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Combined formulation of Acetamiprid and Pyriproxyfen induces Histopathological, Hematobiochemical, and nuclear alterations in Bighead carp (*Hypophthalmichthysnobilis*)

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Acetamiprid and Pyriproxyfen are used as active substances in various insecticides, their combined formulation has remarkable effectiveness for alleviation of pest at broad-spectrum. The study was executed to investigate the mixture toxicity of ACM and PPF on visceral organs, hematology, and histobiochemical parameters of Bighead carp. A total of 108 fish after complete clinical monitoring was arbitrarily distributed into three groups, with each group being replicated twice. Each aquarium contained 12 fish. Fish kept in group T0 was taken as untreated control group with normal diet. Two concentrations of ACM and PPF 2.0 and 4.0mg\L were applied in respective groups T1 and T2 for 26 days. Significant decreasing and increasing trend were noted in hematological parameters in exposed groups ($P<0.05$). Our study results depicted significantly ($P<0.05$) raised values of biochemical parameters as compared to control group. Significantly ($P<0.05$) reduced body weight of exposed fish were measured at day 26 in group treated with 4.0mg\L as compared to unexposed fish. Absolute weight of gills and kidneys were significantly ($P<0.05$) elevated in T2 group (4.0mg\L) at day 13 and 26 as compared to control group. Micrographs of blood smear showed various cellular and nuclear changes at day 26 to 2.0mg/L and 4.0mg\L. Microscopic results of gills and kidney sections showed prominent abnormalities in treated group (4.0mg\L) as compare to control group at day 26. Based on findings of present study, it can be inferred that ACM and PPF exposure to surface water bodies can cause detrimental effects on fish health and physiology.

Keywords: ***Hypophthalmichthys nobilis*, Acetamiprid and Pyriproxyfen, Histopathology, Hematobiochemical parameters**

INTRODUCTION

Pesticides are chemical substances utilized to control pests that pose threats to crops, livestock, or public health (Abaineh et al. 2024). Pesticides play a crucial role in agriculture by enhancing yields and reducing crop losses, their indiscriminate use raises concerns about environmental and health impacts. Pesticides can contaminate soil, water, and air, affecting non-target organisms and ecosystems (Khanet al.2023). Pesticides accumulation cause a detrimental effect on fish fauna (Sreenivasa Rao and Pillala, 2001; Clasen et al. 2018). The effect of pesticides in fish results in fish behavioral changes (Ghelichpour et al. 2020).

Acetamiprid residual concentrations were found in surface water bodies are 2–410 µg/L globally, endangering the survival of aquatic untargeted creatures (Ma et al. 2022). In non-aquatic fauna including humans, acetamiprid application resulted memory dysfunctions, respiratory failure, vomiting, nausea, hypotension, convulsions, muscular weakness and hypothermia (Shamsi et al. 2021). In zebra fish embryos, (Ma et al. (2019) determined that acetamiprid inhibits development and produces morphological deformities. Cossi et al. (2020) demonstrated that acetamiprid largely affects detoxification and antioxidant indicators, showing that toxicity pathways are connected to detoxification and oxidation metabolism in *Biomphalaria stramine*.

Pyriproxyfen can cause the deaths of a variety of nontarget species, including fish residing in aquatic habitats, while controlling mosquitoes (Caixeta et al. 2016). Earlier, PPF values (89.66 ng/L) were found in water samples taken from the river (Belenguer et al.,

2014). Additionally, distinct lethal doses of PPF (LC50) have been studied in a variety of fish species, including rainbow trout (Little et al. 1990), *Labeo rohita* (Naseem et al.2022), and zebrafish embryos (Maharajan et al. 2018). High pyriproxyfen concentrations have been observed to cause congenital defects in embryos (Truong et al. 2016), and erratic swimming in *Xiphophorus maculates* (Caixeta et al. 2016)

Pyriproxyfen act as an effective disrupter in growth and physiological processes of insects at juvenile stages and Acetamiprid work as active neurological disrupter (Elbert et al.,2008; Gasmi et al. 2017) which control variety of insects and pests of commercial crops along with ornamental plants (Kong et al. 2017; Bagri and Jain, 2019).Their combined formulation provides ultra-effective measures against pest controls of a wide range. High dose and prolonged exposure of acetamiprid can increase the risk of toxicity (Phogat et al. 2022).

Pollutants mixed with water bodies through various sources. The aquatic animals intoxicated by toxic chemicals through gills or by consumption of toxic food (Banaee et al.2015), accumulated chemicals ultimately become the part of food chain. Hematological investigation offers a blueprint of health condition and internal environment alterations of the animal (Saravanan et al. 2011; Narra et al. 2015). Serum biochemistry parameters are reliable and useful biomarkers, frequently used to monitor the toxic effects (Gul et al.2017). Histopathological alterations considered as prominent indicators for monitoring the effects of numerous contaminants on aquatic organisms and reflected the overall health status of the whole ecosystem (Drishya et al. 2016).

Bighead carp (*Hypophthalmichthys nobilis*) is a freshwater fish with considerable growth, high resistant to diseases, and with high quality flesh and nutrition, having status in ranked fourth in yearly output among freshwater species cultured in 2012 in China (Marian and Krasznai, 1978). It is a cultivable fish species that may be found in Pakistan's rivers and freshwater lakes (Mahmood et al. 2022). Among other aquatic creatures, fish play a key role in water biomonitoring. Because aquatic life is extremely susceptible to toxicant disintegration, fish has been used as a biological indicator to detect the presence of harmful substance or its impact level on the molecular, cellular, and physiological processes (Sabullah et al. 2015).

The current research was carried out on Bighead carp for ecotoxicological studies. It is a preliminary study to investigate the Bighead carp physiology, hematology, biochemical profile and histopathology under different concentrations of pyriproxyfen and acetamiprid. Additionally, no data is available regarding combined toxicity of ACM and PPF on fish species.

MATERIALS AND METHODS

Ethical approval:

The present research was executed in the laboratory of Department of Zoology (Aquaculture, Genetic Toxicity, and Molecular Biology Laboratory), The Islamia University of Bahawalpur. The ethics of animal handling were strictly followed suggested by institutional Bioethics Committee (IBC) of The Islamia University of Bahawalpur, Pakistan.

Chemical and experimental fish acquired:

Pyriproxyfen (19.80% w/w) and Acetamiprid (18.35% w/w) issued by orange protection pesticide company in dual formulation were acquired from commercial scientific store at Bahawalpur, Pakistan. Healthy individuals of Bighead carp weighing between 100 to 120 grams were procured from local fisheries farm Bahawalnagar, Pakistan. Fish were taken to experimental site in polythene bags occupied with oxygen to ensure the fish survival.

Test management:

Fish undergoes the acclimatization phase for 10 days in glass aquaria having water retaining capacity of 150 liters and dimensions of 14" L × 10" W × 12" H. All the water quality parameters were agreed to international standards of water quality suitable for fresh water fish species. Standard conditions were maintained throughout the experiment. Oxygen was regulated through aerators, and fecal material was removed regularly to avoid ammonia toxicity. After acclimatization level, fish (n=108) were divided equally into three groups (T0-T2) with two replicated units for each group. Total of 12 fish were subjected into100liter water area of each aquarium.

Toxicant and feeding strategy:

Fish of group T0 kept as untreated control group, remaining groups T1 and T2 were exposed to mixture of ACM and PPF @ 2.0mg/L and 4.0mg/L respectively for 26 days. Commercial fish feed of 30% protein were given daily at the 2% of their body weight. Inspection of each aquarium was done on daily basis to find any mortality and clinical ailments. Experiment was divided into two samplings; first sampling was done at day 13 and second sampling was happened at day 26.

Hematology and serum analysis:

At day 13 and 26 blood sample of about 2.5ml was taken from three fish per replicate (n=3) of each group through caudal venipuncture by using disposable 21-gauge hypodermic needle, about 0.5ml blood was collected immediately into anticoagulant EDTA vials for hematological analysis, few drops of fresh blood were taken on glass slides to prepare the thin blood smears for micronuclei assay. 2ml blood was centrifuged at 4000 x g for 5-8 mins for serum separation. Total erythrocytes and leukocytes count, hemoglobin, hematocrit, and platelets values were examined by using hematology analyzer

(Sysmex, Kx21). Serum biochemical parameters like total proteins, glucose, triglycerides, urea, creatinine, ALT, ALP, and AST were assessed by using Fully Automated Veterinary Biochemistry Analyzer (SMT-120: Seamaty, A00120VETEND).

Histopathological analysis and visceral organs measurements:

At day 13 and 26 fish from each replicate of treated and control group were weighed by using digital weight balance (SF-400A), and dissected for removal of gills and kidneys. Collected organs were immediately weighed on Ohaus pioneer analytical balance, and preserved in 10% paraformaldehyde solution, and processed for histological studies according to protocol mentioned by (Afzalet al. 2022). Prepared gills and kidney sections were observed under the digital microscope (Olympus CX43).

Statistical analysis:

Mean±SE values related to whole body and organs measurements, hematology and serum biochemical parameters of each group were calculated by the Tukey multiple comparisons tests in SPSS (15.0), with significant level $P<0.05$.

RESULTS

Clinical responses and visceral organs weight:

Control group (T0) fish was found without any mortality and clinical reactions. Fish of treated groups with low to high doses of ACM and PPF showed moderate to severe clinical indications such as irregular swimming, air gulping, fins darkening, upturned movement, sluggishness, spasms, and excessive mucous secretion from the mouth. With the passage of exposure time severity of clinical signs were increased.

The mean values of whole body weight of fish of group T2 decreased significantly ($P<0.05$) at day13 and 26 as compared to control fish. The absolute weight of gills and kidneys of fish in group T2 at day 13 and 26 increased significantly ($P<0.05$) as compared to untreated fish. The results obtained on body weight and absolute visceral organs weight of bighead carp treated with different doses of ACM and PPF depicted in Table1.

Hematology and serum biochemistry responses:

Significantly ($P<0.05$) decreasing pattern of RBC counts, Hb, and hematocrit contents were measured in fish of group T1-T2 with increase of chemical dose and time. Whereas, platelets, and total leukocyte counts were significantly ($P<0.05$) increased at day 13 and 26 in fish of group T2 as compared to T1 and control group. Variations occurred in blood profile of Bighead carp at different doses of ACM and PPF depicted in Table 2.

The biochemical markers of liver like ALT, AST, and ALP (UL^{-1}) were significantly ($P<0.05$) elevated in fish of group T2 at day 13 and 26 when compared to control group fish. Kidney biomarkers including urea (mg/dl) and creatinine (mg/dl) in fish retained in group T2 were also raised significantly ($P<0.05$) at day 13 and 26 in comparison to untreated fish. The triglycerides (mg/dl) and glucose (mg/dl) contents were also depicted significantly ($P<0.05$) increasing trend in fish of group T1-T2when associated with fish of group T0. Alterations in serum profile of Bighead carp at different doses of ACM and PPF described in Table 2.

Cellular and nuclear responses of erythrocytes:

Regular shaped erythrocytes with normal nuclei were observed in control fish. Micrographs of blood smear of treated fish with 2.0mg/L and 4.0mg/L of ACM and PPF showed various abnormalities at day 26 Figure 1(a)-1(b). Mild to moderate changes in erythrocytes and nuclei shape such as blebbed nuclei, deformed nuclei and spherocytes were noticed in fish of group T1, while prominent irregularities of erythrocytes such as blebbing in nuclei, bilobate nuclei and pear shaped erythrocytes were found in fish of group T2 at day 26.

Histopathological responses:

Histopathological studies of gills sections of exposed fish showed moderate to severe microscopic abnormalities at day 26 Figure 2(a)-2(b). Gills sections of low dose (2.0mg/L) treated fish were found with fused and uplifted lamellae and disruptions. At high dose (4.0mg/L) gills section showed noticeable lesions including disruptions in cartilaginous bar, aneurysm, lamellar degeneration with necrotic epithelium at day 26. Histological kidney alterations such as moderate congestion, necrosis in renal tubules, and enlargement of bowman space were found in fish of group T1 (2.0mg/L). Pronounced alterations in kidney tissues such as severe necrosis of tubules, degeneration of intra tubular lumen, congestion, abnormal sinusoids and visible gap between epithelium and basel lamina of glomerulus were observed in treated fish to high dose (4.0mg/L) of ACM and PPF at day 26. Moderate to severe microscopic changes in kidney sections of treated fish with different doses at day 26 marked in Figure 2(c)-2(d).

Table1: Whole body and absolute weight of visceral organs of fish exposed to different concentrations of ACM and PPF

Parameters/days	Exposed groups		
	Control T0 (0.0mg/L)	T1 (2.0mg/L)	T2 (4.0mg/L)
Whole body weight (g)			
13	114.66 ±4.16	103.25 ±2.63	94.33 ±3.05
26	115.45 ±3.52	96.75±3.42	83.24±2.16*
Absolute weight of gills (g)			
13	4.12 ±0.16	4.25 ±0.24	4.80 ±0.20*
26	4.24± 0.09	4.28 ±0.17	4.89 ±0.64*
Absolute weight of kidney (g)			
13	0.30 ±0.12	0.35 ±0.11	0.40 ±0.08*
26	0.32 ±0.13	0.38± 0.10	0.42 ±0.09*
Mean±SD values with asterisk in each line show significant difference to control group (one way ANOVA, Significant level P<0.05, Tukey multiple comparisons tests in SPSS – 15.0.).			

Table2: Hematobiochemical profile of fish exposed to different concentrations of ACM and PPF

Parameters/days	Exposed groups		
	T0 (0.0mg/L)	T1 (2.0mg/L)	T2(4.0mg/L)
Total erythrocyte counts (10^6/mm^3)			
13	2.65±0.09	1.96±0.08	0.64±0.04*
26	2.50±0.08	1.89±0.03	0.52±0.02*
Hemoglobin (gdL^{-1})			
13	8.75±0.14	7.80±0.09	5.45±0.12*
26	8.56±0.06	7.06±0.10	5.18±0.08*
Hematocrit contents (%)			
13	32.13±1.02	28.96±1.09	19.6±0.43*
26	30.93±2.14	25.72±1.12*	17.43±0.23*
Total Leukocyte counts (10^3/mm^3)			
13	20.66±3.05	35.40±1.03*	48.33±1.08*
26	23.25±2.45	42.16±1.20*	64.10±1.14*
Platelets (10^3/mm^3)			
13	23.30±1.01	30.42±1.06	45.20±0.05*
26	24.12±1.08	38.02±1.14*	68.66±0.10*
Alanine transaminase (UL^{-1})			
13	18.92±0.27	20.87±0.18*	24.08±0.72*
26	19.47±0.57	22.57±0.64*	26.36±0.91*
Aspartate aminotransferase (UL^{-1})			
13	23.75±0.35	34.02±0.23*	56.85±0.24*
26	24.12±0.27	36.55±0.30*	58.45±0.62*
Alkaline phosphatase(UL^{-1})			
13	22.90±0.54	25.12±0.30*	29.13±0.37*
26	23.73±0.033	26.60±0.66*	34.66±0.40*
Urea (mg/dL)			
13	10.13±0.01	12.08±0.13*	15.75±0.25*
26	11.55±0.07	13.21±0.12*	16.96±0.16*
Creatinine (mg/dL)			
13	1.18±0.03	1.86±0.03	2.34±0.02*
26	1.23±0.04	2.06±0.03*	2.50±0.04*
Triglycerides (mg/dL)			
13	141.66±3.51	158.32±1.52	195.08±1.02*
26	145.20 ±2.08	162.66 ±1.08*	212.24±1.15*
Glucose (mg/dL)			
13	37.33±1.15	44.66±1.03*	52.33±1.05*
26	40.0±1.04	48.30±0.93*	56.15±0.10*

Mean±SD values with asterisk in each line show significant difference to control group (one way ANOVA, Significant level P<0.05, Tukey multiple comparisons tests in SPSS – 15.0.).

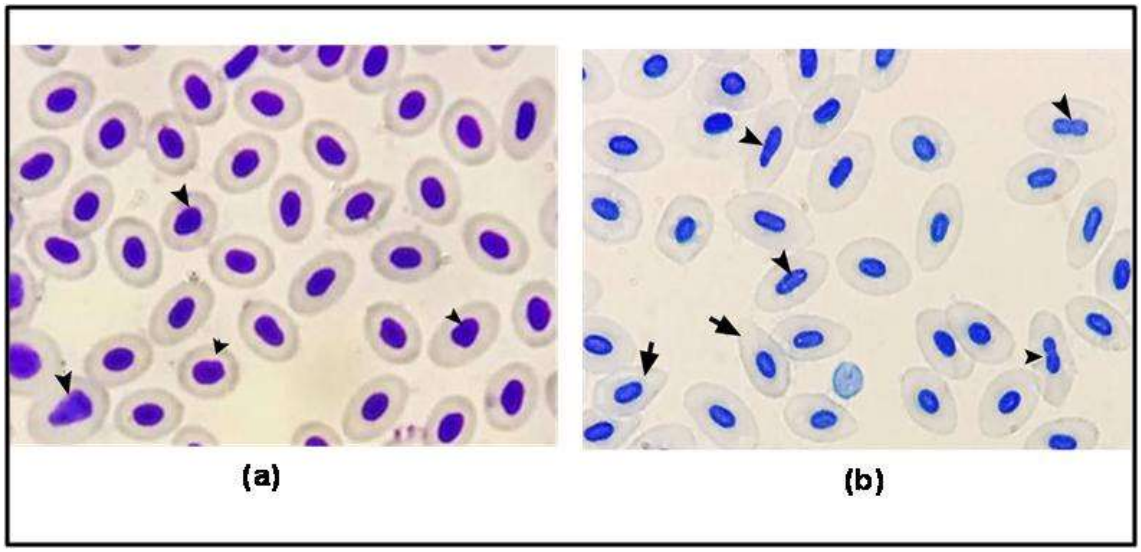

Figure1: Photomicrograph of blood smear of bighead carp (arrowheads and arrows) showing irregularities (a) showing spherocytes, blebbed nucleus and deformed nuclei at day 26 to (2.0mg/L) ACM and PPF. (b) Showing pear shaped cells, bilobed nuclei, blebbed nuclei at day 26 to (4.0mg/L) ACM and PPF. 100x. Field Stain A & B

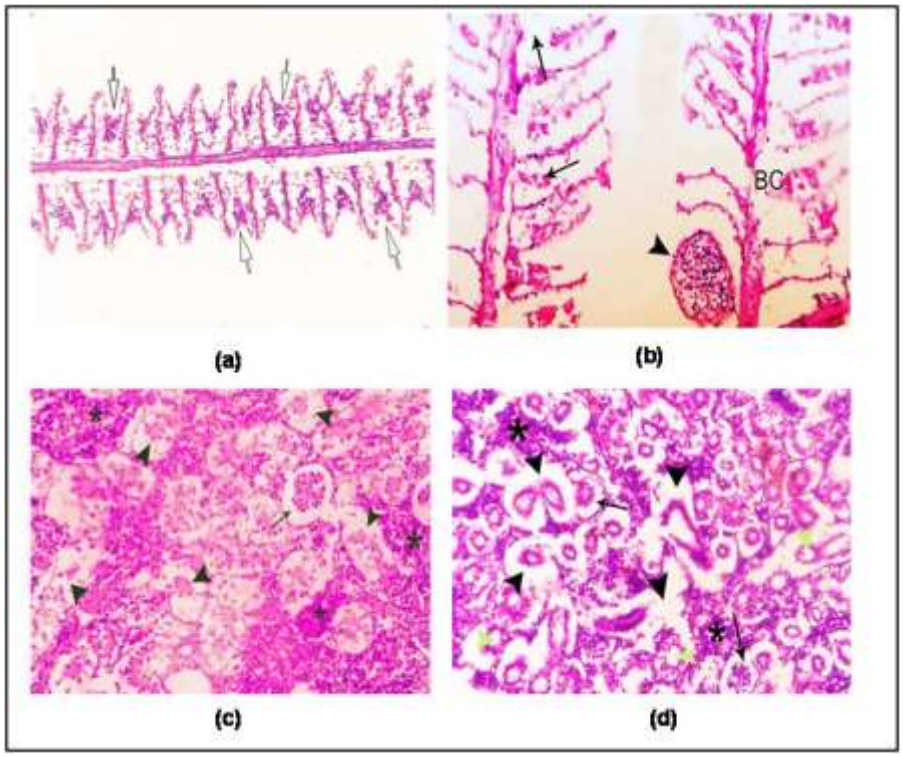

Figure 2: Micrograph of bighead carp (a) gills showing lamellar uplifting and fusion (empty arrows) at day 26 to (2.0mg/L) ACM and PPF. (b) gills showing lamellar necrosis (arrows), aneurysm (arrowhead) and disruptions in cartilaginous bars (BC) at day 26 to (4.0mg/L) ACM and PPF. (c) Kidney section showing congestion (*), renal tubular necrosis (arrow heads), and enlargement of bowman space (arrow) at day 26 to (2.0mg/L) ACM and PPF. (d) Kidney showing severe necrosis of tubules (arrow heads), degeneration of intra tubular lumen (green arrows), severe congestion (*), and visible gap b/w epithelium and glomerulus (arrows) at day 26 (4.0mg/L) ACM and PPF. 40 x. H&E staining.

DISCUSSION

The persistent and inappropriate use of pesticides and insecticides in agriculture sector can cause deleterious effect on aquatic animal's health. The toxic extent of pesticides for aquatic life is a critical concern. Many studies have investigated that exposure to pesticides and insecticides at low levels are mainly related to the induction of adverse effects like immunosuppression, cancer, endocrine disruption, and reproductive disorders. Furthermore, regular use of pesticides can accumulate in the tissues of organisms over time, and become the part of food chain (Yang et al. 2021). Assessing the possible toxicity of ACM and PPF to aquatic environments is essential for mitigate the public health risks. For toxicological evaluation clinical ailments, blood biochemical and histopathological studies are known as reliable biomarkers.

The Bighead carp in the control group was found healthy and no mortality was monitored during experiment while under acetamiprid and pyriproxyfen exposure it revealed multiple irregularities in behavior such as irregular swimming, air gulping, fins darkening, upturned movement, sluggishness, spasms, and excessive secretion of mucous from the mouth and gills. According to (Ghayyur et al. 2021) *Cirrhinus mrigala* showed similar behavioral changes when exposed to combinations of Acetamiprid, Chlorfenapyr and Dimethoate. Similar pattern of fish behavior was also documented by (Ghelichpour et al. 2020) when *Cyprinus carpio* intoxicated to lufenuron. (Ghayyur et al. 2019) revealed similar behavioral responses in *Oreochromis mossambicus* tested with chlorpyrifos.

Our results indicated reduction in body weight of fish with increase of dose and exposure time, (Naseem et al., 2022) reported that different concentration of PPF with increment in exposure time for 30 days lead to drastically reduction in whole body weight of *Labeo rohita* our results are agreed with reported research work. In current study, the absolute weight of gills and kidneys of treated fish were gradually increased with time and dose dependent manner, similar results were observed for *Labeo rohita* visceral organs as they undergoes the different concentrations (3.0mg/L, 6.0mg/L and 9.0mg/L) of PPF for 30 days (Naseem et al. 2022). In earlier research works the data related to combined effect of PPF and ACM on fish body weight and visceral organs weight is scanty.

In current study the blood parameters of fish showed decreasing trend in erythrocytes counts, hemoglobin and hematocrit values with increase of PPF and ACM exposure. Our results are in agreement with results reported by variety of authors on various fish species exposed with different pesticides (Ghayyur et al. 2019; Woryi et al. 2020). Previously, deleterious toxic impacts on blood profile in PPF exposed fish also been documented (Naseem et al. 2022). The reduction in erythrocyte count could be due to the disruption in hematopoietic tissues and breakage of red blood cells under toxic effect (Gul et al. 2017). In present investigation the total leukocytes count and platelets raised significantly with increase of chemical concentration. The increase in leukocytes can be relate to the activation of immune system in response to stress or pollutants, which provides immunity to withstand in unfavorable environment (Malik and Maurya, 2014). Significant increment in total leukocytes & platelets count was recorded by (Ghayyur et al. 2021) in *Cirrhinus mrigala* under treatment of Chlorfenapyr. (Ghayyur et al. 2019) also testified the elevated level leukocytes counts and platelets when *O. mossambicus* were exposed to different concentrations of Chlorpyrifos. (Hussein et al. 2019) found the relevant results on blood profile when investigated the toxic impact of endosulfan on fresh water fish *C. idella.*

In this study, pesticides mixture treated fish revealed considerable enhancement in serum biochemical parameters. ALT, AST, and ALP level increased in investigated fish can be related to the oxidative stress caused by toxicant that can lead to the damaging of hepatocytes cell membrane and leakage of these enzymes to the blood channel (Rahman et al. 2019). The significant elevation in serum ALT, AST, and ALP was seen in *Labeo rohita* when intoxicated to pyriproxyfen. Study indicated the PPF effect on serum biochemical parameters of fish which are accordance to present study results (Naseem et al. 2022). Similar responses in liver enzymes of *C. carpio* and *Oreochromis niloticus* were also noticed when treated with profenofos and deltamethrin respectively (Dawood et al. 2020a). In current experiment the serum urea and creatinine contents were also noticeably increased in exposed fish. Our results are coherent with the findings of (Naseem et al. 2022) when *Labeo rohita* experimented with different doses of pyriproxyfen.

In current assay erythrocytes were found with visible abnormalities like blebbed nuclei, deformed nuclei, spherocytes, bilobed nuclei and pear shaped cells, more or less common changes were detected in bighead carp under the effect of bisphenol A (Akram et al. 2021). Variations in erythrocytes morphology can be more related to oxidative stresses in erythrocytes of fish (Akram et al. 2021).

Our histological results of gills and kidneys sections exhibited that mixture form of acetamiprid and pyriproxyfen with the passage of time induced detrimental changes in histology of bighead carp. Gills sections were found with sloughed lamellae and disruptions in cartilaginous bar, aneurysm, and necrosis. Similar prominent abnormalities in gills sections of *C. mrigala* were spotted under the co exposure of chlorfenapyr, Dimethoate and Acetamiprid Earlier reports also agreed with the similar changes in gills like aneurysm, disruptions, hyperplasia, and necrosis of respiratory epithelium, edema, and lamellar curling in fish

exposed to different toxic chemicals (Dawood et al. 2020b; Jabeen et al. 2021; Naseem et al. 2022). In present study no pathological changes in kidneys section of control group were detected. The treated fish in relation to different doses of chemical showed moderate to severe changes like congestion, necrosis, abnormal sinusoids and renal tubules degeneration were evident. More or less related changes were observed in kidney sections of various fish species in response to toxicants as mentioned by different researchers (Pal and Reddy, 2018).

CONCLUSIONS

This study highlighted the modifications in hematobiochemical, and histopathological profile of investigated fish (Bighead carp). Study clearly demonstrated that combined formulation of Acetamiprid and Pyriproxyfen can causes adverse effects on fish physiology. The findings obtained from this study are primarily valuable to assess the human health risks which can produce by intake of intoxicated fish.

Supplementary materials

Not applicable

Author contributions

All authors contributed equally. They each provided their final consent for publication and acknowledged for all aspects of the work.

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Institutional Review Board Statement

The study was approved by the Bioethical Committee of the Islamia University of Bahawalpur, Pakistan.

Informed Consent Statement

Not applicable.

Data Availability Statement

All of the data is included in the article/Supplementary Material.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this research paper.

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