

Biochemical profile of liver of Indian major carp (*Labeo rohita*) exposed to immunostimulant and bacterial infection

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ABSTRACT

The efficacy of oral feeding diet (supplement) Aqua Fix was studied on the biochemical changes in liver and immune response with regard to biochemical profile against aeromoniasis in *L.rohita*. Three groups(A,B,C) of six months old fish were employed; groups A and B were treated with Aqua Fix for 4 days, on day 5 fish of group B and C were infected with *Aeromonas hydrophila* @ 10⁶CFU/fish(fish of group C were fed with normal diet for 4 days).Controls (group D) were untreated and uninfected. Necropsies were made on day 1, 2,3,4,7 and 15 after infection in fish of group B and C; fish of group A and D were also necropsied on same designated days. Liver protein, carbohydrate, DNA and RNA were estimated following standard methods and results were subjected to statistical analysis and found that the immunostimulant modulated immunity to enhance the level of protein, carbohydrate, DNA and RNA in liver. Stress caused by aeromoniasis led to the decrease of all the above biomolecules in fish which received infection (group C).

Key words: Liver, Immunostimulant , *Aeromonas hydrophila* , *Labeo rohita*.

INTRODUCTION

Aeromonas hydrophila infection was found in both marine and fresh water fishes; it is a causative agent of bleeding in internal organs such as liver, kidney, intestines, and ulcers, tail and fin rot, lesions in gills and skin(Sankar et al.,2000;Hossain et al., 2011). Infected *Labeo rohita* with *A.hydrophila* showed sluggishness with pale and red gills, liquification of the internal organs and marked blackening at the descaled areas(Manoj et al., 2010). Kandeepan (2014) compared the haematological profile and blood glucose and protein levels of four fresh water south Indian teleost fish (*Channa striatus*, *Cyprinus carpio*, *Catla catla* and *L. rohita*). Stress factors such as overcrowding, transport, grading, poor handling and low water quality adversely affect the health of culture fish resulting in immunosuppression and disease outbreaks (Smith et al., 2003; Li et al., 2003; Quesada et al., 2013). Fish immune system can be modulated by the use of herbal extracts (as feed supplement) as alternative chemotherapeutic agents (Raman et al., 2002; Raman, 2007; Kumar et al., 2013). Harikrishan et al., (2005) and Benali et al., (2014) found an increase in the level of Hb, RBC, MCH and MCH-C in fish such as *C. carpio* and *Huso huso* treated

with oat extract supplemented diet. Baba et al., (2016) studied the effect of oat, *Avena sativa* extract against *A. hydrophila* in *C. carpio*. Fishes were fed with various concentrations of oat extract supplemented diet and infected with *A. hydrophila* and found a significant increase in growth, haematological and immunological parameters. Liver and kidney tissues are the primary targets of bacterial accumulation on sepsis and the liver became pale and showed greyish to greenish coloration with foci (Yardimci et al., 2011; Sun et al., 2014). Gangwar et al., (2007) studied various biochemical components of muscles (moisture, fat, protein, amino acid and cholesterol content) of different age groups (6, 18, 36 and 60 months) of *L. rohita*.

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Protein showed maximum percentage in 6 months old fish and minimum in 60 months old fish. Pal *et al.*, (2015) opined that increase of serum protein, albumin and globulin in infected fish was due to the function of non-specific immune response (because the serum protein contains various humoral elements of non-specific immunity) of fish against the bacterial infection.

Various studies indicated that immunostimulants can enhance resistance of fish to several bacterial pathogens including *A. hydrophila*. (Nya and Austin 2009, 2011; Awad and Austin, 2010). A lower mortality on *A. hydrophila* challenge was reported in *C. carpio* fed with *Azadiracta indica* (Harikrishnan *et al.*, 2003), asaxanthin (Jagruthi *et al.*, 2015) and, carotenoids (Anbazahan *et al.*,

2014; Wang *et al.*, 2015). Baba *et al.*, (2016) demonstrated that fishes fed with oat extract supplemented diet remarkably increased the survival rate of *C. carpio* against *A. hydrophila* pathogen. Oat, *A. sativa* extract had a positive effect on the survival rate of common carp due to the active compounds in the extract. Enhancement of growth rate in different fish due to the potential application of herbal extracts has been reported by several authors (MacLennan *et al.*, 2002; Citarasu *et al.*, 2002; Immanuel *et al.*, 2009; Talpur and Ikhwanuddin, 2013; Kanani *et al.*, 2014). Therefore, the present studies were undertaken to assess the biochemical profile in the liver of *L. rohita* fed with immunostimulator (Aqua Fix) and/or *A. hydrophila* infection.

Table 1: Protein (mg/ml) and carbohydrate (mg/ml) content in the liver of experimental and Control during different days of experiment.

(Experimental: **Group A**, immunomodulated, treated with Aqua Fix @ 50 mg / 100 g of feed; **Group B**: immunomodulated and infected with *A. hydrophila* (treated with Aqua Fix @ 50 mg / 100 g of feed and infected @ 10^{-6} CFU/fish); **Group C**: infected with *A. hydrophila* (untreated with Aqua Fix and infected @ 10^{-6} CFU/fish) ; and **Group D**: control (untreated with Aqua Fix and uninfected with *A. hydrophila*) *L. rohita* (6 months old) at various days of experiment)

Day of necropsy	Experimental groups						Control group	
	Group A		Group B		Group C		Group D	
	P	C	P	C	P	C	P	C
1	0.480	1.800	0.450	1.230	0.290	0.800	0.300	1.210
2	0.484	1.820	0.441	1.030	0.265	0.720	0.302	1.213
3	0.492	1.828	0.432	0.940	0.251	0.704	0.301	1.213
4	0.500	1.834	0.421	0.910	0.233	0.682	0.304	1.210
7	0.520	1.840	0.409	0.900	0.210	0.630	0.302	1.213
15	0.580	1.880	0.400	0.810	0.202	0.508	0.301	1.215

Values are expressed in the mean derived from 5 observations.

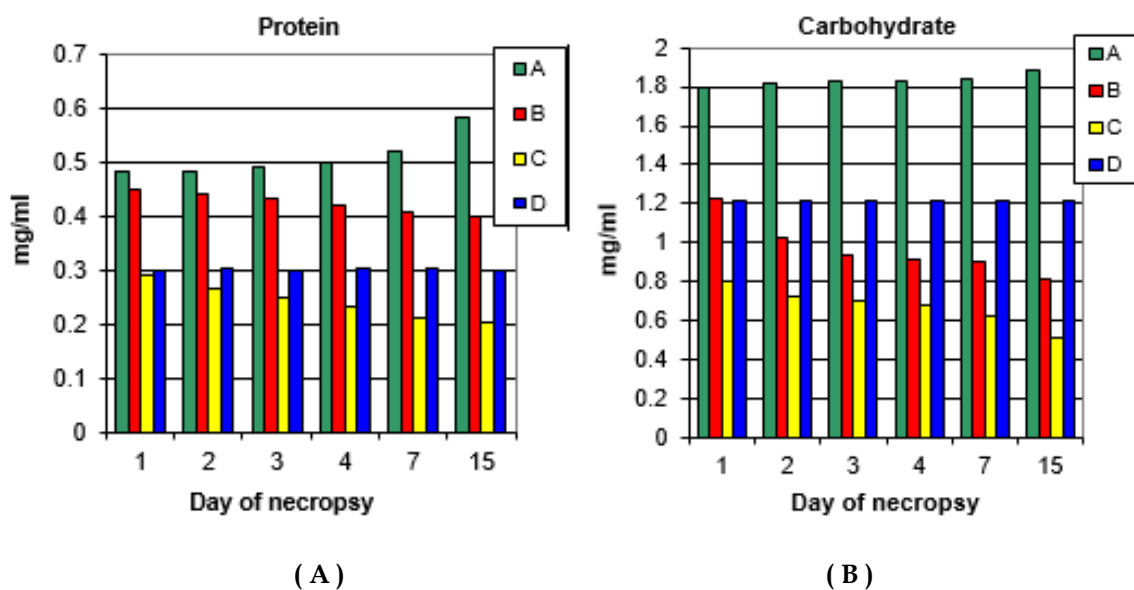


Figure-1 (A): The content of protein (mg/ml) in the liver of experimental (group A, B,C) and control (group D) fish (6 months old) at various days of experiment
(B): . The content of carbohydrate (mg/ml) in the liver of experimental (groupA, B,C) and control (groupD) fish (6 months old) at various days of experiment

Materials and Methods

Three experimental groups of six month old *L. rohita* (12-15 g) were selected for the experimentation. Groups, A (treated with Aqua Fix), B (treated with Aqua Fix and infected with *A. hydrophila*), C (untreated with Aqua Fix but infected with *A. hydrophila*) and one control group, D (untreated and uninfected) of fish (35 in each group) were maintained in optimum conditions. Fish of group A and B fed with a diet supplemented with Aqua Fix (@50mg/100 g of feed) for 4 days; on day 5 fish of group B and C were infected intraperitoneally with *A. hydrophila* @ 10⁶ CFU/fish. Fish of groups C and D were fed with normal diet (without Aqua Fix) during the entire experimental period. Necropsies were made on day 6, 7, 8, 9, 12 and 20 of experiment (for convenience these are described as day 1, 2, 3, 4, 7 and 15). Liver tissue from the experimental and control groups were removed and analysed for protein, carbohydrates, DNA and RNA following Lowry *et al.*, (1951), Nicholas *et al.*, (1956) and Burton (1956) respectively. Results were subjected to student's t-test to find out the significance.

Results and Discussion

Protein activity in liver (Table1, Fig. 1A)

Group A: Fish of group A, which received Aqua Fix showed higher protein levels when compared to that of controls. From day 1 (0.480 mg/ml) to 15 (0.580 mg/ml) of treatment, there was a gradual increase of protein and the increase of protein was significant when compared to other days of infection.

Group B: In case of group B, which received Aqua Fix and infection, there was an increase of protein from day 1 (0.450 mg/ml) to 15 (0.400 mg/ml) when compared to controls (0.300 mg/ml). Although there is a gradual decrease of protein level from day 1 to 15, the content of protein of this group (B) is still higher when compared to that of control.

Group C: In fish of group C, which received infection, lower level of protein were recorded from day 1 to 15 of infection. From day 1 to 15 there is a marked gradual decrease in the level of protein. The estimated protein values are 0.290 mg/ml on day 1, 0.265 mg/ml on day 2, 0.251 mg/ml on day 3, 0.233 mg/ml on day 4, 0.210 mg/ml on day 7 and 0.202 mg/ml on day 15.

The recorded values of protein in group C from day 1 to 15 are lower than that of Aqua Fix treated (group A), Aqua Fix treated and infected (group B) and controls (group D). The protein levels in group B is comparatively lower than that of Aqua Fix treated fish (group A) from day 1 to 15 of experiment.

Carbohydrate activity in liver (Table 1, Fig. 1 B)

Group A: Fish of group A treated with Aqua Fix, the liver carbohydrate content increased slightly from day 1 to 15 of experiment when compared to controls. From the day 1 onwards to the day 15 of Aqua Fix treatment, there was a gradual (slight) increase of carbohydrate content. The recorded carbohydrate values are 1.800 mg/ml, 1.820 mg/ml, 1.828 mg/ml, 1.834 mg/ml, 1.840 mg/ml and 1.880 mg/ml on day 1, 2, 3, 4, 7 and 15 respectively.

Group B: Fish treated with Aqua Fix and infected with *A. hydrophila*, the carbohydrate value is lower than the normal value from day 1 to 15 of experiment (except on day 1). The level of carbohydrates on day 1 (1.230 mg/ml) is higher than normal levels (day 1, 1.210 mg/ml). From day 2 (1.030 mg/ml) to 15 (0.810 mg/ml), there is a gradual and marked decrease of carbohydrates (these values are below normal level).

Group C: In case of group C fish given *A. hydrophila* infection showed lower level of carbohydrates from day 1 to 15 of infection compared to controls. A slight gradual decrease of carbohydrates has occurred from day 1 to 15; these values are below normal level. The estimated carbohydrate values in group C were 0.800 mg/ml, 0.720 mg/ml, 0.704 mg/ml, 0.682 mg/ml, 0.630 mg/ml and 0.508 mg/ml on day 1, 2, 3, 4, 7 and 15 respectively. The carbohydrate value in controls was 1.210 mg/ml on day 1 of experiment. The carbohydrate values in group C were lower than those recorded in group A (Aqua Fix treated) and B (Aqua Fix treated and infected) from day 1 to 15 of experiment.

Aqua Fix treated fish (group A) showed higher carbohydrate values, and Aqua fix + infection treated (group B) and infected (group C) fish showed lower carbohydrate values when compared with controls (except on day 1 in group B). Among the 3 experimental groups (A, B, C), fish treated with Aqua Fix (group A) recorded maximum carbohydrate level in comparison with other groups Band C.

DNA activity in liver (Table 2, Fig. 2A)

Group A: The results of DNA estimation in liver are shown in table 2 and figure 2A. Fish of group A which received Aqua Fix showed increase of DNA during the entire experimental period. The estimated DNA values in group A are 96.0 µg/ml, 99.1 µg/ml, 96.2 µg/ml, 94.0 µg/ml, 96.0 µg/ml and 98.0 µg/ml on day 1, 2, 3, 4, 7 and 15 respectively. All these values are higher than control values (except on day 4).

Group B: In fish of group B, which received Aqua Fix and infection, there was a gradual decrease of DNA from day 1 (63.5 µg/ml) to 15 (50.1 µg/ml). Throughout the experiment; all these values are found lower than control (group D).

Table 2: DNA ($\mu\text{g/ml}$) and RNA ($\mu\text{g/ml}$) content in the liver of experimental and Control during different days of experiment.

(Experimental: **Group A**, immunomodulated, treated with Aqua Fix @ 50 mg / 100 g of feed; **Group B**: immunomodulated and infected with *A. hydrophila* (treated with Aqua Fix @ 50 mg / 100 g of feed and infected @ 10^{-6} CFU/fish); **Group C**: infected with *A. hydrophila* (untreated with Aqua Fix and infected @ 10^{-6} CFU/fish) ; and **Group D**: control (untreated with Aqua Fix and uninfected with *A. hydrophila*) *L. rohita* (6 months old) at various days of experiment)

Day of necropsy	Experimental groups						Control group	
	Group A		Group B		Group C		Group D	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
1	96.0	110	63.5	108	50	92	94.0	94.0
2	99.1	114	62.1	105	48	90	93.9	93.9
3	96.2	120	58.2	103	44	87	94.0	93.8
4	94.0	124	54.2	102	39	84	94.0	93.9
7	96.0	125	52.9	86	34	72	93.9	94.0
15	98.0	130	50.1	59	32	49	94.0	94.0

Values are expressed in the mean derived from 5 observations.

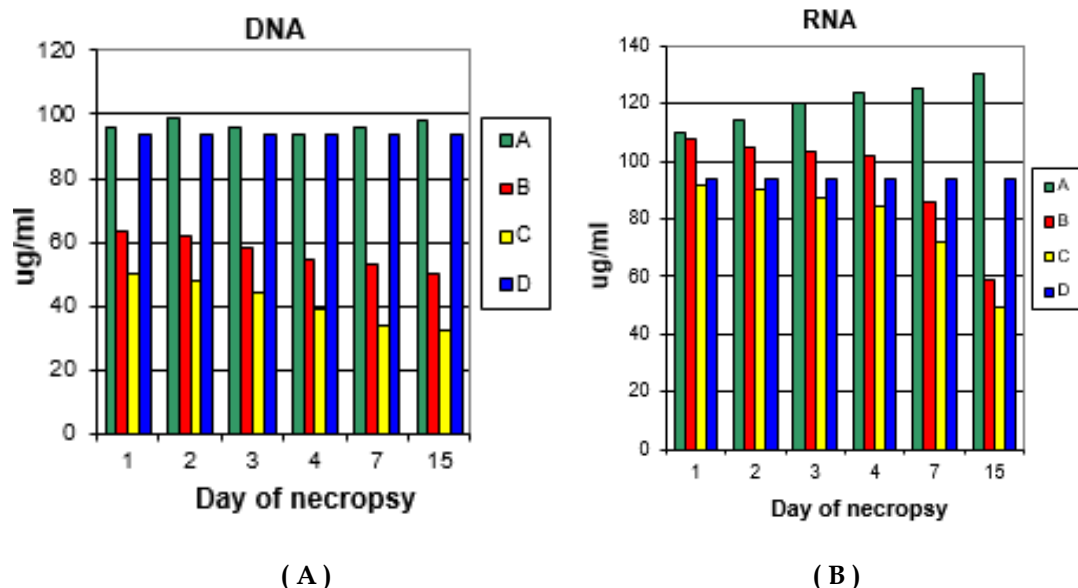


Figure-2 (A): The content of DNA ($\mu\text{g/ml}$) in the liver of experimental (group A, B,C) and control (group D) fish (6 months old) at various days of experiment

(B): The content of RNA ($\mu\text{g/ml}$) in the liver of experimental (group A, B,C) and control (group D) fish (6 months old) at various days of experiment

The recorded DNA values in group B and Dare - 63.5 $\mu\text{g/ml}$ and 94 $\mu\text{g/ml}$ on day 1, 62.1 $\mu\text{g/ml}$ and 93 $\mu\text{g/ml}$ on day 2, 58.2 $\mu\text{g/ml}$ and 94 $\mu\text{g/ml}$ on day 3, 54.2 $\mu\text{g/ml}$ and 93.5 $\mu\text{g/ml}$ on day 4, 52.9 $\mu\text{g/ml}$ and 93.5 $\mu\text{g/ml}$ on day 7, and 50.1 $\mu\text{g/ml}$ and 94 $\mu\text{g/ml}$ on day 15.

Group C: In case of fish of group C which received infection lower values of DNA were recorded from day 1 to 15 of infection when compared with controls. Throughout the experimental period these values stand below normal values. There was a progressive decrease in DNA content in liver of infected group from day 1 to 15 of experiment. The estimated DNA values were 50.0 $\mu\text{g/ml}$ on day 1, 48.0 $\mu\text{g/ml}$ on day 2, 44.0 $\mu\text{g/ml}$ on day 3, 39.0 $\mu\text{g/ml}$ on day 4, 34.0 $\mu\text{g/ml}$ on day 7 and 32.0 $\mu\text{g/ml}$ on day 15.

In comparison with controls, group A showed higher values of DNA and group B and C showed lower values of DNA. When comparison was made among the three experimental groups (A, B, C) liver of immunostimulated fish (treated with Aqua Fix) showed more DNA content than B and C groups. In between the groups B and C, group B (treated with Aqua Fix and infected with *A. hydrophila*) showed more content of DNA than group C (infected with *A. hydrophila*).

RNA activity in liver (Table2, Fig. 2B)

Group A: Table 2 and figure 2B reveal that in fish of group A treated with Aqua Fix, the liver RNA increased markedly from day 1 to 15 of experiment when compared

to controls. The estimated values of RNA on day 1 (110 µg/ml), 2 (114 µg/ml), 3 (120 µg/ml), 4 (124 µg/ml), 7 (125 µg/ml), and 15 (130 µg/ml). All these values are higher than control group D (untreated and uninfected).

Group B: In case of group B, which received Aqua Fix and infection there was an increase from day 1 to 4 and decrease on day 7 and 15; a gradual decrease of RNA was found from day 1 (108 µg/ml) to day 15 (59 µg/ml). Although there is a gradual decrease of RNA from day 1 to 15, the content of RNA of this group (B) is still higher than control group up to day 4 and lower than control group on day 7 (97 µg/ml in group D, 86 µg/ml in group B) and day 15 (98 µg/ml in group D, 59 µg/ml in group B).

Group C: In comparison with controls, fish of group C which received infection showed lower levels of RNA

from day 1 to 15 of experimental period. From day 1 to 15 there is a marked gradual decrease in the content of RNA. Though there was a slight decrease on day 1 (92.0 µg/ml) and 2 (90.0 µg/ml) it was drastically decreased on day 7 (72.0 µg/ml) and 15 (49.0 µg/ml) compared to controls (94.0 µg/ml)

Among the three groups A, B and C, Aqua Fix treated fish (group A) showed enhanced level of RNA from day 1 to 15 with a peak response on day 15 (130 µg/ml) compared to groups B and C. Fish of group B (treated with Aqua Fix and infection) showed lower values compared to group A, and higher values from day 1 to 4 compared to controls (group D) and from day 1 to 15 compared to infected fish (group C). Fish received infection and fed with normal diet (group C) showed below normal values from day 1 to 15.

Table 3. Mean and t-values of protein, carbohydrate, DNA and RNA obtained for liver of experimental (group A, immunomodulated; group B, immunomodulated and infected; group C, infected) and control (group D, unimmunomodulated and uninfected) *L. rohita* (6 months old).

Biomolecules	Groups											
	A		B		C		D					
Protein:												
Mean:	0.509		0.425		0.241		0.301					
t-value	A	D	B	D	C	D	A	B	A	C	B	C
	_____		_____		_____		_____		_____		_____	
	t= 12.64*		t= 15.08*		t= 4.12*		t= 4.57*		t= 12.10*		t= 10.77*	
Carbohydrate:												
Mean:	1.833		0.97		0.674		1.212					
t-value	A	D	B	D	C	D	A	B	A	C	B	C
	_____		_____		_____		_____		_____		_____	
	t= 51.54*		t= 3.71*		t= 12.15*		t= 13.11*		t= 25.44*		t= 3.77*	
DNA:												
Mean:	96		56.8		41.6		93.6					
t-value	A	D	B	D	C	D	A	B	A	C	B	C
	_____		_____		_____		_____		_____		_____	
	t= 3.27*		t= 15.19*		t= 13.7*		t= 16.0*		t= 14.3*		t= 3.4*	
RNA:												
Mean:	120.5		93.8		79		95.5					
t-value	A	D	B	D	C	D	A	B	A	C	B	C
	_____		_____		_____		_____		_____		_____	
	t= 7.30*		t= 0.20 [®]		t= 2.26 [®]		t= 2.97*		t= 5.20*		t= 1.33 [®]	

P value at 5% level of significance is 2.306

*Statistically significant values

[®]Statistically non-significant values

Fish of group A and B showed significant increase of protein when compared with controls and significant decrease of protein in group C when compared with controls (Table 3). There was a significant increase of protein when comparison was made between the groups A and C, B and C and significant decrease in between groups A and B. Carbohydrate showed significant increase in group A when compared with group D (controls); groups B and C showed significant decrease compared with controls. When comparison was made among the three groups there was a significant difference in between groups A and B, A and C, B and C. Group A showed significant increase and groups B and C showed significant decrease when compared with control group D. Also group A showed significant increase in comparison with group B and C. The comparison between group B and C also showed a significant difference. A significant rise of RNA was found in group A in comparison with groups B, C and D. Groups B and C showed significant decrease of RNA when compared with group D (controls). RNA content showed non-significant difference in between groups B and C.

Fish which received immunostimulant (group A) showed enhanced level of liver protein, carbohydrates, DNA and RNA compared to controls (group D) and other experimentals (group B, C). This might be due to the enhancement of resistance of the fish by herbal based Aqua Fix. Pratheepa *et al.*, (2010) and Mohamad and Abasali (2010) reported enhanced resistance and non-specific and specific immune response in *C. carpio* treated with various concentrations of *A. marmelos* (leaf extract) and ten plant extracts supplemented diets. In case of group B (Immunostimulated + infected), the level of protein, carbohydrates, DNA and RNA decreased compared to controls (group D) (except protein) and immunostimulated (group A) fish, and increased compared to infected (group C) fish. Higher increase of tested biochemical constituents in group B compared to group C (except RNA level) might be due to the enhanced resistance by Aqua Fix treatment against *A. hydrophila* infection; it is possible that Aqua Fix modulated the immune system to resist the abnormal changes caused by pathogenic bacteria. Baba *et al.*, (1993) and Gopalakannan and Venkatesan (2006) reported the enhancement of non-specific immune response and resistance to experimental challenge with *A. hydrophila* in *C. carpio* treated with the immunostimulant, levamisole. Infected fish (group C) showed significant decrease of protein, carbohydrates and DNA and non-significant decrease of RNA in liver compared to group B and D (control). The infection stress in the host system might have caused abnormality in the metabolism of protein, carbohydrate, DNA and RNA. Maruthanayagam and Sharmila (2004), Patil and David (2007), Remia *et al.*, (2008) and Vija Kumar *et al.*, (2009) reported altered biochemical variations in *C. carpio* by monocrotophos, hepatotoxic reactions in *L. rohita* by malathion, abnormal biochemical constituents in *Tilapia mossambica* by monocrotophos and altered nucleic acids

and protein content in *C. punctatus* by quinalphos due to stress caused by pesticides.

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Conflicts of Interest

Authors declare that there is no conflict of interests regarding the publication of this paper.

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