

Toxicity Assessment of Acetylsalicylic Acid to a Freshwater Fish *Cyprinus carpio*: Haematological, Biochemical, Enzymological and Antioxidant Responses



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Abstract Pharmaceutical pollution is a global threat to the biosphere causing significant environmental health concern. A wide range of pharmaceuticals (antibiotics, nonsteroidal anti-inflammatory drugs, beta-blockers, etc.) are widely used in

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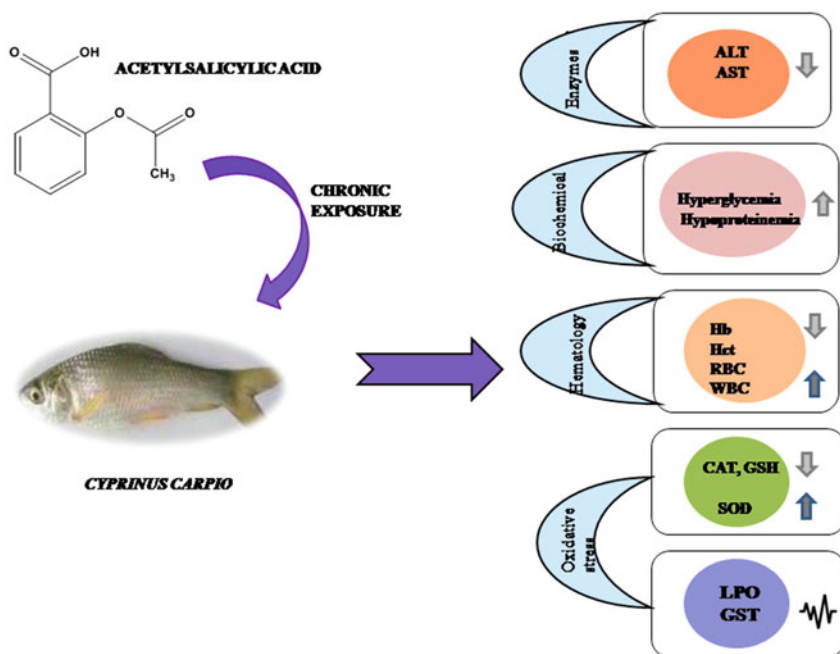
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human and veterinary medicine, agriculture and aquaculture purposes to protect the life against various diseases and to improve human health. The extensive use of these compounds may enter the environment through discharge of domestic waste waters, excretion via water and sewage treatment systems which may affect the aquatic organisms. Aspirin (acetylsalicylic acid, ASA) is one of the most commonly used nonsteroidal anti-inflammatory drug (NSAIDs) worldwide and has been detected in aquatic bodies. Therefore, it is important to gain knowledge about the toxicity of acetylsalicylic acid in aquatic organisms. Here we have administered 100 and 200 mg L⁻¹ of acetylsalicylic acid, to a freshwater fish *Cyprinus carpio* fingerlings, and have studied its effects on haematological, enzymological biochemical and antioxidant parameters. When compared to control, acetylsalicylic acid-treated fish showed a significant ($P < 0.05$) decline in haemoglobin (Hb), haematocrit (Hct) and red blood cell (RBC) levels throughout the study period (12 days). On the other hand, a significant ($P < 0.05$) increase was observed in white blood cell counts (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) values. Acetylsalicylic acid induced a hyperglycaemic condition compared to control, whereas the level of proteins was declined. A significant decrease in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity was noted in acetylsalicylic acid-treated groups (except 21st day in ALT activity and 21st day in AST activity). Significant alterations in various antioxidant parameters such as superoxide dismutase (SOD), lipid peroxidase (LPO), catalase (CAT) glutathione (GSH) and glutathione S-transferase (GST) were observed in ASA-treated groups compared to the control group. From the results, it is noteworthy that the drug ASA even at considerable environmental concentrations causes negative impacts on the health of aquatic organisms. The alterations of these parameters can be effectively used to monitor the impact of pharmaceutical drugs in the aquatic environment.

Graphical Abstract



Keywords Acetylsalicylic acid, Biomarkers, *Cyprinus carpio*, NSAIDs

1 Introduction

Over the past 30 years, environmental pollution has been increasing at an alarming rate. Industrialization, modern agricultural practices and infinite use of pharmaceuticals in medicine release xenobiotics into the environment [1]. Pharmaceuticals are bioactive chemical compounds used in diagnosis, treatment or alleviation of disease, improving health status as well as to revamp normal physiology in organisms [2]. Besides human medication, veterinary sectors (livestock, poultry and aquaculture) rely on the application of pharmaceutical products such as antibiotics and hormones, to promote growth, biological functions and health conditions [3]. Ramesh et al. [4] have reported that about 200 million kg of antibiotics used all over the world flow into water bodies (rivers, lakes and sea) annually causing water pollution.

The indiscriminate use of pharmaceuticals in human and veterinary medicine has caused contamination of aquatic ecosystem [5], perhaps affecting the health of aquatic organisms. Fish and marine mammals which occupy the upper trophic

level of the aquatic food chain are prone to higher levels of toxicity present in the marine or freshwaters [6]. The existence of pharmaceuticals along with their metabolites in water bodies has been reported in many countries [7–9]. Several of these compounds act as endocrine disrupting chemicals interfering with the normal hormonal balance system [10] by mimicking a natural hormone or blocking the binding of endogenous hormone to certain receptors [11] and also induce bone marrow, reproductive and nervous system disorder in living organisms [12, 13].

The major group of pharmaceuticals includes nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, analgesics, lipid regulators, beta-blockers, steroids and related hormones [14, 15]. NSAIDs act by inhibiting cyclooxygenase (COX) enzyme which is responsible for the biosynthesis of various active lipid compounds called prostaglandins [16]. Among the various classes of NSAIDs reported, aspirin, ibuprofen, naproxen and diclofenac are widely prescribed. NSAIDs are weak organic acids having a high affinity for lipids and plasma proteins with various therapeutic potentials.

Aspirin (acetylsalicylic acid, ASA) is one of the most commonly used NSAIDs worldwide [2, 17]. It is medicated to treat minor pains, cardiovascular thrombosis [18], arthritis and related musculoskeletal disorders [19]. ASA with the IUPAC name 2-acetoxy benzoic acid is the NSAID with salicylate chemical group. ASA is widely used as an analgesic and antipyretic agent as it causes cyclooxygenase (COX) inhibition on administration [20]. Kerola et al. [21] reported that ASA is COX-1 specific; COX-1 enzymes are found on the surfaces of platelets and gastric mucosal cells. The acetyl and the salicylate portions of the ASA molecule produce analgesic, antipyretic and anti-inflammatory effects when consumed [22]. The activity of cyclooxygenase (COX-1) is hindered by ASA molecule to decrease the synthesis of precursors of prostaglandins and thromboxanes from arachidonic acid. Therefore, this helps to regulate the production and release of prostaglandins preventing the symptoms of inflammatory responses such as swelling, increased blood vessel dilation, immune response and blood coagulation [23].

The body does not detain the entire dose of drug consumed, as a major portion are transformed to one or more drug metabolites and are defecated as metabolite conjugates or parent compounds through urine and faeces [24, 25]. After consumption, acetylsalicylic acid is rapidly hydrolysed into salicyluric acid and glucuronic acid in the liver and eliminated via urine [26, 27]. The plasma half-life of ASA is dose-dependent and lengthens as the dose increases [28]. Despite its biodegradable potential, it is found in river waters [29, 30]. Schulman et al. [31] reported the presence of ASA in sewage effluents and surface water at maximum loading levels of 1.5 and $\geq 3.1 \text{ g L}^{-1}$, respectively. The concentrations of ASA reported by Philip et al. [32] in surface waters of South Indian zone is about 660 ng L^{-1} , whereas samples of pharmaceutical effluents had about $2,270 \text{ mg L}^{-1}$ of salicylic acid.

ASA exert negative effects on the aquatic ecosystem. Fishes on exposure to salicylates showed hormonal aberrations and delayed response to an acute stressor [33, 34]. ASA is teratogenic in rats [35] and also caused maternal toxicity in rabbits [36]. Exposure of tilapia to ASA induced altered plasma thyroid hormone levels and cortisol levels [34]. Similarly, the freshwater fishes *Cyprinus carpio* and *Danio*

rerio, exposed to salicylic acid, showed an induction in oxidative stress indices [37, 38]. Bioassays performed with acetylsalicylic acid in *Daphnia magna* showed immobilization [39], whereas Cleuvers [40] documented growth inhibition in green alga *D. subspicatus*.

Fishes are potent bioindicators as they occupy different trophic levels in the food chain [41], and the response of fish to pollutants is monitored using several biomarkers [42]. Chronic toxicity analysis using a fish model facilitates better understanding of the impact of any compound on health status of aquatic organisms [43]. Responses of fish biomarkers such as haematology [44], biochemical profile [4], antioxidant status [45], behavioural perturbations [46], neuronal responses [47], metabolomics [48], transcriptomics [49] and toxicokinetics [50] serve as reliable markers in toxicity researches. Marques et al. [51] have reported that chronic effects are much more toxic than acute effects.

Indian drug industry holds third place in terms of volume and 14th in terms of value in and around the globe [52]. In India, analgesics and anti-inflammatory drugs are the two largest groups of over-the-counter drugs in urban areas. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most extensively used class of pharmaceutical agents worldwide [43]. Shanmugam et al. [53] reported the presence of nonsteroidal anti-inflammatory drugs in Indian River waters. The present study focuses on the chronic (100 and 200 mg L⁻¹) effects of acetylsalicylic acid (ASA) in a common carp *Cyprinus carpio* for the period of 21 days by using haematological, enzymological biochemical and antioxidant parameters in gill and liver.

2 Materials and Methods

2.1 Experimental Animal

The handling and testing of individuals were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Organization of Economic Co-operation and Development (OECD). Specimens of *Cyprinus carpio* with an average weight of 6.0 ± 0.5 g and length of 7.5 ± 0.5 cm were procured from Aliyar Fish Farm, Aliyar, and Tamil Nadu in India. Fishes were taken to the laboratory and stocked in a 6' × 4' × 3' size cement tank [42]. Only dechlorinated tap water was used throughout the study period. The physicochemical parameters of water $25.0 \pm 0.5^\circ\text{C}$, pH 7.2, hardness 17.8 mg L^{-1} (as CaCO₃), alkalinity $18.5 \pm 7.0 \text{ mg L}^{-1}$ (as CaCO₃) and dissolved oxygen concentration $6.2 \pm 0.02 \text{ mg L}^{-1}$ were monitored and maintained throughout the study period. The stocked fishes were acclimatized to laboratory conditions for a period of 20 days. During acclimatization, period fish were with fed rice bran, corn flour, and wheat flour and groundnut oil cake once in the day. The water in the aquarium was renewed daily and aerated mechanically. Feeding was ceased 24 h before the commencement of the experiment.

2.2 Test Compounds

The drug acetylsalicylic acid of 99.0% purity (CAS Number: 50-78-2) was purchased from Sigma-Aldrich. AST and ALT kits were purchased from Coral Clinical Systems, India. All the other chemicals and reagents (of analytical grade) used in the present investigation were purchased from HiMedia Laboratories Pvt. Ltd., India. 1 g of acetylsalicylic acid (ASA) was dissolved in 0.9 mL of dimethyl sulfoxide (DMSO) was used to prepare stock solution ($1,000 \text{ mg L}^{-1}$) of ASA. From this stock solution, appropriate quantity was taken and dissolved in experimental tanks to get the desired concentration of toxicity solution.

2.3 Chronic Toxicity Assay

Two different concentrations were selected for testing the chronic toxicity of acetylsalicylic acid such as 100 and 200 mg L^{-1} , and they were grouped as Treatment I and Treatment II, respectively. Three glass aquaria of 100 L capacity were taken and filled with 80 L of water in which one is maintained as control and the other two as Treatment I and Treatment II. The glass tanks were aerated. From the stocking tank, 40 healthy fish fingerlings were randomly selected and introduced into each tank. The toxicant was renewed and the water in the tanks was changed daily, in order to prevent accumulation of faecal matter.

2.4 Blood and Organs Sampling

Fish from control and ASA-treated groups were sacrificed without anaesthetizing, and blood was collected in a heparinized syringe by puncturing dorsal aorta. The collected blood was maintained in sterilized plastic vials. A freshly pooled whole blood sample was used for haematological analysis. The remainder of the blood were centrifuged at 10,000 rpm for 20 min to separate the plasma and transferred to clean vials for biochemical (plasma glucose and protein) and enzymological (AST and ALT) analysis. Subsequently gill and liver tissues were excised out for the study of antioxidant parameters (SOD, LPO, CAT, GST and GSH). The collected tissue samples (liver and gill) were washed with 0.9% NaCl solution dried with filter paper and maintained at -80°C .

2.5 Blood Chemistry Analysis

RBC and WBC count were performed following the methods of Rusia and Sood [54]. Hb count was estimated by the cyanmethaemoglobin method, and Ht was determined by the micro-Ht method of Nelson and Morris [55] using the diagnostic reagent kit (Monozyme India, Ltd., India) at 540 nm using UV spectrophotometer. Erythrocyte indices of fish, viz. MCV, MCH and MCHC, were calculated using standard formulas:

$$\text{MCV (cubic micra)} = [\text{Hct (\%)} / \text{RBC (millions} \times \text{Cu} \times 10^6)] \times 100$$

$$\text{MCH (picograms)} = [\text{Hb (g/dL)} / \text{RBC (millions} \times \text{Cu} \times 10^6)] \times 100$$

$$\text{MCHC (g/dL)} = [\text{Hb (g/dL)} / \text{Hct (\%)}] \times 100$$

2.6 Biochemical Analysis

2.6.1 Estimation of Plasma Glucose and Protein

Plasma protein was estimated following the method of Lowry et al. [56] using bovine serum albumin as standard. Briefly, 0.10 mL of plasma sample from control and ASA-treated groups were added to the reaction mixture (0.90 mL of distilled water and 5 mL of copper tartrate solution (5% copper sulphate, 10% sodium potassium tartrate, 10% sodium sulphate in 0.5 M sodium hydroxide solution)) and kept at room temperature for 30 min. Subsequently, Folin-Ciocalteu phenol reagent diluted in 0.1 N sodium hydroxide was added and incubated at room temperature for 10 min, and the absorbance was read at 720 nm by using UV spectrophotometer. For the preparation of 'Standard' (S) 1.0 mg of bovine serum albumin was added to 10.0 ml of 1N NaOH and made up to 100.0 ml in a solution standard flask. From this, 1.0 ml of solution was taken in 'Standard' tube and mixed with 0.5 ml of Solution- C, kept for 10 min, and then 0.5 ml of Folin phenol reagent was added. The optical density of the 'Standard' (S) was read as mentioned above.

Plasma glucose estimation was performed following the method of Cooper and Mc Daniel [57]. In brief, after exposure, 0.1 mL of plasma samples from each treatment were taken with 5 mL of O-toluidine reagent, and the aliquots were incubated in boiling water bath for 10 min. After incubation, the aliquots were cooled under running tap water. The absorbance was read against blank at 630 nm using UV spectrophotometer.

2.6.2 Estimation of Enzymological Parameters

The enzyme activities of AST and, ALT, were determined by Diagnostic Reagent Kits (Coral Clinical Systems, A Division of Tulip Diagnostics (P) Ltd., India) following the manufacturers' instructions.

2.7 Oxidative Stress Parameters Analysis

2.7.1 Superoxide Dismutase (SOD)

Superoxide dismutase activity was measured by Marklund and Marklund [58]. After the homogenization of tissues in 100 mM Tris-HCl buffer (pH 7.4), the contents were centrifuged at 12,000 rpm for 15 min at 4°C. The obtained supernatant of 50 mL was added to the reaction solution (50 mM Tris-HCl buffer, pH 8.4 with 1 mM EDTA and 2.64 mM pyrogallol), and the absorbance was read at 420 nm in UV spectrophotometer.

2.7.2 Lipid Peroxidation (LPO)

LPO was measured following thiobarbituric acid reactive substances (TBARS) assay [59]. Pooled tissue samples were homogenized in 100 mL ice-cold potassium phosphate buffer (pH 7.4), and 100 mL of 5% trichloroacetic acid (TCA) was added. Shortly the contents were kept undisturbed on ice for 10 min, and 100 mL of 0.67% thiobarbituric acid was added. After centrifugation (2,200 g, 10 min at 4°C), 250 mL of supernatant was incubated in boiling water bath for 10 min preceded by cooling, and the absorbance was determined at 535 nm in UV spectrophotometer.

2.7.3 Catalase (CAT)

The catalase activity was estimated by adapting the method of Sinha [60]. Briefly, the sampled tissues were homogenized manually in Tris-HCl buffer (100 mM, pH 7.4) and cold centrifuged at 12,000 rpm for 15 min. After centrifugation, 100 mL of supernatant with 3 mL of reaction mixture (containing 5% potassium dichromate and acetic acid (1,3) and phosphate buffer (10 mM, pH 7.0)) was incubated in water bath for 20 min, and the absorbance was read at 570 nm in UV spectrophotometer.

2.7.4 Glutathione S-Transferase (GST)

GST activity was measured by the method of Habig and Jakoby [61]. From each treatment, the sampled tissues were homogenized in potassium phosphate buffer (0.1 M, pH 6.5). Subsequently the homogenate is centrifuged at 9,000 g for 30 min at 4°C, and 50 mL of the obtained supernatant is added to 100 mL of the reaction solution (10 mM GSH and 60 mM 1-chloro2, 4-dinitrobenzene). The absorbance of the samples was read at 340 nm in UV spectrophotometer for 5 min.

2.7.5 Reduced Glutathione (GSH)

Reduced glutathione levels were determined by following the method of Ganie et al. [62]. In brief, the tissues were homogenized in 100 mL of ice-cold potassium phosphate buffer (0.1 M, pH 6.5) followed by adding 100 mL of 25% TCA to precipitate the homogenate. Then the precipitate was centrifuged at 3,000 g for 10 min, 4 °C, and 150 mL of supernatant were transferred to aliquots containing reaction mixture (60 mM DTNB and 450 mL of 50 mM potassium phosphate buffer (pH 7.4)). The absorbance of the samples was read at 412 nm in UV spectrophotometer.

2.8 Statistical Analysis

A statistical analysis was performed using SPSS software – Ver.16 statistical package. The results of ASA -treated groups were compared against the control followed by one-way ANOVA and the Duncan's multiple range test at $p < 0.05$ level.

3 Results

3.1 Haematology

The changes in the Hb level of fish *C. carpio* exposed to chronic concentrations (100 mg L⁻¹- Treatment I and 200 mg L⁻¹- Treatment II) of acetylsalicylic acid (ASA) for a period of 21 days were illustrated in Fig. 1. During the above exposure period, Hb level was decreased in both the treatments throughout the study period. A similar trend was also noted for Ht and RBC count in ASA-treated group when compared with their respective control groups ($P < 0.05$). A maximum percent decrease of Hb level was recorded at the end of 14th day in Treatment II (Fig. 2). Similarly a maximum percent decrease of HCT level was noted on 14th day in Treatment II. There was a significant ($P < 0.05$) decrease in the RBC count at the end of 7th, 14th and 21st days of exposure in ASA-treated groups when compared to control groups (Fig. 3). In both the treatments, WBC level of *C. carpio* was elevated when compared to the control group (Fig. 4). Compared with the control groups, ASA-treated *Cyprinus carpio* had significantly higher MCV (Fig. 5), MCH (Fig. 6) and MCHC values (Fig. 7) throughout the study period.

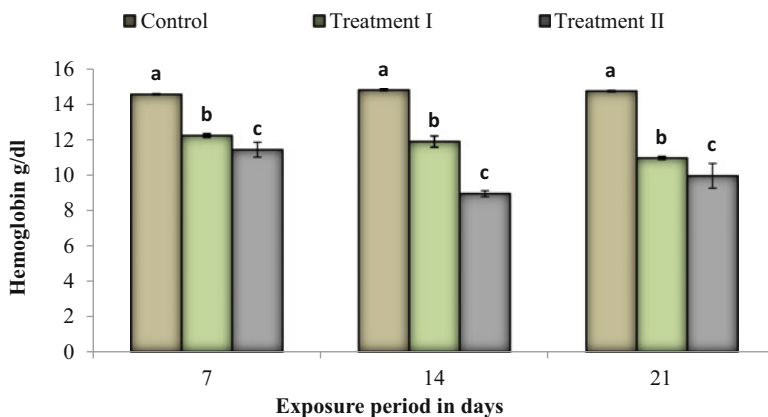


Fig. 1 Hb content of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

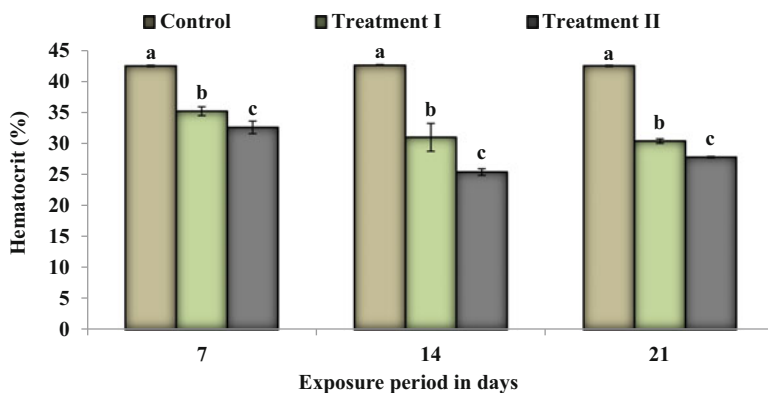


Fig. 2 Hct content of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

3.2 Glucose and Protein Content

Fish exposed to ASA showed a significant increase ($p < 0.05$) in plasma glucose level throughout the study period when compared with the control groups (Fig. 8). However, plasma protein level was found to be significantly lower in ASA-treated fish throughout the study period when compared with the control groups (Fig. 9).

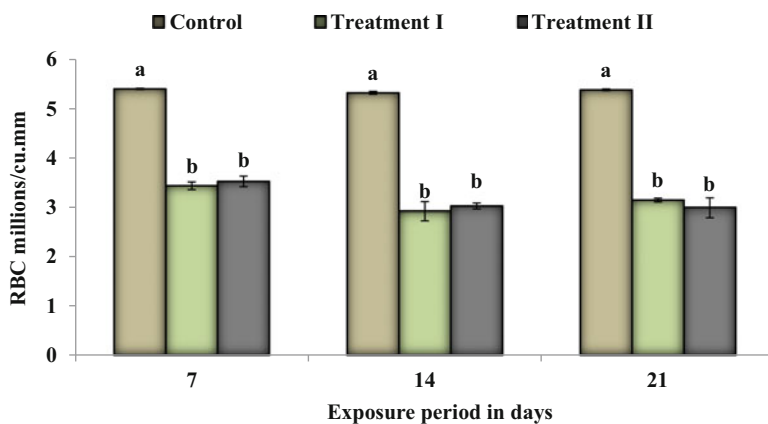


Fig. 3 RBC count of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

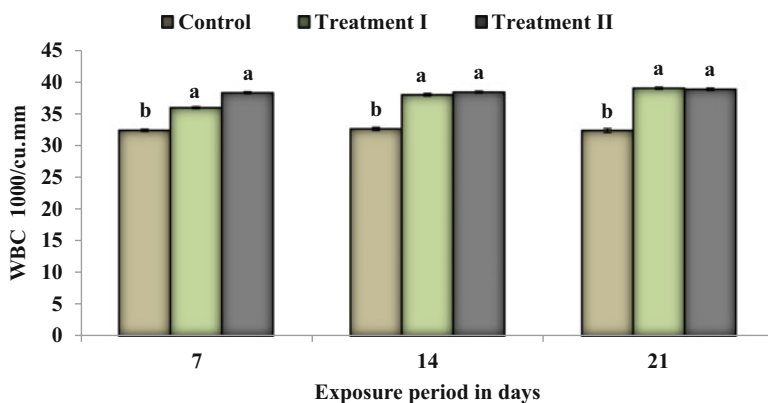


Fig. 4 WBC count of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

3.3 Enzymological Parameters

The changes in the transaminase activities of fish *C. carpio* exposed to chronic concentrations (100 mg L⁻¹- Treatment I and 200 mg L⁻¹- Treatment II) of acetylsalicylic acid (ASA) for a period of 21 days were illustrated in Figs. 10 and 11. AST activity reflected a significant decrease in ASA-treated fish comparable to those obtained in unexposed fish 9 except 21st day in Treatment I. ALT activity was

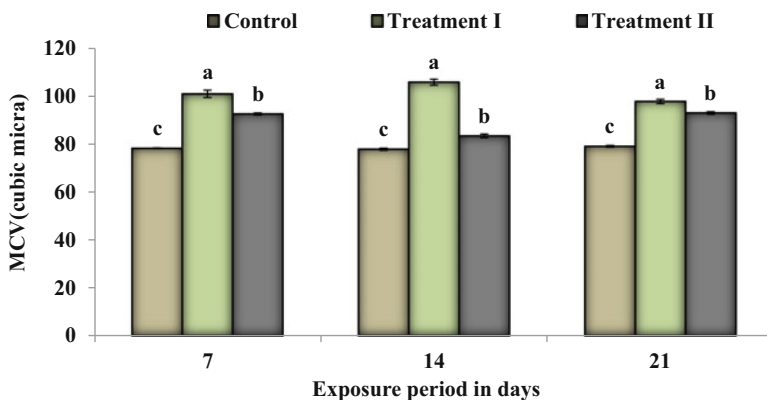


Fig. 5 MCV value of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

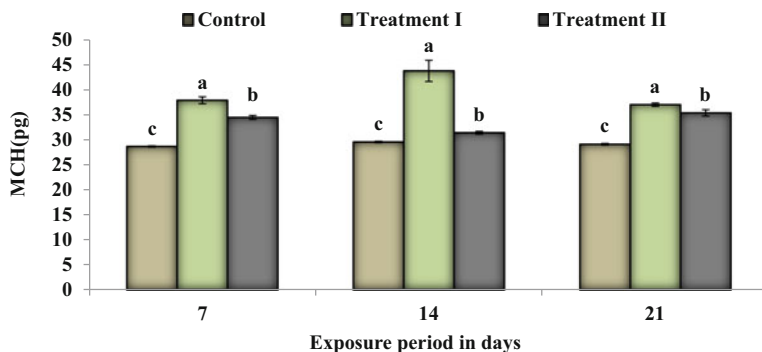


Fig. 6 MCH value of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

also found to be lower in ASA-treated fish throughout the study period when compared with the control groups ($p < 0.05$) except in 21st day of Treatment II.

3.4 Antioxidants

Variations in the antioxidant responses such as SOD, LPO, CAT, GST and GSH levels were presented in Table 1. The SOD activity in gill and liver of ASA-treated group was significantly ($p > 0.05$) increased over the control groups in both the treatments. The LPO activity was found to be elevated in gill at the end of the 14th day, and then it suddenly decreased in the 21st day of Treatment I, whereas it

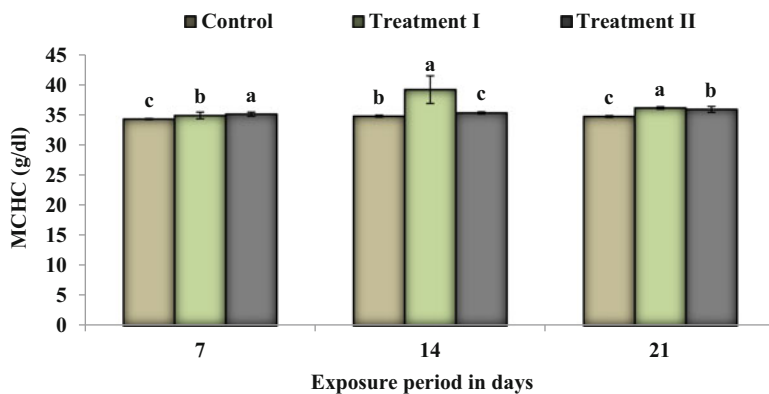


Fig. 7 MCHC value of control and ASA (Treatments I (100 mg L^{-1}) and II (200 mg L^{-1}))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

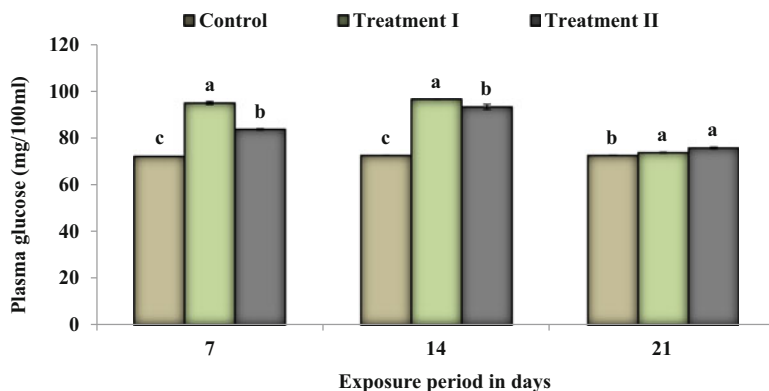


Fig. 8 Plasma glucose level of control and ASA (Treatments I (100 mg L^{-1}) and II (200 mg L^{-1}))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

exhibited a biphasic response in Treatments I and II of the liver. Compared with the control group, the chronic exposure to ASA resulted in significantly lower ($p < 0.05$) CAT activity in gill and liver of *C. carpio*. GST level in gill was significantly ($p > 0.05$) decreased at the end of 21st day exposure in Treatment I and II. However, the gill tissue of fish exposed to Treatment I showed an elevated GST level at the end of the 7th and 14th day. The level of GST in the liver of ASA-treated groups was significantly decreased ($p < 0.05$) compared to the control group except at the end of 7th day in the gill of exposed to Treatment I. The GSH activity in gill and liver of ASA-treated groups was significantly decreased ($p < 0.05$) compared to the control group except at the end of 14th day in Treatment I.

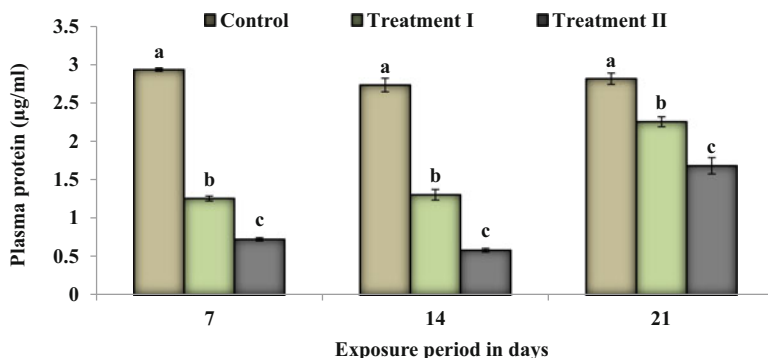


Fig. 9 Plasma protein content of control and ASA (Treatments I (100 mg L^{-1}) and II (200 mg L^{-1}))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

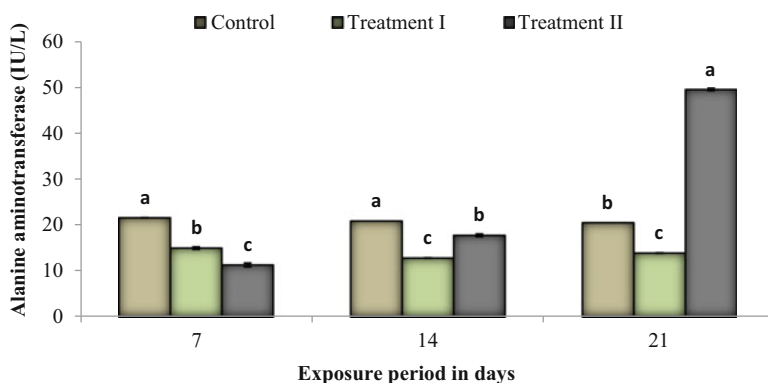


Fig. 10 Plasma ALT activity of control and ASA (Treatments I (100 mg L^{-1}) and II (200 mg L^{-1}))-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

4 Discussion

Increased production and elevated use of pharmaceuticals in human and veterinary medications lead to the discharge of more pharmaceutical compounds into the environment [63]. Despite their presence at very low concentrations, pharmaceuticals are nevertheless preferred for their potency to hinder specific biologic pathways at low levels [64]. Nonsteroidal anti-inflammatory drugs (NSAIDs) and antibiotics are widely prescribed, and their utilization in developed countries is higher than hundred tons per year [65].

Acetylsalicylic acid (ASA) is a nonsteroidal anti-inflammatory drug with analgesic, anti-pyretic and anti-thrombotic properties [20]. Acetylsalicylic acid constitutes about 81% of STPs effluents, whereas salicylic acid constitutes about 99%

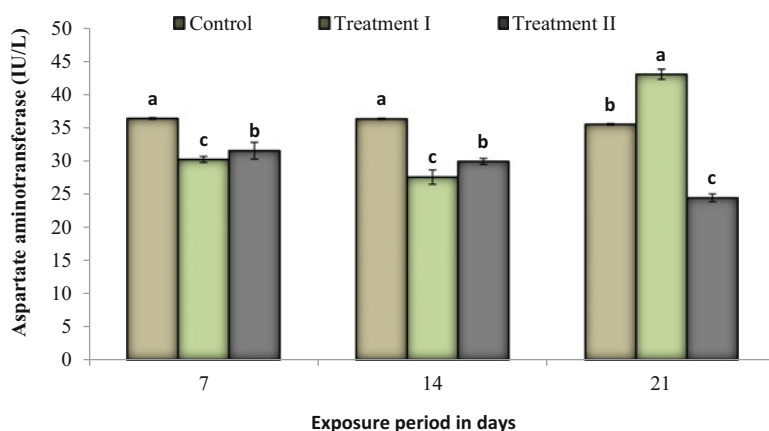


Fig. 11 Plasma AST activity of control and ASA (Treatments I (100 mg L⁻¹) and II (200 mg L⁻¹)-exposed fish *C. carpio* for a period of 21 days. Means in the bars followed by common letters for the experiment are not significantly different ($P < 0.05$) according to DMRT

[66]. Traces of acetylsalicylic acid have been reported in samples of municipal waste waters at levels ranges from 13 $\mu\text{g L}^{-1}$ [67] to 59.6 $\mu\text{g L}^{-1}$ with median levels of 3.6 $\mu\text{g L}^{-1}$ [68]. The adverse reactions of ASA include ulceration, hematemesis, melena [69] and development of ASA resistance in organisms [70]. In addition to the above data, the impact of ASA on fish stress performance was also reported by Van Anholt et al. [34] in Mozambique tilapia, Gravel and Vijayan [71] in rainbow trout and Praskova et al. [72] in zebrafish.

In the current study, the chronic effects of the nonsteroidal anti-inflammatory drug acetylsalicylic acid to the fish *C. carpio* were evaluated under laboratory conditions. During the investigation period (21 days), haematological, biochemical, enzymological and oxidative stress parameters of fish were altered upon exposure to ASA. The two concentrations (100 mg L⁻¹ and 200 mg L⁻¹) of acetylsalicylic acid (ASA) are taken as the Treatment I and II based on the tested concentrations of ASA in *Danio rerio* reported by Praskova et al. [72].

A study of blood biochemistry plays a crucial role in monitoring fish health status, pollution load, stress and disease [73]. In clinical diagnosis laboratories, haematological variables such as Hb, Hct, RBC and WBC counts are extensively used in the prediction of health status [74]. The exposure of *C. carpio* to ASA caused a significant decline in RBC, haemoglobin and haematocrit values. This might have resulted from the inhibition of erythropoiesis process by the drug ASA leading to the anaemic condition of the fish. Similarly, Hemalatha et al. [75] reported that significantly lower values of RBC count and the Hb and Ht levels in *Labeo rohita* exposed to an antimicrobial agent, triclosan. Elevated WBC count indicates the production and circulation of antibodies in the bloodstream which might be due to the stress induced by the drug to the fish [76, 77].

Inflated MCV, MCH and MCHC levels observed in *Cyprinus carpio* exposed to ASA might have resulted from the macrocytic anaemia [78, 79]. Increased red cell

Table 1 Changes in the antioxidant enzymes in a freshwater fish *C. carpio* treated (Treatment-I: 100 mg L⁻¹; Treatment-II: 200 mg L⁻¹) with acetylsalicylic acid

Parameter	Tissue	Exposure period in days	Control	Treatment I	Treatment II
SOD (units/ μ g protein)	Gill	7	01.2 \pm 0.60 ^b	01.59 \pm 0.32 ^b (+32.50)	03.07 \pm 1.74 ^a (+155.83)
		14	01.24 \pm 0.65 ^c	02.23 \pm 0.37 ^b (+79.83)	03.52 \pm 0.14 ^a (+183.87)
		21	01.29 \pm 0.75 ^c	02.06 \pm 1.84 ^b (+59.68)	04.48 \pm 0.44 ^a (+247.28)
	Liver	7	0.46 \pm 0.09 ^b	0.91 \pm 0.28 ^b (+97.82)	02.41 \pm 3.60 ^a (+423.91)
		14	0.47 \pm 0.09 ^c	01.57 \pm 0.50 ^a (+234.04)	01.28 \pm 0.04 ^b (+172.34)
LPO (mole of MDA/g protein)	Gill	21	0.48 \pm 0.10 ^c	01.99 \pm 0.84 ^b (+314.58)	01.27 \pm 0.68 ^a (+164.58)
		7	05.43 \pm 0.15 ^c	07.56 \pm 0.07 ^b (+39.22)	14.74 \pm 4.14 ^a (+171.45)
		14	05.61 \pm 0.15 ^c	08.01 \pm 0.20 ^b (+42.78)	09.74 \pm 0.92 ^a (+73.61)
	Liver	21	05.26 \pm 0.15 ^a	02.16 \pm 0.20 ^c (-58.93)	06.96 \pm 0.27 ^a (+32.31)
		7	04.52 \pm 0.10 ^b	04.54 \pm 0.14 ^b (+0.44)	05.72 \pm 1.15 ^a (+26.54)
CAT (μ mol of H ₂ O ₂ consumed/min/mg protein)	Gill	14	04.44 \pm 0.10 ^a	05.49 \pm 1.01 ^c (+23.64)	04.04 \pm 1.21 ^b (-9.00)
		21	04.54 \pm 0.10 ^b	03.49 \pm 0.42 ^c (-23.12)	06.09 \pm 0.21 ^a (+34.14)
		7	14.34 \pm 0.10 ^a	09.61 \pm 0.12 ^b (-32.98)	05.44 \pm 0.11 ^c (-62.36)
	Liver	14	14.19 \pm 0.09 ^a	08.06 \pm 0.21 ^b (-43.19)	04.02 \pm 0.15 ^c (-71.67)
		21	14.45 \pm 0.10 ^a	03.08 \pm 0.13 ^b (-78.68)	01.23 \pm 0.07 ^c (-91.48)
GST (μ moles of CDNB-GSG/min/mg protein)	Gill	7	20.38 \pm 3.31 ^a	17.26 \pm 0.47 ^b (-15.30)	04.83 \pm 1.03 ^c (-76.30)
		14	20.26 \pm 1.48 ^a	16.04 \pm 1.67 ^b (-20.82)	03.35 \pm 0.21 ^c (-83.46)
		21	20.48 \pm 1.48 ^a	16.17 \pm 2.53 ^b (-21.04)	02.43 \pm 0.05 ^c (-88.13)
	Liver	7	08.12 \pm 0.05 ^b	09.41 \pm 0.04 ^a (+15.88)	08.47 \pm 0.39 ^b (+4.31)
		14	08.09 \pm 0.05 ^b	09.02 \pm 0.19 ^a (+11.49)	07.60 \pm 0.81 ^c (-6.05)
	Gill	21	08.12 \pm 0.05 ^a	07.35 \pm 0.89 ^b (-9.48)	05.80 \pm 1.01 ^c (-28.57)
		7	09.54 \pm 0.06 ^a	09.61 \pm 0.11 ^a (+0.73)	08.04 \pm 0.06 ^b (-15.72)
		14	09.51 \pm 0.05 ^a	04.65 \pm 0.10 ^c (-51.10)	07.89 \pm 0.46 ^b (-17.03)
	Liver	21	09.59 \pm 0.06 ^a	02.56 \pm 0.03 ^b (-73.30)	01.64 \pm 0.03 ^c (-83.33)

GSH (nmole GSH/mg protein)	Gill	7	02.34 ± 0.06 ^a	02.35 ± 0.10 ^a (+0.42)	01.05 ± 0.06 ^b (-55.12)
		14	02.25 ± 0.06 ^a	02.50 ± 0.11 ^a (+11.11)	01.50 ± 0.07 ^b (-33.33)
		21	02.21 ± 0.06 ^a	01.17 ± 0.01 ^b (-47.05)	0.94 ± 0.03 ^b (-57.46)
	Liver	7	03.62 ± 0.19 ^a	02.29 ± 0.05 (-36.74) ^b	02.29 ± 0.11 (-36.74) ^b
		14	03.45 ± 0.18 ^a	02.17 ± 0.04 (-37.10) ^b	01.43 ± 0.11 (-58.55) ^c
		21	03.72 ± 0.19 ^a	01.80 ± 0.11 (-51.61) ^b	01.62 ± 0.08 (-56.45) ^b

Values are expressed as the mean ± S.E five individual observations. Values in the parentheses represent % changes over control. Means within a row bearing same letters are not significantly different ($p < 0.05$) according to DMRT Different letters indicates significant difference at $P < 0.05$.

destruction causes macrocytic anaemia with low haemoglobin content causing dysfunction of organs [80]. Similar results were recorded in *Oreochromis niloticus* exposed to drug sulfamethazine [74] and in *Pangasianodon hypophthalmus* exposed to triclosan [81].

Alterations of plasma carbohydrates levels act as a non-specific hallmark of disturbed physiology as they are considered as the foremost organic nutrients to be degraded in response to in fishes during stress conditions [82]. A rise in the plasma glucose concentration indicates high utilization of glucose to cope the metabolic stress caused by the drugs [83]. Furthermore, the increase in any of the stress hormones catecholamines or corticosteroids may also lead to elevated plasma glucose levels in fishes [84]. Similar observations were reported by Renuka et al. [43] in N-acetyl-p-aminophenol-treated rohu fingerlings, Ambili et al. [85] in oxytetracycline-treated rohu fingerlings and Umamaheswari et al. [86] in amoxicillin-treated rohu fish fingerlings.

Protein serves as the primary energy source to meet energy demand at some point in increased physiological and metabolic activities of fish under stress conditions [87]. The observed decline in plasma protein level in ASA-exposed fishes might be resulted from the free amino acid production and its utilization in TCA cycle for energy production. ASA molecules are weak organic acids having the high affinity for lipids and plasma proteins, and therefore they bind to the circulating free protein molecules causing hypoproteinaemic condition in ASA-treated fishes. Hepatocytes in liver synthesize most of the proteins. The liver is one of the core target organs for the detoxification of toxicants. ASA accumulation in liver causes impaired protein synthesis [88]. This observation is in accordance with the results of Saglam and Yonar [89] who reported a decline in plasma protein content in sulfamerazine-exposed *Oncorhynchus mykiss*.

Any stress condition in fish causes perturbations in enzymatic activities, thus resulting in decreased growth rate and metabolic rate in fishes. Glutamate oxaloacetate transaminase (GOT or AST) and glutamate pyruvate transaminase (GPT or ALT) are two important liver enzymes found in plasma and in various body tissues. Serum ALT and AST level and their ratio are universally accepted clinical biomarkers for analysing liver health [90, 91]. In the present study, there were significant changes in GPT and GOT activity in plasma of *C. carpio* exposed to the chronic concentration of ASA for 21 days. The depleted levels of GPT and GOT in plasma indicate the incapability of destructed hepatocytes to release aminotransferases into the circulatory system [92]. The sudden increase in GPT and GOT activity in plasma might be resulted from the hepatic tissue damage caused by the drug [93]. Similarly, Ramesh et al. [4] documented on the significant alterations in the freshwater fish *Cyprinus carpio* exposed to chloroquine.

Xenobiotics induce oxidative stress in aquatic animals mainly in fishes through free radical and reactive oxygen species (ROS) mechanisms [41]. The liver is the site of detoxification in fishes [94]. The detoxifying mechanism occurs in a sequence involving biotransformation enzymes such as GST and antioxidants such as CAT, SOD, and GPx [95]. The superoxide dismutase (SOD) mediates the first transformation by dismutation of superoxide free radicals (O_2^-) into hydrogen peroxide

(H₂O₂), while catalase (CAT) converts it into water (H₂O) and oxygen (O₂) [96]. In the present study, induction of SOD activity in gill and liver would help to avoid reactive oxygen species generation caused by oxidative damage [97]. Similarly, Matozzo et al. [98] observed an increase of SOD activity in gills of clam *Ruditapes philippinarum* exposed to triclosan treatments.

LPO activity in liver of Treatments I and II showed a biphasic trend, and the values range from 7.56 to 2.16 mol of MDA/g protein and 14.74 to 6.96 mol of MDA/g protein respectively. Significant changes were observed in the LPO activity of gill, liver, and kidney of triclosan-exposed rohu fingerlings [75]. The elevation of LPO level may be due to increased production of ROS, due to ASA stress leading to lipid peroxidation. The significant changes observed in liver LPO level of fish may be due to the persuaded activity of antioxidants, increasing the scavenging of free radicals and reducing MDA production [77].

The CAT and GPx are reactive oxidative species (ROS) reducing enzymes. CAT eliminates hydrogen peroxide, whereas GPx can detoxify hydrogen peroxide and degrades fatty acid peroxides [38]. The depleted levels of CAT may be due to its inactivation by (O₂⁻) or due to the poor detoxifying mechanism as a result of the excess production of hydrogen peroxide. Perhaps Kono and Fridovich [99] explained the inhibition of CAT activity by (O₂⁻) and the synergetic reaction between SOD and CAT. Similarly Ku et al. [100], Alak et al. [101] and Rangasamy et al. [102] observed changes in CAT activity in *Pelteobagrus fulvidraco* exposed to triclosan, in rainbow trout exposed to eprinomectin and in *Danio rerio* exposed to ketoprofen.

GST is one of the indispensable liver enzymes that defend the cell from the ROS toxicity by catalysing the reactive intermediates to reduced glutathione through the process of biotransformation [103, 104]. The fluctuations seen in gill and liver GST activity might be resulted from the defence mechanism developed by the fish against oxidative damage caused by the ASA [105, 106]. Comparably Liao et al. [107] and Zivina et al. (2013) observed an increase in liver GST activity of medaka fish on ketamine exposure and in developmental stages of zebrafish on ASA exposure, whereas Ajima et al. [108] reported for the decreased brain GST activity of the fish exposed to verapamil.

Glutathione (GSH) is one of the important antioxidants capable of preventing damage to cells caused by reactive oxygen species [109]. In the current study, GSH levels in the liver and gill were found to be depleted which indicates its utilization to meet the oxidative stress caused by the drug. Zhang et al. [110] reported that the decline in GSH level may be due to the lack of adaptive mechanisms and GSH oxidation to GSSG. Similar decrease was also noted in *Carassius auratus* after exposure to decabromodiphenyl ether and ethane or their mixture [111] and in *Channa punctatus* after exposure to thermal power plant effluents [112].

5 Conclusion

The present study concludes that acetylsalicylic acid induced alterations in the haematological, biochemical, enzymatic and antioxidant activities of the freshwater fish at chronic concentrations. Therefore it is more rational to affirm that a rise or fall in any of these biomarkers in ASA-exposed fish provides significant information on the overall physiology, metabolism and health status of fish under examination. As a future outlook, we suggest evaluating biodistribution and biotransformation of ASA in fishes as it would provide insight into the biomagnifying potency of pharmaceuticals in aquatic biota.

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