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Sublethal Effects of Ternary Mixtures of Polycyclic Aromatic Hydrocarbons on Selected Liver Biomarkers of *Clarias gariepinus*

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ABSTRACT: The effect of ternary mixtures of polycyclic aromatic hydrocarbons in *Clarias gariepinus* was investigated. Apparently healthy juvenile fish (n = 90) weighing 19.7±1.8 g were exposed to sublethal concentrations of naphthalene, phenathrene and benzo[a]pyrene over a period of 35 days after which liver biomarker analyses were carried out on blood plasma fraction. The observed total plasma protein was 35.1 g/L while the albumin and total bilirubin concentrations were 15.9 g/L and 4.6 µmol/L respectively. The plasma concentrations of the liver enzymes were 25.27 IU/L, 9.37 IU/L, and 28.01 IU/L for alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) respectively. While there were significant declines in plasma total protein and albumin, significant elevation was observed in plasma total bilirubin. Significant increases were also observed in the activities of the liver enzymes, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). Exposed liver and gill sections showed histopathological alteration. There was steatosis accompanied with moderate dense infiltrates in the liver while the gills showed channel dilatations with hyperplasia. Findings from this study suggest that PAH mixtures cause changes in the activities of liver metabolic enzymes as well as alter the normal architecture of liver and gills in exposed aquatic organisms.

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Polycyclic aromatic hydrocarbons (PAHs) are a collection of several hundred carbon-based compounds which contain two or more benzene rings. These compounds are found mainly as complex mixtures. They rarely occur as single compounds (Byeong-Kyu, 2010). PAHs are primarily found in natural sources such as creosote and petrochemicals and also as by-products as incomplete combustion of organic matter (Sorenson and Wichert, 2018). PAHs are ubiquitous in the environment and can be formed from either natural or anthropogenic activities (Sorenson and Wichert, 2018). The dominant sources of PAHs in the environment are from human activity: wood-burning, combustion of fossil and biofuels, mining activities etc. The usual method of risk assessment and management of PAHs has been to examine the effects of single PAH compounds (Altenburger and Greco, 2009). It therefore becomes pertinent to assess the impact of not just single compound contamination but also contamination caused as a result of mixture of these compounds. Due to the rather infinite number of mixture-combinations, and the many different targeted organisms, it is not possible to investigate every individual case. It is therefore of biochemical importance to have models that can assess the toxicity of these compounds. The objectives of this work were to evaluate the effect of PAH mixtures on some liver parameters of the tropical catfish as well as study the possible histopathological alteration in liver and gills of exposed fish.

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MATERIALS AND METHODS

Chemicals: All reagents used were of analytical grade. Benzo[a]pyrene was obtained from Sigma Aldrich (Germany). Phenanthrene was purchased from EGA Chemie KG (Germany). Naphthalene and acetone were obtained from BDH chemicals (UK).

Animals: Juvenile catfish, *C. gariepinus* (n = 90) weighing 19.7±1.8 g were obtained from a commercial fish farm in Aba, South east Nigeria. The fish were acclimatized for 2 weeks in declorinated tap water prior to experimentation. The fish were fed twice daily *ad libitum* with commercial fish feed. Fecal matter and uneaten food were removed daily to prevent contamination of the water.

Sublethal toxicity tests: Stock solutions of the three PAHs were prepared by dissolving each PAH in distilled water taking 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 (OECD, 2019). The 96-h LC_{50} values were 6.6 mg/L for naphthalene, 1.4 mg/L for phenanthrene and 0.0016 mg/L for benzo[a]pyrene. During sublethal studies, fish were exposed to 1/8 and 1/4 of the LC_{50} (corresponding to treatment levels 1 and 2) 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.

Assays: At the end of the exposure period, fish were anaesthetized by means of hypothermia. Blood was then collected from the immobilized fish by caudal vein puncture method as described by Argungu *et al*. (2015) 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. Liver and gill tissues were dissected and place in 10% formal saline solution prior to histopathological investigation**.** Total protein concentration was determined according to the calorimetric method of Lowry et al. (1951). Albumin was determined by the method of Dumas et al (1971. Total bilirubin was determined as described by Jendrassik and Grof (1938). Liver enzymes, alkaline phosphatase (ALP), Alanine transaminase (ALT) and Aspartate transaminase (AST) were determined using standard test kits.

Statistical analysis: Results were expressed as mean ± standard error. Data from the different treatment groups were compared b 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 and the SPSS statistical package (ver. 24.0 SPSS Company, Chicago, IL, USA).

RESULTS AND DISCUSSION

Liver biomarkers were analysed via liver function tests. The effect of PAH ternary mixture on total blood protein is presented in figure 1. The PAH joint mixture significantly affected the total protein concentration. There was a decline ($p < 0.05$) in the total plasma protein concentration at the second level of treatment with the PAH mixtures. The effect of the PAH mixture on albumin are presented in figure 2. The results for albumin concentration closely mirrors that of the total protein. There was a decline ($p < 0.05$) in the albumin concentration at the second level of treatment with the ternary mixture. However the increase at the first level of treatment was not statistically significant. The effect of the ternary mixtures on total bilirubin are shown in figure 3.

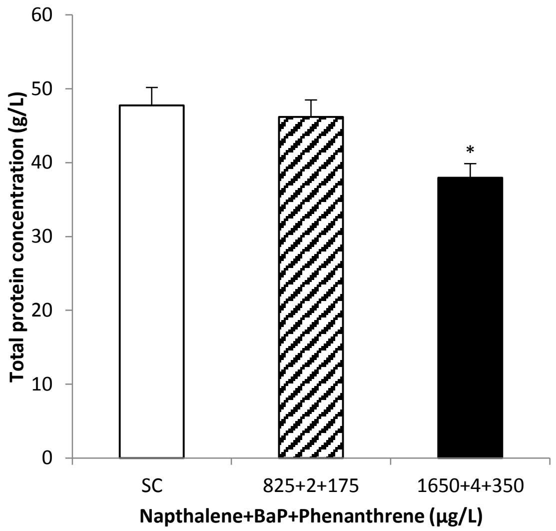

Fig 1. Total plasma protein concentration after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

The data shows that increasing concentrations of the ternary mixtures led to a significant increase ($p < 0.05$) in total bilirubin at the second level of treatment. The activities of the liver enzymes of the fish under investigation are shown in figures 4 to 6. The results show that increasing levels of the ternary mixtures led to concomitant increases in the assayed liver enzymes. ALT and AST were significantly elevated ($p < 0.05$) at both levels of treatment. However, ALP only showed a significant increase at the second level of treatment. The histopathology results of the liver and gill are shown in plates 1 to 4. The liver and gill from the solvent control (plates 1 and 3 respectively) exhibit normal architecture. The liver of fish exposed to the second level of treatment (plate 2) showed extensive steatosis accompanied by very moderate dense infiltrates. The gills of exposed fish at the second level of treatment (plate 4) exhibited tissue hyperplasia with channel dilatation.

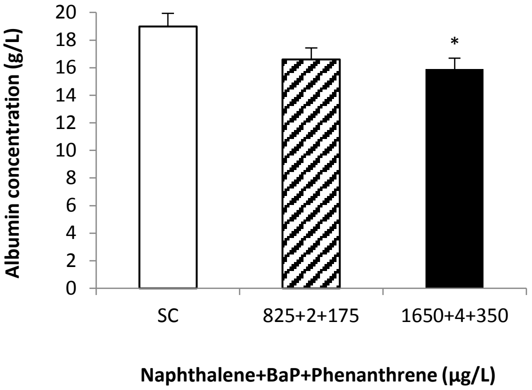

Fig 2. Albumin concentration after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

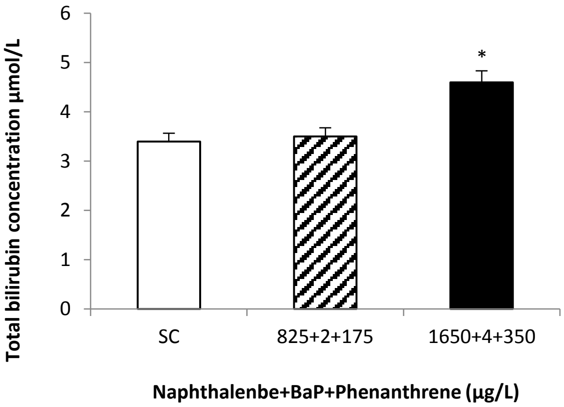

Fig 3. Total bilirubin concentration after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

The present work evaluated the effects of mixed compounds of naphthalene, phenanthrene and benzo[a]pyrene on selected liver and histopathological parameters of the tropical catfish. There was a significant decline ($p < 0.05$) in total plasma protein concentrations of fish exposed to the mixed PAH compounds. The obtained results suggest that PAHs had deleterious effect on the major protein manufacturing organ in the body, being the liver. Proteins form the structural part of most organs and make up enzymes and hormones that regulate body functions (ACBLM, 2020).

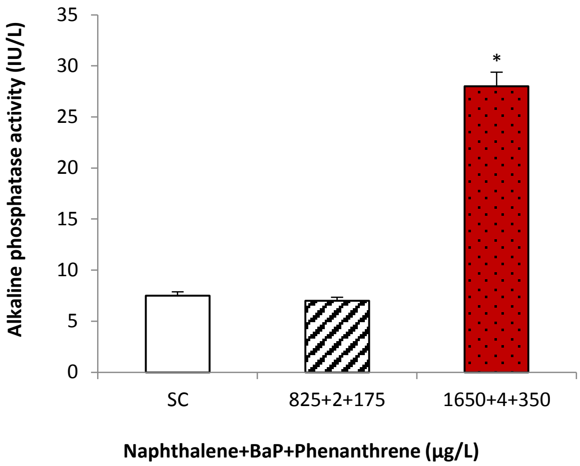

Fig 4. Alkaline phosphatase activity after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

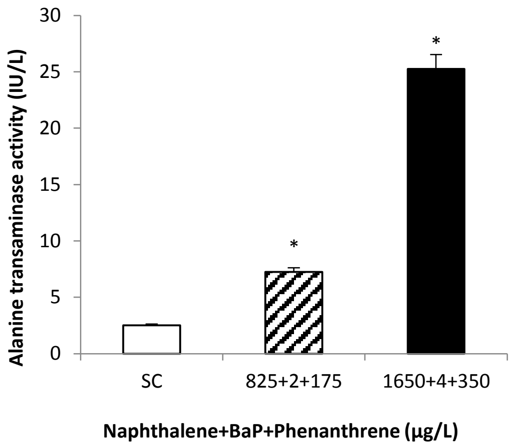

Fig 5. Alanine transaminase activity after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

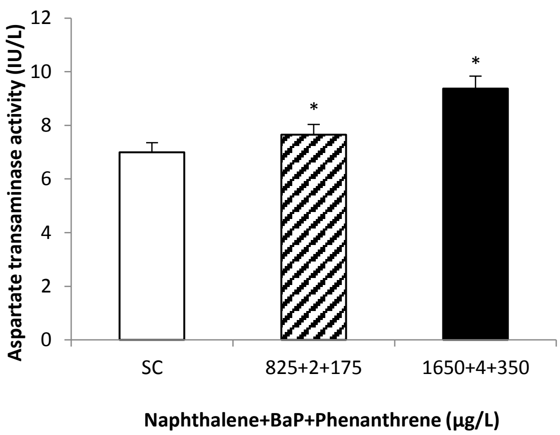

Fig 6. Aspartate transaminase activity after 35 days of exposure to ternary PAH mixture. SC = solvent control; data are expressed as mean±SE; asterisks indicate values that are significantly different from the control value ($p < 0.05$).

Blood protein is recognized as a very good indicator to assess general fish health. Variations in blood protein levels are utilized in investigating PAH contamination (Dey *et al*., 2020). The decrease in total protein in the present study suggests cellular injury that happened in the liver of the fish exposed to PAH toxicants because the liver is a major organ of protein synthesis in organisms (Kori-Sakpere and Ubogu, 2008). In addition, there is also the possibility of a kidney disorder, or a disorder in which protein is not degraded properly (ACBLM, 2020). Albumin, which is a major protein in blood, plays an important role in regulating plasma osmotic pressure and lipid transport. It has been that injury to the liver can affect plasma albumin levels and result in hypoalbuminemia (Farrugia, 2010). The decline in plasma albumin as observed in this study can be attributed to the repression of genes involved in synthesis of albumin (*Souza et al*., 2016). Several experiments have been conducted to show the effect of pollutants on albumin levels. Barisic *et al.* (2019) reported a decrease in serum albumin levels as a result of heavy metals exposure in *Onchorhynchus mykiss*. A similar decline in albumin was observed in response to heavy metals exposure in *Oreochromis niloticus* (Yacoub and Gad, 2012).

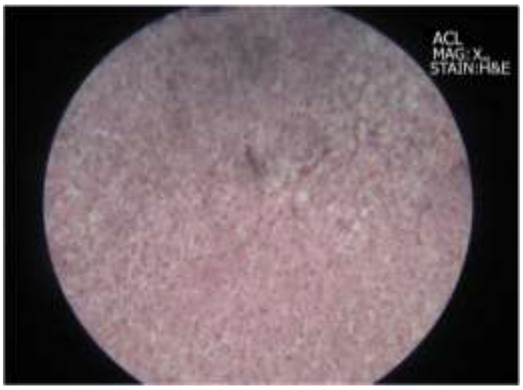

Plate 1: Photomicrograph of a section of the liver from the control group showing normal impression

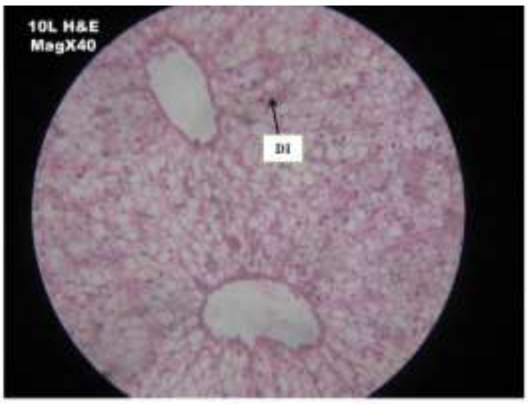

Plate 2: Photomicrograph of a section of the liver from the group exposed to naphthalene, BaP and phenanthrene mixture with steatosis accompanied with moderate dense infiltrates

Plate 3: Photomicrograph of a gill section from the control showing normal impression

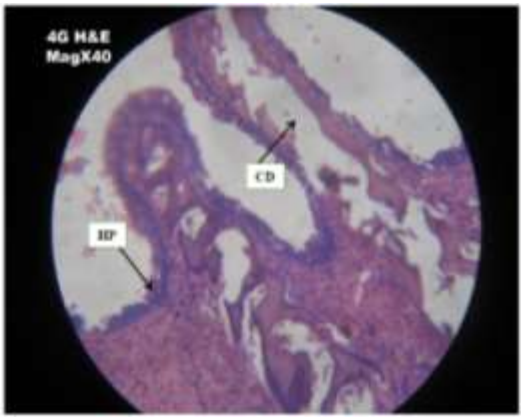

Plate 4: Photomicrograph of a section of the gill from the group exposed to naphthalene, BaP and phenanthrene mixture showing channel dilatation (CD) and hyperplasia (HP)

Khan et al. 2016 reported a markedly decline in both the total protein and serum albumin concentration during acute and chronic toxicity, when grass carp were exposed to atrazine. The findings with exposure to atrazine are in agreement with results obtained in the present study. Bilirubin a molecule that occurs in the catabolic pathway degrades heme in vertebrates. Total bilirubin consists of unconjugated and conjugated bilirubin. Increases in total bilirubin level could be indicative of metabolism problems in the liver such as disruption in bilirubin conjugation, or reduced bilirubin secretion (Greer, 2014). The significant increases in plasma total bilirubin reported in this study could be as a result of damage to the liver cells or increased destruction of erythrocytes. Other researchers have also observed similar findings with respect to total bilirubin levels as reported in the present work. Nwamba et al. (2006) reported increased levels of bilirubin in fishes exposed to crude oil. It was also observed that total bilirubin concentrations was dose-dependant crude oil concentrations. ALP. ALT and AST play essential roles in generating precursors for gluconeogenesis or cellular energy supply (Oner *et* *al*., 2009). ALP which is a hydrolytic enzyme is responsible for elimination of phosphate group from many types of molecules. An increase in ALP could be as a result of bile duct obstruction, consequently affecting the liver (Tamas *et al*., 2002). ALT is responsible for the transamination of alanine and is found at much higher concentrations in the liver compared with other organs (Ozer *et al*., 2008; Shi *et al*., 2010). AST is responsible for the transamination of aspartate. The results show that increasing levels of PAH treatments led to corresponding increases in activities of ALP, ALT and AST. These increases could represent liver damage and alteration in the permeability of the liver membrane, which caused the leakage of these enzymes to the blood stream (Jyothi et al., 2000; Yousef *et al*., 2006). Increases in ALP can be specifically attributed to internal and external hepatic obstruction of the biliary passage (Bachetta *et al*., 2001). Compelling evidence also points to the fact that simultaneous increases in ALT and AST activities of the exposed fish could be as a result of stimulatory effects of PAH on gluconeogesis. It is also possible that the observed elevations in both enzyme activities was as a result of cortisol stimulation, leading to amino acid interconversion for gluconeogenesis (Ronda *et al*., 2018). Histopathological changes are widely used as biomarkers to evaluate the health status of aquatic organisms exposed to toxicants. In the present study, sections of the liver tissues were observed to assess the level of alterations in the liver cells and gills as a result of PAH exposure. The fish in the control group showed normal architecture as they showed no visible alterations. The liver plays a crucial role in fish metabolism. It is also the storage site for many metabolites, especially glycogen. Changes in the liver morphology are important in assessing the metabolic status of fishes (Kolbasi *et al*., 2009). Observed alterations in exposed fish liver in the present study include: hepatic steatosis and moderate dense infiltrates. Cell infiltrates can be observed in damaged tissues. These infiltrates usuallu consist of white blood cells together with other cells of the immune system (Kolbasi *et al*., 2009). The presence of cell infiltrates is a pointer that the liver suffered severe anatomical damage as result of PAH exposure. Mehrnaz *et al*. (2017) observed a dense cell infiltration in liver of fish exposed to phenanthrene. The finding is in agreement with that reported in the present study. In fish, the gills participate in many crucial physiological roles. The biological roles include: respiration, osmoregulation and excretion (Camargo and Martinez, 2007). The gills which are in close proximity with the aquatic environment are very sensitive to variations in the water chemistry and are the primary target of environmental toxicants (Camargo and Martinez, 2007). Alterations found in some of the exposed fish gills include: channel dilatation and hyperplasia. The observed hyperplasia in some of the exposed gills could be an adaptive mechanism to changes in the conditions of the water and was aimed at protecting against excessive penetration of PAH toxicants from water to the blood vessels in the gills. (Strzyzewska *et al*., 2016). Similar morphologic alterations have also been observed by other researchers. In a recent work, Derakshesh (2020) revealed that diclofenac induced the formation of epithelial hyperplasia in exposed *Cyprinus carpio.*

Conclusion: The results from this work show that polycyclic aromatic hydrocarbon mixtures negatively affected some selected plasma parameters and also led to alterations in liver and gill tissues. The findings will enable government and environmental policy makers in the risk assessment and management of PAHs as well as contribute to the growing database of ecological toxicants.

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