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Ameliorative Effect of *Annona muricata* Leaf Extract on Fipronil Induced Liver Biochemical Alterations in *Oreochromis mossambicus*

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Abstract The diverse array of pharmacological properties present in the natural compounds of *Annona muricata* and its wide use in traditional medicine have all been prime area of research focus. The present study was undertaken to focus on the hepatoprotective role of *Annona muricata* leaf extract in *Oreochromis mossambicus* exposed to fipronil toxicity for 15 and 30 days. The study of fipronil toxicity in antioxidant/detoxification enzymes in liver such as Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Acid phosphatase (ACP) and Lactate dehydrogenase (LDH) is an attempt to provide a clear concept of hepatic toxicity of fipronil. Fishes were exposed to 3 sublethal concentrations (1/5, 1/10, 1/15) of fipronil for 15 and 30 days fed with normal and plant extract supplement feed. The antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) showed decreased activity on exposure to fipronil for 15 days and 30 days. The *A.muricata* supplemented group also showed decreased activity significant and dose-dependent but this decrease in activity was less when compared to fipronil exposed groups fed with normal feed. This indicates the stressed condition of *O.mossambicus* on exposure to fipronil that was reduced in *A.muricata* extract supplemented groups indicating the ameliorative effect of leaf extract. The liver enzymes AST, ALT, ALP, ACP and LDH showed increased activity for all concentrations for 15 and 30 days of fipronil exposure. *A. muricata* supplemented groups also showed increased liver enzyme levels but this increase was less when compared with fishes fed with normal feed. The increase in activity was significant and dose-dependent. This reveals liver damage alteration in liver activity when exposed to fipronil whereas fipronil exposed groups fed with plant extract supplement showed a recovery of chronic toxicity as evidenced by the liver enzyme activity.

Keywords: *Annona muricata, fipronil, antioxidant enzymes, hepatic enzymes*

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1. Introduction

The aquatic resources like ponds, rivers, lakes, streams and oceans are valuable avenues for unique and cheap source catering to the demand of population worldwide. Aquatic life is strongly dependent on water quality that keeps the aquatic fauna and flora healthy. The exponential growth of human population with increasing urbanization is exploiting aquatic resources for long term benefits. Anthropogenic additions of chemicals expose or increase environmental stress for aquatic organisms and most of these chemicals are resistant to degradation and persist in the aquatic environment for longer period. Pesticides are undeniable part of modern life contributing to welfare of humans protecting everything from flower gardens to agricultural crops and stored products.

Fipronil is 5-amino-1-[2, 6-dichloro-4-(trifluoro methyl) phenyl]-4[(trifluoromethyl)sulfinyl]-1H-pyrazole-3 carbonitrile. The widespread use of fipronil for management of pest, ectoparasites, ticks and mites [1,2]. Fipronil found that it interferes with the γ-aminobutyric acid (GABA)-gated channels; it disrupts normal nerve influx transmission (e.g., passage of chloride ions) by targeting the GABA-gated chloride channel and at sufficient doses, causes excessive neural excitation, severe paralysis, and insect death. Fipronil exhibits toxic effects on non-target organisms such as aquatic invertebrates [3,4], vertebrates like fish [5] some reptiles [6], birds [7] and mammals [8].

The lethal dose (LD50) and lethal concentration (LC50) values of fipronil for newly emerged Africanized honeybees showing that this insecticide may be harmful to these bees independently of exposure route: LD50 =1.06 ng fipronil/bee and LC50 = 1.27 ng fipronil/lL of food, respectively [9]. The acute oral LD_{50} of fipronil was

calculated as 100.35 mg/kg bw in albino rats [10] and LD_{50} value of fipronil was 99.74 mg /kg b.w. in mice [11].

The metabolites of fipronil are also persistent like the parent compound [12] and its residues have also found to accumulate in fish [13]. The half-life of fipronil in water is 14.5 days. The contamination of surface water by pesticides induces impairment in survival, growth and reproduction of aquatic organisms [14] especially fish that have an important role in food chain of aquatic organisms. The 96 hours LC_{50} of fipronil in *Cyprinus carpio* was estimated as 665 µg/L [5] and 24 hours LC_{50} value in juvenile zebrafish was 220.4µg/L [15]. The LC_{50} of the fipronil 5%SC for 96hr of *Catla catla* was found to be 0.23mg/l [16].

Oreochromis mossambicus is the most cultured fish worldwide after salmon, and carps and is farmed in different culture systems. The wide distribution, extraordinary hardy nature, high stocking density, ease to reproduce, omnivorous nature and adaptability to artificial diet have all contributed in considering *O. mossambicus* for the toxicity studies. A few studies have been conducted to analyze the potential effect of fipronil on *O.mossambicus*. The toxicity of 96 hr LC50 of fipronil on freshwater fish, *Oreochromis mossambicus* was evaluated and was found to be 3.0 mg/L [17].

The pronounced toxic effect of fipronil like neurotoxicity, hepatotoxicity and cytotoxicity both on invertebrates [18] and vertebrates [19] are induced by the underlying mechanism of reactive oxygen species (ROS) [20,21]. Exposure to environmental concentrations of fipronil induces biochemical changes on a neotropical freshwater fish *Prochilodus lineatus* [22]. Fipronil exposure has altered levels of superoxide dismutase and catalase activities in the liver of *Cyprinus carpio* [23]. Male albino rats were exposed to fipronil and glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were significantly decreased in the fipronil exposed group compared to those in the control group and malondialdehyde (MDA) and nitric oxide (NO) levels were markedly increased in the liver, kidney and brain tissues [24]. Fipronil exposure in rats have induced oxidative stress in brain, liver and kidney [25]. Significant alterations in oxidative stress and ROS generation in liver and kidney of male rat was observed on fipronil exposure [26].

Fipronil toxicity on rat liver was studied in serum enzymes AST, ALT and ALP [27]. Fipronil induced hepatorenal enzyme alterations in albino rats [28]. Cytological, morphological and histochemical alterations of liver cells of mice were observed following exposure to different doses of fipronil (15, 25 and 50mg/Kg) [8]. Fipronil exposure on Japanese quail to study the liver enzyme activity of AST, ALT, ALP and LDH for 45 and 60 days were studied [29]. Fipronil toxicity on hepatocyte isolated from rat and its effect on biotransformation was studied [30]. The hepatic oxidative stress induced by fipronil in male mice and its protective effect of antioxidant vitamins E and Vitamin C was studied [25]. Fipronil induced alteration in serum biochemical assays on oral exposure in mice [31].

Fipronil toxicity on *Oreochromis niloticus* exhibited increased serum ALT, AST and damage of vital organs [32]. Fipronil toxicity on serum activity of AST, ALP, ALT, LDH and TNF were studied in at a concentration of 10 mg/L and the protective effect of ginseng was evaluated [33]. Fipronil toxicity was determined on antioxidant parameters and oxidative stress indicators measured in gill and liver tissue of rainbow trout [34].

Annona muricata is used from ancient time for treatment of hypertension, antiarthritis, antidiabetics, antimalarial, anticancerous etc. Studies have shown that the basis of most of the potent therapeutic effects of *Annona muricata* is linked to its antioxidant activity mediated through free radical scavenging. *A.muricata* is a vast source of enzymatic antioxidants like superoxide dismutase and catalase and non-enzymatic antioxidants like Vitamin C and E [35]. Antioxidant activity of methanolic bark extract of *A.muricata* was studied [36]. The stem bark extract of *A. muricata* showed protective effects on the oxidative stress induced by CCl_4 in rat [37]. The hepatoprotective activity of leaf extract of aqueous extract of *Annona muricata* was studied against hyperbilirubinemia induced by acetaminophen [38]. Acetogenesis possess high antioxidant activity and this antioxidant activity is the mechanism for most of the activity observed with *Annona muricata.* The present study focusses on the hepatoprotective role of *A.muricata* leaf extract on *Oreochromis mossambicus* exposed to fipronil.

2. Materials and Methods

2.1. Procurement and Preparation of Fipronil

Technical grade fipronil (99%) was procured from RFCL limited, New Delhi, Art No: P-738N. It was stored under refrigerated conditions. Due to low solubility in water, a stock solution of 100mg was prepared in 5% acetone solution.

2.2. Experimental Animal

Oreochromis mossambicus was collected from culture farms of the state fisheries station, Kerala State Agricultural University, Kochi, India and transported to laboratory in aerated tanks. In laboratory conditions the fish was stocked in larger tanks of 500 litre capacity and was left undisturbed overnight. The fish was given an antibiotic treatment of 0.001% for three days. Water was exchanged every day for first three days followed by exchange of 50% water for every two days. Fish was kept in aerated tanks for two week acclimatization and provided with commercial dry feed pellets.

2.3. Experimental Design

The experiment was carried out in tanks of 50 litre capacity (60x30x30cm). The tanks were filled with 15 ppm potassium permanganate and kept overnight. The tank was properly cleaned with water thrice and chlorine free bore well water was added up to the 40 litre mark. The water in the aquarium was renewed every day and proper photoperiod of 13h light/11h dark was maintained. Fish weighing 30 ± 2.9 g of length 13.62 ± 1.8 cm was used for study. Fish was introduced at a stocking density of 10 fish/ aquarium and feeding was stopped 24 hours prior to start of experiment. Aeration was maintained with an air

stone and a plastic regulator. The tanks were covered by meshed lids.

2.4. Median Lethal Concentration (LC_{50}) Studies

For the study of median lethal concentration, the fish, *O. mossambicus* was exposed for 96 hours to different concentrations of fipronil (2, 3, 4, 5, 6, 7, 8 µg/L) and a control was maintained. The experiment was carried out in three groups of 8 concentrations with each group having three replicates in 24 uniform tanks of 50L capacity holding 40L water. Semi static method was employed and test solution was renewed every day. Mortality and behavioural changes were recorded at 24, 48, 72 and 96 hours. Dead fishes were removed immediately. The mortality in relation to test concentration was maintained and used to determine the median lethal concentration (LC_{50}) for 96 hours using Probit analysis [39].

2.5. Collection of *A.muricata* Leaf

Fresh leaves of *A.muricata* were collected from Kochi, Kerala, India and identified. The leaves were separated from stalk, washed and air dried in a shady place at room temperature. The dried leaves were pulverized, crushed into fine powder, weighed and stored in an air tight container.

2.6. Formulation and Preparation of Experimental Diet

Ingredients included crude protein (fat free, Hi Media Laboratories Ltd), vitamin mix (Hi Media Laboratories Ltd), Sunflower oil (procured locally), carboxymethyl cellulose (CMC) (Hi Media Laboratories Ltd), starch (procured locally), crude fibre (procured locally), Butylated Hydroxy Toluene (BHT) (Hi Media Laboratories Ltd), and Betaine hydrochloride (Hi Media Laboratories Ltd). Crude protein, crude fibre, starch and sunflower oil were mixed together in an earthen vessel (Table 1). The dough after mixing was kept for an hour for proper conditioning and the later steamed for 10 minutes in a pressure cooker. Vitamin mix, Vitamin C, BHT, CMC, and Betaine chloride were mixed after the dough was completely cooled. Later pellets were prepared with the hand pelletizer of 1 mm diameter. The pellets were sun dried for 5 to 6 hours and kept in oven overnight at 50°C for complete drying. The pellets were stored in airtight containers.

Table 1. Composition of experimental diet as expressed in percent

Ingredients	Unit 1	Unit 2
Crude protein	50.00	50.00
Sunflower oil	8.00	8.00
Crude Fibre	4.00	8.00
Starch	30.00	30.00
Vitamin C	0.01	0.01
Vitamin Mix	1.96	1.96
Carboxymethyl Cellulose	2.00	2.00
Betaine Hydrochloride	0.02	0.02
Butylated Hydroxyl Toluene	0.01	0.01
Annona muricata leaf extract	0	4.00
Total Percent	100.00	100.00

2.7. Experimental Setup

The experiment setup in 2 distinct experimental units with each unit having 4 groups - a control and three concentrations of fipronil - $1/5^{th}$, $1/10^{th}$ and $1/15^{th}$ of LC_{50} value. Each group had three replicates. Unit 1 the fishes were fed with normal feed and exposed three different concentrations of fipronil along with the control. Unit 2 in addition to the control the three groups were present that were fed with *A.muricata* plant extract as supplement.

Unit -1

Group A1 - Control + normal feed [NF]
Group A2 - 1/5 fipronil + normal feed [FPN +NF]
Group A3 - 1/10 fipronil + normal feed [FPN+NF]
Group A4 - 1/15 fipronil + normal feed [FPN+NF]

Unit – 2

Group B1 - Control + *A. muricata* plant extract supplement [PF]
GroupB2 - 1/5 fipronil + *A. muricata* plant extract supplement [FPN +PF]
Group B3-1/10 fipronil+ *A.muricata* plant extract supplement [FPN + PF]
Group B4-1/15 fipronil + *A.muricata* plant extract supplement [FPN +PF]
The fishes were collected on day 15 and day 30 of experiment for biochemical analysis.

2.8. Biochemical Analysis

On day 15 and day 30 the experimental fishes were killed by decapitation and liver was dissected out and kept at -20°C until analysis. For biochemical studies the tissue was homogenized for 5 min. in ice-cold 0.1M Tris-HCl buffer solution with pH 7.2 (115 w/v) using a Polytron homogenizer (Polytron model PT 3000, Switzerland) and centrifuged at 5000 RPM for 20 minutes (Remi, India). The supernatant was collected for the antioxidant enzyme studies.

2.9. Determination of Antioxidant Enzymes

2.9.1. Superoxide Dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase was assayed according to a modified procedure [40]. In this method, 1.4 ml aliquot of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer, pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 mM EDTA) was added to 100 µl of the homogenate and incubated at 30°C for 5 minutes. 80 µl of riboflavin was then added and the tubes were exposed to 20W-Philips fluorescent lamps for 10 minutes. After the exposure time, 1 ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and absorbance of the colour formed measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

2.9.2. Catalase (CAT) (EC 1.11.1.6)

Catalase (CAT) was estimated by the method [41]. The reaction mixture 1.5 ml volume contained 1.0 ml of 0.01 M phosphate buffer (PH 7.0) 0.1 ml of tissue homogenate and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).Then the absorbance was measured at 530 nm; CAT activitiy was expressed as n moles of H_2O_2 decomposed/min/mg protein.

2.9.3. Glutathione Peroxidase (GPx) (EC 1.11.1.9)

GPx activity was determined by following the method [42]. To 1.0 mL of phosphate buffer (0.1 M, pH7.4) taken in a tube, 0.5 mL Sodium azide solution (29.25 mg in 15.0 mL of buffer), 0.5 mL of EDTA solution (50.4 mg in 15.0 mL of buffer 0, and 100.0 µL of the enzyme were added and mixed well. To this mixture, 0.5 mL glutathione (36.75 mg in 15.0 mL of buffer) was added and incubated at 37°C for 10 minutes, followed by the addition of 1.0 mL of hydrogen peroxide (freshly prepared by mixing 240 mL of hydrogen peroxide in 40.0 mL of buffer). The control contained all the reagents except the enzyme. After the incubation period, aliquots (1.0 mL) of the samples (both test and control) were taken in a tube to which 2.0 mL of Meta phosphoric acid and 1.0 mL of DTNB (5, 5'- dithio-bis-2- nitrobenzoic acid) reagent were added. The absorbance was then read at 412 nm in a Spectrophotometer. The enzyme activity is expressed as n moles of GSH oxidized/ min/mg protein.

2.10. Estimation of Aspartate Transaminase (AST) Activity

Activity of AST was estimated by the method [43]. 1 ml of substrate was incubated at 37°C for a few minutes and 0.2 ml of serum was added and shaken gently. No serum was added to the control tubes. After 1 h, 0.07 ml of aniline-citrate reagent was added to the sample. 0.2 ml serum is mixed to the control tube after the addition of aniline-citrate reagent. After 20 min, 1 ml of DNPH reagent is added to all the tubes and incubated for another 20 min. 10 ml of 0.4N NaOH is added after the removal of the tubes from the water bath. The absorbance was read at 520 nm after 10 min. A pyruvate standard was prepared and activity was calculated using a standard activity chart.

2.11. Estimation of Alanine Transaminase (ALT) Activity

Activity of AST was estimated by the method [43]. 3 ml of substrate was incubated at 37°C for a few minutes and 0.2 ml of serum was added and shaken gently. After 30 min, 0.07 ml of aniline-citrate reagent was added to the sample. Simultaneously, 0.2 ml serum is added to the control tube after the addition of aniline-citrate reagent. After 20 min 1 ml of DNPH reagent is mixed to all the tubes and incubated for another 20 min. 10 ml of 0.4 N NaOH is added after the removal of tubes from the water bath. Absorbance was read at 520 nm after 10 min. A pyruvate standard was prepared and activity was analysed using a standard activity chart.

2.12. Estimation of Alkaline Phosphatase (ALP) Activity

Activity of ALP was estimated by the method [44]. The tissue homogenate was prepared using sucrose buffer. 1 ml of alkaline buffered substrate was added to each of the test tube marked as test (T), standard (S) and blank (B) and incubated at 37 °C for 2 min. 0.1 ml of distilled water is added to test tube B, 0.1 ml working standard to test tube S and 0.1 ml of enzyme homogenate to test tube T. All the test tubes were incubated at 37 °C for 30 min.10 ml of 0.02 N NaOH and 2 drops of concentrated HCl is added into all the test tubes. Mixed by inversion and read absorbance at 415 nm.

2.13. Estimation of Acid Phosphatase (ACP) Activity

ACP Activity was determined by the method [44]. The tissue homogenate was prepared by using sucrose buffer (0.25 M sucrose). 1 ml of acid buffered substrate was taken in each of the test tube and marked as test (T), standard (S) and blank (B) and incubated at 37°C for 2 min. 0.1 ml of working standard is then added to test tube S, 0.1 ml of distilled water to test tube B and 0.1 ml of enzyme homogenate to test tube T. These test tubes were incubated for 30 min at 37°C. 4 ml of 0.1 N NaOH is then added into all the test tubes. It was mixed by inversion and the absorbance was measured at 415 nm.

2.14. Estimation of Lactate Dehydrogenase (LDH) Activity

Activity of LDH was measured by the method [45]. 2.5 ml phosphate buffer and 0.2 ml of coenzyme were pipette out into the spectrometer cuvette. To this 0.1 ml liver homogenate and 0.3 ml buffered substrate were added. At 30 seconds' interval the absorbance was read at 340 nm for 3 min.

2.15. Statistical Analysis

Results were statistically analysed using one way Anova test using the statistical software SPSS version 20. Data were presented as Mean ± SD. Significantly different means were compared with Tukey's post hoc multiple comparison test. Results were expressed as $p \leq 0.05$ and were considered statistically significant.

3. Results

3.1. Median Lethal Concentration, LC_{50}

Data obtained for 96 hour acute toxicity using static renewal test for studying median lethal concentration, LC_{50} of fipronil to *O.mossambicus* is given in Table 2. The median lethal concentration, LC_{50} was 3.74µg/L calculated using Probit analysis (Figure 1). The LC_{50} values showed an increase with increase in concentration of fipronil.

Table 2. Table showing percent mortality of *Oreochromis mossambicus* exposed to different concentration of fipronil for 96 hours using Probit analysis

Sl.No.	Concentration in µg/L	Log Concentration	No.of Fishes Exposed	No. of Fishes died at 96hr	Percentage Mortality	Probit Mortality
1.	2	0.3010	10	2	20.00	4.158
2.	3	0.4771	10	3	30.00	4.476
3.	4	0.6021	10	5	50.00	5.000
4.	5	0.6990	10	6	60.00	5.253
5.	6	0.7782	10	8	80.00	5.842
6.	7	0.8451	10	9	90.00	6.282
7.	8	0.9031	10	10	100.00	-

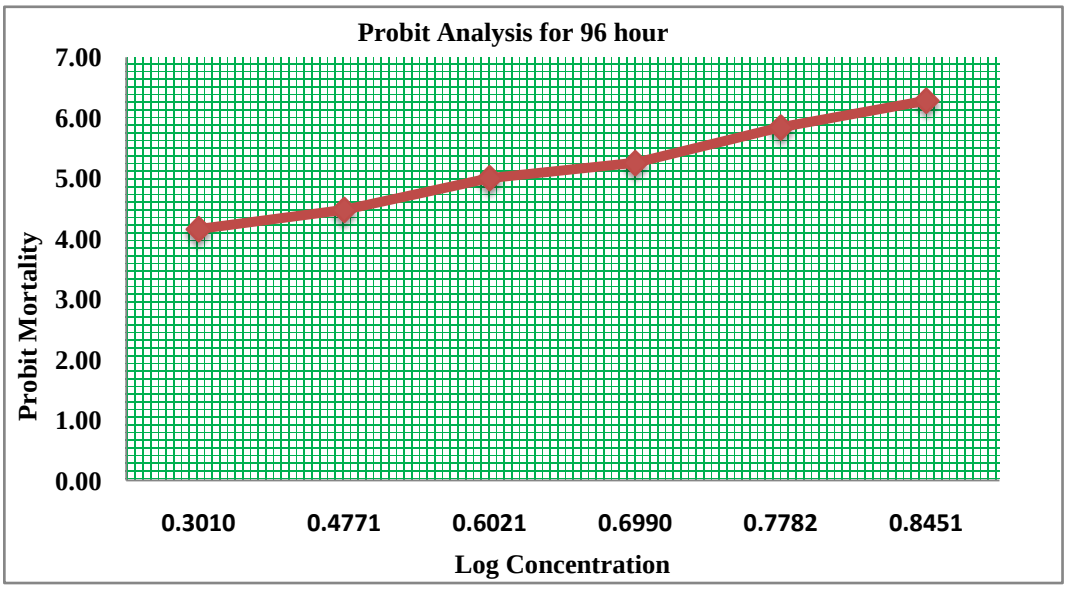

Figure 1. Graphical representation of LC50 of *O. mossambicus* exposed to different concentration of fipronil for 96 hours using Probit analysis

3.2. Analysis of the Antioxidant status in Hepatic Tissues (SOD, CAT, GPx)

Antioxidant enzymes (SOD, CAT, GPx) showed a significant decreased activity in *Oreochromis mossambicus* exposed to both unit 1 (Group A2, A3 & A4) and unit 2 (B2, B3& B4) in all the concentrations of fipronil when compared to control. The fipronil exposed groups-group A2, group A3 and group A4 fed with normal feed exhibited significant ($p<0.05$) decreased enzyme activity in liver The groups, B2, B3 and B4 fed with *A. muricata* plant extract supplement showed significant ($p<0.05$) decreased enzyme activity in liver when compared to control but the activity was significantly ($p<0.05$) higher than the unit 1 groups enzyme activity both for day 15 and day 30. The reduction in antioxidant enzymes activity in liver were dose-dependent (Figure 2, Figure 3 & Figure 4).

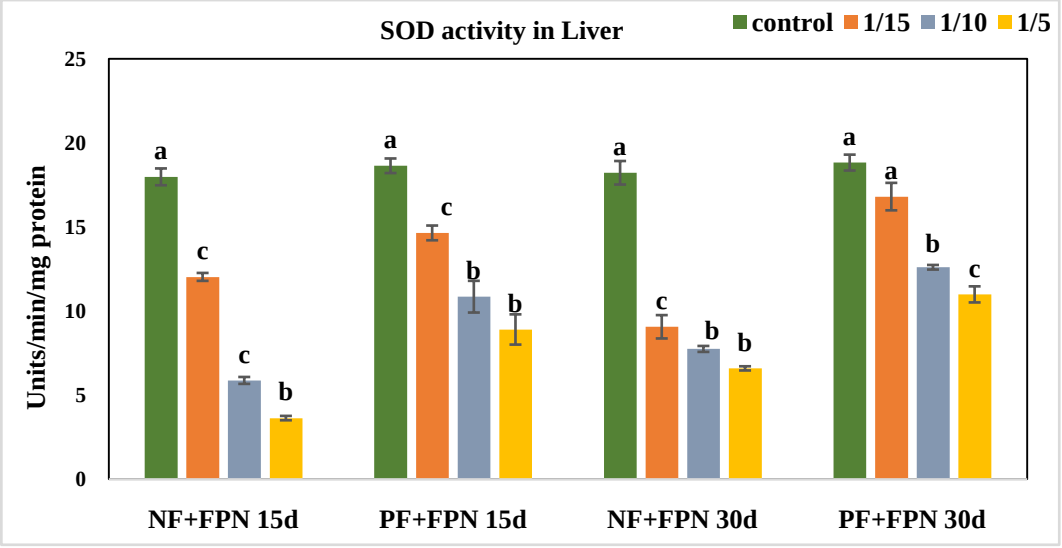

Figure 2. Superoxide dismutase activity in liver tissue of *Oreochromis mossambicus.* Lowercase letters (a, b, c, d) indicate significant differences among the same column within each experimental treatment group, $p < 0.05$. n=20

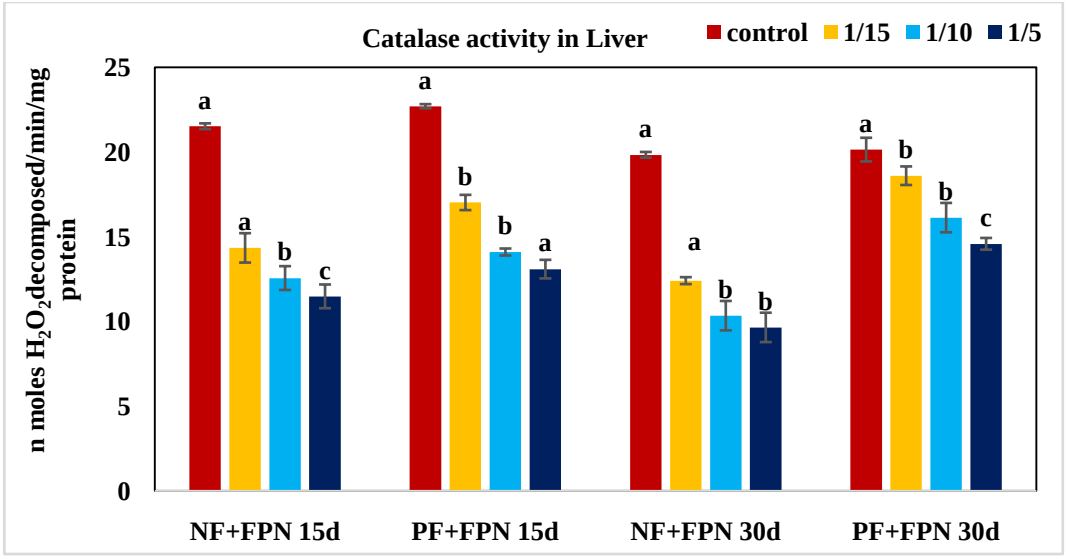

Figure 3. Catalase activity in the liver of *Oreochromis mossambicus*. Values are represented as Mean ± SD. Lowercase letters (a, b, c, d) indicate significant differences among the same column within each experimental treatment group, $p < 0.05$. n =20

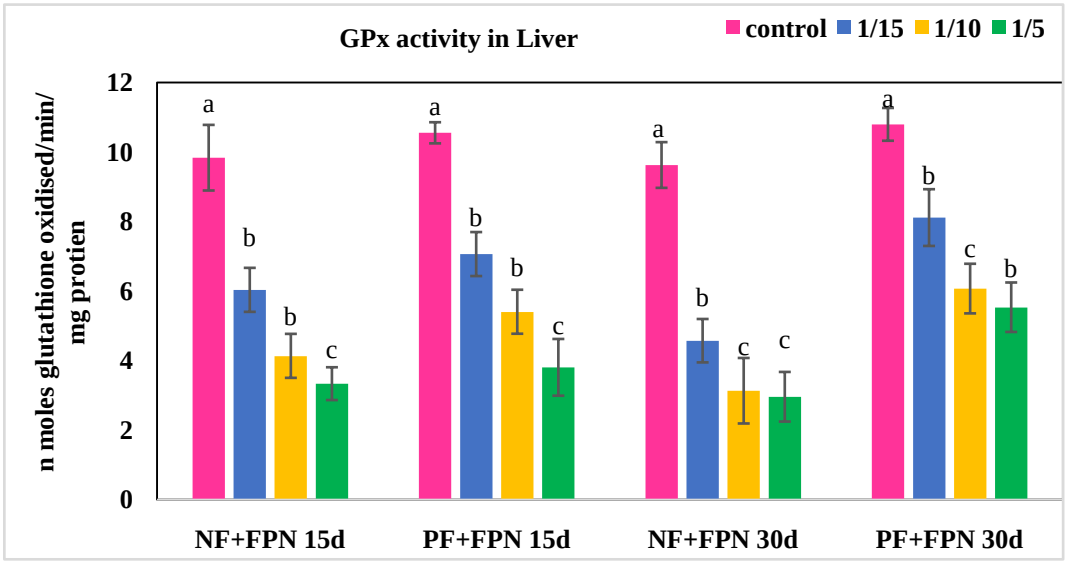

Figure 4. Glutathione peroxidase activity in liver tissue of *Oreochromis mossambicus*. Values are represented as Mean ± SD. Lowercase letters (a, b, c, d) indicate significant differences among the same column within each experimental treatment group, $p < 0.05$. n = 20

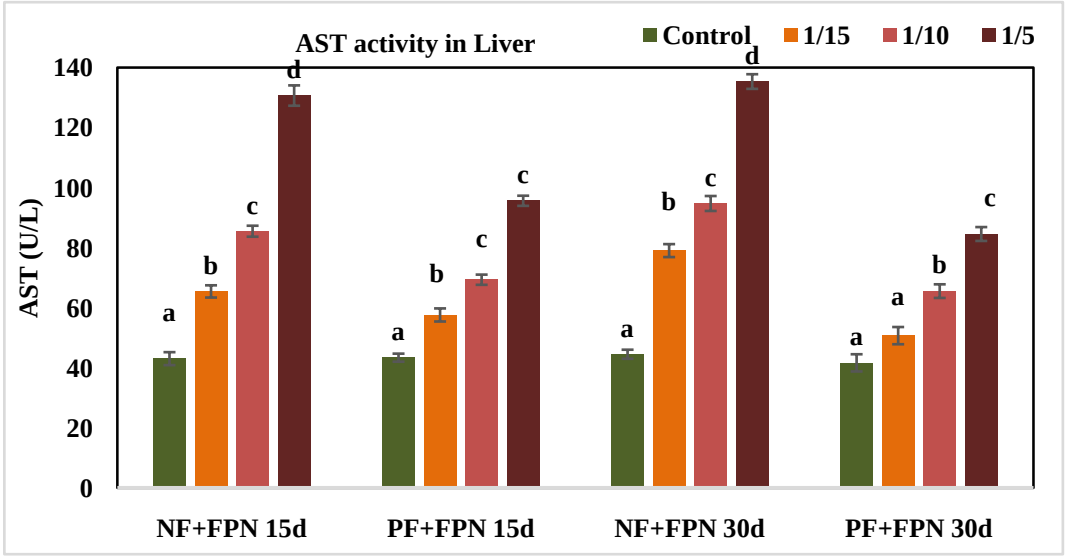

Figure 5. Aspartate transaminase (AST) activity in liver tissue of *Oreochromis mossambicus*. Each bar diagram represented as Mean ± SD. Means having the same letters are not significantly different from each other (One way ANOVA), $P < 0.05$

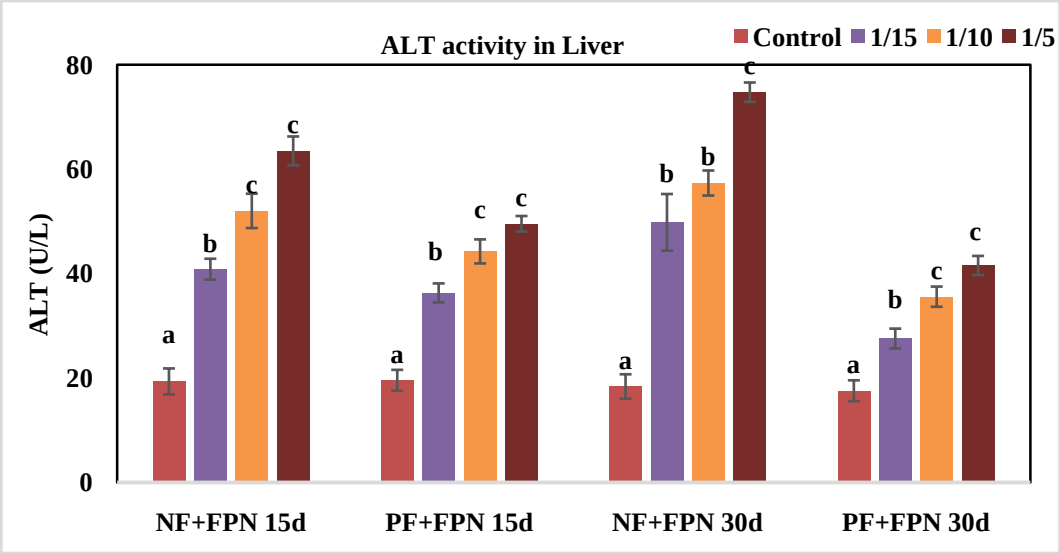

Figure 6. Alanine transaminase (ALT) activity in liver tissue of *Oreochromis mossambicus* Each bar diagram represented as Mean ± SD. Means having the same letters are not significantly different from each other (One way ANOVA), $P < 0.05$

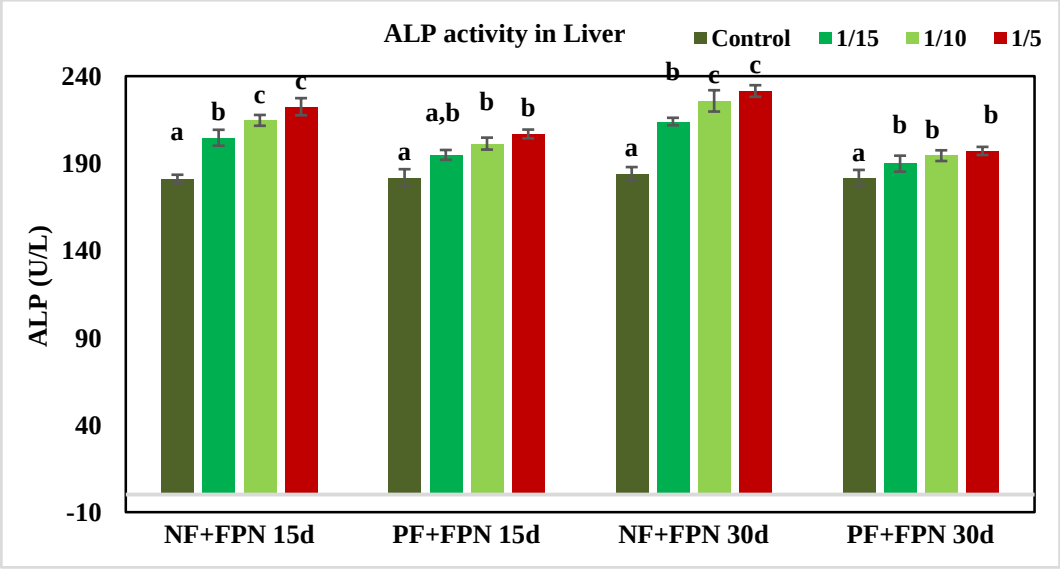

Figure 7. Alkaline phosphatase (ALP) activity in liver tissue of *Oreochromis mossambicus*. Each bar diagram represented as Mean ± SD. Means having the same letters are not significantly different from each other (One way ANOVA), $P < 0.05$

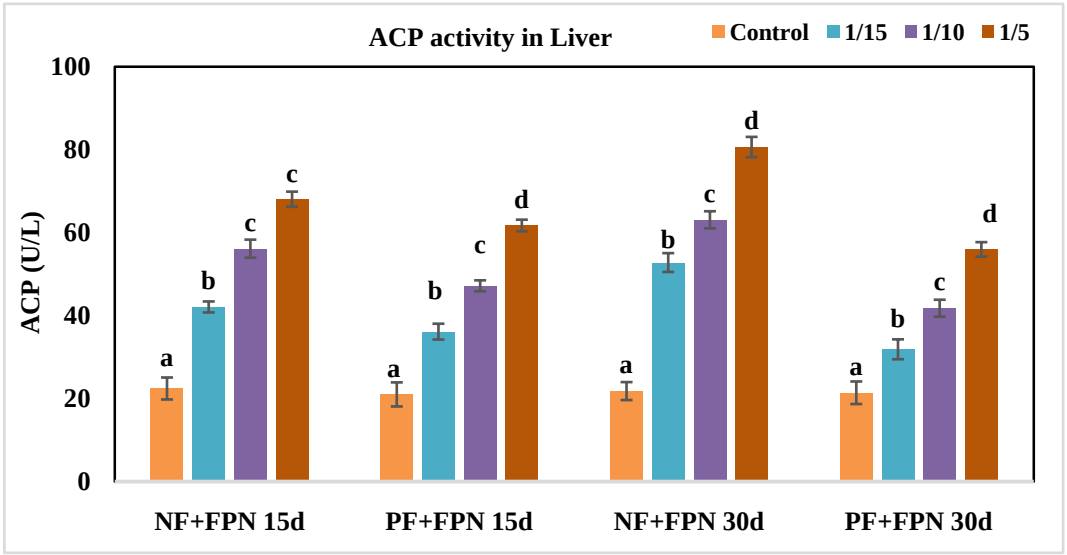

Figure 8. Acid phosphatase (ACP) activity in liver tissue of *Oreochromis mossambicus.* Each bar diagram represented as Mean ± SD. Means having the same letters are not significantly different from each other (One way ANOVA), $P < 0.05$

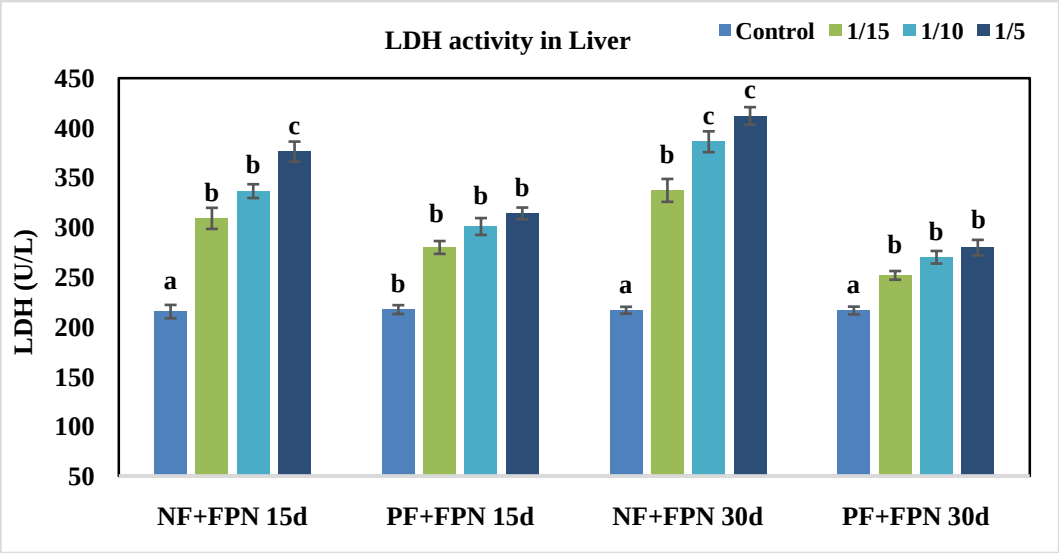

Figure 9. Lactate Dehydrogenase (LDH) activity in liver tissue of *Oreochromis mossambicus*. Each bar diagram represented as Mean ± SD. Means having the same letters are not significantly different from each other (One way ANOVA), P < 0.05.

3.3. Hepatic Enzyme Activity (AST, ALT, ACP, ALP, LDH)

Hepatic enzymes (AST, ALT, ACP, ALP, LDH) activity in *Oreochromis mossambicus* exposed to fipronil showed a significant (P<0.05) increase in activity in all the concentrations of unit 1(Group A2, A3 & A4) and unit 2 (B2, B3& B4) when compared to its control. The fipronil exposed groups - group A2, group A3 and group A4 fed with normal feed exhibited significant (P<0.05) increased enzyme activity in liver. The groups, B2, B3 and B4 fed with *A.muricata* plant extract supplement showed significant (P<0.05) increased enzyme activity in liver when compared to its control but the activity was significantly (P<0.05) decreased than the unit 1 groups both for day 15 and day 30 exposure. The increase in enzyme activity in liver for all the concentrations studied were dose-dependent (P<0.05). (Figure 5, Figure 6, Figure 7, Figure 8 & Figure 9).

4. Discussion

Pesticides are persistent in environment and they reach aquatic ecosystem which is the ultimate reservoir for all anthropogenic activities. The impact of pesticide exposure depend on the concentration of the pesticide, ability to induce adverse effect by penetrating into the organism, by intoxication leading to stressed condition of non-target organism or indirectly altering its physicochemical environment.

Fipronil, phenyl pyrazole finds wide use replacing the organophosphates. The commercial and domestic use of fipronil has increased tremendously in the past two decades. In the present study, the median lethal concentration LC_{50} of fipronil was 3.74µg/L in *Oreochromis mossambicus*. The toxicity of 96 hr LC50 of fipronil on freshwater fish, *O.mossambicus* was found to be 3.0 mg/L [17]. The values showed gradual decrease with increase in exposure time [46]. The adverse effects of fipronil on public health is also of concern and studies have shown fipronil to induce neurotoxicity, nephrotoxicity, hepatotoxicity and cytotoxicity [31,47, 48]. Almost all these toxicities trace the mechanism to oxidative stress [49].

Catalase (CAT) is an endogenous antioxidant enzyme commonly found in biological tissues converting hydrogen peroxide to oxygen and water thereby preventing cell damage [50,51]. The exposure of fipronil induced significant inhibition of catalase activity in liver in a dose-dependent manner. Reduction of CAT changes the redox potential of cell [52] resulting in lower adaptive response to the toxic effects induced by fipronil. The present study recorded a reduction in SOD enzyme activity on exposure to fipronil that might be attributed to utilisation of this enzyme as an antioxidant for the conversion of the free radical formed(O_2 -) to H_2O_2 [2,25,27]. The SOD, CAT and GPx activity are considered as first line defence mechanism of the body that act against reactive oxygen species generated as a result of oxidative stress [53]. Fipronil exhibited dose-dependent inhibition of antioxidant enzymes SOD, CAT and GPx in liver and gill tissues at different doses in in rainbow trout, *Oncorhynchus mykiss* [54]. It is reported that fipronil could result in imbalance in free radical potential of the cell in using cellular macromolecule damage [46,55].

The toxicity of fipronil was confirmed by a significant increase in liver enzymes aspartate transaminase (ALT), alanine transaminase (ALT), acid phosphatase (ACP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). Liver enzymes, such as aspartate aminotransferase and alanine aminotransferase, are considered as the best biomarkers indicating the level of hepatic damage. The increase in these enzyme activities suggest liver dysfunction which leads to increased leakage of these enzymes from hepatocytes due to mitochondrial membrane damage [2]. Also [2] showed alterations in liver enzymes aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) on exposure to fipronil cost pathophysiological changes in liver which is due to generation of free radicals. The toxicity of fipronil to hepatocytes was related to inhibition of mitochondrial activity which decreased ATP synthesis and altered calcium homeostasis leading to cell death [30]. The studies on hepatic oxidative stress induced by fipronil

elevated AST and ALT levels in liver [24]. Concluding that liver is an important target organ in fipronil toxicity. 10mg/L of fipronil expose for 30 days also enhanced AST, ALT, LDH and tumor necrosis factor Alpha eliciting oxidative stress and liver injury in a concentration-dependent manner [33]. Liver acid phosphatase (ACP) and Alkaline phosphatase (ALP) increased in carp on exposure to fipronil [56].

A decreased level of acid phosphatase (ACP) and alkaline phosphatase (ALP) enzyme activity were observed in *Annona muricata* leaf extract supplemented groups indicating the hepatoprotective role of *Annona muricata* against fipronil induced toxicity. Similar results were obtained in CCl_4 induced toxicity in Sprague-Dawley rat model showed increased alkaline phosphatase activity that was reduced significantly when pretreated with *Annona muricata* leaf extract [38] suggesting hepatoprotective mechanism restoring normal liver function. Significant increase in lactate dehydrogenase is due to this rapid conversion of pyruvate to lactate by LDH. Similar increase in lactate dehydrogenase was reported in albino rats on exposure to fipronil for 45 days [2].

Phenolic compounds present in *Annona muricata* other major phytochemical responsible for the antioxidant activity [57]. The decreased level of liver enzymes aspartate transaminase(AST), alanine transaminase(ALT), Alkaline phosphatase (ALP), acid phosphatase (ACP) and lactate dehydrogenase (LDH) in groups with *Annona muricata* supplemented feed extract indicates the restoration of hepato architecture, and the detoxification and metabolic pathways.

5. Conclusion

Based on the outcome of the current study it can be concluded that the fipronil is highly toxic to freshwater fish *Oreochromis mossambicus* as it has an acute toxicity value of 3.74 µg/L. The protective effect of *A. muricata* leaf extract against fipronil toxicity is well evidenced by the increased activity of SOD, CAT and GPx. Results indicated that the supplement with *A. muricata* leaf extract mitigated fipronil induced oxidative damage. The decreased level of liver enzymes Aspartate transaminase(AST), Alanine transaminase(ALT), Alkaline phosphatase (ALP), Acid phosphatase (ACP) and Lactate dehydrogenase (LDH) in groups with *Annona muricata* supplemented feed extract indicates the restoration of hepato architecture, and the detoxification and metabolic pathways.

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