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Original Research

A Preliminary Investigation on the Antihepatotoxic Activity of *Artemisia pallens* Leaves in the Diclofenac-Treated – *Pangasius* Sps

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Abstract

In recent years, diclofenac, a non-steroidal anti-inflammatory drug has been found to be causative for adverse reactions in aquatic fauna and hence it has been chosen for analysing its toxic effect. Being an aromatic herb, *Artemisia pallens* has flavonoids and phenolic compounds that have been employed to check its competency in lessening toxic effects. The fish, *Pangasius* Sp., were treated with lethal concentration (12 mg/mL for 4 days) of diclofenac to induce liver damage and 1% of *Artemisia pallens* extract was added. The vacuolar degeneration in the liver was visually observed using histopathological analysis. The oxidative stress and antioxidant parameters were studied. Noteworthy changes in levels of conjugated dienes, protein sulfhydryl, inorganic phosphorus, superoxide dismutase and catalase activities with their assayed results at $p<0.01$ and $p<0.05$ level of significance when compared to control fish were observed. The stress hormone, cortisol level reduction proved the fact that the action of *Artemisia pallens* has lessened the toxic effects of diclofenac leading to a considerable increase in the lifespan of the fish. The observed changes in the oxidative stress parameters, antioxidant enzymes and cortisol levels further confirmed the hepato-protective effect of *Artemisia pallens* against diclofenac induced toxicity.

Keywords: cortisol level, diclofenac, lipid peroxides, *Pangasius* Sp., antioxidants

Introduction

The pharmaceutical industry has emerged as one of the largest and prominent industries worldwide. Large numbers of pharmaceuticals of different categories are being used to cure and care for human and animal health. In general, pharmaceuticals comprise compounds that include materials extensively used in medicine, agriculture and biotechnology, such as drugs, antibiotics and hormones. Pharmaceutically active compounds (PhACs) are one of the conspicuous classes of pharmaceuticals which reach the aquatic biome through the waste water stream as pharmacologically active metabolites [1]. Due to inefficient removal of PhACs during sewage treatment increased concentrations of PhACs have been reported to be present in different environmental compartments and often the short-term, as well as long-term effects, are obscure. Few kinds of research have been carried out discussing the fate, toxicity and risk assessment of such compounds [2].

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The majority of the research that has been conducted on toxicant mixtures has focused on chemicals of the same class (e.g., pharmaceuticals, metals, or pesticides [3], and there are very few studies investigating mixtures that comprise toxicants of different classes. In the study carried out by Vijaya Geetha and colleagues [4] on the toxic effects of clofibrate and phenol, the significant changes in the lipid peroxidation and total antioxidant capacity were observed proving the fact that there are noteworthy alterations in the oxidative potential of *Pangasius* Sp.

Diclofenac [2-[2-(2,6-dichloroanilino)phenyl]acetic acid] sodium is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory and antipyretic properties. These properties are primarily achieved by its ability to block the enzyme cyclooxygenase, but also by an additional direct effect on hyperalgesia due to the functional down regulation of sensitized peripheral pain receptors. As an analgesic, it has a fast onset and a long duration of action. Compared to other NSAIDs, diclofenac is well tolerated and rarely produces gastrointestinal ulcerations or other serious side effects. Thus, diclofenac is considered as one of few non-steroidal anti-inflammatory drugs of the first choice used in the treatment of rheumatoid arthritis, osteoarthritis, and other illnesses as an analgesic and antipyretic [5]. It is also observed that diclofenac had reduced HSI at 0.2 µg/kg. Thus, the anti-inflammatory drug diclofenac instigated oxidative stress and reduced testosterone levels that can have a negative effect on aquatic organisms [6]. It was found that administration of diclofenac, ibuprofen, or paracetamol may induce immunological and biochemical perturbations, and their toxicity may increase depending on the dose [7]. Quiguo et al., [8] in their research showed that the diclofenac unexpectedly produced a more bioaccumulative and toxic metabolite in invertebrates, fish, and humans that could contribute to enhanced toxicity.

In the study carried out by Pandi et al. [9] lead intoxication caused cell damage but feeding with hydrilla supplemented diet, normal RBC structure was retained. Based on all these literature studies, we have chosen diclofenac for its toxic effect analysis and by introducing the herbal plant *Artemisia pallens* to counteract the oxidative stress effect induced by it in fish model *Pangasius* Sp.

The fish species is also known as *Pangasianodon hypophthalmus*, sutchi catfish, striped catfish or tra fish and has been widely exported due to great acceptability, affordable cost, and white meat, which can replace cod or other more expensive white fish. Currently, sutchi catfish fillets have been exported to over 80 countries worldwide being widely cultured in freshwater systems rendering them valuable as well. Due to its large size, it is not ideal for home aquaria but is sold in many fish stores around the world as small juveniles. In India, fish are consumed for their high protein content. *Pangasius* sp. is one of the top exotic seafood of India. Any significant changes in the number of these fish will affect the economy of India along with studies leading to evidence that these fish are affected by the toxic compounds present in the water.

One of the most native aromatic plants of India is *Artemisia pallens.* It possesses immense medicinal benefits along with anti-microbial properties. In the present communication, the radical scavenging activity of this medicinal herb is discussed. It can be perceived that the DPPH and nitric oxide free radical scavenging activity for the methanol extract of *A. pallens* is found to be noteworthy**.** The antioxidant activity for methanol extract of aerial parts of *Artemisia pallens* wall was assessed, by using radical scavenging assays; 2,2-diphenyl, 1-picryl hydrazyl (DPPH) and nitric oxide. The reduction capability of DPPH radicals was calculated by the decrease in its absorbance at 516 nm induced by antioxidants. This antioxidant property of *Artemisia pallens* has made it the best choice for remediation.

Our research involves determining the lethal concentration of diclofenac followed by the oxidative stress parameters, histopathological analysis, antioxidative stress parameters and estimation of cortisol in the diclofenac and remediator treated *Pangasius* sp., fish model.

Materials and Methods

Sample Collection and Acclimatization of Fish

From the coastal areas, the bred *Pangasius* Sp. were collected and in turn, this was purchased from the local dealers in Chennai. Fish were safely transported to the laboratory in well-sealed polyethene bags containing oxygenated water and subsequently stocked in a large tank with dechlorinated tap water (subsequently reconstituted with salts). The fish exposed was a minimum of one year age and victualised with pellets of 30% protein nourished fish feed. The fish were in the range of 12-15 cm and weighed between 13-14 g. They were fed at regular intervals; the feed had fish meal mixture and worms. They were let to acclimatize in the laboratory environmental conditions for about 15 days. During acclamation, three-fourths of the tank water was replaced every 72 hours in order to maintain a favourable healthy environment. Methylene Blue (also known as methyl thioninium chloride) was added in drops while replacing water. In aquaculture, it serves as an anti-fungal and anti-parasitic and has commonly been used to treat fish eggs to ensure they are not lost to fungal overgrowth. The physicochemical characteristics of tap water reconstituted with salts were maintained, i.e. at room temperature. A total of 25 fish were maintained in five different tanks containing five fish each.

Preparation of Diclofenac For Toxicity Studies

Diclofenac of chemical purity ≥98.0% were purchased as a commercial powder from Sigma Aldrich. After partially dissolving in water, diclofenac was completely dissolved by the addition of few drops of glacial acetic acid. Later on, it was added to the tanks containing fish. For the test group, concentrations of diclofenac in the range of 10 mg, 20 mg, 30 mg, 40 mg and 50 mg were used.

Determination of Lethal Concentration LC_{50}

In conformance to the work done by Vijayageetha et al. [10] to determine the lethal concentration (LC_{50}), an exposure to diclofenac at different concentrations in the range of 7 mg, 14 mg, 21 mg, 28 mg and 35 mg for ~12 litres of water in a fish tank (12 x 6 x 8) inches in dimension was carried out.

Exposure of Drugs to Fish

All the fish were maintained at room temperature with a natural light/dark photoperiod and provided with constant aeration. The fish in tank 1 were maintained as a control without any exposure to drugs. Tank 2 containing five fish was exposed to diclofenac. Food was provided to specimens during the exposure period. The exposed concentration to the fish in the tanks is the lethal concentration of the drug to the *Pangasius* Sp. The fish were anaesthetized after several days according to the lethal concentration and were sacrificed.

Preparation of 1% *Artemisia pallens* solution

For the preparation of 1% *Artemisia pallens* solution; 6 grams of *Artemisia pallens* powder was added to 600 mL of water forming a 1% solution. Following this, 600 mL of 1% *Artemisia* sp. was annexed into a 60 L tank forming a 1 % solution.

Exposure of *Artemisia pallens* to fish

Artemisinin, a sesquiterpene lactone derived from *Artemisia* sps. has been broadly used for treating antiviral diseases [11]. Furthermore, this compound is also notable to procure antibacterial, antifungal, antileishmanial, antioxidant, antitumor, anti-inflammatory activities and other pharmacokinetic properties [12, 13] by which this could be availed to reduce the toxic effects of the subjected chemical compound. Upon implementing this reverberation, the fish were parallelly maintained in other tanks with the same concentration of drugs simultaneously exposing the fish with the medium containing the herbal component, *Artemisia pallens*. This work was carried out for diclofenac infused fish with the herbal component at the concentration of 0.006 mg/mL as well. Later, the fish were anaesthetized and sacrificed, 72 hours after the sacrifice of fish in the tanks containing the fish without herbal components.

Oxidative Stress Parameters

The oxidative stress assay was carried out for different parameters such as lipid peroxide (LPO), conjugated diene (CD), protein sulfhydryl and protein carbonyl, which are as follows.

Assay of Lipid Peroxide (LPO)

LPO was determined by the TBA method [14]. To 100 µL of tissue homogenate, the supernatant was added to tris-HCl buffer solution pH 7.4 until a volume of 1 mL was attained. Samples were incubated at 37ºC for 30 minutes; 2 mL TBA–TCA reagent was added and samples were shaken in a vortex. They were then heated to boiling for 45 minutes, cooled and the precipitate was removed by centrifugation at 3000 rpm for 10 minutes and the absorbance was read at 535 nm against a reaction blank.

Estimation of Conjugated Dienes

For estimating conjugated dienes, test samples (tissue fractions) subjected to oxidative stress were treated with chloroform: methanol mixture (2:1) followed by vigorous vortexing and centrifugation at 2,000 rpm for 10 minutes [15]. The supernatant obtained was discarded along with the proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45ºC. The residue obtained was dissolved in cyclohexane and absorbance was taken at 233 nm against a cyclohexane blank.

Estimation of Protein Carbonyl

The carbonyl content of while protein extracts were measured using the Levine et al., method [16]. Soluble protein (0.5 mL) reacted with 10 mM of DNPH in 2 M concoction of hydrochloric Acid for 1 hour at room temperature and precipitated with 6% of trichloroacetic acid (TCA). The pelleted protein was washed thrice by a resumption in ethanol/ethyl acetate (1:1) and then solubilized in 6M guanidine hydrochloride, 50% formic acid, and subsequently centrifuged at 16,000 × g for 5 minutes to remove any plausible traces of insoluble materials. The carbonyl contents were measured spectrophotometrically at 366 nm. The results were expressed as nanomoles of DNPH incorporated/mg protein based on the molar extinction coefficient of 21,000 M^{-1} cm^{-1}.

Estimation of Protein Sulfhydryl

Protein sulfhydryl was estimated using DTNB method [17]. Diluted tissue homogenate suspension of 0.5 mL was added to 1.5 mL of 0.2 M tris-HCl

and 0.1 mL of 0.01 M DTNB in methanol. The volume was made up to 10mL using methanol and incubated at room temperature for 15 minutes following, sample centrifugation at 300 xg for 10 minutes and absorbance of the supernatant was read at 412 nm.

Assay of Inorganic Phosphorus:

Inorganic phosphorus was estimated using an ANSA reagent [18]. 1 mL of test sample solution was mixed with 0.6 mL of 2.3% molybdate reagent (2.3% of ammonium molybdate in 10 N sulfuric acids). Then 0.2 mL of ANSA solution (0.25 g of 8-anilino-1-naphthalene sulfonic acid (ANSA) in 15% sodium bisulphite solution and 5 mL of 20% sodium sulfite solution) was added to it. The absorbance was measured at 660 nm after 10 minutes of incubation at room temperature.

Extraction of Inorganic Phosphorus from the Fish Liver and Muscle Tissue

The hepatic (liver and muscle) samples were collected from the toxin-induced fish after sacrificing. They were homogenized for 3 minutes in 5 mL of extraction buffer (1.25 mL of conc. perchloric acid, 0.2 mL conc. phosphoric acid diluted to 100 mL with distilled water). The homogenate was further centrifuged at 6000 rpm for 15 minutes at room temperature. The supernatant was separated and filtered and stored till usage.

Histopathological Studies (Modified Protocol of Reza et al.,) [19]

Fish tissue slices collected from the fish exposed to diclofenac and *Artemisia pallens* were immediately fixed in a 10% formalin solution. The tissues were sliced with the help of a microtome (5 µm) and were embedded in paraffin wax. Hematoxylin and eosin staining protocol was followed and the histopathological changes of tissues were visualized under a multi-head microscope.

Antioxidative Stress Parameters

The antioxidative stress assay was carried out using different parameters such as superoxide dismutase and catalase.

Assay of Superoxide Dismutase

The activity of SOD was estimated by monitoring the oxidation of epinephrine according to the procedure of Misra et al., [20]. 2.5 mL of carbonate-bicarbonate buffer (0.3 m, pH 10.2) and 0.5 mL of EDTA (0.6 mm) solution were added to the tubes. Suitably diluted sample 1 and sample 2 were added and the change in absorbance was monitored after adding 0.5 mL of epinephrine (1.8 mm) at 420 mm for 2 minutes at 15 seconds interval using a UV spectrophotometer. The activity was expressed as units/minutes/mg of protein.

Assay of Catalase

The activity of catalase was estimated by the modified method of Hadwan [21]. 23 mL of H_2O_2 – phosphate buffer was pipetted into the cuvette, a required amount of tissue supernatant (cytosolic fraction) was added as enzyme source, and the contents were mixed thoroughly. The decrease in absorbance at 240 nm was recorded every 30 seconds for 3 minutes. The results were expressed as units/mg protein.

Estimation of Cortisol

Cortisol was estimated using the modified protocol from the work done by Marco et al., [22]. Cortisol has been measured using an enzyme-linked immunosorbent assay (ELISA). Blood samples were taken from the caudal peduncle using heparinized syringes to obtain plasma after centrifugation at 10,000 xg for 5 min, maintained on ice until the determination of cortisol concentrations. For the assay, 20 µl of fish plasma samples were added to the plate. Subsequently, 200 µL of enzyme-conjugated to horseradish peroxidase was added into each well. Finally, the wells were gently mixed on a plate mixer at 200 beats.min-1 for 10 min and incubated for 1 h at room temperature. The well contents were briskly eliminated to avoid any residual content. The solution of each well was removed by washing the plate three times with 400 µL of PBS and shaking out the content onto absorbent paper with the aim of removing residual drops that could affect the accuracy and precision of the assay. Subsequently, 100 µL of TMB enzyme substrate was added to each well and incubated for 15 min at room temperature. The enzymatic reaction was visualized by the colour change and was stopped by the addition of 100 µL of 0.5 M phosphoric acid (H_2PO_3). The intensity of the colour is inversely proportional to the concentration of cortisol in the samples. The absorbance was read in a spectrophotometer at 450 nm on a microtiter plate reader within 10 min after the addition of stop solution.

Statistical Analysis

The data were analysed using a commercially available statistical software package (Graphpad software). Students ‘t’ was performed to find out the significance of variations between the control and fish exposed to diclofenac. Results were presented as mean±SD and the *p*-value less than 0.01 and 0.05 were considered to be statistically significant.

Results and Discussion

Oxidative Stress Parameters

Lipid Peroxide and Conjugated Diene

During oxidative stress, ROS are produced. The ROS produced reacts with unsaturated fatty acids that are present in membranes and cause lipid peroxidation. Therefore, increased lipid peroxidation is an indication of a high level of ROS and used extensively to study oxidative stress. In a research by Saleh et al., [23], the garlic extract had significant changes in the lipid peroxidation of the liver exposed to carbon tetrachloride. This was found to be in accordance with our research. Conjugated dienes are the initial peroxidative product and it is considered to be the accurate indicator of lipid peroxidation. Its level in the samples represents the initiation of lipid peroxidation by the reactive oxygen species.

From Fig. 1 and Fig. 2, the level of lipid degradation in terms of LPO and CD was found to be significantly higher in liver tissue exposed to diclofenac of concentration 12 mg /l. The Lipid peroxidation had increased about 3.53 folds in diclofenac treated fish than the control fish, and for conjugated diene values, up to 2.79 folds in diclofenac treated fish than that of control fish were observed of $p<0.01$ level of significance for both the parameters when observed. When *Artemisia pallens* was exposed to the fish, where the LPO has been increased upto 3.53 folds, and the CD increased upto 2.18 folds for diclofenac subjected fish.

The LPO values of fish treated with diclofenac are significantly higher than the LPO values of fish treated with drugs along with *Artemisia pallens* and the control fish. This shows the free radicals scavenging activity of *Artemisia pallens.* Similarly, the CD values of fish treated with diclofenac were significantly higher than that of the CD values of fish treated with drugs along with *Artemisia pallens* and the control fish. The LPO values are reduced up to 65.5% when exposed along with *Artemisia pallens* compared to diclofenac treated fish with a strong indication of no-significance (NS) as the values were inclining to that of the control. Similarly, The CD values were reduced up to 21.7% when exposed along with *Artemisia pallens* compared to diclofenac treated fish significantly with $p<0.05$ level of significance. On a comparative scale, the difference in LPO and CD values for diclofenac and diclofenac + *Artemisia pallens* treated fish showed a difference of nearly 65.5% and 21.74% respectively of $p<0.05$ and $p<0.01$ level of significance. Our results were found to be similar to action of waterborne diclofenac that changed the effects significantly in lipid peroxidation assay when the liver of galaxiid fish inanga (*Galaxias maculatus*) was exposed in combination with cadmium [24].

Protein Sulfhydryl and Protein Carbonyl

ROS causes oxidation of proteins, commonly the amino acids; lysine, arginine and proline. Oxidative cleavage of the peptide backbone via the α-amidation pathway, oxidative cleavage of glutamyl residues, formation of protein-protein cross-linked derivatives, and cell membrane damage by lipid oxidation products give rise to reactive aldehydes and ketones known as protein carbonyls. Protein carbonyl (PC) content

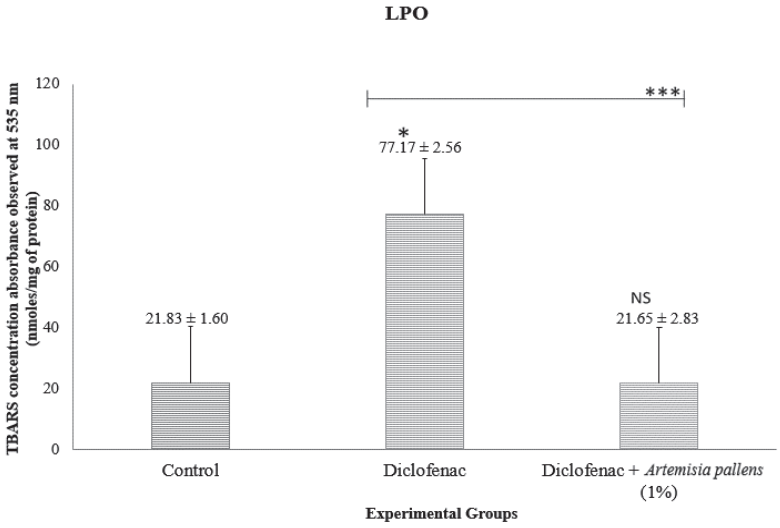

Fig. 1. Level of Lipid Peroxide in hepatic tissue of *Pangasius* sp. exposed to diclofenac (12 mg/mL) LC_{50} concentration.
The statistical significance considered for the LPO values were
*$p<0.01$ representing the LPO values comparison between control and diclofenac treated fish
**NS indicating the collation between control and diclofenac + *Artemisia pallens* (1%) and,
***$p<0.05$ upon comparing LPO values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish; Non-Significant because the diclofenac + *Artemisia pallens*treated fish tends towards the control fish values.

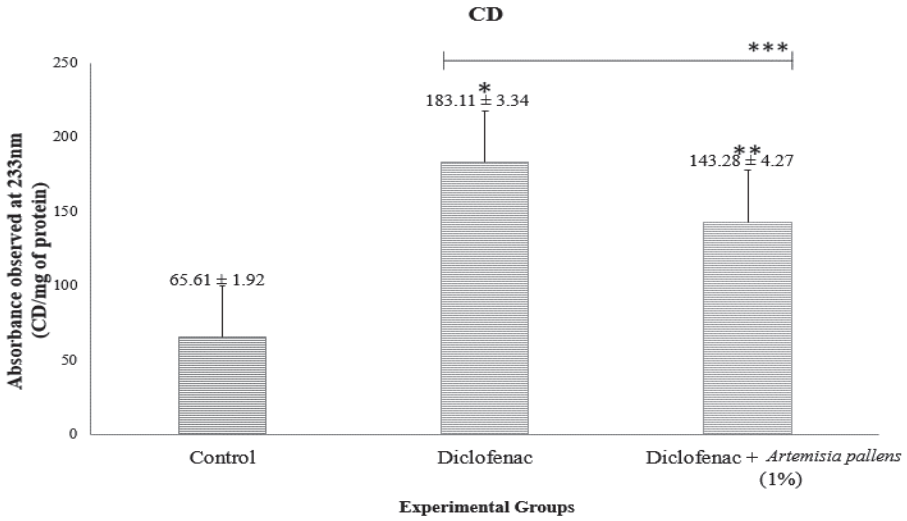

Fig. 2. Level of Conjugated Diene in hepatic tissue of *Pangasius* sp. exposed to diclofenac (12 mg/mL) LC_{50} concentration.
The statistical significance for CD values considered were
*p<0.01 indicating the comparison between control and diclofenac treated fish,
**p<0.05 representing the CD values of comparison between control and diclofenac + *Artemisia pallens* (1%) and,
***p<0.01 for the comparing CD values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish.

in blood and tissues is a reliable indicator of protein oxidation. It is particularly useful in this respect because of its long-lasting stability under suitable storage conditions (i.e. –80ºC).

From Fig. 3, the level of protein damage in terms of protein carbonyls was found to be significantly higher in the liver tissues exposed to a diclofenac concentration of 12 mg /l. Observingly, the carbonyl levels had increased up to 1.0 fold in diclofenac treated fish than that of the control fish of a level of significance of p<0.01. The protein carbonyl values of fish treated with diclofenac were significantly higher than the values for the fish treated with diclofenac along with *Artemisia pallens* and the control fish. When *Artemisia pallens* was exposed to the fish along with the same concentration of diclofenac. The protein carbonyl values reduced up to 0.8% for diclofenac exposed along with *Artemisia pallens* compared to diclofenac treated fish; having no significant as the *Artemisia pallens* treated fish along with diclofenac showing values nearing the

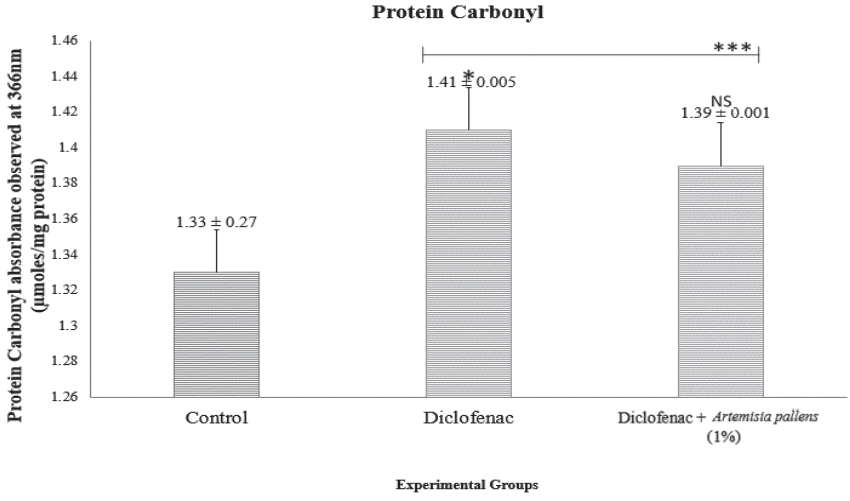

Fig. 3. Levels of Protein Carbonyl in hepatic tissue of *Pangasius* sp. exposed to diclofenac (12 mg/mL) LC_{50} concentrations.
The statistical significance considered for the protein carbonyl values are
* p<0.01 representing the protein carbonyl values comparison between control and diclofenac treated fish,
NS; Non-significant by virtue of the diclofenac + *Artemisia pallens* (1%) treated fish protein carbonyl values tend towards the control fish values, upon comparing control and diclofenac + *Artemisia pallens* (1%) treated fish,
***p<0.01 upon comparing the corresponding protein carbonyl values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish.

control measures, with the difference of 0.78% between diclofenac and diclofenac + *Artemisia pallens* treated fish with $p<0.01$ level of significance.

Protein sulfhydryl damage is measured in terms of free thiols. Free thiols may take part in cell protection against ROS, but at the same time, they are involved in the regulation of many redox-sensitive processes. Low-molecular-weight thiol levels changed in a tissue-specific manner, but in general, their content increased over oxidative stress and levels remained high at recovery. It should be noted that glutathione is the main low-molecular-weightthiol. Therefore, it is possible that L-SH content corresponds roughly to glutathione concentration. The liver is the main source of glutathione in vertebrates and supplies it to other organs. In the hepatocytes of rainbow trout exposed to xenobiotics of 600 μM benzoquinone, GSH content increased suggesting that the glutathione pool responds to stressful conditions enhancing its synthesis and reduction [25].

From Fig. 4, the level of protein damage in terms of PSH was found to be significantly higher in liver tissue exposed to diclofenac 30 mg/L. The sulfhydryl levels of fish treated with drugs are significantly higher than the fish treated along with *Artemisia pallens* and the control fish. PSH had increased up to 12.7 folds in diclofenac treated fish than the control fish with a level of significance at $p<0.01$. When *Artemisia pallens* was exposed to the fish along with the same concentration of drugs, The PSH values reduced up to 64.9% for diclofenac treated fish along with *Artemisia pallens* compared to diclofenac treated fish at $p<0.01$ level of significance. The comparative differences between the drug-treated and the drug + *Artemisia pallens* treated fish were observed to have a 64.899% difference of level of significance as $p<0.05$.

Inorganic Phosphorus

The inorganic and organic phosphorus plays a particularly important role in energy transfer and enzyme systems. In glycolysis, phosphate is a substrate for glyceraldehyde-3-phosphate dehydrogenase and stimulates the activities of hexokinase and phosphofructokinase. If cytosolic phosphate concentration decreases below some critical value, the rates of glycolysis and oxygen consumption should be inhibited. Hence a gradual depletion in different phosphorus contents during spawning is quite justifiable. Since high metabolism is the result of oxidative stress, the organism requires high energy for metabolism during oxidative stress, for which ATP is broken down into ADP and Pi. By measuring the inorganic phosphorus in the cellular content of the specimen, the breakdown of ATP to ADP is indirectly correlated [26].

From Fig. 5, the level of inorganic phosphorus was found to be significantly higher in liver tissue exposed to diclofenac concentration of 12 mg /l. The values for the parameter inorganic phosphorus had increased up to 3.60 folds in diclofenac treated fish than that of the control fish at a level of significance of $p<0.01$. The IP values for fish treated with diclofenac are significantly higher than the IP values for the fish treated with the drugs along with *Artemisia pallens* than the control fish. When *Artemisia pallens* was exposed to the fish with the same concentration of drugs, the IP values were observed to have reduced for diclofenac and *Artemisia pallens* subjugation, with 2.16 folds of reduction observed at $p<0.05$ level of significance. Upon comparing diclofenac treated fish and diclofenac and *Artemisia pallens* subjugated fish alone the difference was observed at 53.71% difference at $p<0.01$ level of significance.

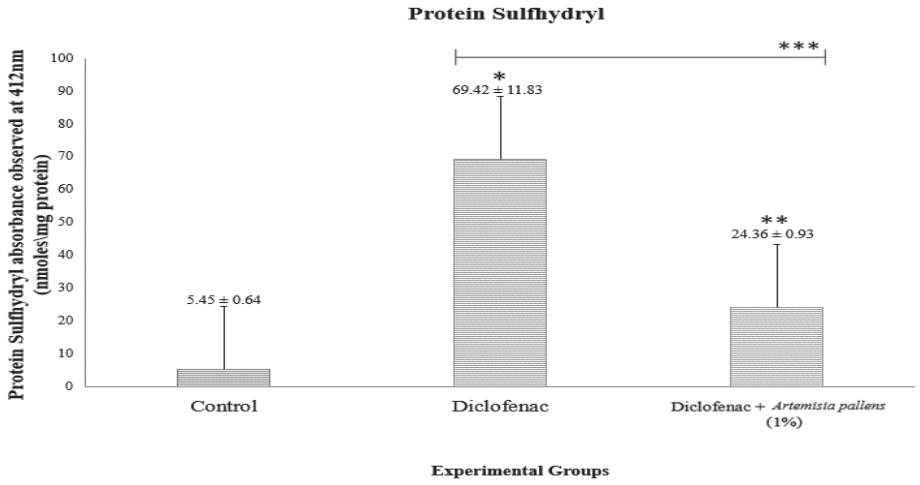

Fig. 4. Levels of Protein Sulfhydryl in hepatic tissue of *Pangasius* sp. exposed to diclofenac (12 mg/mL) LC_{50} concentrations.
Consecutively, the statistical significance for protein sulfhydryl (PSH) values considered were
*$p<0.01$ indicating the comparison between control and diclofenac treated fish,
**$p<0.01$ representing the PSH values of comparison between control and diclofenac + *Artemisia pallens* (1%) and,
***$p<0.05$ for the comparing PSH values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish.

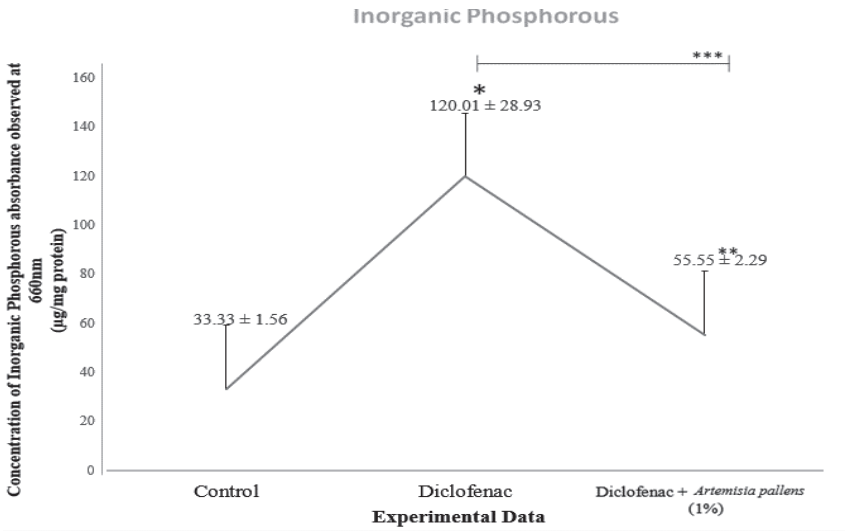

Fig. 5. Inorganic Phosphorus levels in hepatic tissue of *Pangasius* sp. exposed to diclofenac (12 mg/mL) LC_{50} concentrations. The statistical significance considered for inorganic phosphorus (IP) values were
*p<0.01 representing the IP values comparison between control and diclofenac treated fish,
**p<0.05 indicating the collation between control and diclofenac + *Artemisia pallens* (1%) and,
***p<0.01 upon comparing the corresponding IP values of diclofenac and diclofenac + *Artemisia pallens*(1%) treated fish.

Histopathological Studies

Histopathological changes were characterised by early cell infiltration with segmental necrosis and inflammation. The liver tissue that was not exposed to any chemicals and *Artemisia pallens* appeared normal and did not show any changes.

Earlier studies indicated certain histopathological changes such as the ruptured vein, lipid-like vacuolizations, and degenerated hepatocytes with prolonged exposure of (juvenile grass carp) *Ctenopharyn godonidella* for nearly 14 days to BPA, showing the inimical effects on the fish kidney and liver cells [27]. Gupta and his team [28] observed that intake of diclofenac over a period of time was liable for causing hepatotoxicity in rats which were shown by alterations in biomarkers of oxidative stress and liver function in blood and tissue as well as in liver sections. Also in the study carried out by Liaqat and team [29], the infiltrations observed, were similar to ours in the liver cells treated by paracetamol. In the liver sections, hepatocellular necrosis, ballooning and degeneration of hepatocytes clearly indicated the toxic effects of diclofenac. Heeding to the work by Vijaya Geetha et al., [10] the vacuolation and the degeneracies of the *Pangasius* Sps. hepatocytes exposed to diclofenac were significantly observed along with the lesions and similar abscesses.

With this substantiate, from Fig. 6, the liver tissue exposed to diclofenac showed massive increase in inflammation of the hepatocytic cells and degeneration of vacuoles upon subsequent scrutiny. On treatment with *Artemisia pallens,* the degeneration of hepatic cells reduced significantly and the degeneration of vacuoles was highly reduced.

Antioxidant Parameters

Superoxide Dismutase

Oxidative damage resulting from reactive oxygen species (ROS) due to light catalysed reaction. The rise in ROS leads to an increase in O_2 anion, thereby leading to an increase in superoxide dismutase activity. The detoxification of ROS involves a direct reaction with cellular constituents, the lens is equipped with a number of antioxidant enzymes. One of the key enzymes that detoxify the reactive O_2 anion through dismutation to form H_2O_2 is superoxide dismutase (SOD).

The first enzyme measured was SOD, i.e., the enzyme which catalyses the formation of hydrogen peroxide from superoxide anion. Both analysed drugs enhanced the SOD activity in a concentration-dependent manner. The treatment of cells with 0.05 mM and 0.5 mM of diclofenac increased the SOD activity by 15 and 32%, or by 54 and 81%, respectively, as compared with the control. Our research was in accordance with a study on C*ommon Carp (Cyprinus carpio)* showed SOD activity in the different organs assessed after exposure to DCF, ACT and a 1:1 mixture of these pharmaceuticals, SOD activity increased relative to the control group in the brain of specimens exposed to ACT (230.7%), while no significant difference was found with all other treatments [3]. A significant reduction with respect to the control group was observed in the liver of carp exposed to either DCF (74.2%) or the mixture (43%). In gill, a significant increase occurred in specimens exposed to DCF (203.7 %) and a significant reduction (60.4 %) in those exposed to the mixture ($p<0.05$).

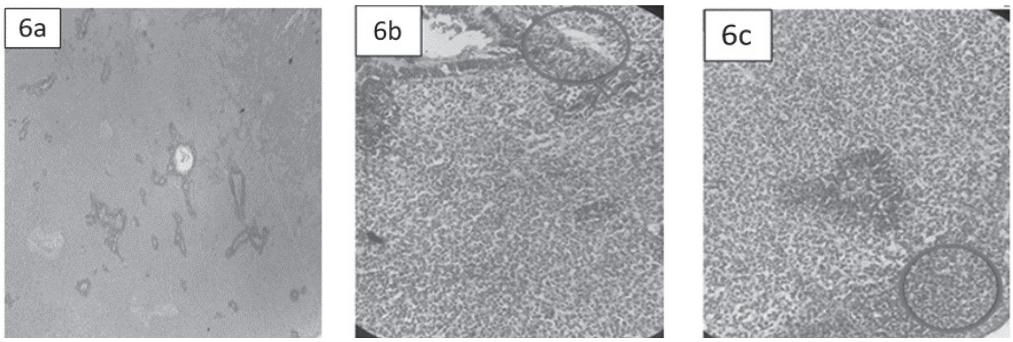

Fig. 6. Histological pictures of the liver of *Pangasius*, normal healthy fish, fish exposed to diclofenac and fish exposed to diclofenac + *Artemisia pallens.*
a) Histopathological image showing normal healthy hepatic cells.
b) Histopathological image showing inflammation of the hepatocytic cells and degeneration of vacuoles when fishes are exposed to drug (diclofenac).
c) Histopathological image showing reduction in cell damage of hepatocytic cells when fishes are treated along with *Artemisia pallens* (diclofenac + *Artemisia pallens*).

From Fig. 7, Superoxide Dismutase (SOD) activity was found to be high in liver tissue exposed to diclofenac 30mg/l and diclofenac 12 mg/l. Superoxide dismutase activity has increased upto 4.9 folds in diclofenac treated fish than the control fish at $p<0.01$ level of significance. The catalase activity was notably decreased both in liver and kidney. Following oxidative stress, catalase is an active and first enzyme that shows alteration [27]. In a similar deduction of *A. scorparia* effects, the essential oils provided a scavenging action of certain enzymes such as; SOD, Ascorbate, guaiacol peroxidase and catalase [30]. Under a similar notion, when *Artemisia pallens* were exposed to the fish along with the same concentration of diclofenac, the sod had increased up to 3.04 folds for diclofenac treated fish than that of the control fish. The sod activity of fish treated with drugs is significantly higher than the values of fish treated with drugs along with *Artemisia pallens* and the control fish. This shows the free radicals scavenging activity of *Artemisia pallens.* The SOD activity reduced up to 22.5 % for diclofenac treated fish along with *Artemisia pallens* compared to diclofenac treated fish of $p<0.01$ level of significance. Consecutively, for the comparison of diclofenac and diclofenac and *Artemisia pallens* subjugated fish, a difference of 37.8% for diclofenac treated fish along with *Artemisia pallens* compared to diclofenac showed a difference of 37.853%, indicating a significant decrease in superoxide dismutase values at $p<0.01$ level of significance.

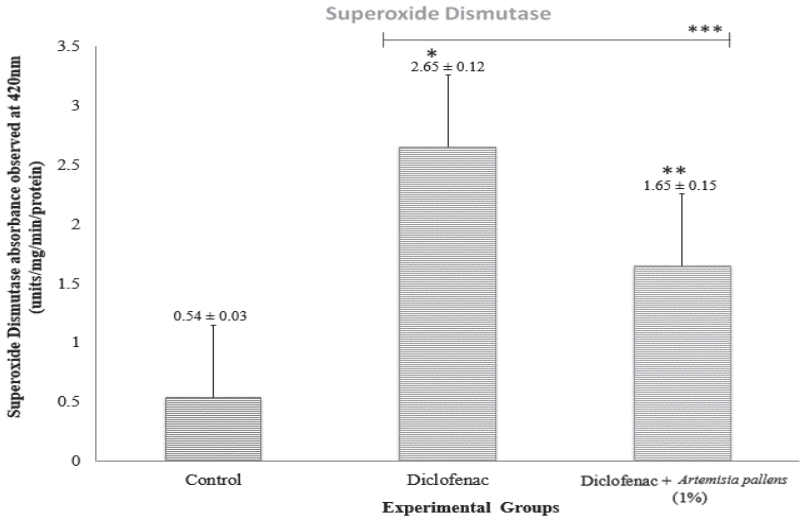

Fig. 7. Level of Superoxide Dismutase in hepatic tissue of *Pangasius* sp. exposed diclofenac (12 mg/mL) LC_{50} concentrations.
The statistical significance considered for superoxide dismutase (SOD) values are
*$p<0.01$ represents comparison between control and diclofenac treated fish ,
**$p<0.01$ indicating the collation between control and diclofenac + *Artemisia pallens* (1%) and,
***$p<0.01$ upon comparing the corresponding SOD values of diclofenac and diclofenac + *Artemisia pallens*(1%) treated fish.

Catalase

Eukaryotic cells have to constantly cope with highly reactive oxygen-derived free radicals. Their defence against these free radicals is achieved by natural antioxidant molecules and also by antioxidant enzymes. The different oxygen-derived free radicals are formed by subsequent univalent reductions of molecular oxygen: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) which is not a free radical hydroxyl radical (OH^-) and then water (H_2O). During respiration, oxygen is directly reduced into water. The increase in superoxide dismutase gives rise to an increase in the production of hydrogen peroxide thereby leads to an increase in catalase activity.

From Fig. 8, Catalase (CAT) activity was found to be high in liver tissue exposed to diclofenac 30 mg/l and diclofenac 12 mg /l. catalase activity has increased up to 3.46 folds in diclofenac treated fish than the control fish. When *Artemisia pallens* were exposed to the fish along with the same concentration of diclofenac, the catalase activity has been increased up to 1.48 folds for diclofenac than the control fish at $p<0.01$ level of significance. The catalase activity of fish treated with drugs are significantly higher than the values of fish treated with diclofenac along with *Artemisia pallens* and the control fish. This shows the free radicals scavenging activity of *Artemisia pallens*. The catalase activity reduced upto 47.93% for diclofenac treated fish along with *Artemisia pallens* compared to diclofenac treated fish samples under a level of significance, $p<0.05$. Between diclofenac and diclofenac + *Artemisia pallens* treated fish, a comparative difference of 57.34%, indicating a significant decrease inprotein catalase values at $p<0.05$ level of significance. In the study carried out by Malachy et al., [31], catalase activity was increased when nila tilapia (*Oreochromis niloticus*) was exposed to diclofenac. A significant level ($p<0.05$) was recorded. Similar catalase activity changes were observed the fish were exposed to compounds like clofibrate, phenol and norfloxacin and later on treated with *Artemisia pallens* [32, 33].

Cortisol

Cortisol is a corticosteroid secreted from the adrenal cortex. Changes in the plasma concentration of adrenocorticotropic hormone (ACTH), a peptide hormone released from the anterior pituitary, regulate the release of cortisol. In turn, the release of ACTH is controlled by the effect of a hypothalamic peptide, corticotropin-releasing factor, on the pituitary. Cortisol is now often regarded as a stress hormone and as an objective marker of stress. The alteration of the plasma cortisol and prolactin level during acute treatment in lead-exposed fish was studied.

From Fig. 9, it can be inferred that the cortisol levels are elevated in the hepatic tissue of the fish exposed to the drugs, diclofenac at the concentration of 12 mg/l. The increased level of cortisol, when treated with the drug, was found to be 3.11 folds greater than the control fish at a $p<0.01$ level of significance. When the specimens were treated along with *Artemisia pallens*, it was found that the stress levels reduced by 1.26 folds *Artemisia* treated fish along with the drug than the fish treated with the drug alone at a level of significance of $p<0.01$, evidently inferring that the cortisol levels of fish treated with diclofenac are significantly higher than the values of the fish treated with diclofenac along with *Artemisia pallens* and the control fish.

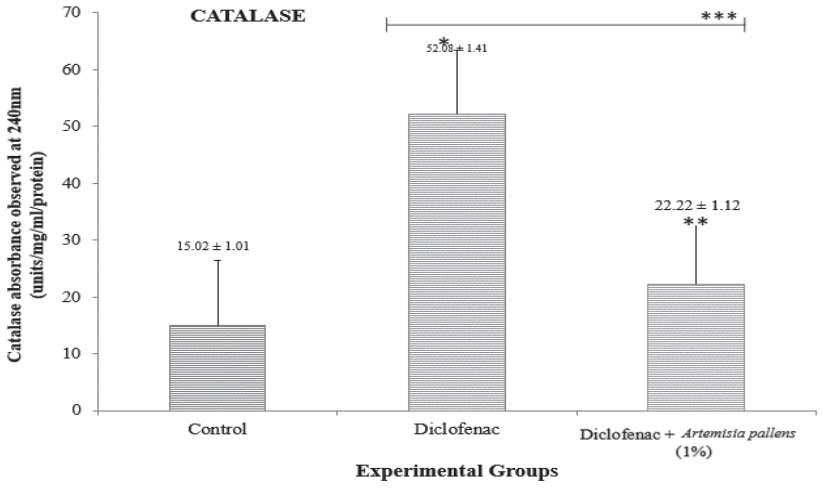

Fig. 8. Level of Catalase in hepatic tissue of *Pangasius* sp. exposed diclofenac (12 mg/mL) LC_{50} concentrations.
The statistical significance for catalase (CAT) values considered were
*$p<0.01$ indicating the comparison between control and diclofenac treated fish,
**$p<0.05$ representing the CAT values of comparison between control and diclofenac + *Artemisia pallens* (1%) and,
***$p<0.05$ for comparing the CAT values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish.

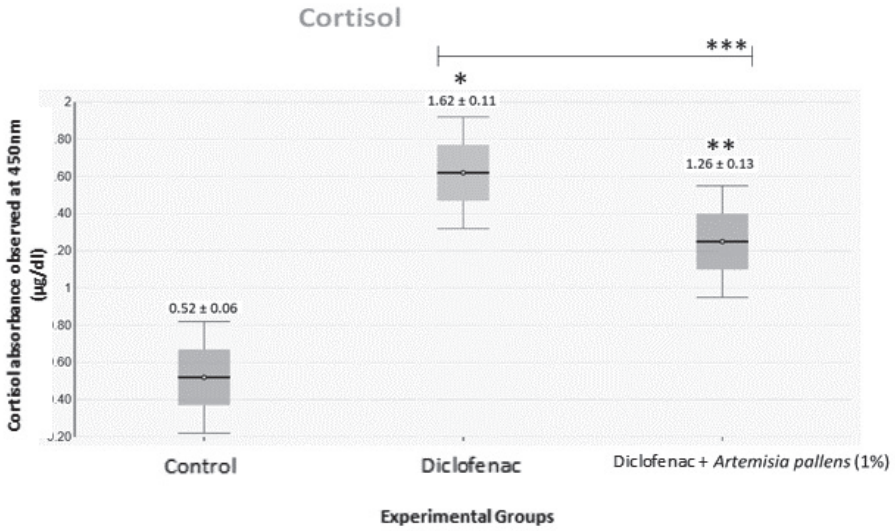

Fig. 9. Cortisol levels in hepatic tissues of *Pangasius* sp. exposed to diclofenac(12 mg/mL) LC_{50} concentrations.
The statistical significance for the cortisol values considered were
*p <0.01 indicating the comparison between control and diclofenac treated fish,
**p<0.01 representing the cortisol values of comparison between control and diclofenac + *Artemisia pallens* (1%) and,
***p<0.05 for comparing values of diclofenac and diclofenac + *Artemisia pallens* (1%) treated fish.

This shows the free radicals scavenging activity of *Artemisia pallens* and as a stress remediator. Upon collating diclofenac and diclofenac + *Artemisia pallens* treated fish values, a comparative difference of 22.22% was observed, indicating a significant decrease incortisol values being positively correlated at p<0.05 level of significance. Artemisinin from *Artemisia* sp has been shown to be widely used for the treatment of malaria. Analogous changes were observed in our previous research on the liver tissues of *Pangasius* Sp exposed to phenol, clofibrate and norfloxacin [32, 33]. Furthermore, artemisinin is known to have antibacterial, antifungal, anti leishmanial, antioxidant, antitumor, and anti-inflammatory activity [12, 13].

Additionally, we extended diclofenac and bioremediator exposure to other organs of the fish. The observable histopathological changes and *in-silico* studies were recorded [34].

Conclusion

The present study revealed that even at lower concentrations of diclofenac, it has the ability to cause lethal effects to aquatic biota in the longer run. It was observed that the toxic nature of diclofenac induced oxidative stress leading to the malfunctioning of metabolisms in the body which resulted in the fatality of the fish. The histopathological analysis visually proved the extent of damage caused by the compound. The investigation on cortisol level showed dramatic elevation in values compared to the control fish. On introducing the remediator, *Artemisia pallens* the toxic effects were mitigated. The flavonoids and phenolic constituents have brought about partial recovery from the oxidative damages that occurred during the exposure of fish to the drugs; thereby giving a locus to imbibe more natural remedial measures as well as traditional herbs to be included in the modern health aids and to inculcate the importance of environmental safety and sustainability in the surroundings. We thereby assert that *Artemisia pallens* have effective mitigation action on the drug, diclofenac in the fish model, *Pangasius* Sp.

Conflict of Interest

The authors declare no conflict of interest.

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