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Sublethal Toxicity of Acetaminophen and Iburophen in *Oreochromis niloticus*: Hematological, Biochemical and Enzymological Parameters

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Abstract: Unsystematic discharge of pharmaceutical waste into the aquatic ecosystem can create serious health problems for aquatic biota and ultimately for the human world as well. The present study was designed to determine the impact of acetaminophen and ibuprofen [commonly used nonsteroidal anti-inflammatory drugs (NSAID)] on hematological, biochemical and enzymological parameters of a freshwater fish *Oreochromis niloticus*. Fish were exposed to acetaminophen (128.6 mg/l^{-1}) and ibuprofen (142.2 mg/l^{-1}) for a period of 28 days and samples were taken every 7th day. Chronic exposure of acetaminophen and ibuprofen altered hematological, biochemical and enzymological parameters. Hematological parameters such as hemoglobin (Hb), red blood cells (RBC) and hematocrit (Hct) values were significantly decreased in drug treated groups whereas, white blood cell (WBC), count was significantly increased in fish treated with acetaminophen and ibuprofen compared with the control group. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values were found to be decreased in acetaminophen treated drugs whereas a significant increase was noted in ibuprofen treated groups. Mean corpuscular hemoglobin concentration (MCHC) values were significantly decreased in fish treated with ibuprofen compared with the control group. Exposure to acetaminophen and ibuprofen resulted in significantly ($P<0.05$) lower values for protein level in the muscle of fish. Glucose level was found to be increased in both drug treated groups. The activity of acid phosphatase (ACP) and alkaline phosphatase (ALP) were significantly increased in the muscle of drug treated fish. Changes observed in this study showed that both ibuprofen and acetaminophen negatively affected the health of the fish and the discharge of both the drugs into the aquatic environment should be monitored and controlled for the benefit of both fish and the human community. Furthermore, changes in these parameters can be effectively used to monitor the impact of pharmaceutical drugs in the aquatic environment.

Keywords: Ibuprofen, Acetaminophen, *Oreochromis niloticus,* Hematological, Biochemical, Hemoglobin,

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Introduction

The environmental impact of pharmaceuticals is a recognized global problem and there is an urgent need to assess their presence in the environment. Pharmaceutical pollution of the environment is caused by the presence of pharmaceutical products and their metabolites, which reach the aquatic environment. These substances enter the aquatic ecosystem primarily through discharge from hospital disposal, rain washing of feces, domestic sewage and sewage treatment plants (STP). Many medications have been detected in the aquatic environment such as ibuprofen, carbamazepine, acetaminophen and many others. Many of these are persistent and personal care products (PPCPs) are being released into rivers on a daily basis. Due to resistance to degradation and lipophilic properties, most drugs persist in the aquatic environment and could negatively affect the biota (Kasprzyk-Hordern *et al*., 2008; Ambili *et al*., 2013; Pereira *et al*., 2020; Ershad *et al*., 2021; Duarte *et al*., 2022).

Acetaminophen is a non-steroidal anti-inflammatory drug (NSAID) and mostly used as an analgesics throughout the world (Kristensen *et al*., 2016; Cedron *et al*., 2020). After administration, acetaminophen reduces the levels of prosta-glandin metabolites in urine (Botting, 2000). Due to over production and usage, acetaminophen is frequently detected in the aquatic water bodies such as drinking and surface water and also in the sewage treatment plant effluents (Kim *et al*., 2007). For example acetaminophen has been detected in the range of 0.066 µg/l in river Elbe (Wiegel *et al*., 2004), 0.264 µg/l and 0.165 µg/l in upstream and downstream Thames, UK (Bound and Voulvoulis, 2006), 10 µg/l in stream water samples, USA (Kolpin *et al*., 2002), 65.4 µg/l in EU (Stuer-Lauridsen *et al*., 2000), 1.95 µg/l in streams of Iowa, USA (Kolpin *et al*., 2004), 3649 ± 851 ng/l in River Kabul and 175 ± 100 ng/l in River Indus in Pakistan (Khan *et al*., 2018). This medication is toxic to aquatic species (Correia *et al*., 2016; Choi *et al*., 2018; Erhunmwunse *et al*., 2021). Acetaminophen has the ability to cause neurotoxicity and to alter the principal route by which the body obtains energy (Nunes *et al*., 2015).

Ibuprofen (IBU) is a non-steroidal anti-inflammatory drug prescribed as analgesic, and antipyretic (Brillas, 2022). Ibuprofen is an analgesic, antipyretic and anti-inflammatory, one of the most widely used and prescribed medicines in the world. Ibuprofen is a persistent surface water pollutant that is resistant to degradation. Similarly ibuprofen has been detected in various aquatic water bodies (Vieno *et al*., 2005; Islas-Flores *et al.*, 2014). For example, ibuprofen has been detected in the range of 0.003–0.1 mg/l in surface water (Kasprzyk-Hordern *et al*., 2008), 1.1–4.8 mg/l in wastewater treatment plant effluents (Gómez *et al*., 2006; Santos *et al.,* 2010), 0.117 mg/l in wastewater and 0.085 mg/l in surface water (Ebele *et al*., 2017) of South Africa. Research shows that this medication is bio-accumulative and cause adverse effects in fish (Bartoskova *et al*., 2013; Mathias *et al*., 2018; Ogueji *et al*., 2018).

For monitoring of emerging contaminants such as PPCPs, a multi biomarker assay are commonly used to monitor the environmental quality and the health condition of the aquatic organisms (Umamaheswari *et al.*, 2019). Analysis of enzymological, biochemical and hematological biomarkers are often used to determine the mode of action of toxicants (Ramesh *et al*., 2018; Shreenidhi *et al*., 2021; Veedu *et al*., 2022). Hematological studies help to understand and monitor physiological and pathological changes. Hematological parameters, e.g., hematocrit (Ht), red blood cell (RBC) count, white blood cell (WBC) count, and hematological indices, e.g., mean cellular volume (MCV), mean cellular hemoglobin (MCH), and mean cellular Hb concentration (MCHC), are the widely used biomarkers to assess toxic stress induced by environmental contaminants like pharmaceuticals (Shahsavani *et al*., 2010; Ambili *et al*., 2013).

Biochemical parameters such as glucose and protein are widely used as a perfect perceptual indicator of environmental stress in fish and widely considered early warning signals (Kavitha *et al*., 2010; Hemalatha *et al*., 2019). Changes in the enzymatic activity of fish exposed to pollutants may be used as potential biomarkers for toxicity assessment (Renuka *et al*., 2018). The enzymes phosphatases play an important role for the regulation of various metabolic processes (Sparks and Brautigan, 1986). Changes in ACP and ALP activity indicate the health condition of the fish under stress condition (Gora *et al*., 2018). Fish models are used extensively in the early stages of pharmaceutical development because they are highly sensitive to pollutants, even at low concentrations. The physiological alterations in fish due to the presence of pharmaceuticals are an important biomarker of environmental pollution and can be used as an effective tool for the evaluation of the aquatic environment (Madureira *et al*., 2012; Renuka *et al*., 2018; Shreenidhi *et al*., 2021).

Based on the literature review, it is recognized that there is a lack of available information on the effect of pharmaceuticals on aquatic organisms, especially in freshwater fish. Hence, in the present study an attempt was made to study the effect of the most commonly used drugs acetaminophen and ibuprofen on hematological, biochemical and enzymological parameters of a fresh water fish, O*reochromis niloticus* in order to use these biomarkers in the field of environmental toxicology.

Materials and Methods

Pharmaceutical drugs and Chemicals

The drugs acetaminophen (purity ≥ 99.0 %) and ibuprofen (purity ≥ 98.0 %) were purchased from Sigma-Aldrich, USA. The other chemicals used in this study were purchased from Himedia, Mumbai, India.

Fish and Acclimatization

Nile tilapia (*Oreochromis niloticus*) has been collected from a fish farm in Poonjar, Kerala, India. The fish (approximately 10.5 cm in length and 35 g in weight) were acclimated to laboratory conditions for approximately 20 days before the start of the experiment. During acclimation, fish were fed once every day. Water supply was provided at least one hour prior to water replacement. Water was changed every 24 h after supply. A healthy environment was maintained for fish during acclimation and the trial period. The physic-chemical characteristics of tap water, such as temperature, pH, dissolved oxygen, and total hardness, were estimated according to the American Public Health Association (1998) and are -- temperature (26.1 ± 1.0 °C), pH (7.2 ± 0.1), dissolved oxygen (7.0 ± 0.02 mg l^{-1}) and total hardness (17.0 ± 0.5 mg l^{-1}) which is suitable for the survival of the fish. The fish stock was maintained at natural photoperiod to ensure sufficient oxygen for the fish and the environment is devoid of any accumulated metabolic wastes.

Sub- lethal toxicity study

The sub-lethal toxicity (LC_{50}) for acetaminophen and ibuprofen has been obtained from Folarin *et al*. (2018) and Saravanan *et al*. (2012). For the sub-lethal toxicity studies, three glass tanks were taken and filled with water. Then 1/10th of the LC_{50} value of the drug acetaminophen (128.6 mg/l^{-1}) and ibuprofen (142.2 mg/l^{-1}) were added to the tanks following dissolution of the drugs in boiling water. A toxic-free water tank was maintained to provide control. Subsequently, 15 healthy fish were placed in each tank. Experiments were carried out during a period of 28 days with a sampling frequency of 7 days. Three replicates were also maintained simultaneously for each drug and control groups. At the end of every 24 h, the test water was renewed, and the freshly prepared drugs solution was added to maintain the drugs at a constant level.

Sampling

Every seven days, live fish from drug treated and control groups were sacrificed and blood was taken from the heart and caudal regions using syringes and needles. Blood samples from the control and drug treated groups were expelled into their respective heparin plastic vials and placed under frigid cold conditions. Subsequently, the pooled blood sample was used for hematological studies. After blood collection, fish were washed with double distilled water, blotted dry with absorbent paper, and muscle tissue was removed and stored at -80°C for biochemical and enzymological assays. The collected muscle tissue was homogenized with potassium phosphate buffer (pH 7.0, containing 0.5 mM EDTA) in ice-cold condition, centrifuged at 12000 g for 20 min at 4°C and the supernatant was used for the assays.

Hematological studies

Heart and caudal peduncle regions of fishes were used to take blood using 5 ml graduated syringe, causing minimum stress. The blood is used for the estimation of hemoglobin, hematocrit, erythrocyte and leukocyte counts.

Determination of Total RBC and WBC count

Total count of RBC and WBC was done with the help of a Neubauer haemocytometer, as described by Rusia and Sood (1992).

Determination of Hemoglobin and Hematocrit

Hemoglobin amount of blood was estimated using Acid Hematin method. It is also called Sahli's method. Hematocrit estimation was estimated according to the procedures of Nelson and Morris (1989).

Determination of MCV, MCH and MCHC

Erythrocyte indices, such as MCV, MCH, and MCHC, were also calculated according to standard formulas as follows:

MCV = Hematocrit X 10/ RBC count in million

MCH = Hemoglobin (g/100 ml) X 10 / RBC in million

MCHC = MCH X 100/ MCV.

Biochemical analyses

Determination of Glucose and Protein: Glucose content in the muscle of the fish was estimated according to O-toluidine method (Cooper and McDaniel, 1970). Protein content in muscle was estimated following Lowry *et al*. (1952) method.

Enzymological analyses

Determination of ACP and ALP: ACP and ALP were estimated using Wootton's method (1964). Citrate buffer of 0.5 M at pH 4.8 was used along with substrate buffer for ACP estimation. For ALP estimation, Glycine NaOH buffer of 0.5 M at pH 10 was taken.

Statistical Analysis

The statistical data analysis was made between control and the drugs treated groups and the mean value of three individual observations was taken for each variable along with the standard deviation of the sample mean. The resulting data were analyzed statistically to determine their significance using one way analysis of variance (ANOVA).

Results

In this study, until day 7, RBC count was slightly increased, followed by a steady decrease in the case of acetaminophen treated group (Fig. 1). In ibuprofen-treated fish, the RBC count was decreased significantly ($p < 0.05$) compared to control. The white blood cell count (WBC) was significantly ($p < 0.05$) increased throughout the study period in both drugs exposed fish when compared to control groups (Fig. 1). We observed that Hb and Hct level was significantly ($p < 0.05$) decreased in both the treatment groups (Fig. 1). The maximum increase in Hb and Hct level was observed in individuals treated with acetaminophen.

When compared to the control, a significant decrease ($p< 0.05$) in the level of MCV was observed in the case of acetaminophen treated group (Fig.1). However, in the ibuprofen treated group a significant increase ($p< 0.05$) in the MCV value was observed with a slight decrease on day 14 (Fig. 1). A significant decrease ($p< 0.05$) in the MCH value was noticed in acetaminophen treated group (Fig.1). An inverse tendency was observed in the ibuprofen-treated group on day 21 and 28,

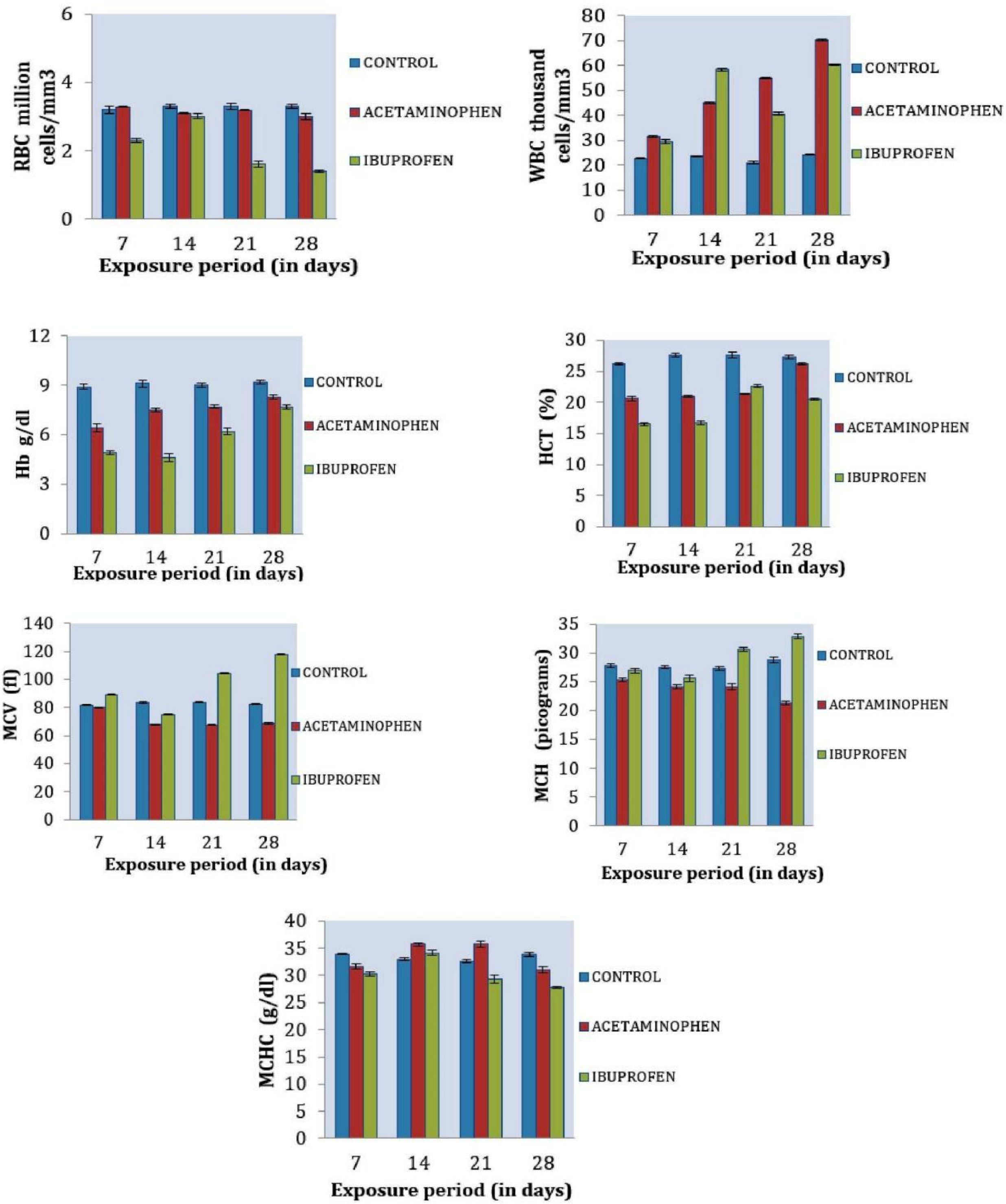

Fig. 1: Hematological parameters of *Oreochromis niloticus* exposed to sublethal concentration.

where the value was significantly increased ($p < 0.05$). In the case of MCHC value, a mixed trend was noticed in both the treatment groups (Fig. 1).

The level of glucose in the muscle of *O. niloticus* exposed to sublethal concentration showed a progressive significant increase

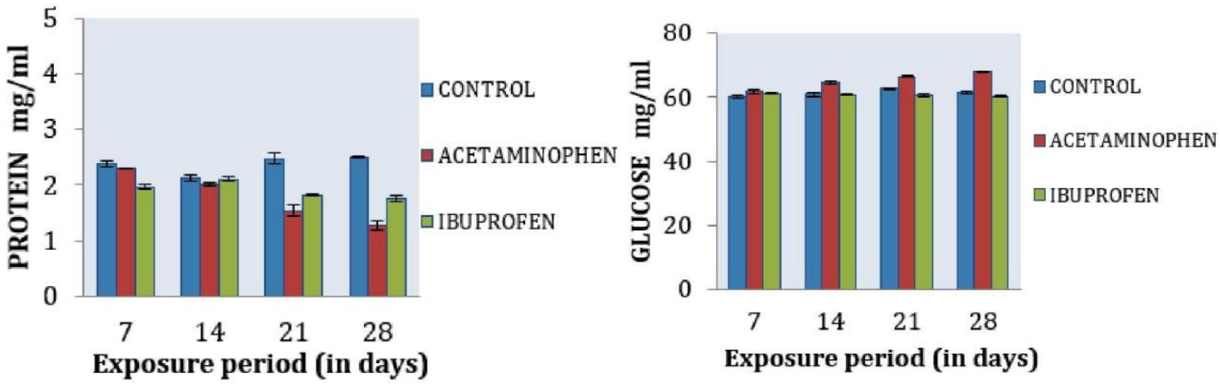

Fig. 2: Effects of Acetaminophen and Ibuprofen on biochemical parameters of *Oreochromis niloticus.* Data are mean + S.E. of five experiments.

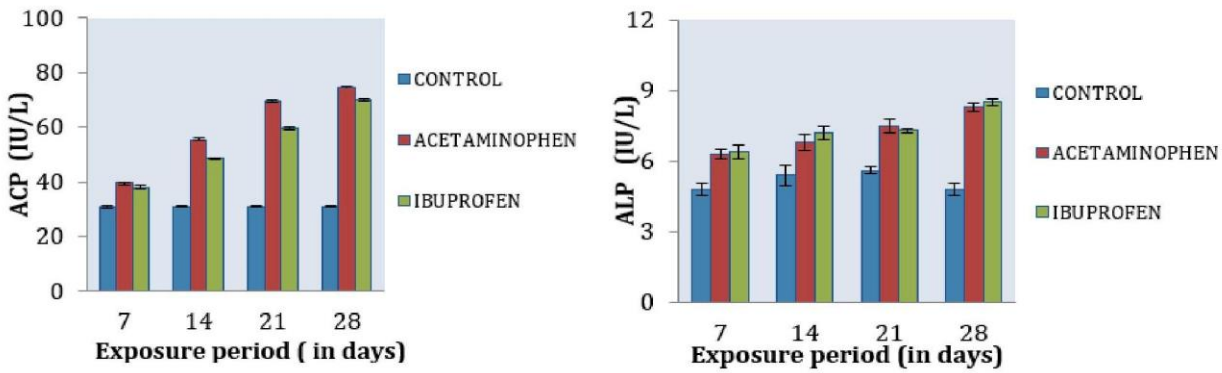

Fig. 3: Changes in the muscle AST and ALT activity of *Oreochromis niloticus* exposed to sublethal concentration of Acetaminophen and Ibuprofen for 28 days. Data are mean + S.E. of five experiments.

$p<0.05$) throughout the study (Fig. 2) in the acetaminophen treated group. For ibuprofen, there was a significant decrease in glucose levels ($p < 0.05$). Over the experimental period, protein level was declined significantly ($p < 0.05$) for the acetaminophen group (Fig. 2). In the ibuprofen treated group, a small increase (7.1%) in the level of protein was observed on day 14, followed by a continuous decrease in the remaining days of study.

An increase in ACP and ALP activity was observed in the muscle of the drugs treated fish (Fig. 3). While comparing the two drugs, a more significant increase in ACP activity was observed in the case of the drug acetaminophen ((Fig. 3).

Discussion

The extensive use of pharmaceutical drugs for the improvement of human health leads to contamination of aquatic water bodies which may pose a serious threat to aquatic organisms (Mezzelani *et al*., 2018; Uumamaheswari *et al*., 2019; Martyniuk *et al*., 2022). Acetaminophen and ibuprofen are most commonly used drugs throughout the world and their residues have already been reported in various environmental metrics (Sung *et al*., 2014; Renuka *et al*., 2018; Chopra and Kumar, 2020). Acetaminophen is one of the most used pharmaceutical drugs to reduce fever and pain (Antunes *et al*., 2013). The higher concentration of acetaminophen in the aquatic environment serves as aquatic pollutant and cause adverse effects in aquatic organisms (Kim *et al*., 2012; Sung *et al*., 2014; Vo *et al*., 2019; Cedron *et al*., 2020; Erhunmwunse *et al*., 2021; Panchamoorthy *et al*., 2022). Among the over the counter medicine, ibuprofen is highly prescribed throughout the world. Due to their persistence and bio-accumulative potential, ibuprofen has been reported to cause adverse effect on aquatic organisms (Sung *et al*., 2014; Mathias *et al*., 2018; Ogunwole *et al*., 2021; Peñuela and Martínez-López , 2021; Narayanasamy, 2022).

In general, hematological parameters are routinely used to detect the physiological status of animals such as fish due to their close association with the external environment (Adhikari *et al*., 2004). Furthermore, physiological disturbances in fish exposed to environmental contaminants can be assessed by blood parameters (Osman *et al*., 2010). To assess the sublethal toxicity of contaminants, hematological parameters are commonly used as indicators of aquatic pollutants (Remya *et al*., 2015). In the present study, the significant decrease in RBC count can be attributed to anemic condition in fish due to drug toxicity. Anemia may also be due to the inhibition of erythropoiesis due to the effect of medications. Furthermore, disturbances in osmoregulation caused by the drug toxicity may also cause a decline in RBC count (Uumamaheswari *et al*., 2019). Similarly, the significant decrease in Hb content in the drug treated fish indicates the impact of drug on the enzyme system responsible for hemoglobin synthesis. Swelling of RBCs or the reduction in the rate of formation of RBCs could decline the Hct level in the organisms (Uumamaheswari *et al*., 2019). The decrease in RBC and Hb level in *C. gariepinus* treated with chloramphenicol indicate impaired erythropoietic process due to drug toxicity (Nwani *et al*., 2014). The reduction in the hematological parameters such as RBC, Hb and Hct may also indicate the direct toxicity of the drugs which may cause destruction of hematopoietic centers (Rezaei *et al*., 2020). Furthermore, pharmaceutical drugs due to their lipophilic properties may cross the membrane and the disruption of membrane may also leads to a decline in RBC count (Iftikhar and Hashmi, 2021).

An increase in the values of WBC can be due to leukocytosis, which is used as an adaptive mechanism to fight the stress caused by the drug (Sweety *et al*., 2008). The significant increase in WBC in ibuprofen treated fish indicates induced stimulatory effects on the immune system of the fish (Ogueji *et al*., 2018). The significant increase in WBC count in *C. gariepinus* treated with chloramphenicol indicates immune and protective mechanism of the fish due to drug toxicity (Nwani *et al*., 2014). Alterations in hematological parameters such as RBC, WBC, Hb and Hct have also been reported in catfish *Clarias gariepinus* treated with ciprofloxacin, amoxicillin and ampicillin (Faisal, 2003), in *O. mykiss* exposed to carbamazepine (Li *et al.,* 2010), in *Oreochromis niloticus* exposed to chloramphenicol (Shalaby *et al*., 2006), in zebra fish *Danio rerio* exposed to chronic concentrations of ibuprofen (Bartoskova *et al*., 2013) and in *Cyprinus carpio* treated with acetylsalicylic acid (Siddeswaran *et al*., 2020).

In the present study, the decline in the values of MCV and MCH in the acetaminophen treated fish group can be due to the impairment in the uptake of oxygen, which can be caused by gill damage due to drug exposure (Ambili *et al*., 2013). Significant decrease in MCV, MCH and MCHC has been reported in fish exposed to varying concentrations of pharmaceutical drugs (Velisek *et al*., 2009; Li *et al*., 2010) which may be due to defective Hb biosynthesis in the fish (Ogueji *et al*., 2018). Due to erythrocyte swelling, Hct levels increase, which can lead to a reduction in MCHC levels (Satyanarayanan *et al*., 2011). The significant decrease in hematological parameters such as Hb, Hct and MCV and increase in MCHC value in *Rhamdia quelen* exposed to paracetamol indicate the anemic condition of the fish due to paracetamol toxicity (Guiloski *et al*., 2017). Rise in the values of MCV and MCH in the iburophen treated fish group may be caused by anemic conditions prevalent in the fish due to problems in the gaseous exchange through the gills (Caravalho and Fernandes, 2006).

The glucose content of fish may be considered the best biological indicator to study the stress conditions in fish (Wedemeyer, 1969). In the present study an increase in the values of glucose content can be attributed to the increase in energy demand due to stress conditions induced by the drug (Ramesh and Saravanan, 2008). The significant elevation of glucose level may indicate metabolism of the drugs which may have induced hyperglycaemia due to increased rate of glycogenolysis or gluconeogenesis (Ogueji *et al*., 2017). Similar observation has also been reported in Indian major carp *Cirrhinus mrigala* exposed to ibuprofen (Saravanan *et al*., 2012). Furthermore, the increase in the concentration of circulating catecholamines or corticosteroids may also leads to hyperglycemic condition in fish (Min and Kang, 2008).

The introduction of the drug led to an alteration in food consumption. Proteins are therefore used to meet the energy requirements of detoxification processes to reduce stress (Neff, 1985). The reduction of protein quantity is mainly due to increased energy demand to maintain homeostasis. In the present study, the reduction in plasma protein level may be due to inhibition of protein biosynthesis or utilization of the protein as an energy source to repair the damaged cells caused by the drug (Ogueji *et al*., 2017). Furthermore, detoxification and excretion pathways during xenobiotic biotransformation may cause depletion of protein in the liver (Ogueji *et al*., 2017). Liver disorder due to toxicant stress may also lead to a decrease in protein levels (Lavanya *et al*., 2011).

Biomarkers play a vital role to assess the toxic effect of environmental contaminants on aquatic organisms (Hamza-Chaffai, 2014). Among the biomarkers, estimation of enzymatic activities may provide valuable tool to indicate the adaptive changes of aquatic organisms to stressors such as pharmaceutical drugs (Tomanek *et al*., 2011; Freitas *et al*., 2016). In the present study, there were significant changes in enzyme biomarkers. Acid phosphatases are hydrolytic lysosomal enzymes and have a role in certain detoxification functions. Excretion, ionic conduction, proliferation and water sorption are also mediated by ACP (Terra and Ferreira, 2012). A significant increase in the level of ACP in both drugs treated fish groups can be due to cell necrosis and more supply of phosphate group for energy metabolism. This can have an effect on fish health and reproduction (Alexander *et al*., 2013). An elevation of ACP activity in various tissues may be due to glycogenolysis and enhanced breakdown of phosphatase to release energy in view of impaired ATPase system during cadmium stress (Suresh *et al*., 2016). Hence, the overall increase in ACP values can be attributed to the toxic effect of the drugs on the fish.

ALP activity can be used as a marker of hepatic dysfunction and a measure of the cell membrane's functionality (Lovatto *et al*., 2015). The enzyme ALP is involved in synthesis of protein, carbohydrate metabolism and growth ((Suresh *et al*., 2016). An increase in ALP values in treated groups may be due to increased intake of phosphate by fish (Edori *et al.*, 2013). The increase in liver ALP activity may be a response to overcome the drug toxicity or the protective role of the organs and tissues that synthesise enzymes (Chinnadurai *et al*., 2022). High levels of ALP may also be associated with tissue necrosis (Barse et al., 2006; Lin *et al*., 2011). The elevation of liver enzymes in human and animals when exposed to paracetamol indicate the liver damage caused by paracetamol (Kumar *et al*., 2004). Furthermore, an increase in ACP and ALP activity can be interpreted as a shift, which emphasise on energy break down pathway from normal ATPase system which includes phosphorylation (Tilak and Mary, 2009). In the present study the significant increase in the level of ALP in both drugs treated fish can be due to protective role of the fish against the drug toxicity or damage of the muscle tissue.

Conclusion

Acetaminophen, and ibuprofen provoked significant alterations in a number of indicators that were investigated. Over the course of the current investigation, it was discovered that both medications have negative impacts on a variety of enzymological, biochemical, and haematological parameters in tilapia, *Oreochromis niloticus.* The findings of this study raise concerns about the environmental dangers that are caused by the discharge of drugs in an incorrect manner. Due to the fact that non-selective releases of pharmaceutical effluents continue to occur from all directions, there is a continued need for additional research and data in this particular field.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the PG and Research Department of Zoology, Alphonsa College Pala, Kottayam, Kerala.

Author Contributions

Josy Jose: Conceptualization, investigation, experiments, original draft writing; *Ambili T.R.*: Resources, experiments, supervision, formal data analysis, data curation; *Manju Elizabeth Kuruvila*: Conceptualization, investigation, writing - review and editing; *Elezabeth Basil*: Formal data analysis, data curation, validation; *Sojomon Mathew*: Data curation, data editing, data analysis. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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