Original Research Article

Effects of PAH mixtures on haematological, hepatic and oxidative stress biomarkers in *Clarias gariepinus.*

ABSTRACT

Haematological, hepatic and oxidative stress parameters in juvenile *Clarias gariepinus* exposed to a combined ternary mixture of naphthalene, phenanthrene and benzo[a]pyrene were investigated under static-renewal bioassay conditions. The test organisms were exposed to two levels of treatments of the mixed compounds for a period of 35 days. Red blood cell (RBC) count, haemoglobin (Hb) concentration and haematocrit (Hct) were significantly lowered in exposed fish compared to the control. Significant decreases were also observed in mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). White blood cell (WBC) and platelet (PLT) counts were significantly raised in exposed organisms compared to the control. There were significant reductions in total plasma protein and bilirubin. While increases in superoxide dismutase (SOD) and catalase (CAT) were significant, that of glutahthione s-tranferase (GST) was not significant.

Key words: biomarkers, naphthalene, phenathrene, benzo[a]pyrene, haematological, hepatic, oxidative stress

INTRODUCTION

Risk assessment is usually performed on the toxicity of single compoundsrather than mixtures [1]. However, chemicals often appearas complex mixtures in the environment. One group of such chemicals that occur as complex mixtures are polycyclic aromatic hydrocarbons (PAHs). PAHs which are organic in origin are ubiquitous environmental pollutants. Some PAH compounds have been classified as mutagens, teratogens and carcinogens by the U.S. Environmental Protection Agency [2].

Naphthalene a widely distributed PAH in air and aquatic environments forms a major constituent of petroleum fuels [3]. Anthropogenic activity has been implicated in the abundance and distribution of naphthalene and is regarded as a major contributor of the aquatic environment [4]. Phenanthrene is one of the most abundant PAH compounds in the aquatic environment. The United States Environmental Protection Agency (USEPA) regards phenanthrene as among the 16 PAHs on its priority pollutant list [5]. Benzo[a]pyrene is a five-ring PAH that is synthesized by the incomplete combustion of organic compounds. In recent times, many authors have confirmed it as possessing carcinogenic properties [6].

Wildlife exposure to PAHs through natural and anthropogenic activities can produce a variety of biochemical and physiological effect. These responses termed biomarkers serve as indicators of biotic stress in exposed organisms [7]. Biomarkers can be defined as indicators in biological systems or samples ofmeasurable changes at the molecular, biochemical, cellular, physiological or behavioral levels in response to foreign compounds.Utilization of

biomarkers in ecotoxicology serves as early warning signals that appear before measurable effects on population dynamics occur [8].

There is limited data onthe toxicity of joint PAH compounds in aquatic organisms [9]. Therefore, the need arises to conduct and document toxicological effects ofthese mixed compounds on experimental organisms in order to establish appropriate guidelinesfor environmental and health risk assessments. *Clarias gariepinus* was model organism of choice, due its ecological [10] and commercialimportance in Nigeria [11].

The aim of this study was to investigate the effects the combined mixture of naphthalene, benzo[a]pyrene and phenanthrene on haematological, hepatic and oxidative stress indices in Clarias gariepinus. These indices could serve as biomarkers at the biochemical and molecular levels

2. MATERIALS AND METHODS

2.1 Reagents

Benzo[a]pyrene was purchased from Sigma Aldrich (Germany). Naphthalene and acetone were purchased from BDH chemicals (UK). Phenanthrene was purchased from EGA Chemie KG (Germany).

2.2 Experimental animals

Juvenile *C. gariepinus* (n = 90) weighing 19.7±1.8 g were obtained from a commercial fish farm in Aba, Nigeria. Fish were acclimatized for 14 days in tap water prior to experimentation and fed twice daily *ad libitum* with commercial fish feed. Fecal matter and excessive feed was removed daily to prevent contamination of the water and growth of mould.

2.3 Sublethal toxicity tests

Stock solutions of the three PAHs were prepared by dissolving each PAH in distilled water with acetone as solvent carrier. Test solutions were prepared by dilution of stock solutions in tap water. The acute LC_{50} values of the three PAHs were determined in a pilot study using a semi-static method [12]. The 96-h LC_{50} values were 1400 µg/L for phenanthrene, 6600 µg/L for naphthalene and 16 µg/L for benzo[a]pyrene. During sublethal studies, fish were exposed to combined mixtures of 1/8 and 1/4 of the LC_{50}corresponding to treatment levels 1 (T1) and 2 (T2) of the respective PAHs. A solvent control was included in the experimental design. Fish were kept in groups of 10 in 30L plastic tanks containing the test solutions. Experiments were performed in triplicates. Period of exposure lasted 35 days

2.4Assays

At the end of the exposure period, fish were anaesthetized by hypothermia. Blood was collected from the immobilized fish by caudal vein puncture method as described by Argungu*et al.* [13] using a 5ml sterile disposable syringe with a 22 gauge needle. The blood was transferred to EDTA tubes and transported to the lab for analysis.

2.4.1 Haematology

Haematological parameters were determined using automated haematology analyzer machine (Mindray BC 2300, USA).

2.4.2 Total protein and albumin

Total protein concentration was determined according to the calorimetric method of Lowry *et al*. [14]. Albumin was determined by the method of Dumas [15]. Total bilirubin was determined as described by Jendrassik and Grof [16]. Both parameters were expressed in g/L.

2.4.3 Oxidative stress enzymes

The method of Misra and Fridovich [17], was used to determine plasma superoxide dismutase (SOD) activity (U/mL) by measuring the inhibition of autooxidation of adrenaline at pH 10.2 and 30 °C. Catalase activity (kU/L) was determined according to the method of Sinha [18] by measuring the reduction of dichromate in acetic acid to chromic acetate at 570 nm. Glutathione S-transferase (GST) activity (U/L) was determined by the method described by Habig*et al*. [19] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.

2.5 Statistical analysis

Results are expressed as mean ± standard error. Data from the different treatment groups were compared by a one-way analysis of variance (ANOVA) followed by a Scheffes test to determine statistically different groups. All differences were considered significant at $p < 0.05$. Statistical analysis was performed using Microsoft Excel.

3. RESULTS

Table 1 shows the results for the haematological parameters. The results shows that ternary mixtures of PAH had negative impact on the studied parameters. There were statistical significant ($p < 0.05$) decreases in RBC count, Hb concentrations and Hct compared to the control. Observed reductions in the red blood cell indices were also statistically significant ($p < 0.05$) with mean corpuscular haemoglobin (MCH) showing a concentration dependant decrease pattern.

The effect of the ternary mixture on plasma total protein and albumin is shown in figure 1. There was a concentration dependant decrease in both total protein and albumin. The observed decreases were statistically significant.

There was a statistically significant ($p < 0.05$) increase in bilirubin of exposed fish compared with the solvent control.

The result for the oxidative stress enzymes is shown in figures 3 to 5. There were increases in the activities of all three enzymes. However while the increases in the activities of SOD and CAT were statistically significant ($p < 0.05$), the increase in activity of GST was not statistically significant.

Table 1: Haematological parameters of *C. gariepinus* exposed to sublethal concentrations of PAH mixtures. Means not sharing the same letter (a, b or c) are statistically different at $p < 0.05$

Parameter	Control group	Ternary mixture	concentration
RBC (x10^{9}cell/L)	2.41±0.06^a	2.3±0.14^a	2.09±0.18^b
Hb (g/L)	102.66±3.71^a	94±4.0^a	93±2.9^b
Hct (%)	34.84±2.38^a	29.6±0.5^b	22.4±0.6^c
MCV (fL)	147.5±2.5^a	141.3±1.2^b	140.6±1.95^b
MCH (pg)	43.3±0.24^a	41.16±1.15^b	39.1±1.11^c
MCHC (g/L)	305.33±4.4^a	279.75±2.2^b	279±1.1^b
WBC (x10^{9}cell/L)	135.56±2.98^a	147.67±3.22^b	155.15±1.05^c
PLT (x10^{9}cell/L)	12.66±1.33^a	17±2.2^b	17.25±2.2^b

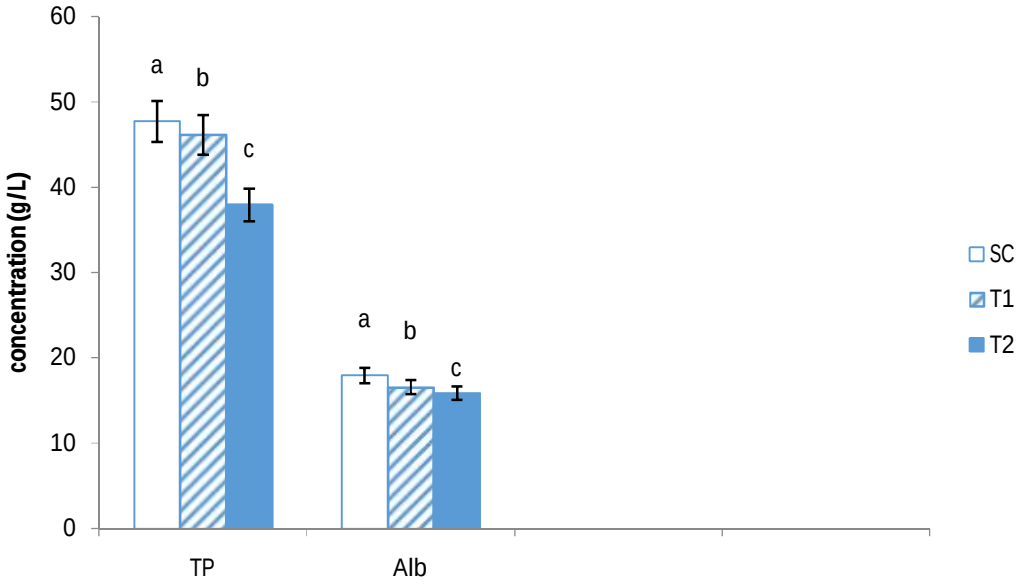

Figure 1: Total protein and albumin concentrations in fish exposed to ternary PAH compounds. Means not sharing the same letter (a, b or c) are statistically different at $p < 0.05$

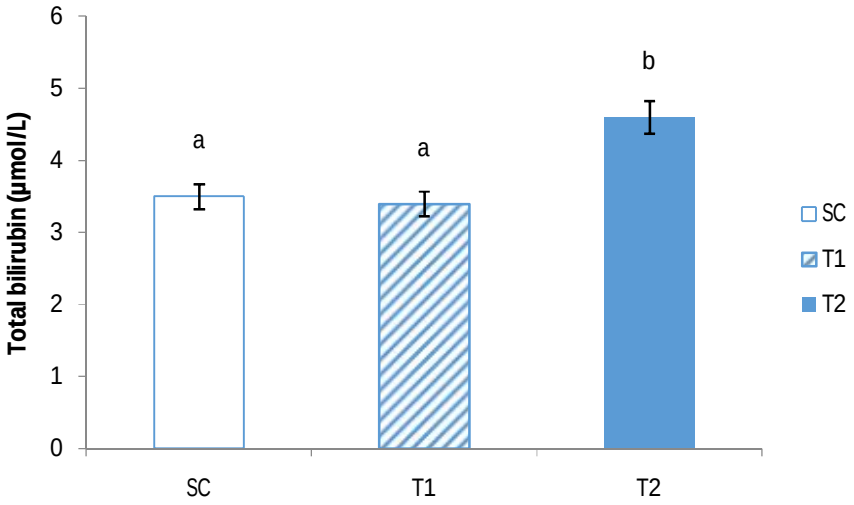

Figure 2: Bilirubin concentration in fish exposed to ternary PAH mixtures. Means not sharing the same letter (a or b) are statistically different at $p < 0.05$

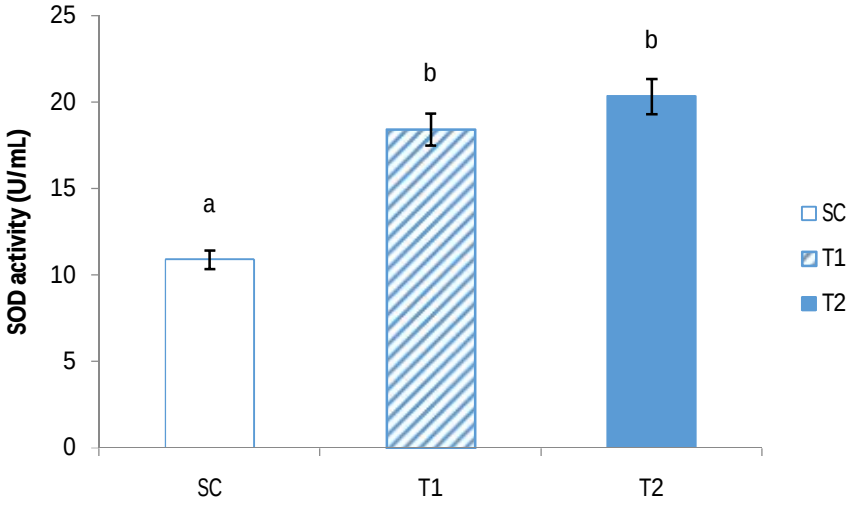

Figure 3: SOD activity in fish exposed to ternary PAH mixtures. Means not sharing the same letter (a or b) are statistically different at $p < 0.05$

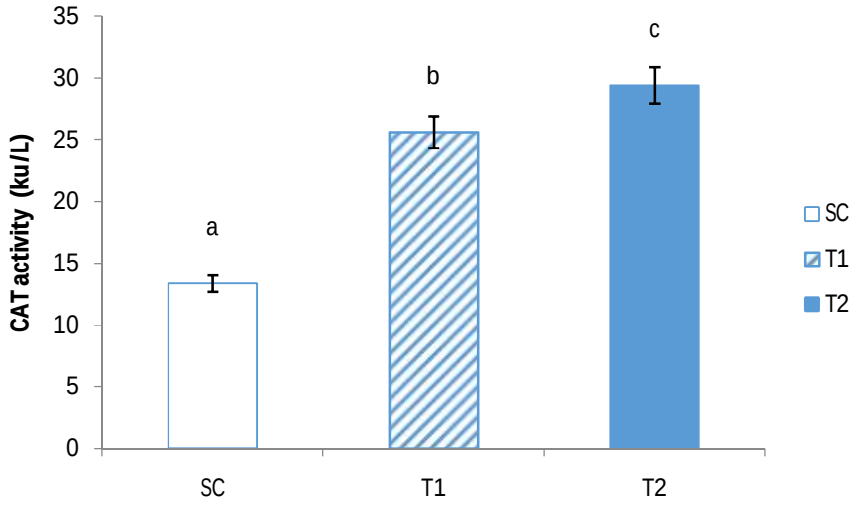

Figure 4: CAT activity in fish exposed to ternary PAH mixture. Means not sharing the same letter (a, b or c) are statistically different at $p < 0.05$

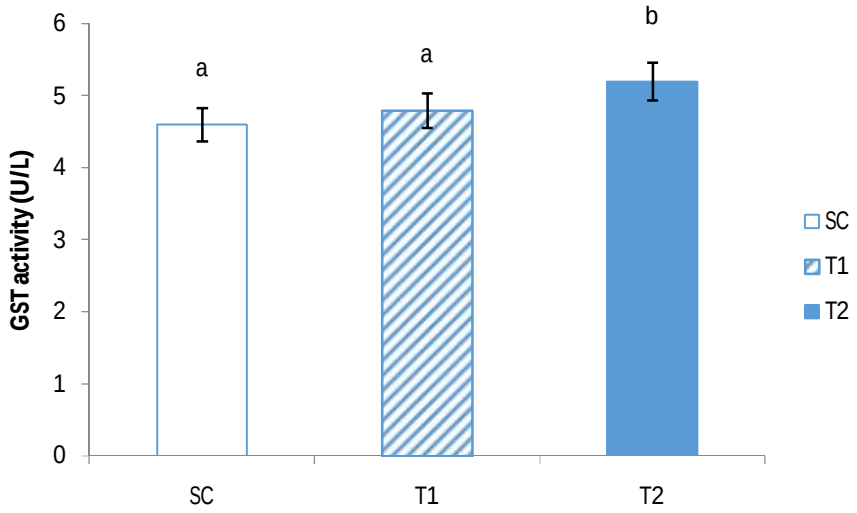

Figure 5: GST activity in fish exposed to ternary PAH mixtures.Means not sharing the same letter (a or b) are statistically different at $p < 0.05$

4. DISCUSSION

In the biomarker studies of fish to toxicants, finding early physiological signs is of important concern. Haematological parameters are suitable biomarkers for evaluating the potential risk of xenobiotics [20]. Many researchers have also identified changes in haematological parameters as bioindicators of toxicants [20]. In the present study mixed PAH compounds led to statistically significant ($p < 0.05$) declines in the studied haematological parameters.

The capacity of toxic chemicals to induce anaemia is well understood. Dey*et al.* [21]reported in a recent study that *A. testudineus* on exposure to varying concentrations of naphthalene exhibited a significant reduction in RBC count in comparison to the control. Usually, a decrease in RBC count is indicative ofpoor iron metabolism [22]. In an earlier work, the authors also revealed a similar trend with *A. testudineus* exposed to anthracene [21].

As with RBC count, many authors have demonstrated decreases in haemoglobin concentration of fishes exposed to toxic chemicals. Haemoglobin which is a conjugated protein containsheme as prosthetic group and globin as the apoprotein [23]. Haemoglobin concentration can serve as a sensitive bioindicator of alterations in challenging ecological conditions.Chavez-Veintemilla [24]showed that haemoglobin concentration in the tambaqui exposed to phenanthrene was the most affected blood parameter in the fish. The decrease in haemoglobin concentration has been linked to a reduction in its synthesis or by increases in its destruction. Mehrnaz*et al.* [25]also reported significant reductions in hemoglobincontent of *A.latus* exposed to mixtures of polycyclic aromatic hydrocarbons.

In addition to altering erythrocyte count and haemoglobin levels, toxic chemicals have been shown to affect haematocrit.Dey*et al.* [21] reported a significant decreasesin haematocrit of*A. testudineus*on exposure to naphthalene. Decreases in haematocrit (which measures the volume of packed red blood cells relative to the whole blood) can be caused by the deformation or inhibition in the synthesis of red blood cells [26].

The observed increase white blood cell count in exposed fish points strongly to the toxicity of PAHs to the immune system. White blood cells which are part of the immune system participate in both the innate and humoral immune responses. These cells circulate in the blood and mnobilized in response to toxicants, injury or pathogens [27]. Ramesh and Saravanan [28]have reported increases in white blood cell count of *L. rohitato*on exposure to the insecticide, deltamethrin.

Platelets which are cytoplasmic fragments derived from megakaryocytes [29] play crucial roles in the inflammatory process. Under challenging conditions that cause physical injuries to vascular tissues, platelet counts are increased to ameliorate haemorrhage [21].

Total bilirubin consists of unconjugated and conjugated bilirubin. Increased total bilirubin level is indicative of metabolic problems in the liver such as reduced hepatocyte uptake or impaired bilirubin conjugation [30].Nwamba*et al.* [31] demonstratedincreased levels of bilirubin in fish exposed to crude oil as that seen in the present study. The authors observed that total bilirubin concentrations increased with increasing concentration of crude oil.

Total protein (including albumin) is recognized as an excellent indicator to investigate fish health, and alterations in blood protein concentrations are crucial in identifying PAH intoxication [21]. The observed decrease in total protein and albumin in the present study suggests cellular injury thatoccured in the liver of the fish exposed to the PAH mixture because the liver is the major site of protein synthesis in organisms [32]. Khan *et al*. [36] reported significant reductions in both the total protein and plasma albumin levels in grass carp exposed to atrazine under both acute and subchronic conditions. The finding is in agreement with results obtained in the present study.

Oxidative stress manifests when there is an imbalance between the generation of reactive oxygen species (ROS) and the capacity of the biological system to mop up the oxidizing agents [33]. In the present study, PAH mixtures were found to induce the expression of the antioxidant enzyme systems. Jiffa*et al*. [34]have reported significant elevation in SOD and CAT activities in *L. japonix*exposed to benzo[a]pyrene. Palanikumar *et al*. [35] also reported increases in GST activity in fish exposed to benzo[a]pyrene. These findings are in concordance with the results obtained in the present study.

5. CONCLUSION

Joint mixtures of PAHs adversely affected the general health of *C. gariepinus.* There were observed alterations in the observed haematological, hepatic and oxidative stress parameters. These parameters could serve as bio-indicators of fish in challenging ecological situations. There is need to carry out further studies especially at the genetic level to study the real time effect of PAHs on gene expression.

ETHICAL APPROVAL

Animal ethics committee approval was obtained before the commencement of experiments.

References

1. Qian L, Zhang J, Chen X, Qi S, Wu P, Wang C. Toxic effects of boscalidin adult zebrafish (*Daniorerio*) on carbohydrate and lipid metabolism. Environ.Pollut.2019; 247: 775–782.

2. USEPA (United States Environmental Protection Agency). PAHs: Integrated risk information system. 2011. Accessed 1 December, 2019.
Available
http://www.epa.gov/IRIS.

3. Honda M, Suzuki N. Toxicities of Polycyclic Aromatic Hydrocarbons for Aquatic Animals. Int. J. Environ. Res. Public Health. 2020;17(4): 1363.

4. Alderman SL, Dilkumar CM, Avey SR, Farrell AP, KennedyCJ, Gillis TE.Effects of diluted bitumen exposure and recovery on the seawater acclimation response of Atlantic salmon smolts.Aquat.Toxicol.2020; 221: 105 – 109.

5. Sinaei M, MashinchianA. Polycyclic aromatic hydrocarbons in the coastal sea water, the surface sediment and Mudskipper Boleophthalmusdussumieri from coastal areas of the Persian Gulf: Source investigation, composition pattern and spatial distribution. J. Environ. Health Sci. Eng. 2014;12(1): 59-60.

6. Derakhshesh, N., Negin, S., AbdolAli, M. and Mahmoud H. Exposure of liver cell culture from the orange-spotted grouper, *Epinepheluscoioides*, to benzo[a]pyrene and light results in oxidative damage as measured by antioxidant enzymes. Chemosphere.2019;226: 534 – 544.

7. Heintz RA. Chronic exposure to polynuclear aromatichydrocarbons in natal habitats leads to decreased equilibriumsize, growth, and stability of Pink Salmon populations. Integr.Environ. Assess. Manag.2017;3: 351–363.

8. Munoz I, Sabater S. Integrating chemical and biological status assessment: assembling lines of evidence for theevaluation of river ecosystem risk. ActaBiolo.Colomb.2014; 190: 25–34.

9. WHO (World Health Organisation). WHO Guidelines for indoor air quality: selected pollutants. Europe:WHO; 2010.

11. EsenowoIK, Ugwumba OA. Growth response of catfish(*Clarias gariepinus*) exposed to water-soluble fraction ofdetergent and diesel oil. Environ. Res. J. 2010;4: 298–301.

10. Farombi EO, Adelowo OA, Ajimoko YR. Biomarkers of oxidative stressand heavy metal levelsas indicators of environmental pollution in African catfish Clarias gariepinus from Nigeria Ogunriver. Int. J. Environ.Res. Public Health. 2007; 4(2): 158 – 165.

12. OECD. Test No. 203: Fish acute toxicity test, OECD guidelines for the testing of chemicals, Section 2., Paris: OECD Publishing; 2019.

13. Argungu LA, Siraj SS, Christanus A. A simple and rapid method for blood collection from walking catfish.Iranian J. Fish. S. 2015; 16(3): 934 – 944.

14. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein determination with the Folin phenol reagent. J. Biol. Chem. 1951; 193: 265 – 275.

15. Dumas BT. Diagnostic reagent kit for *in vitro* determination of total protein and albumin in serum. Clin.Chim.Acta.1971; 31: 87 – 96.

16. Jendrassik L,Grof P. Simplified photometric methods for the determination of bilirubin. Biochem. J. 1938; 297: 81-89.

17. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase.J. Biol. Chem. 1972;247: 3170 – 3175.

18. Sinha AK. Colorimetric assay of catalase.Anal.Biochem.1997; 47: 389–394.

19. Habig WH, Pabst MJ, Jakoby WB. Glutathione transferases, the first enzymatic step in mercapturic acid formation.J. Biol. Chem. 1974; 249: 7130–7139.

20. Jee JH, Kim SG, Kang JC. Effects of phenanthrene on growth and basic physiological functions of the olive flounder *Paralichthysolivaceus*. J. Exp. Mar. Biol. Ecol. 2004; 304(2004): 123 – 136.

21. Dey S, Palas S, Niladri S. Dose specific responses of *Anabas testudineus* (Bloch) to anthracene (PAH): Haematological and biochemical manifestation. EmergContam.2019; 5: 232 – 239.

22. Chowdhury MJ,Mcdonald, DG, Wood CM. Gastrointestinal uptake and fate of cadmium in rainbow trout acclimated to sublethal dietary cadmium. AquatToxicol. 2004; 69: 149 − 163.

23. Chhabra N. Biochemistry for Medics. New Delhi: Jaypee Brothers; 2013.

24. Chávez-Veintemilla C, Val A. Effects of phenanthrene on the amazonian fish Tambaqui*Colossomamacropomum*: LC_{50}, growth and haematology. Environ. Ecol. Res.2019; 7(5): 293 – 302.

25. Mehrnaz S, Negin S, Mohammad TR. Effect of phenanthrene on the tissue structure of liver and aminotransferase enzymes in YellowfinSeabream (*Acanthopagruslatus*) Iran. J. Toxicol. 2017; 11(4): 201 – 207.

26. Eriegha OJ, Omitoyin BO, Jani EK.Evaluation of Haematological and Biochemical Parameters of Juvenile *Oreochromisniloticus*after Exposure to Water Soluble Fractions of Crude Oil.J. Appl. Sci. Environ. Manage. 2017; 21(6): 1041 – 1045.

27. Tigner A. Sherif A, Ian M. Histology, White Blood Cell. 2021.Accessed 11August, 2021. Available
https://www.ncbi.nlm.nih.gov/books/NBK563148/

28. Ramesh M, Saravanan M. Haematological and biochemical responses in a fresh water fish *Cyprinuscarpio* exposed to chorpyrifos. Int. J. Integr. Biol.2008; 3(1): 80 – 84.

29. Valli VEO. Hematopoietic system. In: Maxie, MG. (Ed.), Kennedy and Palmer's Pathology of Domestic Animals. St. Louis, MO: Saunders/Elsevier; 2007.

30. Greer JP.Wintrobe's Clinical Hematology. ESP: Wolters Kluwer Health Adis; 2014.

31. Nwamba HO, Achikanu CE, Onyekwelu KC. Effect of crude oil and its products on bilirubin of african catfish *Clarias gariepinus*. Anim. Res. Int. 2006; 3 (3): 531 – 533.

32. Kori-Siakpere O, Ubogu EO.Sublethal haematological effects of zinc on the freshwater fish, *Heteroclarias sp*. Afr.J. Biotechnol. 2008;7: 2068 – 2073.

33. Sawyer DB, Siwik DA, Xiao L, Pimental D. Role of oxidative stressin myocardial hypertrophy and failure. J. Mol. Cell.Cardiol.2002; 34(4): 379 – 388.

34. Jifaa W, Yu Zhiminga Y, Xiuxiana, S, Youa W. Response of integrated biomarkers of fish (*Lateolabraxjaponicus*) exposed to benzo[a]pyrene and sodium dodecylbenzenesulfonate.Ecotoxicol, Environ. Saf.2006; 65(2006): 230–236.

35. Palanikumar N, Kumaraguru CM, RamakritinanM.Biochemical response of anthracene and benzo[a]pyrene in milkfish *Chanos chanos* L. Ecotoxicol. Environ. Saf. 2011; 75: 187–197.

36. Khan AA, Muhammad MS, Munawar SA. Quantitative determination of lethal concentration LC_{50} of atrazine on biochemical parameters; total protein and serum albumin of freshwater fish Grass Carp (*Ctenopharyngodonidella*). Pol. J. Environ. Stud.2016; 25(4): 1555–1561.