

World News of Natural Sciences

An International Scientific Journal

WNOFNS 65 (2026) 15-35

EISSN 2543-5426

Effects of Lethal and Sub-Lethal Diclofenac Exposure on Enzymatic Activities in *Heteroclaris* and the Ameliorative Role of Neem Leaf Supplementation

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ABSTRACT

This study examined the lethal and sub-lethal effects of diclofenac on juvenile hybrid catfish (*Heteroclaris*) over 8, 15, and 30 days of exposure, focusing on behavioural and biochemical responses in blood, gill, and liver tissues. Exposed fish showed dose-related behavioural disturbances such as erratic swimming, surface gasping, and altered pigmentation, with effects becoming more pronounced over time. Biochemical analyses revealed significant changes in aspartate aminotransferase (AST), alanine aminotransferase (ALT), acetylcholinesterase (AChE), lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA). Enzyme responses varied across tissues and exposure durations, indicating physiological stress and oxidative imbalance. Dietary supplementation with neem leaf (*Azadirachta indica*) reduced several of the observed biochemical disruptions and improved behavioural stability in exposed fish, suggesting a protective role against diclofenac-induced toxicity. These findings demonstrate that diclofenac can adversely affect aquatic organisms even at sub-lethal concentrations and highlight the potential of plant based dietary additives as a mitigation strategy. The study emphasizes the ecological risks posed by pharmaceutical contaminants in freshwater systems and supports the need for improved environmental monitoring and management.

Keywords: Diclofenac, Biochemical analysis, *Heteroclaris*, Aquatic toxicity, Neem *Azadirachta indica*, Pharmaceutical contaminants

1. INTRODUCTION

There is a growing awareness among ecologists that behavioural variation and alterations are important indicators of individual performance, ecosystem functioning, and species evolution. In ecotoxicology, such behavioural changes are often caused by contaminants present in natural systems, especially emerging pollutants like pharmaceuticals. Standardized ecotoxicological tests, such as those recommended by the Organization for Economic Co-operation and Development (OECD), rarely consider ecologically relevant behaviours or their impacts on food webs and ecosystem processes. Integrating ecological perspectives into ecotoxicology is particularly valuable, as many pharmaceutical compounds are biologically active even at low concentrations and can modify behaviours of ecological significance (Ziemba, 2004; Woodward et al., 2007; Fatta-Kassinos et al., 2010; Gill et al., 1991).

Pharmaceutical substances enter aquatic ecosystems through several pathways, including human excretion, industrial discharge, disposal of unused drugs, and effluents from hospitals and wastewater treatment plants. Some drugs are metabolized in the body, while others remain unchanged and are excreted into sewage systems. Wastewater monitoring studies have detected pharmaceuticals continuously in surface waters and sediments worldwide, confirming their persistence and ecological risk.

Although environmental concentrations of pharmaceuticals are often low (ng – µg/L), sub-lethal effects on aquatic organisms are increasingly reported. Behavioural endpoints, such as activity, feeding, boldness, social interactions, and swimming patterns, are often more sensitive to pharmaceutical exposure than traditional endpoints such as mortality or reproduction. (Stakelberg et al., 2007; Scott et al., 2004; Cooper et al., 2008; Melvin & Rodriguez, 2012).

Studies on zebrafish and other teleosts have shown significant disruptions in locomotor activity, social behaviour, and risk-taking even at environmentally relevant concentrations of antidepressants, analgesics, and other drug classes (Ziemba, 2004; Woodward et al., 2007; Fatta-Kassinos et al., 2010; Gill et al., 1991).

Bioaccumulation further complicates the assessment of pharmaceutical exposure. Many pharmaceuticals and their metabolites accumulate in aquatic organisms and can persist in predators for extended periods, with behavioural consequences that may not synchronise with exposure duration. Despite low water concentrations, many compounds reach tissues at levels sufficient to interfere with neurological and physiological processes, which are not captured by risk assessments focusing solely on water concentrations.

Species-specific responses to pharmaceuticals can disrupt ecological interactions and affect ecosystem functioning differently across taxa. Behavioural, biochemical, and physiological endpoints provide complementary insights into the ecological risks of pharmaceutical contamination. In this context, the present study investigates the acute toxicity of diclofenac, a widely used non-steroidal anti-inflammatory drug, on juvenile hybrid catfish (*Heteroclarias*).

Specifically, it examines the median lethal concentration (LC50), behavioural alterations, enzymatic activities, and the potential ameliorative effect of neem leaf supplementation on biochemical disturbances in gill, liver, and blood tissues. This study contributes to understanding the ecological risks of pharmaceutical contaminants in freshwater systems and highlights strategies for mitigating their impacts (Daughton & Ternes, 1999).

2. MATERIALS AND METHODS

2. 1. Experimental Setup

Juvenile hybrid catfish (*Heteroclarias*) were obtained from Raji Fish Farm in Ilorin, Kwara State, Nigeria, and transported to the laboratory in well-aerated black plastic containers filled with water from the farm. To minimize stress and transport-related mortality, fish were not fed the day prior to transportation. Upon arrival, they were transferred to 30 L plastic aquaria containing dechlorinated water maintained at 24 °C and acclimatized for fourteen days. During acclimatization, fish were fed commercial feed twice daily at 09:00 and 17:00 hours. Water was renewed daily to remove uneaten feed and fecal matter, maintain water quality, and reduce the risk of infection. Fish health was monitored by observing feeding patterns, swimming activity, and any behavioural or physical abnormalities.

2. 2. Collection and Processing of Neem Leaf

Fresh, mature neem leaves (*Azadirachta indica*) were harvested from trees within the University of Ilorin campus, Ilorin, Nigeria. The leaves were carefully selected to ensure they were free from visible disease or pest damage and were transported to the laboratory in clean containers. In the laboratory, the leaves were thoroughly rinsed with clean water to remove adhering dust and debris, then spread out on clean trays and air-dried at room temperature under shade to prevent photodegradation and loss of bioactive compounds. The leaves were turned regularly to promote uniform drying and prevent fungal growth. After complete drying, the leaves were manually separated from the stalks and pulverized into fine powder using a clean mortar and pestle. The powdered neem leaf material was stored in airtight cellophane bags at room temperature until incorporation into the experimental diets, following established procedures for herbal supplementation in aquaculture studies (Daughton & Ternes, 1999).

2. 3. Experimental Feed Formulation

Experimental diets were prepared by incorporating varying concentrations of neem leaf powder (0–5%) into a basal feed. Feed ingredients were ground, mixed with water, and extruded using a minced meat machine. Pellets were air dried and broken into sizes suitable for juvenile *Heteroclarias*. This ensured uniform distribution of neem leaf supplementation across treatments.

Table 1. Fish Feed Experimental Diet Formulation.

Constituents	0.0%	1.0% (20g)	2% (40g)	3% (60g)	4% (80g)	5% (100g)
Fish Meal	23.3	23.3	22.3	21.3	21.3	22.3
Maize	16.0	15.0	15.0	15.0	15.3	14.0
Groundnut cake	26.0	26.0	26.0	26.0	25.0	25.0
Toasted soya	26.0	26.0	26.0	26.0	26.0	25.0

DCP	1.5	1.5	1.5	1.5	1.5	1.5
Oil	5.5	5.5	5.5	5.5	5.5	5.5
Vitamin	0.20	0.20	0.20	0.20	0.20	0.20
Bone Meal	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.20	0.20	0.20	0.20	0.20	0.20
Methionine	0.30	0.30	0.30	0.30	0.30	0.30
Lysine	0.5	0.5	0.5	0.5	0.5	0.5
Neem Leaves	0.0	1.0	2.0	3.0	4.0	5.0

Table 1, shows the dietary supplement made using the technique of with minor modifications. The fish feed was grinded with a grinder, hydrated with water and mixed properly before extruded through a minced-meat machine. The mixture was air dried until it got hard and then was broken into small pellets.

2. 4. Range-Finding Test

A preliminary trial was carried out to identify appropriate sub-lethal concentrations of Diclofenac for subsequent exposure experiments. The drug was first dissolved in distilled water to obtain a working stock solution, which was then serially diluted to produce a range of test concentrations.

Juvenile *Heteroclaris* were randomly assigned to separate aquaria containing these concentrations, with each group consisting of ten fish. The fish were observed over a short screening period for signs of stress and mortality. The pattern of survival obtained from this initial assessment was used to select exposure levels that would not cause immediate death but could induce measurable physiological and biochemical responses during the 8-, 15-, and 30-day experimental periods.

2. 5. Chronic Toxicity and Ameliorative Test

Chronic exposure experiments were conducted over 8, 15, and 30 days to evaluate the toxic effects of Diclofenac at both lethal and sublethal concentrations. Juvenile *Heteroclaris* were exposed to a range of Diclofenac concentrations (0, 40, 80, 120, 160, and 200 mL), with two replicates per concentration.

Concentrations at the higher range (e.g., 160 and 200 mL) were expected to cause mortality (lethal effects), and mortalities were recorded daily throughout the exposure period. Lower concentrations (e.g., 40–120 mL) were considered sublethal and were used to assess biochemical and physiological responses.

To evaluate the potential ameliorative effects of neem leaf supplementation, fish in the sublethal treatment groups were fed neem supplemented feed twice daily. Aquaria were cleaned daily, and toxicant solutions were renewed to maintain consistent exposure conditions.

2. 6. Biochemical Assays

At the end of each exposure period, surviving fish were humanely sacrificed, and blood, gill, and liver tissues were collected. The samples were transported to the biochemical laboratory at the University of Ilorin Teaching Hospital (UITH) for analysis. Enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), acetylcholinesterase (AChE), superoxide dismutase (SOD), malondialdehyde (MDA), Glutathione Peroxidase (GPX), and catalase were measured using established protocols (Ziemba, 2004; Woodward et al., 2007; Fatta-Kassinos et al., 2010; Gill et al., 1991). Tissue samples were homogenized in sucrose solution, centrifuged, and the supernatant analyzed spectrophotometrically at appropriate wavelengths.

2. 7. Statistical Analysis

Data were analyzed using one way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) for separation of means, using SPSS version 16. Statistical significance was accepted at $P < 0.05$. All results are presented as mean $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

3. 1. Behavioral Responses

Behavior provides a valuable perspective linking the physiology and ecology of an organism to its environment. It encompasses quantifiable actions regulated by the central and peripheral nervous systems and reflects cumulative expression of genetic, biochemical, and physiological processes essential for survival, including feeding, reproduction, and predator avoidance (Little & Brewer, 2005; Zha et al., 2021).

In this study, juvenile *Heteroclinus* exposed to diclofenac exhibited noticeable distress behaviours, indicating pharmaceutical-induced toxicity. Observed responses included erratic swimming, frequent surfacing accompanied by gasping for air, and colour changes, whereas control fish maintained normal behavioural patterns. Prior to mortality, fish were observed turning their ventral side upward, displaying pronounced respiratory distress and lethargy, consistent with behavioural disruptions documented in pharmaceutical exposure research (Zha et al., 2021; Ortiz-Alemán et al., 2025). Increasing diclofenac concentrations progressively weakened fish, ultimately leading to death.

Importantly, fish that received dietary supplementation with neem leaf exhibited improved behavioural responses, suggesting an ameliorative effect of the supplement. This protective effect was evident in chronic exposure experiments and aligns with recent evidence that herbal dietary supplements can mitigate oxidative and stress responses in fish exposed to environmental contaminants (Daughton & Ternes, 1999).

3. 2. Water Quality Parameter.

Throughout the experimental period, key water quality parameters, including temperature, pH, and dissolved oxygen, were continuously monitored to maintain stable conditions across all experimental groups. Table 2 summarizes the water quality parameters during the chronic exposure period, reflecting the controlled maintenance of the experimental system for all treatment groups.

Table 2. Mean water quality parameters during chronic exposure of *Heteroclaris* exposed to varying concentrations of Diclofenac over 30 days.

	Group pH	TSD (mg/L)	TSS (mg/L)	COD (mg/L)	BOD (mg/L)	CD (us/amp)	TB (mg/L)	DO (mg/L)	Temp (°C)
A	6.10 ±0.06d	28.47 ±5.77d	127.00 ±5.77c	2.92 ±0.06a	3.20 ±0.06c	202.0 ±0.06c	85.0 ±5.77l	6.4 ±0.06b	29.40 ±0.77c
B	5.30 ±0.06b	32.80 ±5.77b	199.00 ±5.77c	4.03 ±0.06a	6.40 ±0.06a	224.2 ±0.06a	99.0 ±5.77c	6.8 ±0.06d	28.2 ±0.77b
C	6.60 ±0.06c	28.50 ±5.77c	107.00 ±5.77a	2.68 ±0.06c	2.80 ±0.06b	190.8 ±0.06b	79.0 ±5.77d	6.5 ±0.06a	30.0 ±0.77a
D	6.80 ±0.06d	28.47 ±5.77d	129.00 ±5.77d	3.39 ±0.06f	3.40 ±0.06a	206.5 ±0.06c	90.0 ±5.77c	6.6 ±0.06f	29.0 ±0.77d
E	6.10 ±0.06c	28.57 ±5.77c	137.00 ±5.77b	3.87 ±0.06d	3.80 ±0.06f	211.0 ±0.06f	93.0 ±5.77a	6.7 ±0.06c	28.6 ±0.77c
F	5.40 ±0.06f	28.30 ±5.77f	139.00 ±5.77f	4.01 ±0.06c	4.40 ±0.06c	214.0 ±0.06d	94.0 ±5.77b	6.9 ±0.06c	28.5 ±0.77f

Mean with the same superscript in the column are not significantly different ($P < 0.05$)

TDS = Total dissolved solid, TSS = Total suspended solid, COD = Chemical oxygen demand, BOD = Biological oxygen demand, DO = Dissolved oxygen, TB = Turbidity, CD = Conductivity, Temp. = Temperature.

3. 3. Estimation of LC_{50}

The median lethal concentration (LC_{50}) of Diclofenac for juvenile *Heteroclaris* was estimated based on observed mortality over the chronic exposure periods of 8, 15, and 30 days. Results indicated that concentrations above 10.8 ppm caused significant mortality, whereas lower concentrations primarily induced sub-lethal effects, including behavioral alterations and biochemical changes. This finding aligns with previous studies reporting Diclofenac's acute and chronic toxicity in freshwater fish, confirming its potential to impair physiological functions at elevated concentrations.

3. 4. ALT Enzyme Activity in Blood, Gills, and Liver

Figure 1 illustrates alanine aminotransferase (ALT) activity in the blood, gills, and liver of juvenile *Heteroclaris* after 8, 15, and 30 days of Diclofenac exposure.

After 8 days, ALT activity in the liver was higher than in the blood and gills across most groups. A noticeable elevation occurred in groups D and E, while the control and lower-dose groups showed comparatively reduced values. Blood ALT activity remained relatively low with slight fluctuations among groups. Gill ALT activity also showed minor variations but remained lower than liver values. Overall, the liver exhibited the highest ALT activity at this early exposure period.

At 15 days, ALT activity increased markedly, particularly in the liver where a strong peak was observed in group D, followed by a decline at higher concentrations. Blood ALT activity

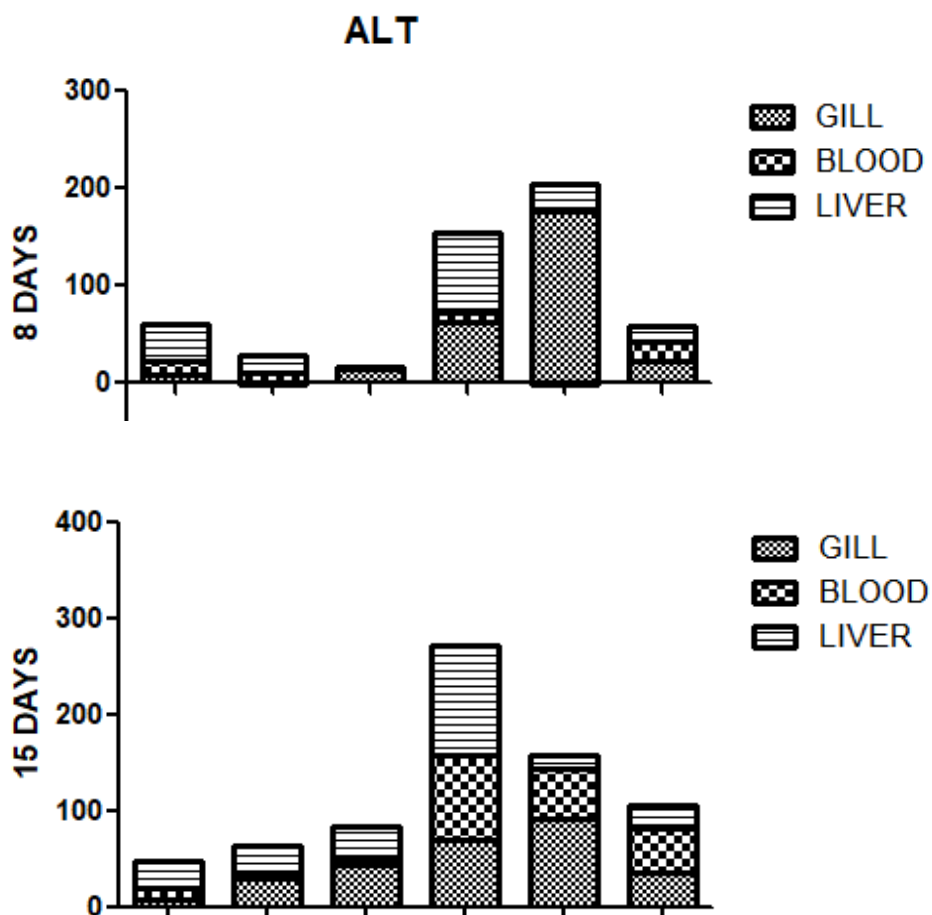
rose moderately compared to 8 days but remained below liver levels. Gill ALT activity showed gradual increases across treatment groups before declining in the final group. At this exposure duration, the liver clearly displayed the highest ALT activity, indicating intensified hepatic response during mid-term exposure.

After 30 days, ALT activity shifted in distribution among tissues. Blood ALT levels became more pronounced compared to earlier exposure periods, with higher values observed across several treatment groups. Liver ALT activity declined relative to the 15-day peak but remained elevated compared to initial levels. Gill ALT activity showed modest fluctuations without a consistent concentration-dependent pattern. At this stage, blood exhibited the highest ALT activity overall, suggesting prolonged systemic stress associated with extended Diclofenac exposure.

When comparing across exposure durations, the maximum ALT activity occurred at 15 days, indicating that enzymatic response peaked during mid-term exposure before redistributing among tissues with longer exposure.

Dietary supplementation with neem leaf did not produce a consistent or significant ameliorative effect on ALT activity, as enzyme patterns largely followed exposure trends rather than dietary treatment.

ALT



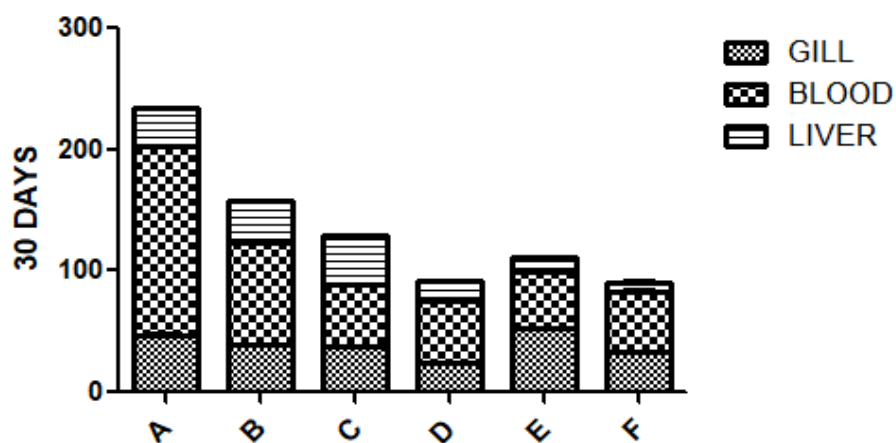
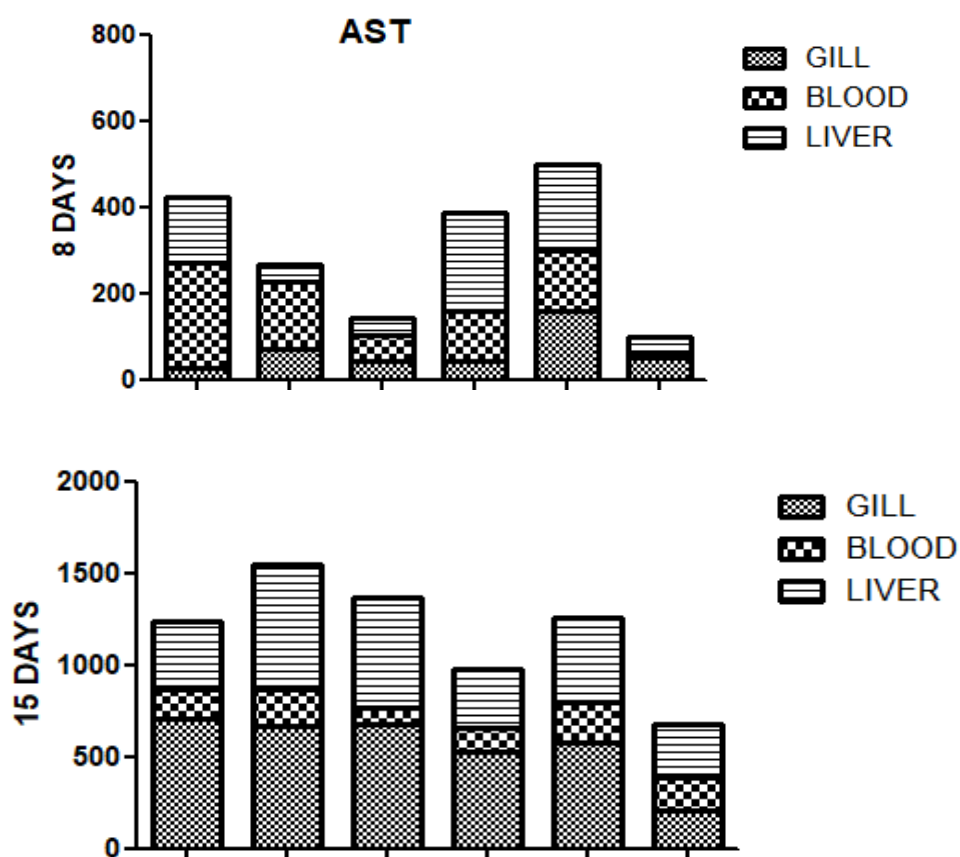


Figure 1. Biochemical analysis of ALT activity on gill, blood and liver in juvenile *Heteroclinus* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

3. 5. AST Enzyme Activity in Blood, Gills, and Liver

AST



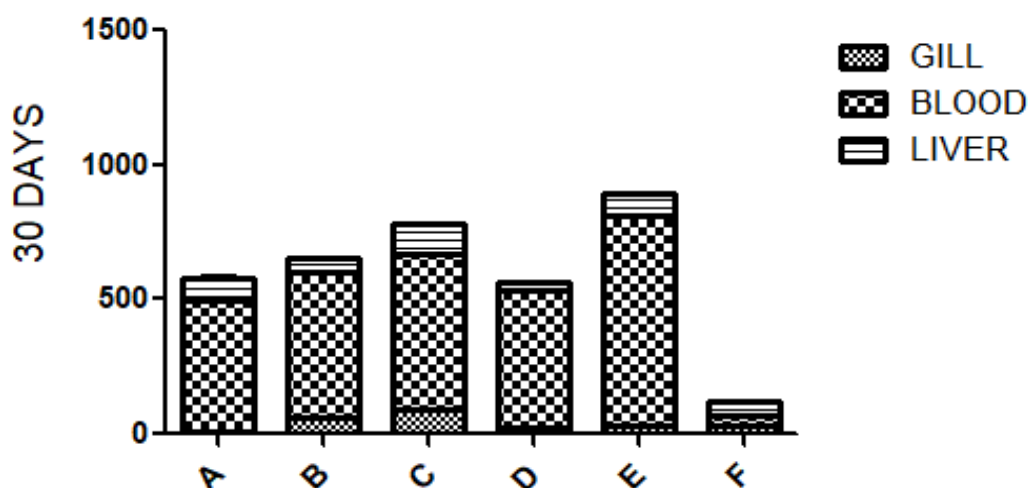


Figure 2. Biochemical analysis of AST activity on gill, blood and liver in juvenile *Heteroclarus* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

Figure 2 shows aspartate aminotransferase (AST) activity in the blood, gills, and liver of juvenile *Heteroclarus* after 8, 15, and 30 days of Diclofenac exposure.

After 8 days, AST activity in blood was highest in the control group and lower in all exposed groups. Gill AST activity peaked in group E, while the remaining groups showed markedly lower values. Liver AST activity decreased across the first three groups, rose in groups D and E, and declined in the last group. Overall, blood exhibited the highest AST activity at this exposure period.

At 15 days, liver AST activity increased in group B relative to the control, declined in groups C and D, rose again in group E, and decreased in group F. Gill AST activity showed no significant differences among the first three groups ($P > 0.05$), followed by slight reductions in groups D and E and a pronounced decline in the last group. Blood AST activity remained relatively low with minor fluctuations. The gills displayed the highest AST activity among the organs at this exposure duration.

After 30 days, blood AST activity gradually increased with rising concentration, except for slight reductions in groups D and F. Gill AST activity was negligible in the control, increased across treatment groups, declined in groups D and E, and rose again in the last group. Liver AST activity alternated between decreases and increases across the groups, starting slightly higher in the control. At this time point, blood showed the highest AST activity overall.

When comparing across exposure periods, the maximum AST activity was observed at 15 days, indicating peak enzymatic response during mid-term Diclofenac exposure.

3. 6. Acetylcholinesterase (ACHE) Activity in Blood, Gills, and Liver

Figure 3 shows ACHE activity in the blood, gills, and liver of juvenile *Heteroclarus* following 8, 15, and 30 days of Diclofenac exposure. After 8 days, blood exhibited the highest ACHE activity, particularly in group C, though differences across groups were not statistically significant.

ACHE

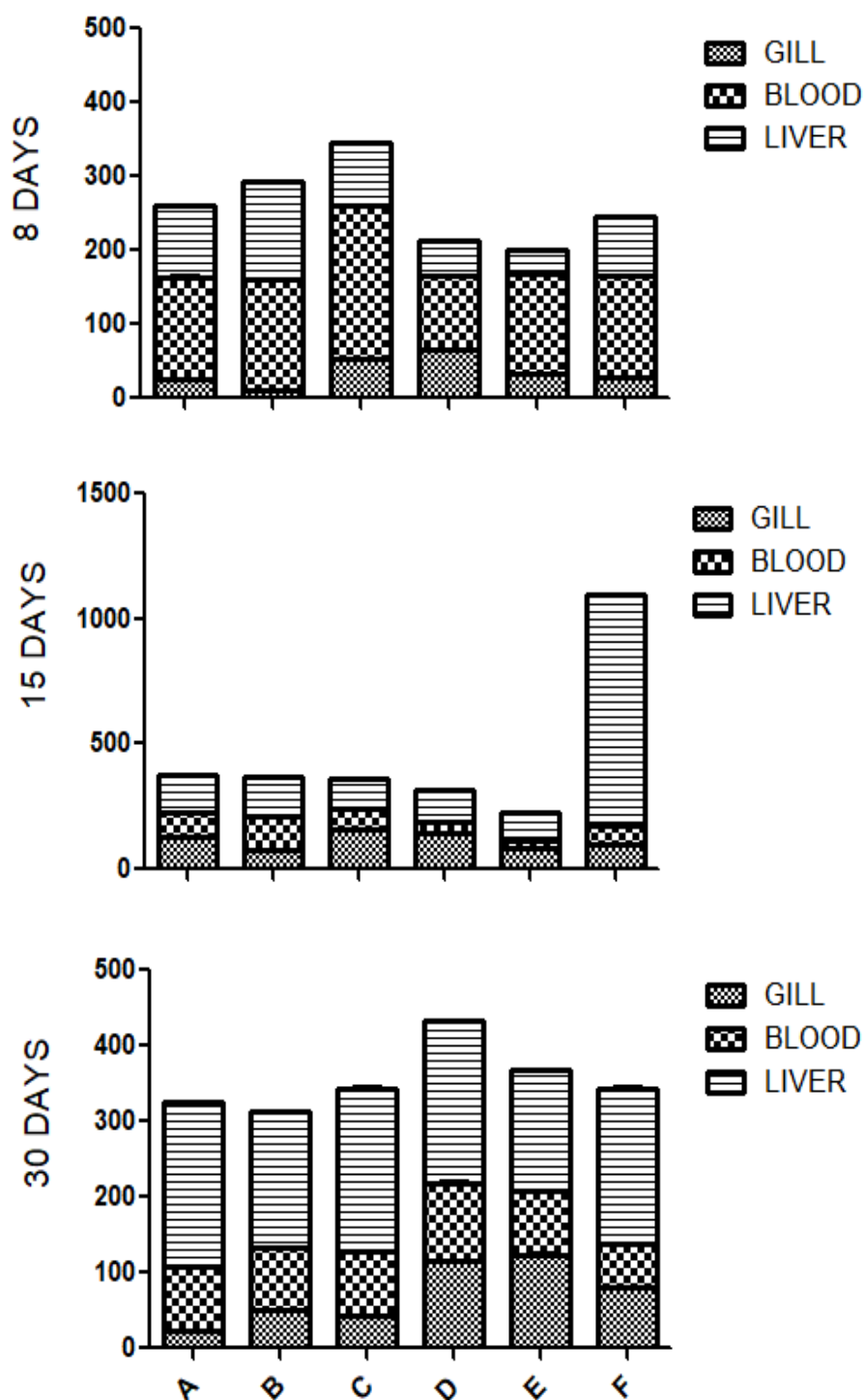


Figure 3. Biochemical analysis of ACHE activity on gill, blood and liver in juvenile *Heteroclinarias* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

Liver activity increased in group B before declining progressively with higher concentrations. At 15 days, ACHE activity remained low in blood and gills across most groups. In contrast, liver activity increased sharply in the last group, likely due to the highest Diclofenac concentration.

Following 30 days, ACHE activity in blood and gill showed minor, non-significant fluctuations, while liver activity experienced a significant increase in some groups and a decrease in others. Across both 15- and 30-day exposures, the liver consistently displayed the highest enzyme activity, with the maximum overall

ACHE activity observed at 30 days, indicating cumulative enzymatic response to prolonged exposure. These patterns align with previous studies reporting decreased ACHE activity in fish exposed to toxicants (Aguiar et al., 2001; Hassanein, 2002).

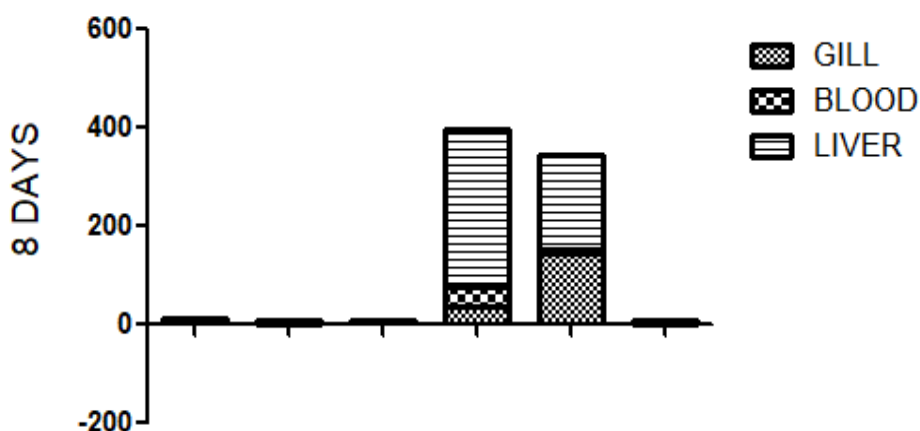
3. 7. Lactate Dehydrogenase (LDH) Activity in Blood, Gills, and Liver

Figure 4 shows LDH activity in the blood, gills, and liver of juvenile *Heteroclinus* after 8, 15, and 30 days of Diclofenac exposure.

After 8 days, LDH activity in the gills and blood remained generally low across most groups. However, a sharp rise in liver LDH activity was observed in groups D and E, while the other groups showed minimal changes. Gill LDH levels were slightly elevated in exposed groups compared to the control but remained far lower than liver values. At this exposure duration, the liver exhibited the highest LDH activity, suggesting early metabolic stress in hepatic tissue.

At 15 days, LDH activity increased markedly in all tissues compared to day 8. The most pronounced elevations were observed in groups D and E, particularly in the liver and blood. Gill LDH activity also rose but to a lesser extent. The control and lower exposure groups showed only minor changes. Overall, the liver again showed the highest LDH activity, followed by blood, indicating progressive tissue stress and increased anaerobic metabolism with continued exposure.

LDH



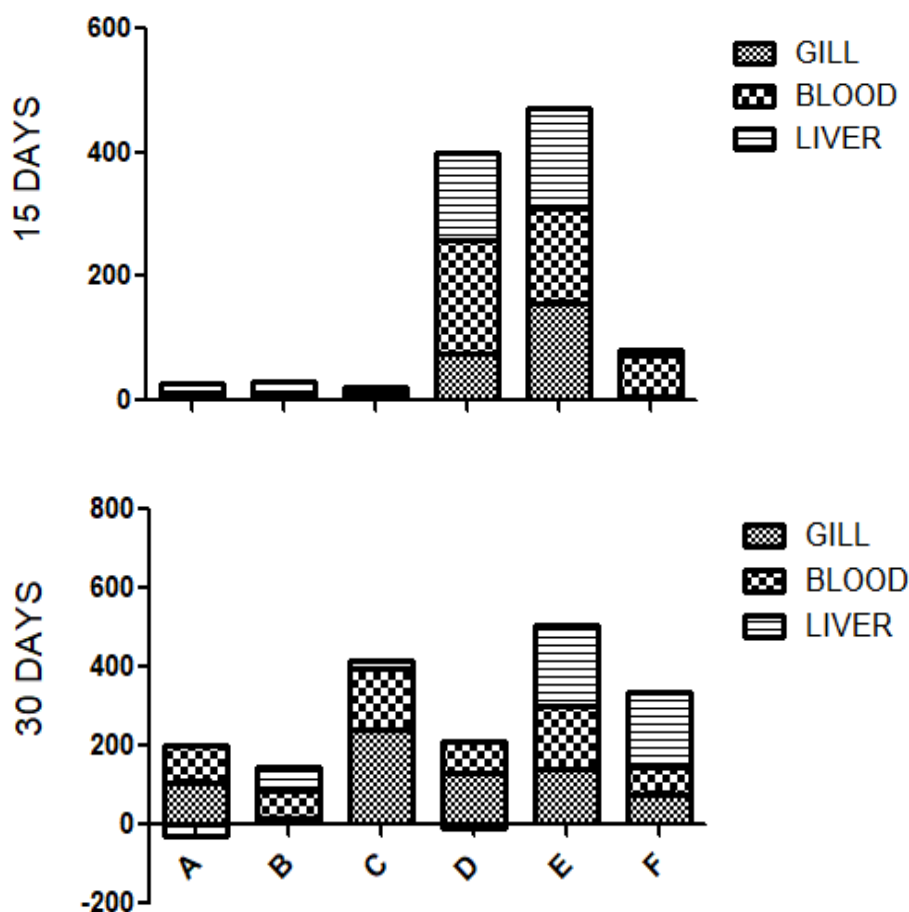


Figure 4. Biochemical analysis of LDH activity on gill, blood and liver in juvenile *Heteroclinarias* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

After 30 days, LDH activity was elevated across nearly all treatment groups, indicating cumulative toxic effects. Liver activity remained high, especially in groups E and F. Blood LDH showed moderate increases across concentrations, while gill activity fluctuated but remained elevated compared to earlier exposure periods. Unlike earlier time points, LDH distribution among tissues was more variable, but the liver still demonstrated the most consistent and pronounced elevation. When comparing exposure durations, LDH activity was lowest at 8 days, increased substantially at 15 days, and remained high at 30 days. This pattern suggests progressive tissue damage and metabolic disturbance due to prolonged Diclofenac exposure. Elevated LDH levels across tissues indicate cellular stress and membrane damage, supporting its role as a biomarker of toxicant induced tissue injury.

3. 8. Malondialdehyde (MDA) Activity in Gill, Liver, and Blood

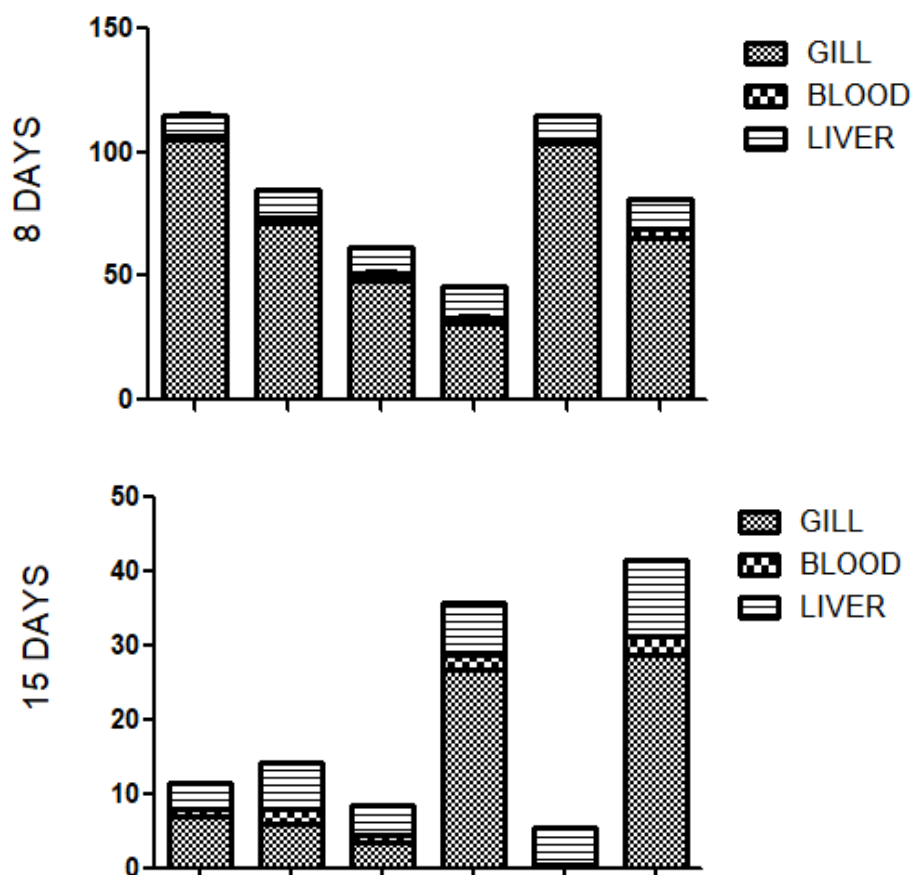
Figure 5 illustrates MDA activity in the gill, liver, and blood of juvenile *Heteroclinarias* following 8, 15, and 30 days of Diclofenac exposure.

After 8 days, gill activity was highest among the tissues, decreasing with declining concentration before rising again in the fifth group. Liver activity remained relatively stable, while blood activity was very low.

At 15 days, liver activity increased from the control to the second group, possibly due to the ameliorative effect of neem supplementation, then decreased across subsequent groups except for peaks in groups D and F. Gill activity showed minimal changes in the first three groups but rose sharply in the fourth and last groups, indicating oxidative stress, with the gill remaining the tissue with the highest MDA levels.

By 30 days, gill MDA activity was highest in the control, decreased in the second group, increased slightly in group C, stabilized in the next group, and then decreased progressively in groups E and F. Liver activity rose slightly in the first two groups, potentially reflecting low neem leaf concentrations followed by fluctuating increases and decreases at higher concentrations. Blood activity remained stable in the first two groups, rose in group C, and declined in groups D and E to rise slightly in the last group. Overall, the highest MDA activity was observed in fish exposed for 30 days, highlighting cumulative oxidative stress over prolonged exposure.

MDA



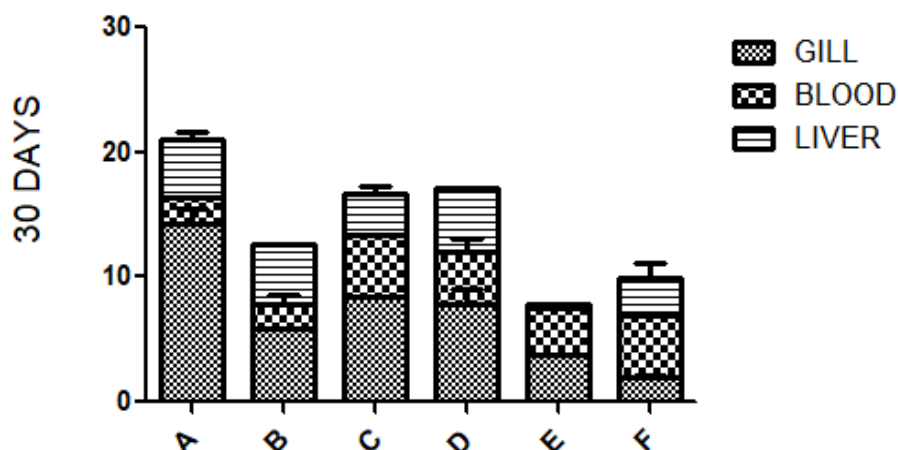


Figure 5. Biochemical analysis of MDA activity on gill, blood and liver in juvenile *Heteroclinarias* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

3. 9. Superoxide Dismutase (SOD) Activity in Gill, Liver, and Blood

Figure 6 presents superoxide dismutase (SOD) activity in the gills, blood, and liver of juvenile *Heteroclinarias* exposed to Diclofenac for 8, 15, and 30 days.

After 8 days, SOD activity was moderately elevated across all tissues, with only slight differences between groups. Gill, blood, and liver activities were relatively balanced, although groups with neem supplementation showed marginally higher liver and blood SOD levels compared to the control. This indicates an early antioxidant defense response to Diclofenac-induced oxidative stress.

At 15 days, SOD activity showed a more distinct pattern. Blood SOD activity increased noticeably, particularly in group B, where the highest overall SOD level was recorded. Liver and gill activities also rose slightly in some groups but remained lower than blood levels. Compared to day 8, the antioxidant response appeared more pronounced in the blood, suggesting increased systemic oxidative stress during mid-term exposure.

After 30 days, SOD activity declined in most tissues compared to earlier exposure periods. The highest SOD levels were observed in group C, especially in the liver and blood, while groups E and F showed reduced activity. Gill SOD levels remained relatively low across the groups except group C.

This reduction after prolonged exposure may indicate exhaustion or suppression of antioxidant defense mechanisms over time.

Comparing exposure durations, SOD activity was highest at 8 days, increased in certain groups at 15 days, and declined by 30 days. This trend suggests an initial activation of antioxidant defenses followed by a possible depletion or impairment of the system under prolonged toxic stress.

Variations among groups imply that neem supplementation may have provided partial modulation of oxidative stress responses.

Overall, SOD activity patterns support its role as a biomarker of oxidative stress, reflecting both the onset and progression of Diclofenac-induced physiological disturbances.

SOD

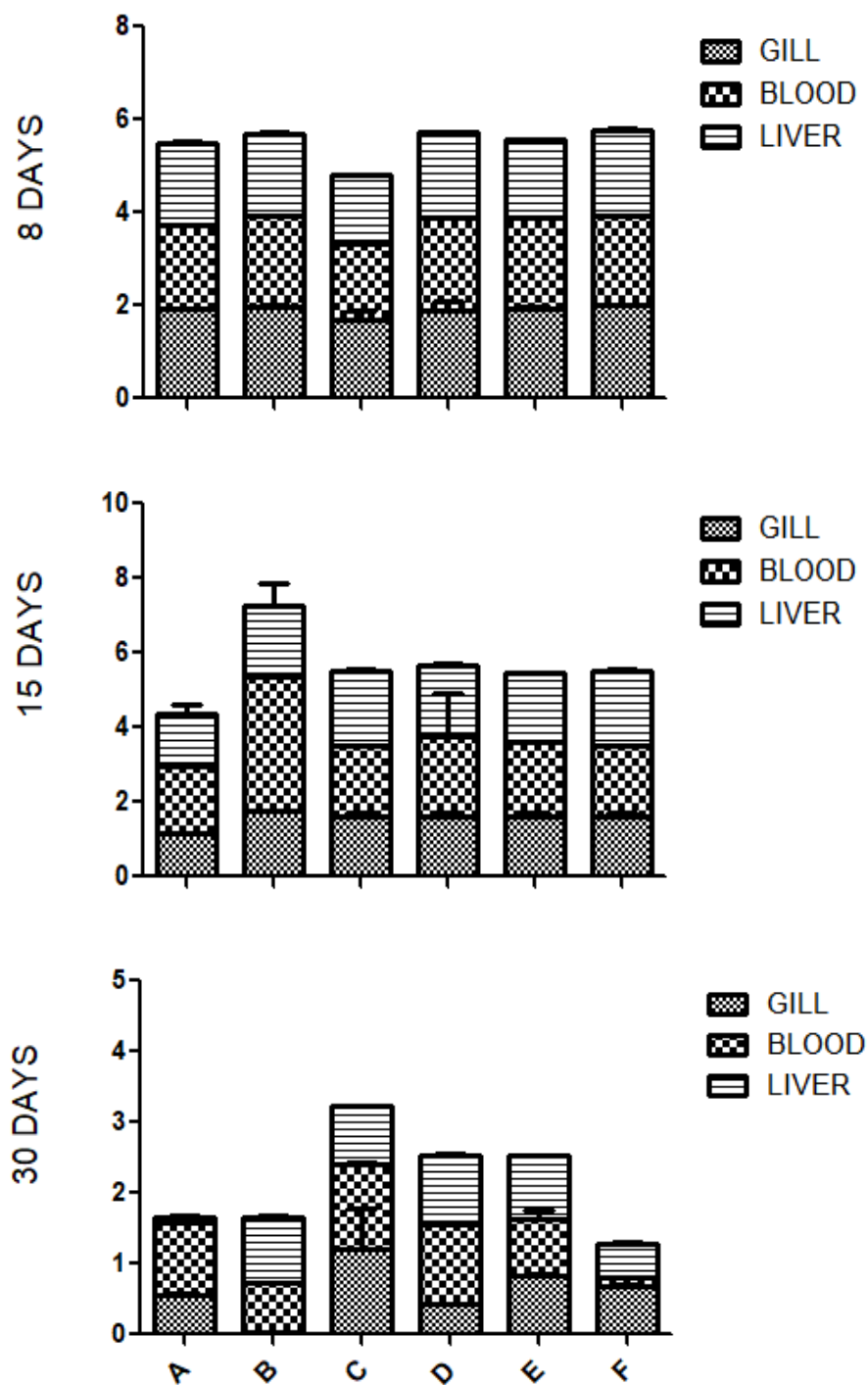


Figure 6. Biochemical analysis of SOD activity on gill, blood and liver in juvenile *Heteroclinarias* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

3. 10. Glutathione Peroxidase (GPX) Activity in Gill, Liver, and Blood

Figure 7 shows GPX activity in the gill, liver, and blood of juvenile *Heteroclinus* following 8, 15, and 30 days of Diclofenac exposure.

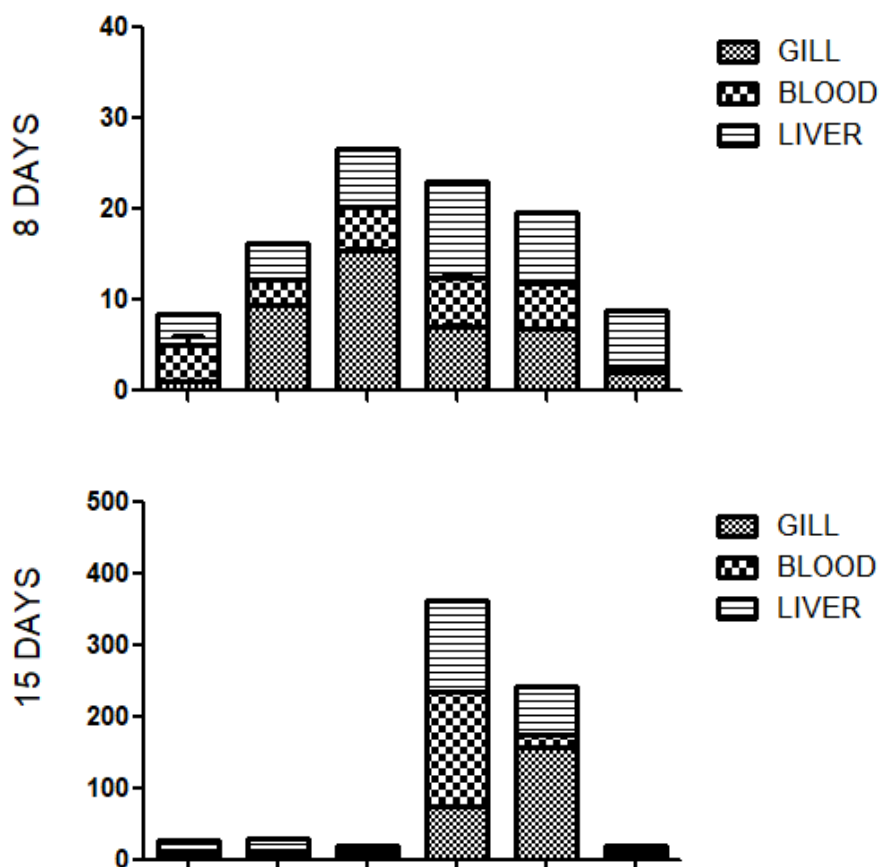
After 8 days, GPX activity was highest in the gill, making it the organ with the greatest activity, while liver remained relatively constant and blood showed slightly negligible activity.

At 15 days, GPX activity was low across most groups, except for significant increases in groups D and E. In this period, liver and blood peaked in group D, and gill activity was highest in group E, suggesting activation of antioxidant defenses in response to oxidative stress. Activity generally decreased in later groups, likely due to high hydrogen peroxide accumulation.

During 30 days of exposure, blood GPX activity increased steadily from the control to group D, then declined in the last two groups. Liver activity was highest in the control and gradually decreased with increasing neem leaf concentration. Gill activity was highest in the control, generally decreasing with concentration except in group E.

Overall, GPX activity was highest in the gill for 8 and 15 days exposure, while in 30 days it was highest in blood. Enzyme activity was generally greater in fish exposed for 8 days, indicating early oxidative response.

GPX



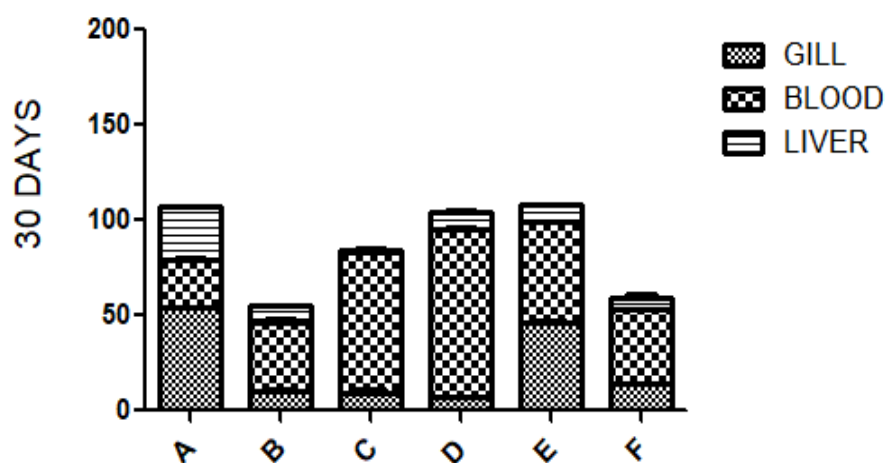


Figure 7. Biochemical analysis of GPX activity on gill, blood and liver in juvenile *Heteroclinarias* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

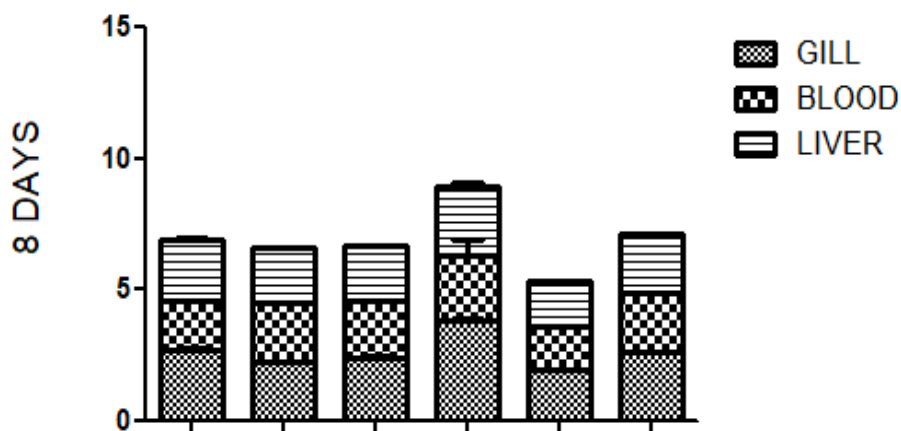
3. 11. Catalase (CAT) Activity in Gill, Liver, and Blood

Figure 8 presents the catalase (CAT) activity in the gill, liver, and blood of juvenile *Heteroclinarias* exposed to a sub-lethal concentration of Diclofenac for 8, 15, and 30 days.

Across all exposure periods, catalase activity was generally highest in the liver, followed by the gill, while the blood consistently showed the lowest activity, indicating tissue-specific antioxidant responses.

After 8 and 15 days of exposure, catalase activity remained relatively similar across the treatment groups, with values largely ranging between 5 and 10 units. Only minor variations were observed among groups, suggesting limited short-term effects of Diclofenac exposure and dietary supplementation on catalase activity during these periods.

CAT



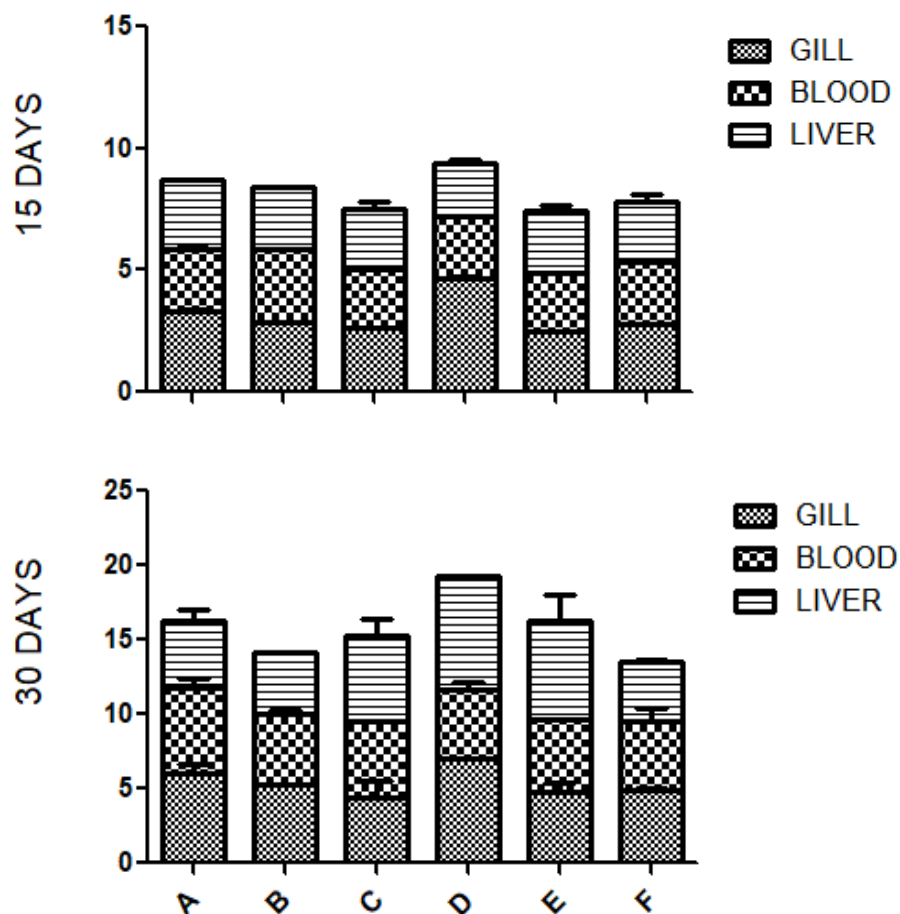


Figure 8. Biochemical analysis of CATALASE activity on gill, blood and liver in juvenile *Heteroclaris* exposed to sub-lethal concentration of Diclofenac (10.8 mg/l) and fed with supplementary diet mixed with ameliorative neem leaf for 8, 15 and 30 days.

Following 30 days of exposure, catalase activity increased noticeably across all tissues, particularly in the liver, where values generally ranged between 10 and 20 units. This indicates a stronger antioxidant response with prolonged exposure duration. Variations among treatment groups were evident, with group D recording the highest catalase activity, while groups B and F showed comparatively lower activity levels, suggesting differential effects of neem leaf concentration on enzymatic response.

Overall, the results demonstrate that catalase activity in juvenile *Heteroclaris* is both time and tissue dependent, with prolonged exposure enhancing antioxidant enzyme activity, particularly in the liver, while dietary treatments modulate the magnitude of this response.

4. CONCLUSION

Observations from this study indicate that diclofenac exerts significant toxic effects on the biochemical parameters of juvenile *Heteroclaris*, demonstrating its harmful impact on

aquatic organisms. At low concentrations (1–3%), neem leaves acted as an ameliorative agent, mitigating tissue damage caused by diclofenac. However, at concentrations exceeding 3%, neem leaves themselves exhibited toxic effects. These findings underscore the importance of careful management and disposal of pharmaceutical effluents to prevent harm to aquatic ecosystems.

Acknowledgements

The author gratefully acknowledges the contributions of colleagues who participated in different aspects of the undergraduate research project from which this study was derived. Their collaborative efforts during data collection and experimental procedures are sincerely appreciated. Special appreciation is extended to my project supervisor for academic guidance, constructive feedback, and supervision throughout the course of the study. I also acknowledge the support and encouragement of friends and mentors who contributed intellectually and morally during the research process.

References

- [1] Aguiar, C., Santos, A., & Rocha, E. Effects of phenol on the acetylcholinesterase activity of the fish *Brycon cephalus*. *Bulletin of Environmental Contamination and Toxicology*, 67(4), 2001, 525–531
- [2] Breton, R., & Boxall, A. B. A. Occurrence and fate of human pharmaceuticals in the environment. *Reviews of Environmental Contamination and Toxicology*, 175, 2003, 29–66
- [3] Cheesbrough, M. District laboratory practice in tropical countries (Part 2). Cambridge University Press, 2005.
- [4] Cooper, E. R., Siewicki, T. C., & Phillips, K. Preliminary risk assessment database and risk ranking of pharmaceuticals in the environment. *Science of the Total Environment*, 398, 2008, 26–33
- [5] Daughton CG, Ternes TA. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect.* 1999, 107 Suppl 6(Suppl 6): 907-38. doi: 10.1289/ehp.99107s6907
- [6] Duarte IA, Reis-Santos P, Fick J, Cabral HN, Duarte B, Fonseca VF. Neuroactive pharmaceuticals in estuaries: Occurrence and tissue-specific bioaccumulation in multiple fish species. *Environ Pollut.* 2023; 316(Pt 1): 120531. doi: 10.1016/j.envpol.2022.120531
- [7] Fatta-Kassinos, D., Vasquez, M., & Nikolaou, A. Environmental fate of pharmaceuticals in water systems. *Environmental International*, 36, 2010, 733–743
- [8] Gill, T. S., Tewari, H., & Pande, J. In vivo and in vitro effects of cadmium on selected enzymes in different organs of the fish *Barbus conchoniis*. *Comparative Biochemistry and Physiology Part C*, 100, 1991, 501–505
- [9] Hassanein, M. A. Changes in acetylcholinesterase activity of fish exposed to pesticides. *Journal of Environmental Science and Health, Part B*, 37(1), 2002, 1–9

- [10] Hernández-Zamora, M., Michelangeli, M., Bertram, M. G., & Martin, J. M. Evidence of the impacts of pharmaceuticals on aquatic animal behaviour (EIPAAB): A systematic map and open-access database. *Environmental Evidence*, 14, 2025, Article 4. <https://doi.org/10.1186/s13750-025-00357-6>
- [11] Jee, J. H., Masroor, F., & Kang, J. C. Responses of cytochrome P450, metallothionein, and antioxidant enzymes in olive flounder (*Paralichthys olivaceus*) exposed to benzo[a]pyrene. *Environmental Toxicology*, 20, 2005, 223–232
- [12] Johnson, K. J., Adekunle, A., & Ogunyemi, A. Bioaccumulation of pharmaceuticals in freshwater predators: Implications for aquatic ecosystems. *Environmental Pollution*, 189, 2014, 56–63
- [13] Klaminder, J., Fick, J., & Brodin, T. Ecological effects of neuroactive pharmaceuticals on freshwater fish. *Aquatic Toxicology*, 150, 2014, 133–141
- [14] Little, E. E., & Brewer, S. K. Neurobehavioral toxicity in fish. *Environmental Toxicology and Chemistry*, 24(2), 2005, 295–305
- [15] Melvin, S. D., & Rodriguez, J. Behavioral effects of pharmaceuticals in fish. *Environmental Toxicology and Chemistry*, 31, 2012, 1227–1236
- [16] Melvin, S. D., & Wilson, S. P. The utility of behavioral endpoints in aquatic toxicology. *Environmental Toxicology and Chemistry*, 32(1), 2013, 1–9
- [17] Mousa, A. Effects of chemical stressors on biochemical biomarkers in fish. *Journal of Environmental Science and Health, Part B*, 39, 2004, 123–131
- [18] Nikolaou, A., Meric, S., & Fatta-Kassinos, D. Occurrence patterns of pharmaceuticals in water and wastewater environments. *Analytical and Bioanalytical Chemistry*, 387, 2007, 1225–1234
- [19] Reitman, S., & Frankel, S. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *American Journal of Clinical Pathology*, 28, 1970, 56–63
- [20] Scott, G. R., & Sloman, K. A. The effects of environmental pollutants on complex fish behaviour. *Environmental Biology of Fishes*, 71(4), 2004, 369–392
- [21] Shalaby, A. M., Khattab, Y. A., & Abdel Rahman, A. M. Effects of garlic (*Allium sativum*) and chloramphenicol on growth performance of Nile tilapia. *Journal of Veterinary Science*, 8(1), 2007, 83–88
- [22] Stakelberg, M. J., Young, T. M., & Martin, J. D. Occurrence of endocrine-disrupting compounds in aquatic environments. *Environmental Science & Technology*, 41, 2007, 146–152
- [23] Stock, J., & Dormandy, T. L. The autoxidation of human red cell lipids induced by hydrogen peroxide. *British Journal of Haematology*, 20, 1971, 95–111
- [24] Topp, E., Monteiro, S. C., & Beck, A. Runoff of pharmaceuticals from agricultural soils amended with manure. *Environmental Toxicology and Chemistry*, 27(1), 2008, 123–132

- [25] Woodward, G., Perkins, D. M., & Brown, L. E. Climate change and freshwater ecosystems: Impacts across multiple levels of organization. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362, 2007, 209–223
- [26] Ziemba, R. Behavioral variability as an ecological indicator. *Ecological Indicators*, 4(3), 2004, 287–294