822). J Anim Plant Sci 26: 514-22.

Faheem M, Jahan N, Khaliq S, *et al*., 2018a. Validation of reference genes for expression analysis in a teleost fish (*Catla catla* Hamilton) exposed to an endocrine disrupting chemical, bisphenol A. Rendiconti Lincei 29:13-22.

Faheem M, Jahan N, Khaliq S, *et al*., 2018b. Modulation of brain kisspeptin expression after bisphenol-A exposure in a teleost fish, *Catla catla.* Fish Physiol Biochem

Faheem M, Khaliq S and Lone KP, 2017a. Disruption of the reproductive axis in freshwater fish, *Catla catla*, after bisphenol-A exposure. Zool Sci 34:438-44.

Faheem M, Khaliq S and Lone KP, 2017c. Non-Monotonic Endocrine-Disrupting Effects of Bisphenol-A on Vitellogenin Expression in Juvenile Freshwater Cyprinid, *Catla catla.* Pak J Zool 49: 1531-4.

Faheem M, Khaliq S, Ahmad HU, *et al*., 2017b. Bisphenol-A (BPA) alters plasma thyroid hormones and sex steroids in female Pakistani major carp (*Catla catla; Cyprinidae*). Pak Vet J 37:326-30.

Flint S, Markle T, Thompson S, *et al*., 2012. Bisphenol A exposure, effects, and policy: A wildlife perspective. J Environ Manage 104:19-34.

Hadi AA, Shokr A and Alwan S, 2009. Effects of aluminum on the bio-chemical parameters of fresh water fish *Tilapia zillii.* J Sci Appl 3:33-41.

Hamed HS and Abdel-Tawwab M, 2017. Ameliorative effect of propolis supplementation on alleviating bisphenol-A toxicity: Growth performance, biochemical variables and oxidative stress biomarkers of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. Comp Biochem Physiol 202:63-9.

Ishibashi H, Watanabe N, Matsumura N, *et al*., 2005. Toxicity to early life stages and an estrogenic effect of a bisphenol A metabolite, 4-methyl-2, 4-bis (4- hydroxyphenyl) pent-1-ene on the medaka (*Oryzias latipes*). Life Sci 77:2643-55.

Lee CM, Park M, Kim H-M, *et al*., 2007. The expression patterns of estrogen responsive genes by bisphenol A in the wild Medaka (*Oryzias sinensis*). Mol Cell Toxicol 3:185-9.

Livak KJ and Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCt method. Methods 25:402-8.

Mandich A, Bottero S, Benfenati E, *et al.,* 2007. In vivo exposure of carp to graded concentrations of bisphenol A. Gen Comp Endocrinol 153:15-24.

Maradonna F, Nozzi V, Valle DL, *et al.,* 2014. A developmental hepatotoxicity study of dietary bisphenol A in *Sparus aurata* juveniles. Comp Biochem Physiol 166:1-13.

Matozzo V, Gagne F, Marin MG, *et al*., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. Environ Int 34:531-45.

Mekkawy IAA, Mahmoud UM, Wassif ET, *et al*., 2011. Effects of cadmium on some haematological and biochemical characteristics of *Oreochromis niloticus* (Linnaeus, 1758) dietary supplemented with tomato paste and vitamin E. Fish Physiol Biochem 37:71-84.

Mihaich E, Rhodes J, Wolf J, *et al*., 2012. Adult fathead minnow, *Pimephales promelas*, partial life-cycle reproductive and gonadal histopathology study with bisphenol A. Environ Toxicol Chem 31:2525-35.

Negintaji A, Safahieh A, Zolgharnein H, *et al*., 2018. Short-term induction of vitellogenesis in the immature male yellowfin seabream (*Acanthopagrus latus*) exposed to bisphenol A and 17 β-estradiol. Toxicol Ind Health 34:119-27.

Ololade IA and Oginni O, 2010. Toxic stress and hematological effects of nickel on African catfish, *Clarias gariepinus*, fingerlings. J Environ Chem Ecotoxicol 2:14-9.

Organization for Economic Cooperation and Development. 1992, Fish acute toxicity test. Test Guideline 203. OECD Guidelines for the Testing of Chemicals. Paris, France.

Roberts RJ, 1978. Fish pathology. Bailliere tindall London, pp:489.

Sayed AE-H and Hamed HS, 2017. Induction of apoptosis and DNA damage by 4-nonylphenol in African catfish (*Clarias gariepinus*) and the antioxidant role of *Cydonia oblonga.* Ecotoxicol Environ Saf 139:96-101.

Sayed AE-H, Mekkawy IA and Mahmoud UM, 2012. Histopathological alterations in some body organs of adult *Clarias gariepinus* (Burchell, 1822) exposed to 4-nonylphenol, zoology. In: Garcia MD (ed) zoology. InTech, West Palm Beach pp:163-84.

Sayed AE-H, Mekkawy IAA and Mahmoud UM, 2011. Effects of 4-nonylphenol on metabolic enzymes, some ions and biochemical blood parameters of the African catfish *Clarias gariepinus* (Burchell, 1822). Afr J Biochem Res 5:287-97.

Sohoni P, Tyler CR, Hurd K, *et al.,* 2001. Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). Environ Sci Techno 35:2917-25.

Suzuki K, Ishikawa K, Sugiyama K, *et al*., 2000. Content and release of bisphenol A from polycarbonate dental products. Dent Mat J 19: 389-95.