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Evaluation of Acute and Subacute Toxicity of Synthesized Calcium Aluminate Nanoparticles in Common Carp *Cyprinus carpio*

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ABSTRACT

In the present study, a simple and convenient synthesis of $CaAl_2O_4$ nanoparticle was reported. The static renewal bioassay was conducted to determine the lethal toxicity of $CaAl_2O_4$ nanoparticle and sublethal exposure reveals the effect of nanoparticle on the haematology and biochemical parameters of the test species *Cyprinus carpio*. The acute toxicity (96hrs LC50) of $CaAl_2O_4$ NPs was observed between 25- 225ppm. As a novel attempt, our study showed the impact of $CaAl_2O_4$ nanoparticles in acute toxicity and biochemical parameters of freshwater fish *Cyprinus carpio.*

Introduction

Aluminium is the third most abundant element in the earth's crust and aluminium oxide nanoparticles are extensively used in a number of applications in food, material, cosmetics, medicine, chemical and biological sciences (Hoffmann et al., 1995; Nowack and Bucheli, 2007). $CaAl_2O_4$ is a novel nanoparticle and this is the first report on the toxicity of calcium aluminate nanoparticle in fish *Cyprinus carpio*.

The calcium aluminate nanoparticle was synthesized by solution combustion method using acetamide as fuel. Calcium aluminate nanoparticle was characterized by Scanning Electron Micrograph (SEM), X-Ray Diffraction (XRD) and UV-absorption spectroscopy and tested for its efficiency in degrading selected azo dyes under solar irradiation in our previous studies (Bhavya et al., 2015). As a continuation of the degradation studies, our $CaAl_2O_4$ was also tested for its impact on the LC_{50} lethal study and its sublethal effect of on some haematological and biochemical parameters of a freshwater fish *C. carpio* was estimated.

Literature pertaining to toxicity study of nanoparticle in fish as a model organism is limited Particularly, toxicity assay on adult fish focused on carbon-based nanoparticles Some of the studies about the toxicity of metal oxide NPs such as TiO_2, ZnO NPs on fishes have concentrated the early developmental stages and enzymatic studies (Suganthi et al., 2015). Being a novel attempt, this study was framed to evaluate the acute toxicity and haematological effects of $CaAl_2O_4$ NPs on freshwater fish *C. carpio*.

Materials and methods

$CaAl_2O_4$ NP Synthesis

$Ca(NO_3)_2.4H_2O$ (6.49 g) and $Al(NO_3)_3.9H_2O$ (20.63 g) were dissolved in a minimum quantity of water along with acetamide (5.90 g) in a silica crucible (with a volume of 100 cm^3). The resulting mixture was introduced into the muffle furnace which was preheated to 600°C. The combustion reaction for the synthesis of $CaAl_2O_4$ by the redox mixture method (acetamide) is given below:

$$11Ca(NO_3)_2+22Al(NO_3)_3+ 40CH_3CONH_2 \longrightarrow 11CaAl_2O_4 + 80CO_2 + 100H_2O + 64N_2$$

Experimental animals and $CaAl_2O_4$ NPs suspension preparation

Fish with an average weight: 18.5±2 gm, length: 10±2 cm was collected from the State Fisheries Department, Division of fish breeding, Bhadra Reservoir Project, Shimoga, Karnataka state, India. They were safely brought to the laboratory in well-packed polythene bags containing aerated water. Fish were given a bath for a minute in 0.5% $KMnO_4$ for prevention of any disease outbreak. The fish were placed in plastic tubs for a period of 12 days to get acclimatized to the laboratory conditions and were normally fed once a day with commercial fish feed during acclimatization and test periods (Wang et al., 2011; Karthigarani and Navaraj, 2012). The sonicated nanoparticles exposed to the fish groups in respective concentrations and control group maintained separately.

Acute lethal assay

Acclimatized fishes were separated into groups and exposed to different concentrations of sonicated $CaAl_2O_4$ nanoparticles for acute lethal concentration studies. Feeding was terminated 24 hours before the commencement of the test and during the experimental period to avoid absorption of NPs by food or faecal materials (Karthigarani and Navaraj, 2012). Dead fishes were removed immediately to avoid contamination of the exposure solutions when observed and mortality recorded at every 24hours.

Experimental Procedure for Subacute Toxicity Test

Sublethal concentration was fixed based on the LC_{50} obtained by the toxicity evaluation of nanoparticles. Fish were divided into 3 groups (n=12).

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The first group of fishes served as control and other two groups served as experimental groups. Test medium was renewed for every 24 hours with their respective test concentrations. While transferring the fishes special care was taken to avoid any injury during handling.

Haematology and biochemical estimation in the serum sample

Fishes were exposed to sublethal concentrations for 7, 14 and 21 days. At the end of exposure time, the blood was collected from both the test and control fish by means of cardiac puncture.

Blood profile such as Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin (Hb) estimated using a whole blood sample. Serum biochemical estimations *viz.*, glucose, protein, cholesterol, creatinine, SGOT and SGPT were done by Robonik kit (diagnostic reagent) manufactured by Robonik India Pvt. Ltd., Navi Mumbai.

Statistical analysis

The lethal (LC_{50}) and sublethal concentration of exposure values for $CaAl_2O_4$ NPs was calculated using SPSS 16. All the values were expressed as Mean±SD. Data obtained from each exposure day were tested for the significant difference using ANOVA (one-way analysis of variance with a Turkey's post hoc test). $p<0.05$ and $p<0.01$ were considered statistically significant.

Results

The acute toxicity of $CaAl_2O_4$ NPs to *C. carpio* increased with particle concentration, demonstrating a dose dependency. LC50 value of $CaAl_2O_4$ NP was found to be 97.169 ppm $CaAl_2O_4$ nanoparticles (Table 1&2, Fig 1) in three replicates.

In the present investigation, RBC was slightly increased ($p>0.05$) group II (14.9%) and group III (14.3%) for day7 compared to control. In group II there was a significant ($p<0.05$) decrease was observed at the end of the exposure day21 (-24.0%) and in the group III exposed fish shows significant ($p<0.05$) decrease compared to group I. The percent change is-23.8 and-23.5 was observed at day 14 and 21 respectively. WBC shows the significant increased levels in both the group II (29.3%, $p<0.05$) and group III (36.3%, $p<0.01$) compared to group I at the end of day 7.

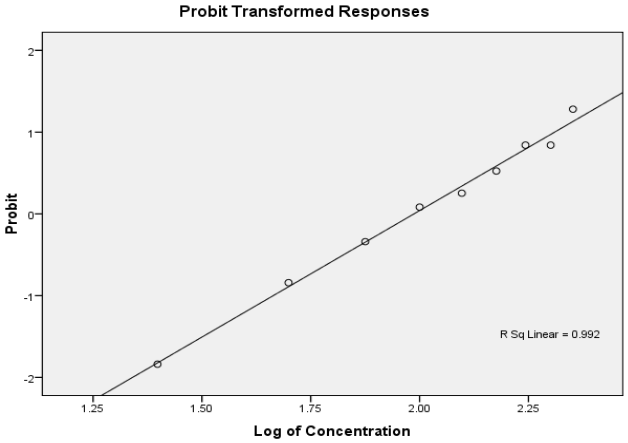

Figure 1. Probit analysis Graph showing LC50 of $CaAl_2O_4$ NPs in *C. carpio.*

But in the case of 14 days of exposure, the WBC level showed increase of 7.7% for group II and a decrease of -14.8% for group III which was a not significant over the group I. There was a significant ($p<0.05$) decrease at end of 21 days of exposure periods for both the group II and group III, the percent change are -26.4% and -27.8% respectively. Haemoglobin content shows a general decrease over 21 days of exposure periods. Initially, there were no significant changes in the Hb content for 7 days of exposure whereas the decrease was significant ($p<0.05$) after 14 days exposure periods. For 21 days of exposure to the nanoparticle, we observed highly significant ($p<0.01$) decrease for both the sublethal concentrations compared to control. Decreased percent are, -7.3, -20.9 and -24.4 for 7, 14 and 21 days respectively for group II fish. Group III exposed fish shows percent decrease of Hb content over the control are, -8.3, -22.4 and -31.6 group III at day 7, 14 and 21 respectively (Table 3, Fig 2a-2c).

A marked rise in the serum glucose level was observed in the treated fish than that of the control after 14 days of the exposure period. An initial insignificant decrease in glucose was observed for group II and group III for 7 days of exposure. A significant increase of 29.2 and 40.8 percent was observed for the group II fish after day 14and 21 respectively.

Table 1. Mortality of *C. carpio* at 96h after treatment of different concentration of $CaAl_2O_4$ NP.

No.	Conc. (ppm)	Log Conc.	No. of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	25	1.398	10	0	0.359	-0.029	0.036
2	50	1.699	10	2	1.891	0.109	0.189
3	75	1.875	10	4	3.656	0.014	0.366
4	100	2.000	10	5	5.152	0.178	0.515
5	125	2.097	10	6	6.308	-0.308	0.631
6	150	2.176	10	7	7.177	-0.177	0.718
7	175	2.243	10	8	7.824	0.176	0.782
8	200	2.301	10	8	8.309	-0.309	0.831
9	225	2.352	10	9	8.673	0.327	0.867

Table 2. Estimated $CaAl_2O_4$ NP concentration values and confidence limits.

Probability	Estimate (Conc.)	95% Confidence Limits for Concentration	
		Lower Bound	Upper Bound
0.01	16.822	4.423	30.079
0.05	28.119	10.403	43.815
0.1 _LC_{10}	36.978	16.353	53.742
0.2	51.52	28.07	69.328
0.3	65.439	41.026	84.146
0.4	80.275	55.965	100.668
0.5_LC_{50}	97.169	73.26	121.551
0.6	117.619	92.984	151.368
0.7	144.285	115.564	198.761
0.8	183.265	143.74	283.48
0.9	255.339	188.262	479.269
0.99_LC_{99}	561.292	339.991	1753.045

The decrease was highly significant ($p<0.01$) in group III fish *i.e.*, 40.8 and 49.5 for 14 and 21 days respectively. The value of protein content in group II shows non-significant change at day 7 (3.9%) and 14 (-9.3) and at day 21 there was a significantly ($p<0.05$, -21.0%) decreased amount of protein was observed. Whereas group III shows a significant decrease at 14 and 21 days, the observed percent change is -27.0 and -26.1 respectively. The serum cholesterol level of fishes exposed to group II showed an increase (3.0%) at day 7 and a slight decrease (-18.9%) at day 14 was not significant ($p>0.05$). The decreased (-21.7%) level of protein was significant ($p<0.05$) at the end of the day 21 over the control value. In group III significantly ($p<0.05$) decreased percent change was observed when compared to group I are -29.7% and -26.7% was observed for day 14 and 21 respectively. Level of serum creatinine in the group II exposed fish shows a non-significant change throughout the period of exposure the observed percent change is -3.4, 8.5, 18.6 exposed to 7, 14, 21 days respectively. Whereas in group III fish significant increased level of creatinine was found to be 26.0%, 40.5% for day 14 and 21day respectively. A general increase in the SGOT activity was observed for the exposure periods. In group II fish, the increase was not significant ($p>0.05$) for day 7 (9.3%) and day 14 (14.7%) but shows significantly increased (30.0%, $p<0.05$) at the end of the exposure day21 compared to group I. In group III fish, the increase in SGOT level was significant ($p<0.05$ and $p<0.01$) for 14 and 21 days and the increased percent are 40.0% and 38.9% respectively. An elevated level of transaminase was slightly increased ($p>0.05$) in both the exposure throughout the experiment compared to group I. The activity of SGPT is significantly ($p<0.05$) increased at the end of the day 14 and 21 in group III the percent increase was found to be 28.8 and 29.1 respectively (Table 3, Fig 2d-2i).

Discussion

The mean LC_{50} value of $CaAl_2O_4$ NP on *C. carpio* was found to be 97.169 mg/L (Table 2). Vidya et al., 2017, reported the acute toxicity of aluminium oxide (Al_2O_3-NPs) on the freshwater fish, *Oreochromis mossambicus* for 96h. Mortality of fish for Al_2O_3 NPs was found to be 40mg/L. Juhel et al., 2011, exposed the aquatic plant Lemna minor to Al_2O_3 NPs and found that they mediated growth enhancement. In contrast, Al_2O_3 NP induced morphological changes, cytotoxicity, and oxidative stress in Chinook salmon (CHSE- 214) cells (Srikanth et al., 2015). Moreover, the toxicity of Al_2O_3 NP was shown by exposing *C. dubia* to different NP concentrations up to 120 $\mu g.L^{-1}$ (Pakrashi et al., 2013).

The insignificant changes in RBC count may be attributed to the amounts of Al accumulated by the erythrocytes (during 7days), which were not sufficient to initiate significant effects on the RBC count (Ali, 2013). The increase in WBC count can be correlated with an increase in antibody production which helps in the survival and recovery of the fish exposed to sublethal concentrations of toxicant (*Joshi et al., 2002*). But in contrast, Reduction in leukocyte counts (*i.e.*, leucopenia) was observed in *Channa punctatus* after chronic exposure of monocrotophos (Singh and Srivastava, 1993).

Table 3. Haematology and biochemical parameters of *C. carpio* exposed to sublethal concentrations of $CaAl_2O_4$ NP concentrations for varying periods.

$CaAl_2O_4$	Exposure Day	Group I	Group II		Group III	
		Control	1/10th conc	%Change	1/5th conc	%Change
RBC (Cellx10^6.mm^{-3})	7day	4.38±0.41	5.03±0.66	14.9	5.00±0.56	14.3
	14day	4.53±0.33	4.06±0.51	-10.2	3.45±0.37	-23.8
	21day	4.63±0.57	3.52±0.37	-24	3.54±0.56	-23.5
Hb (g/dl)	7day	12.51±0.76	11.60±1.13	-7.3	11.48±1.33	-8.3
	14day	11.31±1.12	8.95±0.74	-20.9	8.78±0.90	-22.4
	21day	11.74±1.31	8.88±0.87	-24.4	8.03±0.88	-31.6
WBC (Cellx10^3.mm^{-3})	7day	10.64±0.98	13.75±1.26	29.3	14.50±1.57	36.3
	14day	10.45±1.11	11.25±1.50	7.7	8.90±1.01	-14.8
	21day	11.35±1.43	8.35±1.08	-26.4	8.20±1.02	-27.8
Glucose (mg/dl)	7day	85.50±89.47	80.75±12.50	-5.6	78.50±11.12	-8.2
	14day	79.75±8.88	103±12.57	29.2	112.25±10.37	40.8
	21day	80.25±11.59	113±12.25	40.8	120±16.77	49.5
Protein (g/l)	7day	2.07±0.20	2.15±0.29	3.9	1.97±0.23	-5
	14day	2.21±0.30	2.01±0.21	-9.3	1.61±0.24	-27.2
	21day	2.28±0.26	1.80±0.22	-21	1.68±0.22	-26.1
Cholesterol (mg/dl)	7day	109±15.85	112.25±15.59	3	107.38±13.24	-1.5
	14day	113.75±15.46	92.25±11.44	-18.9	80±12.06	-29.7
	21day	108.50±12.34	85.00±10.20	-21.7	79.50±9.40	-26.7
Creatinine (mg/dl)	7day	1.25±0.13	1.27±0.14	-3.4	1.78±0.18	15.2
	14day	1.39±0.09	2.21±0.30	8.5	2.14±0.18	26
	21day	1.34±0.10	2.04±0.28	18.6	2.89±0.25	40.5
SGOT (U/L)	7day	22.00±2.94	22.13±2.39	9.3	23.50±2.08	5.2
	14day	23.75±3.77	33.25±4.03	14.7	39.00±4.76	40
	21day	20.77±3.86	43.38±3.25	30	45.50±6.03	38.9
SGPT (U/L)	7day	15.75±2.63	16.75±1.69	8	21.25±1.50	12.5
	14day	14.50±1.29	20±0.82	10.1	29.50±3.79	28.8
	21day	18.50±2.65	26.25±1.71	10.8	27.25±3.59	29.1

Results are represented as Mean ± SD (n=4)

'+' denotes per cent increase over control

'-' denotes per cent decrease over control

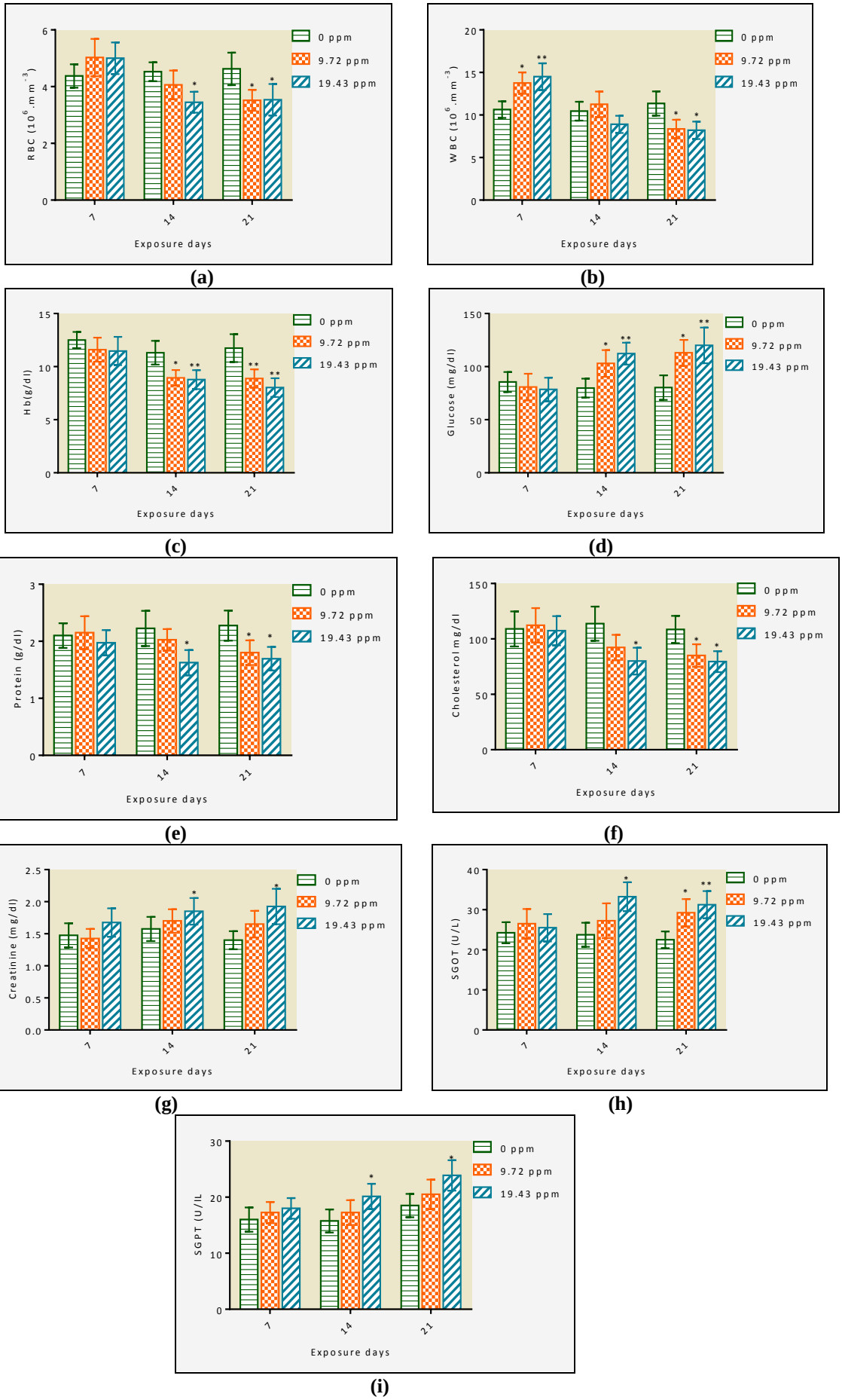

Figure 2. Haematology and biochemical parameters *C. carpio* exposed to sublethal concentrations of $CaAl_2O_4$ NP for varying periods [(a) RBC, (b) WBC, (c) Hb, (d) Glucose, (e) Protein, (f) Cholesterol, (g) Creatinine, (h) SGOT and (i) SGPT] **P<0.01, *P<0.05.

The present study demonstrated the obvious toxic effect of $CaAl_2O_4$ NP on the haemoglobin of *C. carpio*. The decreased haemoglobin concentration represents the reduced supply of adequate oxygen to the tissues and resulted in the decline of physical activities (Nussey et al., 1995). The above results are in agreement with earlier works that reported a significant decrease in haemoglobin of flounder, *Pleuronectes flesus* (Johansson-Sjobeck and Larson, 1978) and other freshwater fishes exposed to heavy metals (Shalaby 2001; Vutukuru, 2005).

Serum glucose levels were significantly higher in fish exposed to $CaAl_2O_4$ NP as compared to the control groups (Table 3 and Fig. 2d). The depletion of liver glycogen (glycogenolysis) and the rise in blood glucose levels was reported in *T. zillii* as a consequence of water pollution (Abdelmeguid et al., 2002). Blood glucose levels have long been used as indicators of stress in fish. It is generally thought that, under conditions of stress, hyperglycemia may provide additional energy during times of high metabolic need such as a fight or flight response (Goss & Wood, 1988). Consequently, we suggest that $CaAl_2O_4$ NP affects glucose dynamics in *C. carpio* in order to obtain more energy to withstand and overcome the existing stress condition.

Protein level changes in the serum sample of fish exposed to $CaAl_2O_4$ NP represented in Fig. 2e. *Das et al.*, 2004, stated that the reduction of protein content in serum occurs due to shrinkage and lysis of RBCs causing plasma dilution and/or protein catabolism where structural protein converts to energy. *Singh et al., 1993*, who attributed the decline in protein content to an alteration in the nucleic acids in the *H. fossilis* exposed to sublethal concentrations of aldrin. Lynch et al., 1969, correlated the reduced level of serum protein to either its excessive loss due to nephrosis or to reduce protein synthesis due to liver cirrhosis. Blood serum protein is a fairly labile biochemical system, precisely reflecting the condition of the organism and the changes happening to it under the influence of internal and external factors (Shalaby et al., 2006). Thus, the influence of toxicants on the total protein concentration of fish has been taken into consideration in evaluating the response to stressors and consequently the increasing demand for energy.

The level of cholesterol in the serum is a good parameter for studying the influence of stressors (Gluth and Hanke, 1984; Öner et al., 2008). Several studies report an increase in blood cholesterol levels during stress (Wedemeyer and McLeay, 1981; Gluth and Hanke, 1984). In the present study, cholesterol increase was insignificant for the initial period of exposure in both treated groups. But the cholesterol level was significantly decreased after 14 and 21 days of exposure periods (Fig. 2f).

In this study, it was found that serum creatinine had increased to a considerable level in group II and group III compared to the control group (Fig. 2g). The elevation in serum creatinine level is considered as a significant marker of renal dysfunction (Rudenko et al., 1988). Many previous studies have shown that aluminium hydroxide (mainly antacid) has a protective effect on the progress of renal dysfunction (Sanai et al., 1991). *Murali et al., 2018*, reported Al_2O_3 NPs treated fishes showed degeneration of renal tubules, presence of melanomacrophages and sinusoidal space along with necrotic cells in the hematopoietic tissue, aggregation of blood cells, membrane damage in the blood cells, vacuolation, increased space in between glomerulus and enlarged Bowman's capsule in their experiment.

In this work, treatment with $CaAl_2O_4$ NP resulted in a significant increase in the activities of serum AST and ALT as compared with control (Fig. 2h & 2i). AST and ALT belong to the serum non-functional enzymes which are normally localized within the cells of liver, heart, gills, kidneys, muscle and other organs. It is also considered to be important in assessing the state of the liver and some other organs (Verma et al., 1981). Their presence in blood plasma may give information on tissue injury or organ dysfunction (Wells et al., 1986). Herein, we attribute the increase in AST and ALT after nanoparticle exposure to the hepatocellular degeneration and destruction in other tissues. Therefore, the increases of these enzymes in plasma are indicative of liver damage and thus alterations in liver function.

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