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Acute Toxicity and Ethological Changes in an Indian Freshwater Catfish, *Clarias Batrachus* (Linnaeus) Exposed to Silver Nanoparticles

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Abstract: ***This is an initial report on the acute toxicity and ethological changes in an Indian freshwater catfish Clarias batrachus (Linnaeus) exposed to silver nanoparticles. The work was conducted by the use of static renewal and probit analysis method during 2018. The 24, 48, 72 and 96 hr LC_{50} values were calculated 346.20, 237.33, 201.28, 171.16 mg L^{-1} respectively. The findings suggest that Clarias batrachus has more capacity to resist silver nanoparticles toxicity in comparison to other fishes. The fish intoxicated to 1%, 2%, 3%, 4% and 5% of 96hr-LC_{50} dose of silver nanaoparticles showed various ethological changes. Buccal movements, opercular movements and the forced swimming of fish increased significantly in response to silver nanoparticles. Number of feeding attempts in silver nanoparticles intoxicated fish reduced significantly while irregular responses were observed regarding their attempt to form school/shoal. The silver nanoparticles concentrations showed strong relationship with opercular movement followed by forced swimming > buccal movement > rate of feeding > schooling/shoaling behaviour in Clarias batrachus. As a whole, exposure of fishes to silver nanoparticles showed significant effects on all studied behaviour. So it can be concluded that the silver nanoparticles is toxic to fishes and its excess use should be avoided.***

Keywords: Acute toxicity, Ethological changes, *Clarias batarchus*, silver nanoparticles

1. Introduction

Over the last decade, large number of research has focused silver nanoparticles toxicity. The toxicity of silver nanoparticles depends on the type of aquatic organisms, nature of particle and exposure conditions [1]-[2]. Increased utility of silver nanoparticles effects into the bioaccumulation of these particles in the environment. Its toxicity ranges from μgl^{-1} to mgl^{-1} among aquatic organisms inclusive of bacteria, plants, fungi, algae, invertebrates and major carps and other fish [3]-[5].

Fish being one of the mannequin animals is ill affected via silver nanoparticles, therefore concern of potential jeopardy to aquatic organism increases [6]. Behaviour offers an essential linking the physiology and ecology of living organisms and its environment. Such studies of silver nanoparticles with Indian catfishes are lacking.

Clarias batrachus (Linnaeus) regionally called as magur is one of the important Asiatic fish. It is taken as a model fish to describe alimentary canal of a teleost and selected as a test animal in many laboratories [7]. This fish has least concern category in term of conservation status.

The study will be helpful to evaluate suitability of environmental conditions for fish, relative sensitivity of fish and variation in health of the fish due to toxicity of silver nanoparticles.

2. Materials and Methods

Clarias batrachus (Linnaeus) (body weight: 46.0-68.0g, total length: 18.0-22.0cm and standard length: 16.2-19.2cm) were purchased from the local sources. They were acclimatized for a fortnight in Departmental Laboratory of Zoology, VKS University, Arrah.

The silver nanoparticle (size 60nm, TEM; Product No. 730815) was procured from Sigma Aldrich. It was stored at 6^0C temperature in a freeze. Stock suspension of the uncoated powder was done using dechlorinated tap water to get various concentrations of the test solution. Calculated amount of silver nanoparticle was dissolved in 1 L of the dechlorinated tap water to obtain appropriate mixtures to obtain stock solutions. From stock solution, solutions of desired concentrations were made for experimental purposes.

The investigation was performed using static renewable method in controlled laboratory conditions following ethics of the Department and University. Physico-chemical parameters of experimental water were recorded daily following standard methods [8].

Finney method was used to calculate LC_{50} dose [9]. After that, 1%, 2%, 3%, 4% and 5% of 96hr-LC_{50} dose of silver nanoparticles were selected for ethological studies.

Statistical analysis was done with Graph Pad Prism 5 software. Data were entered in a Microsoft Excel spreadsheet. All the entries were double checked for any possible key board error. The observations were expressed in mean for each group. The data were used for group analysis followed by its linear regression.

3. Results and Discussion

Certain physico-chemical parameters such as temperature (25.0±1.0), pH (7.84±0.08), dissolved oxygen (4.56±0.64mg/L), total alkalinity (334.00±8.62mg/L) and total hardness (190.66±8.66mg/L) were recorded daily at exposure times of 24, 48, 72 and 96 hours.

(A) Acute Toxicity Test

Finney method [9]**:** It is a parametric maximum likelihood method in which after calculating percent mortality, net/corrected percent mortality from 10% to 100% were calculated. Then, values of empirical probit from 3.72 to 8.22 (Table-1) were noted from Fischer and Yates table depending upon the straight line obtained in the graph. Values of empirical probit were applied in the calculation of expected/provisional probit from 3.70 to 6.40. The values of working probit (from 4.90 to 6.20) and weighing coefficient (from 0.302 to 0.627) were calculated to determine the values of mean and deviation of the dose of silver nanoparticles and mortality.

Table 1: Probit analysis for acute toxicity of silver nanoparticles in *Clarias batrachus*

Dose of silver nano particles (mg/L)	Log dose of silver nano particles (mg/L)	Number of fish exposed	Mortality of fish	% of mortality of fish	Net/corrected mortality of fish	Empirical probit	Expected/Provisional probit	Working probit	Weighing Coefficient	nw	nwx	nwy	nwx^2	nwy^2	nwxy
	x	n		p			Y	y	w						
0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0
100	2.000	10	1	10	10	3.72	3.70	4.90	0.336	3.36	6.72	16.46	13.44	80.67	32.93
150	2.176	10	3	30	30	4.48	4.80	5.40	0.627	6.27	13.64	33.86	29.69	182.83	73.67
200	2.301	10	6	60	60	5.25	5.60	5.80	0.558	5.58	12.84	32.36	29.54	187.71	74.47
250	2.398	10	9	90	90	6.28	6.20	6.00	0.370	3.70	8.87	22.20	21.28	133.20	53.24
300	2.477	10	10	100	100	8.72	6.40	6.20	0.302	3.02	7.48	18.72	18.53	116.09	46.40
	-	-	-	-	-	-	-	-	-	21.93	49.55	123.60	112.48	700.50	280.71

96hr-LC_{50} = Antilog 2.234= 171.4mg/L of silver nanoparticles.

Finally, the median lethal concentration was calculated to be LC_{50} = Antilog 2.234= 171.4 mg/L of silver nanoparticles.

The median lethal dose of silver nanoaprticles ranged from 0.202mg/L in *Hypophthalmichthys molitrix*, 0.34mg/L in *Hypophthalmichthys molitrix* and 0.53mg/L in *Carassius auratus*, 20mg/L in *Carassius auratus gibelio*, 100mg/L in *Oreochromis niloticus* and *Labeo rohita*, 164.02mg/L in *Clarias batrchus* and 250mg/L in *Danio rerio* [10]-[16]. These findings suggest that *Clarias batrachus* has capacity to resist silver nanoparticles toxicity more in comparison to most of the other fishes. The difference in LC_{50} dose among the same species depends upon the body weight, age, sex and feeding conditions of the test fishes; ambient conditions, water temperature and regional variations [10]-[11].

(B) Ethological Observations:

Fish behaviour under stress conditions provides important information for aquaculturists and ichthyologists [17]. Methods of observing and quantifying the ethological changes are potential alternatives to assess stress, disease, water pollution and toxic material in ambient water [18].

The controlled *Clarias batrachus* behaved in regular manner. They were active with their properly managed swimming. The test fish showed various ethological changes at 1%, 2%, 3%, 4% and 5% of 96hr-LC_{50} dose of silver nanoparticles. The type, rate and duration of ethological changes elevated with increased concentrations of silver nanaoparticles.

It was observed that ethological changes of *Clarias batrachus* at the lowest concentration of silver nanaoparticles cause insignificant changes. However, among that treatment, at first, the restlessness; while in the subsequent treatment, the fierce changes of the behaviour of exposed fish was noted. Ethological changes of fish are the main sensitive indicator of stress induced by chemicals [19]. Any variation in the behaviour of fish indicates the deterioration of water quality, as fish are the bioindicator and thus environmental suitability index and the cost of survival [20].

As *Clarias batrachus* were introduced in to the experimental solutions, they become excited and showed erratic swimming, jerky movement, rolling the body, convulsion for 20-30 minutes, loss of equilibrium difficulty in breathing and lethargic at 8.75 mg/L of silver nanaoparticles. They tried to soar outside the aquarium occasionally, try to jump out of aquarium and then gradually settled down at the bottom.

Body colour of fish was observed lighter with the increasing concentration as well durations of the treatment. It has been suggested that hypersecretion of epinephrine during stress condition inhibits the action of MSH resulting in pale body colour [21]. A similar mechanism may be operable in the present study indicating an influence of the pituitary as well.

Abundant discharge of mucus was seen over the body. Mucus on the skin plays an important position in the protection of fishes from the harmful toxic consequences of chemicals. .The enormous secretion of mucus by the fish is perhaps to coat the body to reduce contact with the toxic environment and get relief from the irritation caused by silver nanoparticles and may lead to mortality of the fish [22]-[23].

Aside from these observations, certain fundamental ethological elements such as schooling/shoaling behaviour, buccal movement, rate of feeding, opercular movements and forced swimming were observed and discussed in detail.

(I) Schooling/Shoaling behaviour:

Normal fish maintained a school/shoal. When startled, they formed a close-fitting school/shoal. They were sensitive to light and moved to the bottom of the tank. In sub lethal treatment, the schooling/shoaling behaviour of the fish was slowly disrupted from very first day. No specific trend to form a school/shoal was observed in 1.75 to 8.75mg/L silver nanoparticles exposed fishes. The number of fish in a school/shoal ranged beyond 2$\pm$1 in accordance with 7$\pm$3 (Table-2; Plate I-Figure 1) and showed 0% to 100% variation. Dose of silver nanoparticles and tendency to form a school/shoal in relation to time has insignificant relationship as:

(A) 24hours: $y = 0.212x + 1.904$ ($F = 0.978^{NS}$)
(B) 48hours: $y = 0.114x + 3.000$ ($F = 0.583^{NS}$)
(C) 72hours: $y = 0.098x + 4.095$ ($F = 0.123^{NS}$)
(D) 96hours: $y = 0.277x + 2.285$ ($F = 0.651^{NS}$)

Application of two-way ANOVA concludes that the durations of exposure have insignificant ($p>0.05$) effect on tendency to form a school/shoal. But, the dose of silver nanoparticles bear a significant ($p<0.05$) effect on tendency to form a school/shoal in this fish (Table 2).

The disruption of schooling/shoaling behaviour due to the stress of the toxicant might be results in increase in the rate of swimming [24]-[25].

(II) Buccal Movement:

The buccal movements in fishes exposed to 1.75 to 8.75mg/L silver nanoparticles were found to be increased significantly from 96$\pm$4 to 124$\pm$8 min^{-1}. The percent of increment was 3.105% to 29.17% (Table-2; Plate I-Figure 2). Dose of silver nanoparticles and buccal movements in relation to time bear significant relationship:

(A) 24hours: $y = 2.840x + 93.90$ ($F = 29.41^{**}$)
(B) 48hours: $y = 3.151x + 95.04$ ($F = 109.4^{***}$)
(C) 72hours: $y = 2.840x + 95.23$ ($F = 52.72^{**}$)
(D 96hours: $y = 2.971x + 96.00$ ($F = 91.00^{***}$)

Application of two-way ANOVA deduced that the durations of exposure have significant ($p<0.05$) effect on buccal movements. But, the dose of silver nanoparticles bear a highly significant ($p<0.001$) effect on buccal movements of this fish (Table 2).

(III) Rate of Feeding

Rate of feeding attempts was reduced significantly in 1.75 to 8.75mg/L silver nanoparticles exposed fishes. Rate of feeding attempts decreased from 9$\pm$1 to 2$\pm$1 per 5 min. The observation showed -14.00% to -77.77% decreases (Table-2; Plate I-Figure 3). Dose of silver nanoparticles and rate of feeding attempts in relation to time has following significant relationship:

(A) 24hours: $y = -0.571x + 6.666$ ($F = 52.50^{**}$)
(B) 48hours: $y = -0.653x + 6.523$ ($F = 14.12^{*}$)
(C) 72hours: $y = -0.734x + 7.381$ ($F = 19.60^{*}$)
(D) 96hours: $y = -0.587x + 6.238$ ($F = 09.85^{*}$)

The application regarding two tailed F test (=ANOVA) conjectured that the different intervals over exposure has insignificant ($p>0.05$) effect on rate of feeding. The evaluation of analysis further inferred that the variation in dose of silver nanoparticles has a highly significant ($p<0.001$) effect on rate of feeding of this fish (Table-2).

It has been reported in *Cyprinus carpio* that, the nerves of terminal buds present in the lip was sensitive to high concentration of benthiocarb, fenitrothion and isoprothiolane [26] and petrilachlor [25]. Food intake of fish is influenced by ecological factors and hunting behavior of fish. Reduced food intake might also influence the ability of prey capture by the fish in addition to the loss in appetite. Contaminated aquatic media reduces the food intake capacity of the larvae and affecting their proper development and then growth.

(IV) Opercular Movement

The opercular movement increased significantly in comparison to controlled one. Rapid opercular movements are indicative of respiratory inconvenience. The opercular movement in fishes exposed to 1.75 to 8.75mg/L silver nanoparticles were found to be increased significantly from 54$\pm$2 to 69$\pm$7 min^{-1} at treated concentrations. The percent of increment was 1.75% to 21.84% (Table-2; Plate I-Figure 4). Dose of silver nanoparticles and opercular movement in relation to time has following significant relationship:

(A) 24hours: $y = 0.914x + 53.67$ ($F = 336.0^{***}$)
(B) 48hours: $y = 1.012x + 54.24$ ($F = 90.80^{***}$)
(C) 72hours: $y = 1.257x + 54.67$ ($F = 55.24^{**}$)
(D) 96hours: $y = 1.355x + 55.90$ ($F = 89.08^{***}$)

The application of two tailed F test (=ANOVA) deduced that the different intervals over exposure is significant ($p<0.01$) effect on opercular movement. The analysis further confirmed that the variation in dose of silver nanoparticles also has a highly significant ($p<0.001$) effect on opercular movement of this fish (Table-2).

Therefore, it might be inferred that sub lethal exposure to silver nanoparticles adversely effects respiration in fishes similar to the conclusions made with atrazine [27] and pretilachlor [25]. The increased opercular activity in the test fishes might be due to stressful toxic environment along with sensory stimulus to increase the opercular movement for proper ventilation of gills to cope up with hypoxia [28]-[29].

(V) Forced Swimming reactions:

A significant increase in the number of forced swimming reactions was observed which increase with increasing from 1.75 to 8.75mg/L of silver nanoparticles. Rate of forced swimming reactions was increased from 0 to 12$\pm$4 per 5 min (Table-2; Plate I-Figure 5). Dose of silver nanoparticles and rate of forced swimming reactions in relation to time has following significant relationship:

(A) 24hours: $y = 1.278x + 1.095$ ($F = 33.37^{**}$)
(B) 48hours: $y = 1.290x + 0.523$ ($F = 97.01^{***}$)
(C) 72hours: $y = 1.192x + 0.619$ ($F = 112.60^{***}$)
(D) 96hours: $y = 1.273x + 1.095$ ($F = 78.67^{***}$)

The application regarding two tailed F test (=ANOVA) conjectured that the different intervals over exposure has insignificant ($p>0.05$) effect on forced swimming reactions. The evaluation of analysis further inferred that the variation in dose of silver nanoparticles has a highly significant ($p<0.001$) effect on forced swimming reactions of this fish (Table-2).

Forced swimming in *Clarias batrachus* are result of stressful condition [30].

The silver nanoparticles concentrations showed strong relationship with opercular movement followed by Forced swimming > buccal movement> rate of feeding > shoaling behaviour in *Clarias batrachus* with R^2 value of 0.959; 0.943; 0.937; 0.817 and 0.115 respectively. Therefore the dose of silver nanoparticles has more impact on ethological changes in comparison to duration of the exposure period. These findings are in confirmatory with the results of in cadmium intoxicated *Channa punctatus* [31].

Table 2: Quantitative Behavioural modifications in *Clarias batrachus* exposed to different sub-lethal concentration of silver nanoparticles.

	Dose of silver nanaoparticles (mg/L)	Duration of treatment				F test
		24hr	48hr	72hr	96hr	
Tendency of shoaling (per 5min)	Control	0	2±1	2±1	0	0.37^{NS} and 3.15* (for n_1=3 and n_2=15; 0.5=3.3, 0.1=5.4 and 0.01=9.3) (for n_1=5 and n_2=15; 0.5=2.9, 0.1=4.6 and 0.01=7.6)
	1.75	5±2	4±2 (100%)	4±2 (100%)	5±2	
	3.50	3±1	3±1 (50%)	7±2 (250%)	3±2	
	5.25	2±1	5±2 (150%)	4±2 (100%)	4±2	
	7.00	3±1	4±2 (100%)	3±1 (50%)	7±3	
	8.75	4±2	3±1 (50%)	2±1 (0%)	2±2	
	F at 0.5=7.7, 0.1= 21.2 and 0.01=74.1 (for n_1=1; n_2=4)	0.778^{NS}	0.853^{NS}	0.123^{NS}	0.651^{NS}	
Buccal movement (min^{-1})	Dose of silver nanaoparticles (mg/L)	Duration of treatment				F test
		24hr	48hr	72hr	96hr	
	Control	96±4	97±4	97±5	96±4	3.87* and 191.7*** (for n_1=3 and n_2=15; 0.5=3.3, 0.1=5.4 and 0.01=9.3) (for n_1=5 and n_2=15; 0.5=2.9, 0.1=4.6 and 0.01=7.6)
	1.75	99±5 (3.12%)	100±4 (3.10%)	100±5 (3.10%)	101±4 (5.15%)	
	3.50	103±5 (7.29%)	104±4 (7.22%)	105±6 (8.25%)	109±5 (13.54%)	
	5.25	107±6 (11.46%)	111±5 (14.32%)	108±6 (11.34%)	109±5 (13.54%)	
	7.00	109±6 (13.54%)	114±5 (17.53%)	112±7 (15.46%)	115±6 (19.79%)	
	8.75	124±8 (29.17%)	124±6 (27.84%)	124±7 (27.84%)	124±7 (29.17%)	
	F at 0.5=7.7, 0.1= 21.2 and 0.01=74.1 (for n_1=1; n_2=4)	29.41**	109.4***	52.72**	91.00***	
Rate of Feeding (per 5min)	Dose of silver nanaoparticles (mg/L)	Duration of treatment				F test
		24hr	48hr	72hr	96hr	
	Control	7±1	8±1	9±1	8±1	2.30^{NS} and 68.68*** (for n_1=3 and n_2=15; 0.5=3.3, 0.1=5.4 and 0.01=9.3) (for n_1=5 and n_2=15; 0.5=2.9, 0.1=4.6 and 0.01=7.6)
	1.75	6±1 (- 14.29%)	5±1 (- 37.50%)	5±1 (- 44.44%)	4±1 (- 50.00%)	
	3.50	4±1 (- 42.86%)	3±1 (- 62.50%)	4±1 (- 55.55%)	3±1 (- 62.50%)	
	5.25	3±1 (- 57.14%)	2±1 (- 75.00%)	3±1 (- 66.66%)	2±1 (- 75.00%)	
	7.00	3±1 (- 57.14%)	2±1 (- 75.00%)	2±1 (- 77.77%)	2±1 (- 75.00%)	
	8.75	2±1 (- 71.43%)	2±1 (- 75.00%)	2±1 (- 77.77%)	2±1 (- 75.00%)	
	F at 0.5=7.7, 0.1= 21.2 and 0.01=74.1 (for n_1=1; n_2=4)	52.50**	14.12*	19.60*	9.85*	
Opercular movement (min^{-1})	Dose of silver nanaoparticles (mg/L)	Duration of treatment				F test
		24hr	48hr	72hr	96hr	
	Control	54±2	55±2	55±3	57±3	28.33**and 81.67*** (for n_1=3 and n_2=15; 0.5=3.3, 0.1=5.4 and 0.01=9.3) (for n_1=5 and n_2=15; 0.5=2.9, 0.1=4.6 and 0.01=7.6)
	1.75	55±5 (1.85%)	56±5 (1.82%)	58±4 (5.46%)	58±5 (1.75%)	
	3.50	57±5 (5.55%)	57±5 (3.64%)	58±5 (5.46%)	60±4 (5.26%)	
	5.25	58±6 (7.4%)	59±5 (7.28%)	60±5 (9.10%)	62±5 (8.77%)	
	7.00	60±7 (11.1%)	61±6 (10.92%)	63±5 (14.56%)	65±5 (14.04%)	
	8.75	62±7 (14.8%)	64±6 (16.38%)	67±5 (21.84%)	69±7 (21.05%)	
	F at 0.5=7.7, 0.1= 21.2 and 0.01=74.1 (for n_1=1; n_2=4)	336.0***	90.00***	55.24***	89.08***	
Forced Swimming (per 5min)	Dose of silver nanaoparticles (mg/L)	Duration of treatment				F test
		24hr	48hr	72hr	96hr	
	Control	0	0	0	0	2.50^{NS} and 177.2*** (for n_1=3 and n_2=15; 0.5=3.3, 0.1=5.4 and 0.01=9.3) (for n_1=5 and n_2=15; 0.5=2.9, 0.1=4.6 and 0.01=7.6)
	1.75	5±1	4±1	3±1	5±1	
	3.50	5±1	4±1	5±1	5±2	
	5.25	9±2	8±2	8±2	8±2	
	7.00	8±2	9±2	8±2	10±2	
	8.75	13±3	12±4	11±3	12±4	
	F at 0.5=7.7, 0.1= 21.2 and 0.01=74.1 (for n_1=1; n_2=4)	33.37**	97.01***	112.6***	78.67***	

Plate I

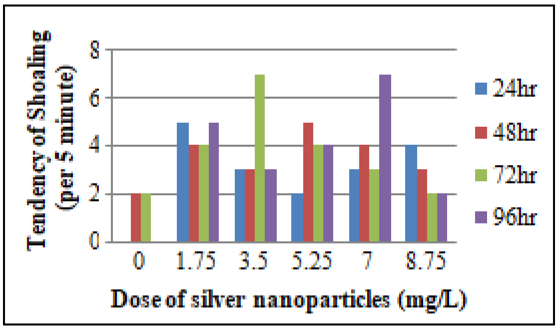

Figure 1: Variation in schooling/shoaling of *Clarias batrachus* under silver nanoparticles toxicity

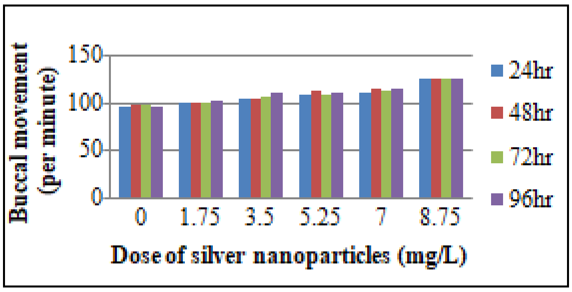

Figure 2: Variation in buccal movement of *Clarias batrachus* under silver nanoparticles toxicity

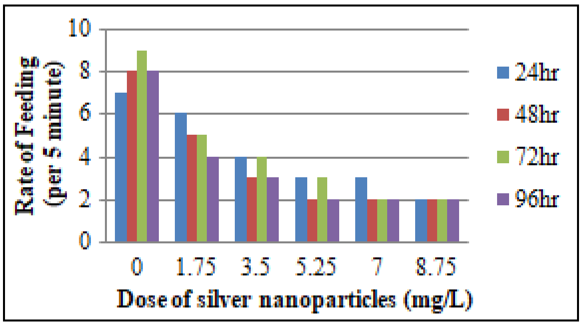

Figure 3: Variation in rate of feeding of *Clarias batrachus* under silver nanoparticles toxicity

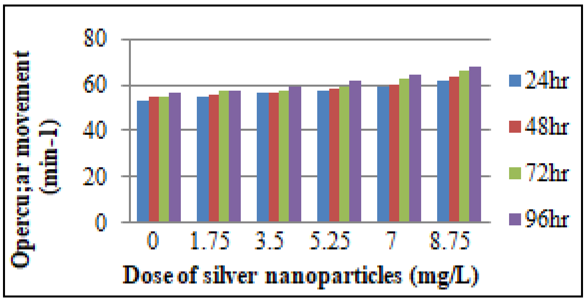

Figure 4: Variation in opercular movement of *Clarias batrachus* under silver nanoparticles toxicity

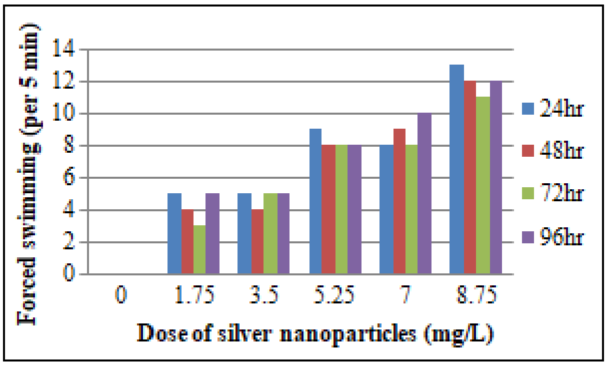

Figure 5: Variation in forced swimming of Clarias batrachus under silver nanoparticles toxicity

4. Conclusion

It may be inferred that for ecotoxicological work, determination of LC_{50} dose is one of the basic step. Finney probit method was used to determine the dose of 96hr-LC_{50} and its value was found 171.16 mg/L of silver nanoparticles. The observation suggests that *Clarias batrachus* has more capacity to resist silver nanoparticles toxicity in comparison to other fishes. The fish intoxicated to sublethal dose of silver nanaoparticles showed various ethological changes. Therefore, it can be concluded that the silver nanoparticles is toxic to fishes and its excess use should be avoided.

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