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Effects of diclofenac on hormonal level of *Heteroclarias* after acute 96 h exposure

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ABSTRACT

This study was conducted to assess the effect of Diclofenac on the brain and serum hormones of *Heteroclarias*. The acute 96h test was carried out using graded concentrations of 4.00 mg/L, 8.00 mg/L, 12.00 mg/L and 16.00 mg/L. At various concentrations of the toxicant, uncoordinated behaviours such as erratic swimming, loss of balance and gasping for air by exposed fish was observed. In terms of endocrine disruptions, the study found that diclofenac exposure led to alterations in hormone levels. Follicle stimulating hormone (FSH) showed varying responses, with both increases and decreases in different concentration groups. Luteinizing hormone (LH) exhibited no significant changes, while prolactin levels increased in higher diclofenac concentrations, potentially in response to changes in electrolyte levels. Thyroid stimulating hormone (TSH) levels in the brain increased, likely due to decreased negative feedback from reduced serum thyroxine (T4) levels. In the serum, estradiol levels significantly decreased in the 4.00mg/L diclofenac group, while progesterone and testosterone levels decreased in multiple groups. Overall, this study highlights the ecological implications of diclofenac contamination in aquatic ecosystems, emphasizing the need for further research to mitigate pharmaceutical contaminant effects and protect aquatic biodiversity. Understanding the complex interactions between pharmaceuticals and aquatic organisms is crucial for sustainable aquaculture practices and ecosystem preservation.

***Keywords*:** Diclofenac, acute 96 h exposure, hormone, heteroclarias, *Heteroclarias*

1. INTRODUCTION

Pharmaceuticals are being produced and consumed at a growing rate, resulting in their introduction into aquatic ecosystems. Many pharmaceuticals flowing into wastewater plants are

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not degraded before they reach water bodies (Hong *et al*., 2019). As a result of their ubiquitous presence in aquatic ecosystems, personal care products and pharmaceuticals have become increasingly recognized as environmental contaminants. This has drawn attention from the public as residual pharmaceuticals in streams, groundwater, lakes and even drinking water has been reported around the globe (Furlong et al., 2017; Kay et al., 2017; Yao et al., 2018). The occurrence of these pharmaceuticals in the aquatic aquatic ecosystem is a cause for concern as it has potential impacts on aquatic organisms and also humans (Ngubane *et al*., 2019).

Among these pharmaceuticals, diclofenac has received particular attention from the scientific and regulatory communities (McRae et al., 2018). Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) with worldwide consumption estimated to be around 940 tons per year (Zhang et al., 2008). Partly due to these high consumption rates, diclofenac has been detected in environmental samples such as surface, ground, and even drinking water from at least 50 countries (Aus Der Beek et al., 2016), with some global wastewater and surface waters having concentrations ranging from low ngL-1 to low μgL-1 (Acuña et al., 2015). Therefore, the presence of diclofenac in aquatic environments, even in minute quantities has raised concerns about its adverse effects on aquatic organisms even as it offers therapeutic benefits to humans.

In African aquaculture, Heteroclarias is an important species formed by crossing *Clarias gariepinus* with *Heterobranchus longifilis*. It shows fast growth and has high capacity to grow in unfavourable farming conditions, efficiently utilizes diverse feeds and has increased resistance to diseases (Sobczak *et al*., 2022). They are served as delicacies in various cultures and are highly relished.

Hormones are substances produced and released by cells to affect other cells. They are transported through the blood to the tissue on which it has specific effect and are critical towards maintaining proper function. Hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone and prolactin are hormones that are associated with reproduction, and they are secreted from the anterior pituitary. Other hormones like thyroid hormones (TH) are mostly found in the serum of the blood and are concerned with growth and are consistently used as potential biomarkers in fish (Zaccaroni *et al*., 2009). NSAIDs exposure can cause disruption of endocrine system by alteration of aromatase activity which might subsequently influence sex hormone balance (Ji et al., 2013).

Fish has been used in numerous experiments as they can serve as sensitive bioindicators for exposure to aquatic pollutants (Van der Oost *et al*., 2003). In this study *Heteroclarias* was used as the experimental model. It has been used frequently in studies on the effects of toxins on ecosystems (Adamu 2009; Oyoroko and Ogamba 2017; Owolabi *et al*., 2021).

Understanding the potential endocrine-disrupting properties of diclofenac in *Heteroclarias* is not only critical for the preservation of aquatic ecosystems but also for the sustainable development of aquaculture practices in regions where this species plays a significant role as the major economically important non-target species that are very much affected by the aquatic pollution are fish (Thangavel *et al*., 2005). While diclofenac's impact on aquatic biota has garnered attention in recent years, there remains a paucity of comprehensive studies, particularly concerning its effects on hormonal systems in fish species such as *Heteroclarias*. Hormonal disruption in aquatic organisms is a subject of substantial ecological significance as it can influence various physiological processes, reproductive success, and overall population dynamics. Therefore, the present study aims to investigate the effects of diclofenac on hormonal regulation in *Heteroclarias* following acute 96-hour exposure.

2. MATERIALS AND METHODS

2. 1. Experimental setup

The test for toxicity of diclofenac was performed on *Heteroclarias*. The experimental fish were obtained from a commercial fish farm, Raji farm, in Ilorin, Kwara state. They were transported in a well aerated plastic jerry can to the Department of Zoology early in the morning to avoid mortality due to stress from heat. Upon arrival, they were sorted into 60litres plastic aquarium bowls with dechlorinated water pumped from a borehole and were not fed on that day so as to avoid death due to stress. The fish were then acclimated to the test condition for 14 days (USEPA, 1994). They were fed thrice each day with Durante commercial feed having a pellet size of 1.8 mm. The water was changed every morning to prevent spread of infection by the polluted water and to ensure the water quality was kept standard. The health status and behaviour of the fish were monitored throughout the experiment.

2. 2. Diclofenac

Diclofenac potassium manufactured by Shandong Xier Kangtai Pharmaceutical Co., Ltd, Shandong China with Batch Number 180575 and NAFDAC Registration Number B4-6381 was purchased from Feolu Pharmacy under the trade name "Ostomed". Each tablet of the drug contained 50 mg of the active ingredient, Diclofenac potassium.

2. 3. Range finding test

The range finding test was carried out to determine the appropriate concentration of toxicant to be used for the definitive test. It was conducted after the acclimatization period, and it lasted for 72h. A stock solution was prepared by dissolving 30g of the toxicant in 3000ml of distilled water. This was carried out in five different plastic aquaria each containing 5L of water with one serving as the control while the others had varying concentrations of the toxicant measured out from the stock solution.

2. 4. Acute toxicity test

Static toxicity test was applied in determining the 96h LC_{50} of Diclofenac to *Heteroclarias.* This was carried out using four different concentrations with two replicates each containing ten fishes. Concentrations of 40 ml, 80 ml, 120 ml and 160 ml were added into 10L of water from a stock solution of 15g of the toxicant dissolved in 1500 ml of distil water. Due to coagulation, the toxicant was renewed each day after every 24h and mortality was recorded with the fishes considered dead when the movement of the opercula ceased, and they did not respond to gentle probing. After 96h, the fishes were sacrificed, and the blood and brain were collected and taken for analysis.

2. 5. Hormonal analysis

During the sacrifice, blood of the fishes was collected into heparinized bottles by the laceration of the caudal arteries of the selected fishes. The brains of the fishes were also extracted and stored in bottles containing sucrose solution. These were taken to the biochemical laboratory at the University of Ilorin Teaching Hospital (UITH) for analysis. The quantitative estimation of serum prolactin level was carried out by the EIA method (Uotila *et al.*, 1981) using Omega diagnostic net.

2. 6. Statistical analysis

Analysis of variance (ANOVA) was carried out using computerized IBM SPSS Statistics 20 application and Duncan multiple range test was used to carry out level of significance test 95% ($P < 0.05$) between control and experimental means.

3. RESULTS AND DISCUSSION

3. 1. Behavioural effect

Changes in the behaviour and state of the fish exposed to diclofenac were observed daily during the test. The exposed fish showed erratic swimming, tonic convulsion, sudden quick movements, increase in opercula ventilation, respiratory distress as the fish kept swimming to the surface to gasp for air.

The fish attempted to escape from the aquaria by jumping consistently. Subsequently, loss of balance while swimming was noticed while bleaching of skin colour and whitish slimy discharge were observed. These changes were observed to increase with respect to the concentration of exposure.

The fish became slow, lethargic, and hardly responded to gentle probing. Physicochemical properties of water used in the present study are tabulated in Table 1. In this study, certain behavioural alterations were observed after fish exposure to Diclofenac like those observed by Padma (2018).

These alterations could be because of the neurotoxic properties of the drug (onzalez-Rey & Bebianno, 2014). Surfacing and gasping for air may be means of coping with the reduction in dissolved oxygen after the exposure to the toxicant. Other behavioural changes observed such as erratic swimming may be due to an avoidance syndrome the fish developed in reaction to the toxicant (Aziz *et al*., 2014; Nassef *et al*., 2010; Schwarz *et al*., 2017)

Table 1. Mean water quality parameter during acute exposure of *Heteroclarias* to varying concentrations of Diclofenac for 96 h

Conc. (mg/L)	Temp (°C)	CD (us/amp)	pH	DO
0.00	26.10 ± o.17^{c}	1.43 ± 1.15^{a}	7.06 ± 0.02^{a}	7.20 ± 0.17^{c}
4.00	26.3 ± 0.16^{b}	1.45 ± 1.15^{a}	6.98 ± 0.02^{a}	6.90 ± 0.12^{d}
8.00	26.2 ± 0.17^{c}	1.48 ± 1.73^{b}	6.95 ± 0.01^{b}	6.85 ± 0.02^{b}
12.00	26.3 ± 0.23^{d}	1.48 ± 0.58^{c}	6.95 ± 0.02^{a}	6.80 ± 0.06^{c}
16.00	26.0 ± 0.06^{a}	1.46 ± 0.58^{c}	6.90 ± 0.02^{a}	6.74 ± 0.02^{b}

Results are means of two replicates ± SEM (Standard Error Mean). Means along the same column with the same superscript are not significantly different ($p>0.05$).
DO: Dissolved Oxygen
CD: Conductivity

3. 2. Hormonal activity

After the exposure, the levels of the hormones in the brain and serum shown in Figures 1 and 2 were affected by the exposure to diclofenac.

The level of follicle stimulating hormone (FSH) in the brain shows significant ($P < 0.05$) increase and decrease among the various concentrations with a high level of 0.72 ± 0.02 mlU/ml observed in 4.00 mg/L group while a low level of measured at 0.23 ± 0.02 mlU/ml was observed in the group exposed to 8.00 mg/L.

After this, a steady increase in the level of FSH was recorded as the concentration of the toxicant increased when compared to the control (0.00 mg/L) group.

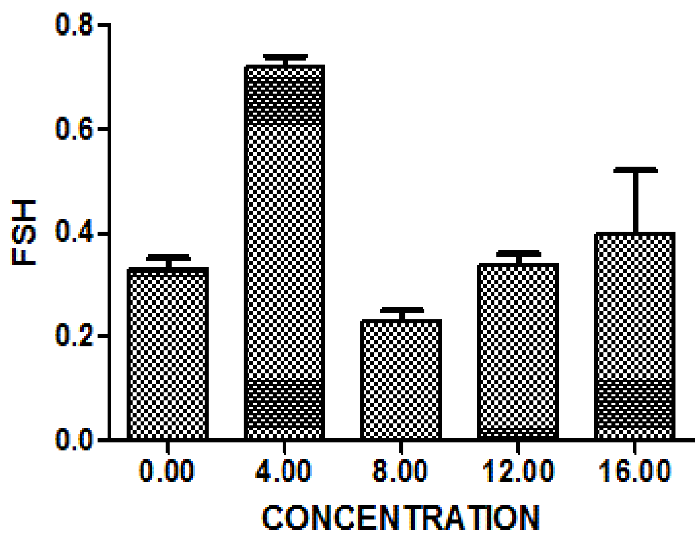

Figure 1. Hormonal levels of FSH in the brain of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

The level of luteinizing hormone (LH) in the brain showed no significant ($P > 0.05$) changes among the varying concentrations of the toxicant exposure with a high level of 0.32 ± 0.01 mlU/ml recorded in the 4.00 mg/L group.

The FSH is involved in the initiation of gametogenesis and regulation of gonadal growth, whereas LH mainly regulates gonadal maturation and spermiation/ovulation. The elevated levels recorded in some concentrations may be an indication of FSH stimulatory effect on its own receptor mRNA as a result of estrogenic activity of Diclofenac while the rise in LH is attributed to normal endocrine system disruption (Chitra & Revathy, 2019).

The level of prolactin in the brain also showed no significant ($P > 0.05$) changes among the varying concentrations of the toxicant exposure having a high of 2.23 ± 0.58 mg/L recorded in the 4.00 mg/L group. Prolactin is one of the main osmoregulatory hormones in fish maintaining the plasma electrolyte levels mainly by controlling gill epithelium permeability.

The increase in prolactin recorded in higher concentrations of the toxicant may be due to a response to a drop in plasma electrolytes (Wendelaar Bonga *et al.*, 1987). The decrease in prolactin levels in some groups may be due to stressful conditions as reported by Waring *et al.* (1996).

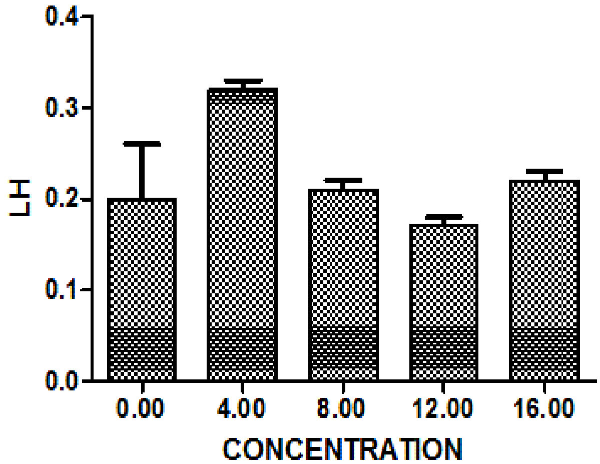

Figure 2. Hormonal levels of LH in the brain of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

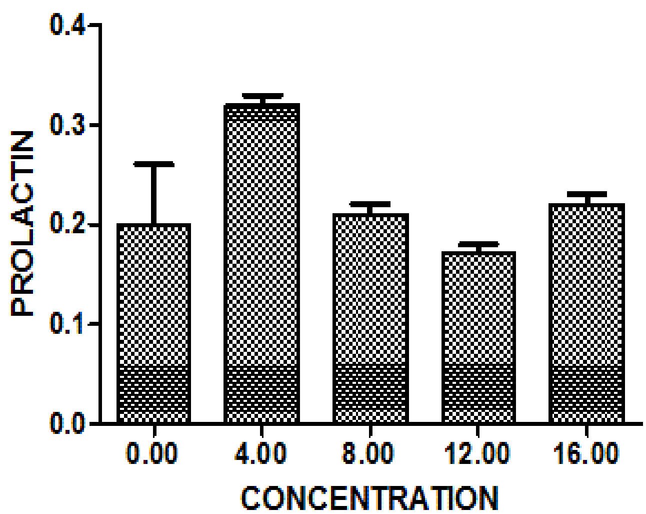

Figure 3. Hormonal levels of Prolactin in the brain of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

The level of thyroid stimulating hormone (TSH) is highest in the control group (0.59 ± 0.01 mlU/ml). insignificant (P > 0.05) increases and decreases were recorded among the other groups. The increase in brain TSH recorded with the increase in concentration of Diclofenac may be attributed to the reduced negative feedback from the hypothalamus and pituitary due to decreased levels of circulating T4 in the serum (Liqin *et al.*, 2010).

These in part shows that the alteration of hormonal levels in the brain is independent of the varying concentrations of toxicant the fish were exposed to.

The levels of estradiol in the serum shows the most significant (P < 0.05) decrease in the 4.00 mg/L group. It is significantly (P < 0.05) lower in the 16.00 mg/L group while in the other groups, insignificant (P > 0.05) changes were recorded. A decrease in the level of estradiol was recorded in line with Chitra *et al*., (2019).

This indicates the failure of steroidogenic enzymes for the conversion of testosterone into the potent female hormone, estradiol. This is in contrast with Vicentini *et al*., (2022) who reported that estradiol levels were not altered in Neotrpical fish exposed to Cadmium. Raibeemol & Chitra, (2020) also reported a decrease in estradiol levels in male and female *Pseudetroplus maculatus* exposed to chlorpyrifos, which they stated could be due to several factors such as, alteration in the process of steroidogenic biosynthesis pathway, downregulation of transcriptional factor expression, cholesterol transporters, and/or alteration of enzymes involved in cytochrome P450 system.

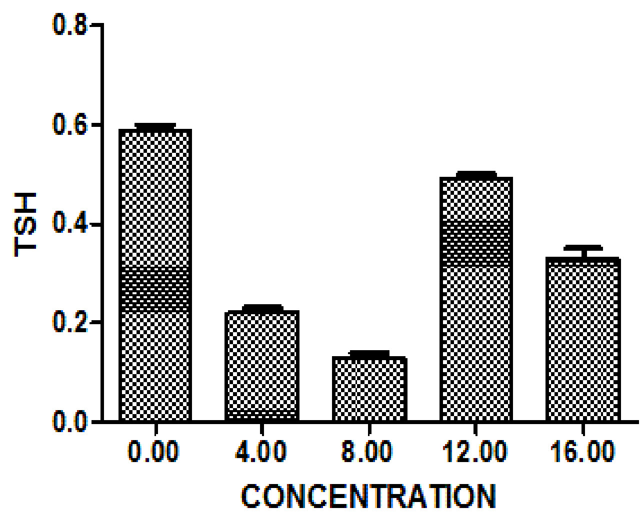

Figure 4. Hormonal levels of TSH in the brain of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

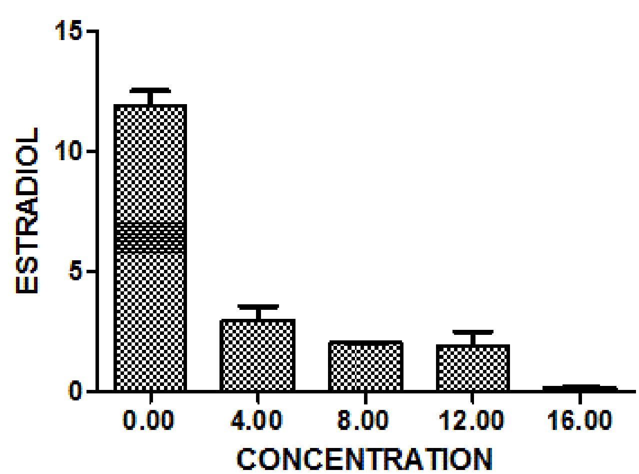

Figure 5. Hormonal levels of Estradiol in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96h.

The level of progesterone in the serum is highest in the control and decreases significantly ($P < 0.05$) in the 4.00 mg/L groups. There are insignificant ($P > 0.05$) changes recorded in the other groups. A significant decrease in Progesterone and testosterone levels was recorded and this agrees with Abbassey et al., (2023) in their study on Tilapia fish. This was also reported in other species exposed to bisphenol S (Ji et al., 2013) and cadmium (Gárriz et al., 2019). Progesterone plays an important role in spermatocyte and oocyte maturation, the production of seminal fluid, and ovulation (Lubzens *et al*., 2010). The decrease in progesterone is similar to that of (Chaube & Gautam, 2022) but in contrast with (Kennedy & Janz, 2022; Opute *et al*., 2020).

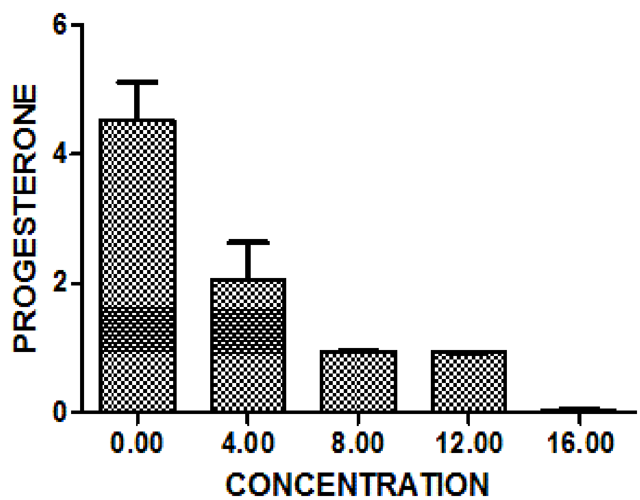

Figure 6. Hormonal levels of Progesterone in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

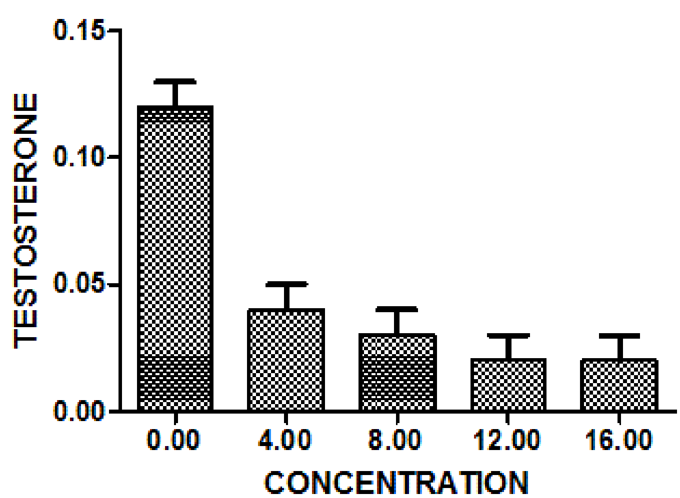

Figure 7. Hormonal levels of Testosterone in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96h.

Changes in the testosterone levels of the serum recorded were insignificant (P > 0.05) from the control group. Testosterone hormone plays an active role in the maturation stage of the gonads at the end of the reproductive cycle and acts as an intermediate product in synthesizing estradiol (Barannikova et al., 2002). The reduced testosterone hormone concentration might be due to the disruption of enzymatic reactions that synthesize the hormone (Muliari et al., 2019). Concentration of estradiol hormone in fish is related to the synthetization of testosterone hormone and a decreased testosterone level can cause the decreased level of estradiol (Carnevali et al., 2018).

Thyroid stimulating hormone (TSH) in the serum showed insignificant (P > 0.05) changes from the control group. Thyroid stimulating hormone (TSH) is a member of the vertebrate glycoprotein hormone family, which also comprises the pituitary and chorionic gonadotropins. TSH stimulates the thyroid gland to produce the THs namely thyroxine (T4) and tri-iodothyronine (T3). Despite the decrease of T3 and T4, the levels of TSH also decreased, which refers to the failure in hypothalamic-pituitary-thyroid axis. (Alkaladi *et al*., 2021)

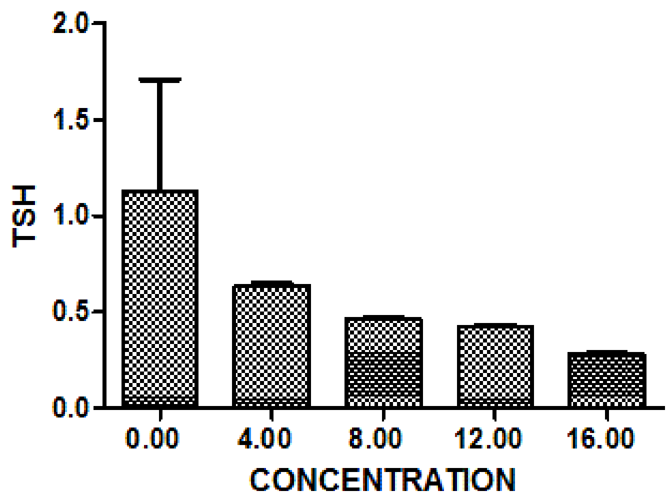

Figure 8. Hormonal levels of TSH in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

Changes in triiodothyronine (T3) and thyroxine (T4) showed significant (P < 0.05) changes with increases from the level recorded from the control group.

The thyroid hormones (THs), namely tri-iodothyronine (T3) and thyroxine (T4), are the principal endocrine hormones and play a vital role in various physiological processes of vertebrates (Subhash Peter 2011). In fishes, thyroid hormones regulate osmoregulation, growth, development, skin pigmentation, reproduction, metamorphosis, behaviour and all aspects of protein and lipid metabolism (Yu *et* al., 2015; Lascasana *et al*., 2010). T3 and T4 regulate growth, development and metabolism, and are involved in more biological actions than any other hormone (Liu and Chan, 2002).

The observed reduction in T3 could be taken to suggest that the fish adaptively reduces its metabolic rates thereby indirectly reducing the impact of the toxicant (Thangavel *et* al., 2005). Decrease in serum TSH led to similar alterations in T4 and T3 levels which corresponds with the study of Alkaladi *et al*., (2020).

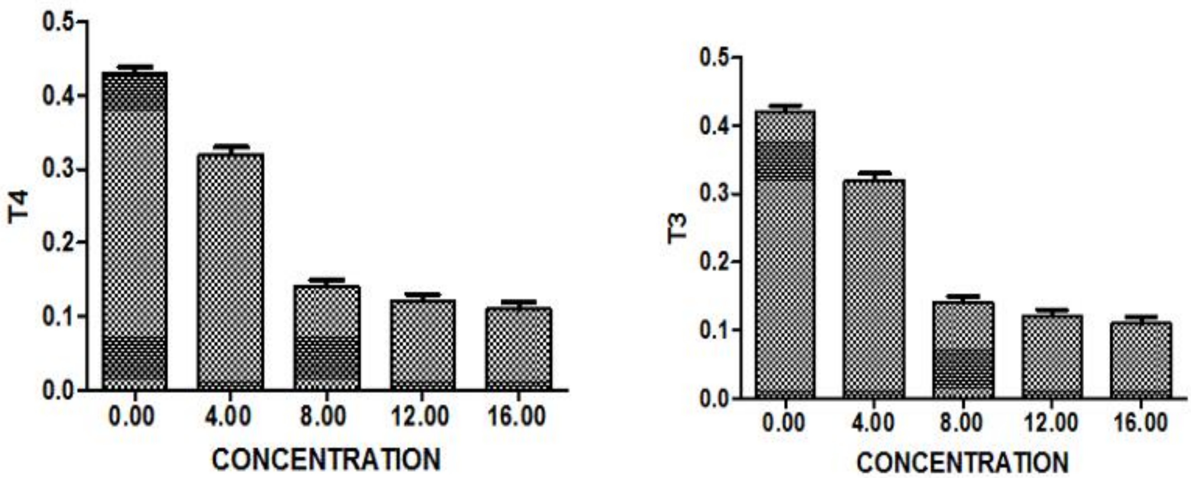

Figure 9. Hormonal levels of T4 and T3 in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac after 96 h.

4. CONCLUSION

In conclusion, this study on the effects of Diclofenac exposure on *Heteroclarias* has revealed significant behavioral and hormonal disruption, shedding light on the potential ecological consequences of pharmaceutical contamination in aquatic ecosystems. These findings collectively emphasize the impact of Diclofenac on both behavior and hormonal function in fish, exposing the ecological implications and the need for continued research to curb pharmaceutical contaminant effects in aquatic ecosystems and protect aquatic organisms.

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