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AMELIORATIVE EFFECT OF *ARTHROSPIRA PLATENSIS* (SPIRULINA) DIETARY SUPPLEMENTATION AGAINST FLUORIDE TOXICITY IN THE FRESHWATER FISH, COMMON CARP (*CYPRINUS CARPIO* L)

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ABSTRACT: Aim of the present study was to investigate the ameliorative effect of cyanobacteria, *Arthrospira platensis* (Spirulina) dietary supplementation against the fluoride (F) toxicity in the freshwater fish, common carp (*Cyprinus carpio* L) with reference to growth performance, digestive enzyme activities, haematological parameters, and F accumulation in muscular tissues. The study parameters were studied after 30 days of supplementation. For the study, fingerlings of *C. carpio* L were purchased from a local fish market, and then acclimatized for a total period of 28 days. A total of 120 acclimatized fingerlings were allocated into five experimental groups (GI, II, III, IV, and V) each with 24 fingerlings. Each group was further divided into three replicates containing 8 fingerlings each. Group I and II were provided control diet and served as control and toxic control, respectively, whereas group III, IV, and V were provided with 5 gm, 7.5 gm, and 10 gm of *A. platensis* /kg of supplemented diet, respectively. All groups except for Group I were exposed to 5% of the LC_{50} of F (NaF @ 33.75 mg/L). The results revealed that, exposure of sub-lethal concentration of F (33.75 mg/L) adversely affected the growth and feed utilization parameters, inhibited the digestive enzyme activities, altered the haematological parameters, and increased the accumulation of F in the muscular tissues of group II (GII) compared to control. However, *A. platensis* dietary supplementation significantly ($p<0.05$) improved F induced alterations of growth and feed utilization parameters, digestive enzyme activities, haematological parameters, and reduced the accumulation of F in the muscular tissues in group III, IV, and V compared to group II (GII). In conclusion, *A. platensis* dietary supplementation restored the F induced alterations and thus mitigated the F toxicity. Fish fed 10 gm of *A. platensis* /kg supplemented diet showed the best response; hence it is the optimum dose.

Keywords: Amelioration; *Arthrospira platensis* (Spirulina); Common carp (*Cyprinus carpio* L); Dietary supplements; Fluoride; Freshwater fish; Toxicity

INTRODUCTION

Fluoride (F) is ubiquitously present in the soil, air, and aquatic environments in varying amounts. The maximum permissible limit of F in drinking water for humans is 1.5 mg/L as per guidelines of the World Health Organization.[1] In India, except in few eastern states, almost all the ground-drinking water sources of 23, out of 37 states and union territories are contaminated with F and most of them have F beyond the threshold level 1.0 or 1.5 mg/L.[2] Presence of abnormally high F concentration in groundwater in the country is due to natural cause of higher abundance of F-bearing minerals in the host rocks and sediments.[3] F in non-polluted surface water is almost negligible or present in fractions or traces (0.01–0.3 ppm).[1,4] If the humans and domestic animals drink a water having F more than this level for long time, then it becomes toxic and harmful for health and causes a serious disease called fluorosis in them. Most of the studies on chronic F toxicosis have been conducted in man[5-6] and

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diverse domestic animals.[7-10] However, in few experimental studies, especially in diverse species of fishes, F induced adverse changes have also been reported. [12-16]

Arthrospira platensis (also called Spirulina) is a filamentous cyanobacteria,[17] widely distributed in tropical and sub tropical regions of Asia, America, and Central Africa.[18] It contains more than 60% protein[19] with a considerable number of essential amino acids,[20] bioactive components like gamma linolenic acid, vitamins, minerals, and phytopigments.[18,21,22] Several studies in recent past reported the anti-oxidative,[17,23,24] immunomodulatory,[23,25] anti-inflammatory,[23,26] hepatoprotective,[25,27] and metallo-protective[27] properties of *A. platensis*. However, limited information is available on the effect of *A. platensis* dietary supplementation against F toxicity in aquatic vertebrates. The fresh water fish, common carp (*Cyprinus carpio* L.) is an important species in freshwater aquaculture. It is widespread and cultivated in most parts of the world including India. Therefore, the present study was considered to investigate the effect of *A. platensis* dietary supplementation against F-induced alterations of growth, digestive enzyme activities, haematological parameters, and accumulation of F in the muscular tissues in the freshwater fish, *C. carpio* L.

MATERIALS AND METHODS

Experimental Design: The experimental study was conducted as per the internationally accepted laboratory animal use and care, and guidelines (Guiding principles in the use of animals in toxicology, adopted by the Society of Toxicology in 1989), and as per the guidelines of the Institutional Animal Ethics Committee, University of Calcutta, West Bengal, India. The 96 hr LC_{50} values of F to fingerlings of *C. carpio* L (Cyprinidae: Cypriniformes) were determined in an earlier study.[28] In this experiment, 5% of the 96 hr LC_{50} i.e., 33.75 mg/L of NaF was used as the sub-lethal dose. The fingerlings of *C. carpio* L (Figure 1) were purchased from a local fish market of Naihati city, North 24 Parganas, West Bengal, India, and then brought to the laboratory in plastic bag with sufficient oxygen. The collected fingerlings were stocked in a large glass aquarium (150L capacity), and acclimatized them for a total period of 28 days. During this period, fingerlings were fed commercial diet at 3% of the body weight daily and the water was aerated continuously. A total of 120 acclimated fingerlings of *C. carpio* L (mean weight 10.135±0.305 gm, mean length 8.855±0.063 cm) aged three months old were randomly allocated into 5 experimental groups (Group I, II, III, IV, and V) each with 24 fish. Each group was further divided into three replicates with 8 fish per replicate, which were reared in small glass aquaria with 21 L of F free surface water. Group I & II were received control diet and served as control and toxic control respectively, whereas, group III, IV, and V were provided with 5 gm of *A. platensis*/kg, 7.5 gm of *A. platensis*/kg, and 10 gm of *A. platensis*/kg supplemented diet respectively. All groups except for Group I were exposed to 5% of the LC_{50} of F (NaF @ 33.75 mg/L) for 30 days. Water was aerated continuously with the replacement of water in every alternate day. These fingerlings were provided feed @ 3% of their body weight at 9.00 hr daily.[29] Standard method (APHA) [30] was followed to estimate the physico-chemical parameters of water

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(Table 1). The SPADNS spectrophotometric method was followed to estimate the F content in water as well as in fish muscle.[30]

Figure 1: Fingerlings of common carp, *Cyprinus carpio* L

Table 1: Physico-chemical parameters of the water (as per APHA, 2012).[30] Values are means ± SEM, n = 3 per treatment group).

Water quality parameters	Values
Dissolve Oxygen	4.146±0.260 mg/L
Free Carbon dioxide	4.633±0.120 mg/L
Total Alkalinity	192.333±4.333 mg/L
Total Hardness	125±1.154 mg/L
Water Temperature	30.333±0.440°C
pH	7.466±0.145
Fluoride	0.813±0.008 mg/L

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Experimental Diet: The source of *A. platensis* was the 'Spirulina capsules', manufactured by the Surya Herbal Ltd, India (Figure 2).

Figure 2: 'Spirulina capsules' manufactured by Surya Herbal Ltd, India.

Table 2: Experimental diets including ingredients (gm/kg) and proximate compositions.[31] Values are in mean±SE, (n=3 per sample)

Ingredients(gm/Kg)	Control diet (gm/kg)	5 gm/kg *A. platensis* diet	7.5 gm /kg *A. platensis* diet	10 gm /kg *A. platensis* diet
GNO Cake[1]	600	600	600	600
Fish Meal[2]	200	200	200	200
Rice Bran[3]	100	100	100	100
Vit. & Min. Premix	10	10	10	10
Wheat Flour	90	85	82.5	80
A. platensis[4]	-	5	7.5	10
Proximate composition				
Crude Protein	40.673±0.014	40.190±0.037	40.546±0.050	40.266±0.012
Crude fat	8.656±0.017	8.456±0.017	8.203±0.014	8.100±0.017
Fiber	6.140±0.036	6.206±0.023	6.306±0.034	5.793±0.021
Ash	8.093±0.023	8.130±0.011	8.243±0.034	8.430±0.011
Moisture	11.546±0.024	11.803±0.014	11.506±0.040	10.430±0.005
Nitrogen free extract[5]	24.856±0.014	25.220±0.011	25.626±0.040	26.970±0.011
Gross energy(kcal/100 gm feed)[6]	372.592	369.959	370.891	374.144

Composition of vitamin & mineral mixture(premix): Each 1 kg contains Vitamin A 8,00,000 IU, Vitamin D_3 80,000 IU, Vitamin E 0.6g, Nicotinamide 1.2 g, Cobalt 2.2g, Copper 4.7g, Iodine 0.6g, Iron 2.2g, Magnesium 6.5g, Manganese 3.3g, Potassium 0.2g, Sodium 0.04g, and Zinc 10g; [1] Ground nut oil (GNO) cake contains 55.43% proteins and 14.45% fat; [2] Contains 51.65% proteins and 7.6% fat.; [3] Contains 9.25% proteins and 8.3% fat; [4] Contains 60% proteins; [5]NFE (Nitrogen Free Extract)=100-(Protein+Fat+Ash+Crude fiber); [6]GE (Gross Energy): Estimated according to NRC (1993)[32] as 4.64, 9.44 and 4.11 Kcal/gm for protein, fat and carbohydrate respectively.

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The maximum tolerable limit of *A. platensis* to *C. carpio* L fingerlings was determined for a period of 14 days following Organization for Economic Cooperation and Development (OECD) guideline, and the value was 20 gm/kg. *A. platensis* dietary supplementation at 0, 5, 7.5 and 10 gm/kg was incorporated to prepare one basal or control diet and three *A. platensis* containing diet (Table 2) which were analyzed by standard methods.[31,32]

Haematological parameters: After the end of the 30 days of feeding trial, blood was collected by cutting the caudal peduncle using a sharp knife, after anaesthetized with clove oil in a watch glass containing EDTA. The erythrocyte and leukocyte of blood were counted using an improved Neubauer haemocytometer. Whereas, haemoglobin content of the blood was measured using Sahli's haemoglobinometer[33].

Digestive enzyme activities: The crude digestive enzyme extract was prepared from the intestinal tissues of fishes by homogenization using ice cold distilled water (1:10 w/v) and centrifugation at 15,000 rpm for 30 minutes at 0^{o}C. The supernatant was used for the determination of three digestive enzymes like amylase, [34]protease, [35] and lipase.[36]

Statistical Analysis: One way Analysis of Variance (ANOVA) followed by Tukey's post hoc test was done to compare the means between the experimental groups by a computer program (SPSS version 20).

RESULTS AND DISCUSSION

.The results of the present study showed that exposure of sub-lethal concentration of F significantly ($p<0.05$) reduced the growth and feed utilization parameters in group II (GII) or toxic control, compared to group I(GI) or control (Table 3). F probably inhibited the growth performance in fish via anorexia which caused reduction of feed intake,[37] higher accumulation in bones,[38] and by inhibiting the enzymatic activity resulting in the disruption of metabolic processes.[39,40] Several previous studies also reported the similar results in which F inhibited the growth performance in fish.[37-40]

A. platensis dietary supplementation significantly ($p<0.05$) restored F-induced alteration of most of the growth and feed utilization parameters in group III, IV, and V towards the control except viscerasomatic index (Table 3). *A. platensis* is rich with protein (60–70%) containing essential amino acids which reduced F bioavailability, and thus ameliorates F toxicity.[41,42] The chlorophyll content of *A. platensis* has the ability to alleviate different toxic substances.[43] In addition, *A. platensis* is rich with vitamins (vitamin B_1, B_2, B_3, B_6, B_9, B_{12}, vitamin C, vitamin D, vitamin E, and provitamin A or β–carotene) and minerals (potassium, selenium, sodium, chromium, calcium, magnesium, copper, iron, manganese, phosphorus, and zinc), that are responsible for the higher growth performance in fish.[20,44] *A. platensis* dietary supplementation also reduces copper toxicity in carp,[45] diazinon in Nile tilapia,[17] and lead nitrate in cat fish.[21]

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Table 3: Effect of *A. platensis* dietary supplementation on the fluoride induced alteration of growth and feed utilization parameters in the freshwater fish, common carp (*C. carpio* L.) for 30 days. Values are in mean±SE, (n=3 per sample) .

	GI	GII	GIII	GIV	GV
IBW	9.87 ± 0.174^{b}	9.23 ± 0.112^{c}	10.7 ± 0.0921^{a}	9.85 ± 0.0576^{b}	10.7 ± 0.0658^{a}
FBW	10.9 ± 0.164^{b}	10 ± 0.114^{c}	11.6 ± 0.0988^{a}	10.8 ± 0.0549^{b}	11.8 ± 0.0191^{a}
WG	0.979 ± 0.00924^{b}	0.766 ± 0.00208^{c}	0.922 ± 0.0101^{b}	0.969 ± 0.007^{b}	1.13 ± 0.0468^{a}
ITL	8.95 ± 0.0722^{bc}	8.7 ± 0.104^{c}	9.57 ± 0.0636^{a}	8.84 ± 0.0311^{bc}	9.09 ± 0.0729^{b}
FTL	9.16 ± 0.0857^{bc}	8.86 ± 0.101^{c}	9.82 ± 0.07^{a}	9.13 ± 0.0352^{bc}	9.41 ± 0.0667^{b}
LG	0.217 ± 0.0203^{cd}	0.165 ± 0.00404^{d}	0.246 ± 0.0182^{bc}	0.297 ± 0.00491^{ab}	0.329 ± 0.0116^{a}
FI	4.14 ± 0.0728^{b}	3.88 ± 0.0468^{c}	4.49 ± 0.0386^{a}	4.14 ± 0.0241^{b}	4.45 ± 0.0335^{a}
WG%	9.93 ± 0.244^{a}	8.3 ± 0.0845^{c}	8.63 ± 0.0748^{bc}	9.84 ± 0.109^{ab}	10.6 ± 0.507^{a}
SGR	0.336 ± 0.00833^{a}	0.284 ± 0.00321^{c}	0.296 ± 0.00231^{bc}	0.335 ± 0.00367^{ab}	0.358 ± 0.0161^{a}
FCR	4.23 ± 0.0998^{b}	5.06 ± 0.0512^{a}	4.87 ± 0.042^{a}	4.27 ± 0.0479^{b}	3.97 ± 0.183^{b}
PER	0.591 ± 0.0144^{a}	0.494 ± 0.00493^{b}	0.513 ± 0.00433^{b}	0.585 ± 0.00677^{a}	0.633 ± 0.0296^{a}
HSI	0.501 ± 0.13^{b}	0.515 ± 0.0817^{b}	0.95 ± 0.0287^{ab}	1 ± 0.0937^{a}	1.16 ± 0.144^{a}
VSI	5.92 ± 0.524	5.88 ± 1.35	7.13 ± 0.553	7.53 ± 0.546	7.34 ± 0.414
ISI	5.18 ± 0.778^{ab}	5.07 ± 0.394^{ab}	5.65 ± 0.316^{a}	6.36 ± 0.45^{a}	3.04 ± 0.507^{b}

Different superscripts in a row are differ significantly ($p<0.05$)
Abbreviations: IBW: Initial Body Weight (gm), FBW: Final Body Weight (gm), WG: Weight Gain (gm),

ITL: Initial Total Length (cm), FTL: Final Total Length (cm), LG: Length Gained (cm), FI: Feed Intake (gm),
FCR(Feed Conversion Ratio)= Consumed Feed/ Final weight-Initial weight),
PER(Protein Efficiency Ratio)= Weight Gain/ Protein intake,
SGR(Specific Growth Rate)= 100 x (ln final weight-ln initial weight) /days,

Wt gain % (Weight gain %)= (Final body wt-Initial body wt)/ Initial body wt x 100

HSI(Hepatosomatic Index)=Liver weight (g)/Body weight (g) x100,

VSI(Viscerasomatic Index)=Viscera weight/Body weight x100,

ISI (Intestosomatic Index)=Weight of intestine/Body weight x100

The digestive enzymes are known to play a pivotal role in the nutrient digestion. The results of the present study showed that digestive enzyme activities were significantly ($p<0.05$) decreased in group II (GII or toxic control) compared to group I (GI or control) (Figures 3–5). The result of the present study is in consistent of some previous studies in which F adversely affected the digestive enzyme activities in animals.[46,47] However, *A. platensis* dietary supplementation significantly ($p<0.05$) improved the digestive enzyme activities in group III, IV, and V compared to the group I (control) and group II (toxic control), which in turn might have resulted in the efficient absorption of nutrients into the blood, and improved the growth performance in fish (Figures 3–5). Recently, researchers [48] also reported the stimulation of intestinal digestive enzyme activities by *A. platensis* in *C. carpio* L.

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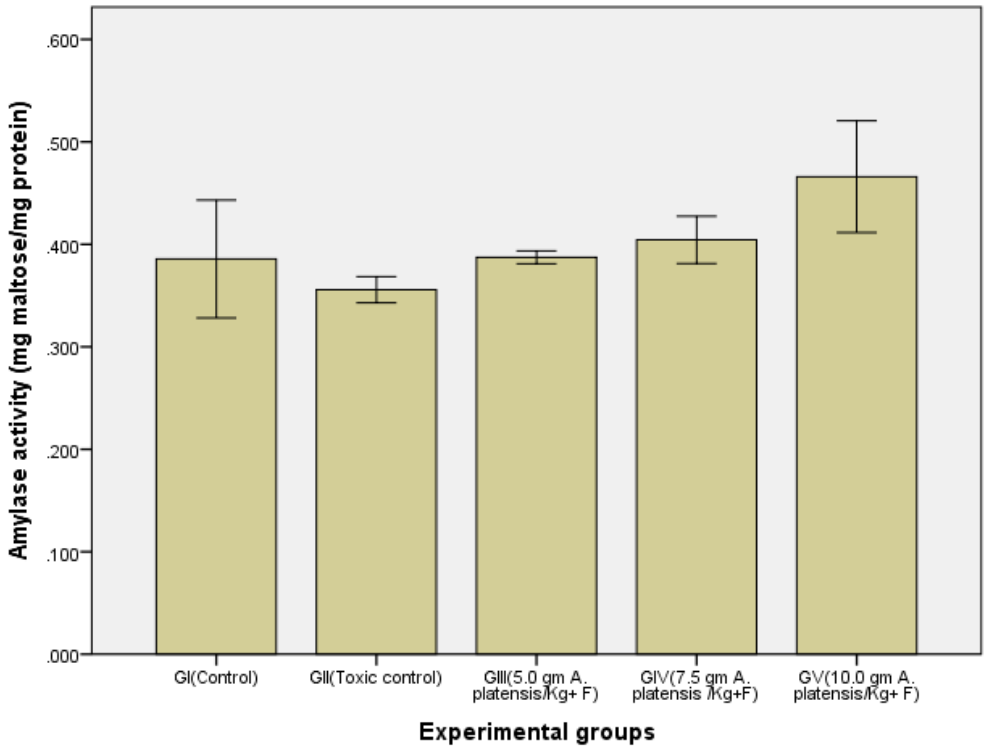

Figure 3: Bar diagram showing the effect of *A. platensis* dietary supplementation on amylase activity in fish exposed to sub-lethal concentration of fluoride.

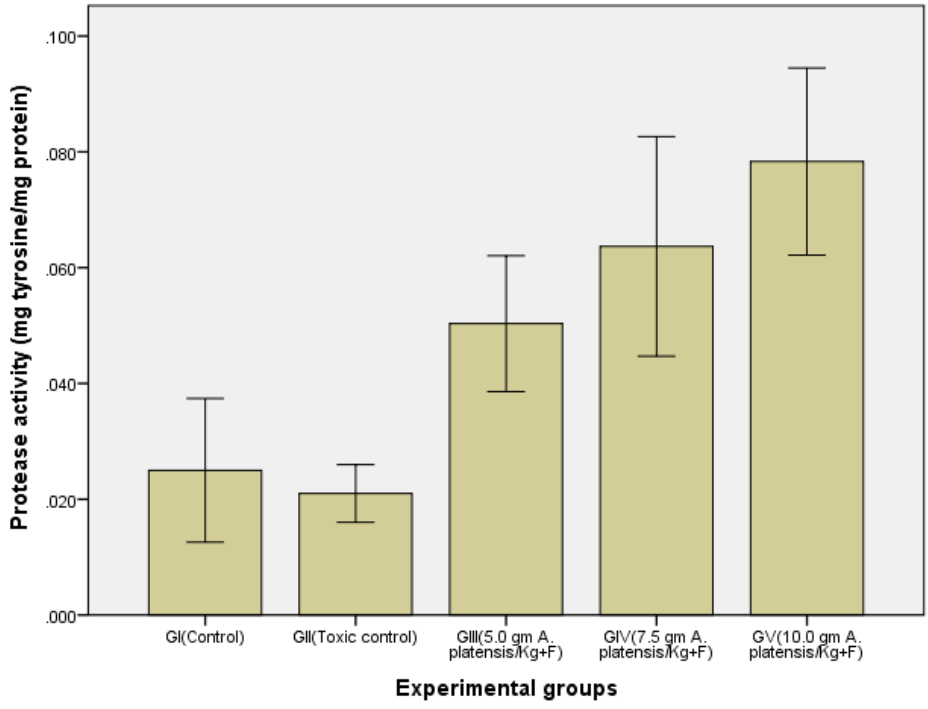

Figure 4: Bar diagram showing the effect of *A. platensis* dietary supplementation on protease activity in fish exposed to sub-lethal concentration of fluoride.

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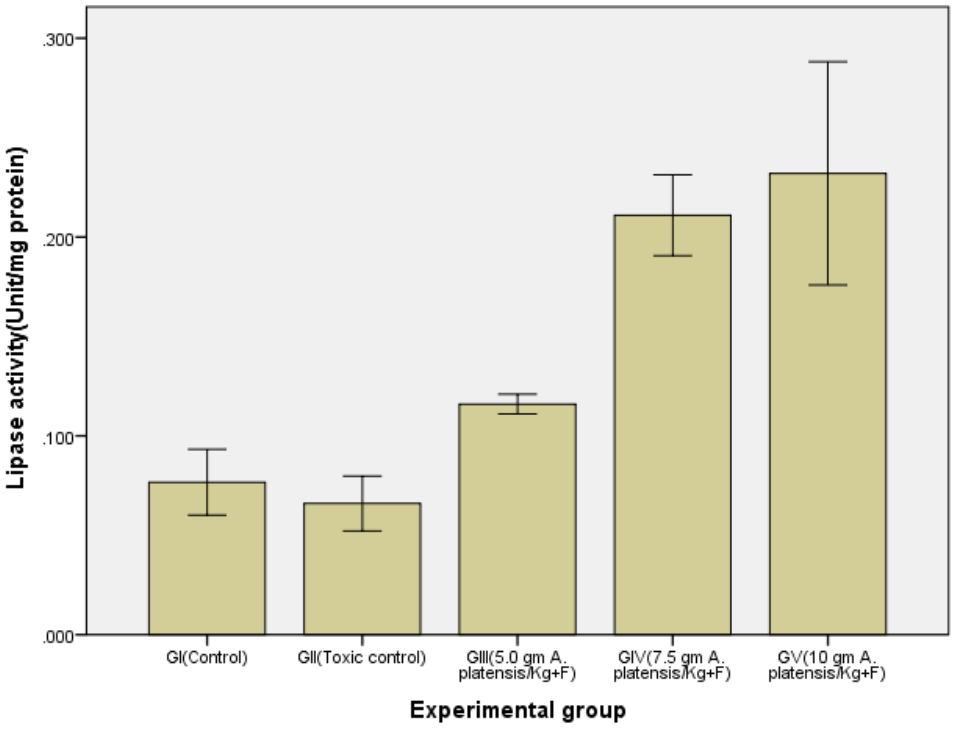

Figure 5: Bar diagram showing the effect of *A. platensis* dietary supplementation on lipase activity in fish exposed to sub-lethal concentration of fluoride.

Present study revealed the detrimental effect of F in haematological parameters by decreasing the erythrocyte count and haemoglobin content in the blood of group II (GII or toxic control) compared to group I (control) (Table 4). F possibly damaged the erythrocyte membrane and/ or adversely affected the erythropoietic tissue resulting in the alteration of the haematological parameters.[49, 50] The results of the present study is in agreement with the study of previous workers[50-52] who have reported the detrimental effect of F on haematological parameters in animals.

Table 4: Effect of *A. platensis* dietary supplementation on the fluoride induced alteration of haematological parameters in the freshwater fish, common carp (*C. carpio* L.) for 30 days. Values are mean ±SE, n=3 per sample

	GI	GII	GIII	GIV	GV
RBC ($x10^6$cells/mm^3)	2.34 ± 0.0348^{b}	1.75 ± 0.117^{c}	1.83 ± 0.0186^{c}	1.81 ± 0.0521^{c}	2.85 ± 0.161^{a}
WBC ($x10^3$cells/mm^3)	193 ± 1.56^{c}	235 ± 3.44^{a}	123 ± 2.7^{e}	144 ± 0.866^{d}	214 ± 1.13^{b}
Hb (gm/dl)	6.94 ± 0.0872^{ab}	6.17 ± 0.203^{c}	6.37 ± 0.0882^{bc}	6.57 ± 0.233^{ac}	7.23 ± 0.145^{a}

Different superscripts in a row are differ significantly ($p<0.05$)

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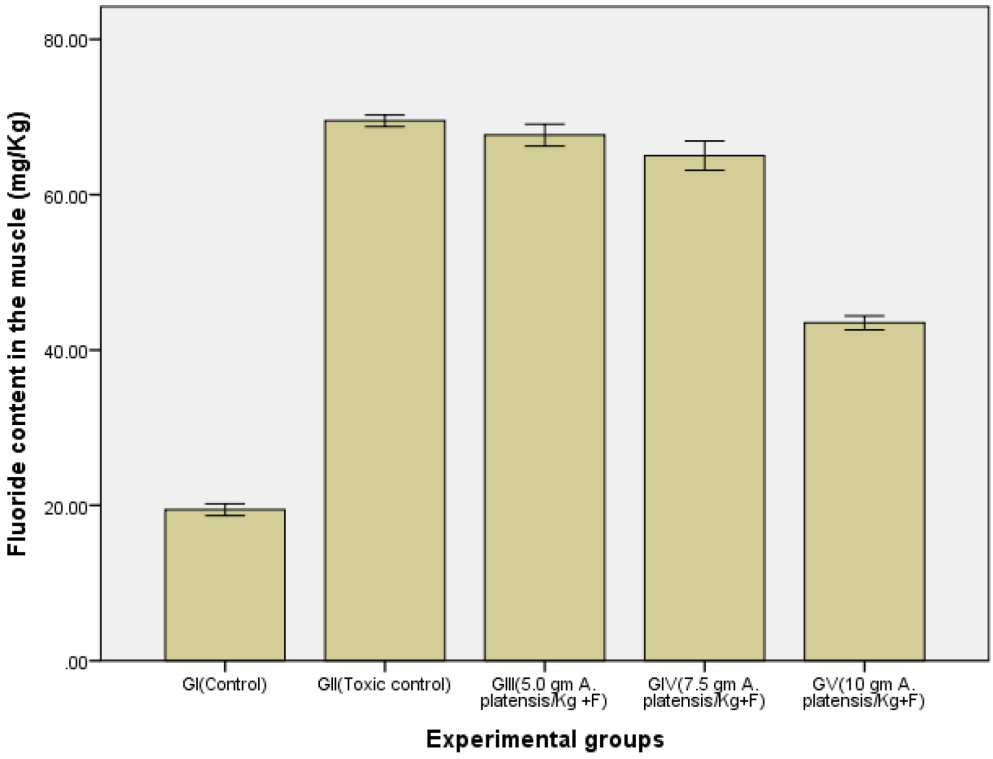

Figure 6: Bar diagram showing effect of A. platensis dietary supplementation on fluoride accumulation in the muscular tissues in fish exposed to sub-lethal concentration of fluoride.

However, *A. platensis* dietary supplementation improved the haematological parameters by significantly ($p<0.05$) increasing erythrocyte count and haemoglobin content in group III, IV, and V compared to group II (GII) (Table 4), demonstrating the ameliorating effect of *A. platensis* against F toxicity. In an earlier study, the protective effect of *A. platensis* dietary supplementation against F toxicity induced alteration of haematological parameters has also been reported in *Gambusia affinis* and Wistar albino rats.[51,52] Owing to the rich content of phycocyanin, *A. platensis* dietary supplementation increased the production of erythropoietin hormone, resulting in the enhancement of erythrocyte count as well as haemoglobin content.[43]

A. platensis dietary supplementation significantly ($p<0.05$) reduced the accumulation of F in the muscle tissues of group III, IV, and V compared to group II (GII) (Figure 6). It is well established that diet rich with ascorbic acid (vitamin C) and calcium (Ca) can reduce the F absorption.[53-56] The ascorbic acid as well as calcium rich nature of *A. platensis* decrease the absorption of F, thus reduced the accumulation of F in the muscular tissues. The accumulation of copper in *C. mrigala* is also prevented by *A. platensis* dietary supplementation.[45] In the recent study, it has also reported the reduction of bio-accumulation of heavy metals in the Nile tilapia using a plant based dietary supplement, Indian lotus leaves.[57]

CONCLUSIONS

A. platensis dietary supplementation almost restored the F-induced alteration of growth performance, digestive enzymes activities, and haematological parameters nearest to control by preventing the accumulation of F in muscular tissues of fish, and thus ameliorates F toxicity. Fish fed 10 gm of *A. platensis*/kg showed the best response, hence considered as the optimum dose. The present findings are important to add to the existing knowledge of F toxicity and its improvement through dietary supplementation.

CONFLICT OF INTERSTS

The authors declare that they have no conflict of interest.

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