Acute and sublethal toxicity of Calcium Magnesiate nanoparticle to the *freshwater fish, Cyprinus carpio*

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Abstract :The present study was aimed to assess the toxicological potential of synthesized $CaMgO_2$ NP in the freshwater fish *Cyprinus carpio*. Acute toxicity was determined by Probit analysis of Finney. Solution combustion method was used to synthesize $CaMgO_2$ nanoparticle and Scanning Electron Micrograph (SEM), X-Ray Diffraction (XRD) and UV-Vis absorption spectroscopy were used to characterize the $CaMgO_2$ nanoparticle. Sublethal exposure reveals the effect of $CaMgO_2$ NP on the haematology and biochemical parameters of the test species *Cyprinus carpio*. As a novel attempt, our study showed the impact of $CaMgO_2$ nanoparticles in acute toxicity and biochemical parameters of freshwater fish *Cyprinus carpio*.

IndexTerms- $CaMgO_2$, *Cyprinus carpio*, LC50, Toxicity

I. INTRODUCTION

By definition, manufactured nanoparticles are particles in which at least one dimension smaller than 100nm in size. Nanomaterials have unique properties as compared to the same material in a conventional formulation. The application of one type of nanoparticle, the metal oxide nanoparticle, has accelerated in the last decade (Mehdi Ghobadian *et al.,* 2015).

The widespread use of nanoparticles results in their inevitable release into the general environment and they will find their way into terrestrial, aquatic and atmospheric environments where their behaviour and ultimate end are largely unknown (Sovová *et al.,* 2009). A major concern of nanotechnology is environmental health and safety, as many in this field consider the properties of engineered nanomaterials to be potentially hazardous to the environment and human beings (Junchao *et al.,* 2013).

Environmental pollutants, such as metals, pesticides, and other organics, pose serious risks to many aquatic organisms (Graham R. Scott and Katherine A. Sloman, 2004). There has been an increasing awareness that aquatic pollution and other anthropogenic impacts on water resources may have the potential to damage natural fish stocks. The agricultural and industrial wastes partially treated or without treatment are being discharged into surface water (Mohammad and Authman, 2011).

The fishes are quite sensitive to the contaminated water since the pollutants significantly damage their physiological and biochemical processes (Nemesok *et al.,* 1987). Acute toxicity tests are generally used to determine the concentration of a toxicant that produces a specific adverse effect on a specified percentage of test organisms in a short span of time. The most common acute toxicity test is acute lethality test (Sivakumar *et al.,* 2014).

Some studies have investigated the toxic effects of magnesium oxide NPs using model organisms such as fish. *(*Kovrižnych *et al.,* 2013;Thomas *et al.,* 2014*).* But calcium magnesiate as a novel nanoparticle, this is the novel attempt to assess the effect of synthesized $CaMgO_2$ on the haematology and biochemical parameters along with acute toxicity in fish *C. carpio* which is the continuation of our previous photocatalytic studies (Bhavya *et al.,* 2016).

II. $CaMgO_2$ NP Synthesis

The calcium magnesiate nanoparticle was synthesized by solution combustion method using urea as fuel. Metal nitrates such as $Ca(NO_3)_2.4H_2O$ (14.18g) and $Mg(NO_3)_2.6H_2O$ (15.38 g) along with fuel NH_2CONH_2 (12g) were dissolved in silica crucible (with the volume of 100 cm^3) using the minimum quantity of distilled water. The homogeneous solution was then introduced into the muffle furnace for calcination which was preheated to 450˚C. The reaction mixture undergoes combustion to form the product with the release of CO_2, N_2 and H_2O. The product obtained was crushed in a mortar to make it amorphous. According to propellant chemistry, the reaction is as follows:

$3Ca(NO_3)_2 + 3Mg(NO_3)_2 + 10NH_2CONH_2 \longrightarrow 3CaMgO_2 + 10\,CO_2 + 20H_2O + 16N_2$

The synthesized nanoparticle was characterized by Scanning Electron Micrograph (SEM), X-Ray Diffraction (XRD) and UV-Vis absorption spectroscopy and tested for its efficiency in degrading selected azo dyes under solar irradiation in our previous studies (Bhavya *et al.,* 2016). As a continuation of the degradation studies, $CaMgO_2$ was also tested for its impact on the LC50 lethal study and its sublethal effect of on some haematological and biochemical parameters of a freshwater fish *C. carpio* was estimated.

III. Materials and methods

3.1 Experimental animals and lethal assay

The freshwater fish *Cyprinus carpio* (18.5 ± 2.0g) were procured from the State Fisheries Department, Bhadra Reservoir Project, Bhadravati and acclimatized to laboratory conditions for about twelve days before the commencement of the experiment. During acclimatization and test periods, fish were fed with CP 9932 herbivorous fish feed once a day (Wang *et al.,* 2011). The sonicated nanoparticles exposed to the fish groups in respective concentrations and control group maintained separately.A preliminary study was then conducted to determine the 96-h LC 50 of $CaMgO_2$ NP for common carp according to Finney's Probit Analysis (Finney, 1971). The sublethal test was performed by semi-static bioassay method. The acclimatized fishes were divided into 3 groups. The first group of fishes served as control (0.0ppm), Group II was exposed to one-tenth of LC 50 (21.24ppm) concentration and Group III have exposed to one-fifth of LC50 (42.48 ppm) concentration of NP. The experiment for each concentration of NP was performed for a period of twenty-one days. A blood sample was collected from control and exposure groups at 7, 14 and 21 days. No mortality was observed during the experiment.

3.2 Hematology 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. Whole blood was used for the estimation of RBC, WBC and Hb. RBC and WBC counts were calculated using haemocytometer (Rusia and Sood, 1992). Hb content was estimated by the cyanmethemoglobin method (Blaxhall and Daisley, 1973). Serum glucose was quantified by GOD-POD method (Trinder, 1969). The serum total protein was measured by the Biuret method (Henry *et al.,* 1974). Total cholesterol was estimated by COD-PAP enzymatic test (Allain *et al.,* 1974). The creatinine level was determined according to Jaffe's method (Jaffe, 1886). The serum activities of alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT) were determined according to the IFCC method (Reitman and Frankel, 1957). All the biochemical estimation was measured using spectrophotometric method (Prietest-Touch Semi-Automated Chemistry Analyzer (Indian) with reagents provided in standard analyses kits (Robonik India Pvt. Ltd.).

3.3 Statistical analysis

The lethal (LC_{50}) and sublethal concentration of exposure values for $CaMgO_2$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.

IV. Results

In this study the acute toxic effect of $CaMgO_2$ on the test species, *Cyprinus carpio* was determined by the probit analysis (*Finney, 1971*). Table 1 and Fig. 1 show the relationship between the $CaMgO_2$ concentration and the mortality rate of *Cyprinus carpio* according to probit analysis. The mean LC_{50} value of $CaMgO_2$ NPs on *C. carpio* was found to be 212.409 mg/L.

In the present investigation, in both the sublethal treatment RBC count was decreased non-significantly ($p>0.05$). in group II exposure 4.68%, -10.87%, -13.64% changes were observed whereas in group III -2.00%, -15.22%, -19.32% changes were obtained when compared to control at the end of day 7, 14 and 21 respectively.

WBC shows the significant ($p<0.05$) increased levels in both the group II and III compared to group I. The increased percent are 31.71, 42.86 and 42.12 for group II and 39.02, 49.08 and 41.10 for group III respectively for 7, 14 and 21 days of exposure time. There are no noticeable changes in the control fish.

In group II, there was a non-significant ($p>0.05$) increase in the Hb content for 7 and 14 days of exposure whereas a decrease($p>0.05$) was observed at the end of 21 days exposure and the observed percent changes are 3.39, 1.40 and -8.70 respectively for group II exposed fish. Whereas in group III, the decreased level of Hb content was not significant ($p>0.05$) when compared to control. the change in percent with respect to the group I are -6.94, -8.18, -8.70 for 7, 14 and 21 days respectively.

Increase in the serum glucose level was not significant throughout the experimental period ($p>0.05$) than that of the control. Percent increase was found to be 4.73, 10.76 and 9.84 in group II and 2.18, 6.94, 13.11 in group III was observed for 7, 14 and 21 days of exposure periods respectively.

Changes in serum protein levels of fish from sublethal studies show a non-significant ($p>0.05$) change for both group II and group III compared to group I. Percent changes in the protein level for 7, 14 and 21 days for group II and group III are, -0.83, -4.85, -8.53 and 4.17, 5.83, -6.20 respectively (Fig. 2e).

In the present investigation, there was an increase ($p>0.05$) in the cholesterol levels for the initial period of exposure. The percent increase is 4.65 and 4.07 for group II and group III compared to group I for 7 days of exposure. Whereas for the next exposure periods, we have observed a decrease in the percent cholesterol. Decreasedpaercent for group II are, -12.70, -11.39, and group III are, -13.49 and -15.70 for 14 and 21 days respectively (Fig. 2f).

In the present investigation, there was a general increase in the creatinine levels except for a slight decrease in group III fish at 7 days of the exposure period. Changes in the creatinine levels are not significant ($p>0.05$) throughout the experimental period. The increased percent for group II are, 6.42, 3.75 and 9.76 for 7, 14 and 21 days respectively. Similarly, percent change in serum creatinine for group III fish exposed for 7, 14 and 21 days are -3.72, 7.50, and 13.82 respectively (Table 3 and Fig. 2g).

The activity of GOT/AST in the serum of the test fish exposed to the NP was presented in Table 3 and Fig. 2h. Enzyme activity was found to be increased in both group II and group III compared to group I for all the three exposure periods. The highest increase was recorded for day 14 and 21 in group III and the percent increase was 32.85 ($p<0.05$) and 46.43 ($p<0.01$).

The activity of GPT/ALT in the serum of the test fish exposed to the NP was presented in Table 3 and Fig. 2i. Increase in the SGPT levels was observed overexposure periods in both the sublethal exposures except for an insignificant decrease in group III at 7 days exposure period. In group II fish, the increase ($p>0.05$) was recorded as 2.07 and 26.29 at 7 and 14 days whereas 40.30% ($p<0.05$) was recorded for day 21. The group III fish, the increase in SGPT was significant ($p<0.05$) for 14 (38.97%) and 21 (39.30%) days.

Table1. Mortality of *Cyprinus carpio* at 96h after treatment of different concentration of $CaMgO_2$ NP

No.	Conc. (ppm)	Log Conc.	No. of Subjects	Observed Responses	Percent Mortality	Expected Responses	Residual	Probability
1	100	2	10	1	10	0.9	0.1	0.09
2	125	2.097	10	2	20	1.726	-0.026	0.173
3	150	2.176	10	3	30	2.679	0.321	0.268
4	175	2.243	10	4	40	3.651	0.049	0.365
5	200	2.301	10	4	40	4.573	-0.273	0.457
6	250	2.398	10	6	60	6.141	-0.441	0.614
7	300	2.477	10	7	70	7.306	-0.306	0.731
8	350	2.544	10	8	80	8.13	-0.13	0.813
9	400	2.602	10	9	90	8.701	0.599	0.87

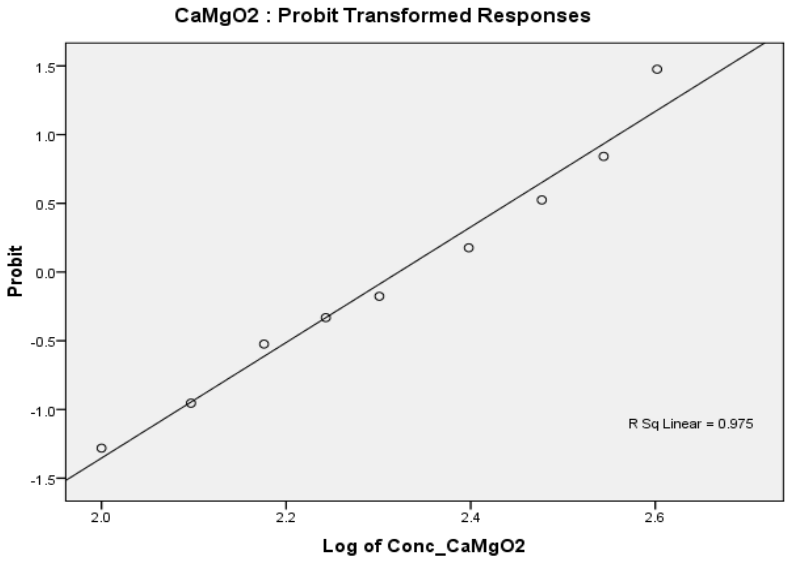

Figure 1. Probit analysis Graph showing LC50 of $CaMgO_2$ NPs in *Cyprinus carpio*

Table 2. Estimated $CaMgO_2$ NP concentration values and confidence limits

Probability	Estimate (Concentration)	95% Confidence Limits for Concentration	
		Lower Bound	Upper Bound
0.01	57.491	23.681	85.254
0.05	84.307	44.405	113.164
0.1_LC_{50}	103.396	61.85	132.103
0.2	132.384	91.641	160.623
0.3	158.209	120.243	187.143
0.4	184.231	149.194	216.766
0.5_LC_{50}	212.409	178.501	254.286
0.6	244.898	208.272	305.88
0.7	285.178	240.284	381.037
0.8	340.808	279.264	501.204
0.9	436.358	339.033	743.758
0.99_LC_{99}	784.784	524.765	1944.647

Table3. Haematology and biochemical parameters *C. carpio* exposed to sublethal concentrations of $CaMgO_2$ NP for varying periods

$CaMgO_2$	Exposure Day	Group-I	Group-II		Group-III	
		Control	21.24 ppm	%Change	42.48 ppm	%Change
RBC (Cellx106.mm-3)	7day	2.25±0.33	2.35±0.26	4.68	2.20±0.37	-2
	14day	2.3±0.38	2.05±0.21	-10.87	1.95±0.13	-15.22
	21day	2.2±0.29	1.90±0.27	-13.64	1.78±0.17	-19.32
WBC (Cellx103.mm-3)	7day	7.18±0.85	9.45±1.37	31.71	9.98±1.37	39.02
	14day	6.83±0.99	9.75±1.26	42.86	10.18±1.43	49.08
	21day	7.30±0.87	10.38±1.38	42.12	10.30±1.25	41.1
Hb (g/dl)	7day	12.82±1.55	13.25±1.71	3.39	11.93±1.68	-6.94
	14day	12.53±1.23	12.70±1.70	1.4	11.50±1.73	-8.18
	21day	11.50±1.29	10.50±1.29	-8.7	10.50±1.29	-8.7
Glucose (mg/dl)	7day	68.75±8.54	72.00±7.79	4.73	70.25±8.77	2.18
	14day	72±10.61	79.75±5.91	10.76	77.00±4.76	6.94
	21day	76.25±8.54	83.75±4.35	9.84	86.25±4.79	13.11
Protein (g/dl)	7day	3.00±0.35	2.98±0.42	-0.83	3.13±0.1	4.17
	14day	2.58±0.33	2.45±0.29	-4.85	2.73±0.28	5.83
	21day	3.23±0.42	2.95±0.47	-8.53	3.03±0.1	-6.2
Cholesterol (mg/dl)	7day	86±11.58	90±10.80	4.65	89.5±13.70	4.07
	14day	94.50±10.54	82.5±11.90	-12.7	81.75±7.37	-13.49
	21day	98.75±10.31	87.5±8.66	-11.39	83.25±6.8	-15.7
Creatinine (mg/dl)	7day	1.95±0.17	2.07±0.25	6.42	1.88±0.17	-3.72
	14day	2±0.22	2.08±0.3	3.75	2.15±0.31	7.5
	21day	1.85±0.27	2.03±0.13	9.76	2.1±0.18	13.82
SGOT (U/l)	7day	13.38±2.21	15.5±3.11	15.89	15.75±1.71	17.76
	14day	17.13±2.32	19.50±2.52	13.87	22.75±2.50	32.85
	21day	14.00±1.41	17.25±1.50	23.21	20.50±3.11	46.43
SGPT (U/l)	7day	6.03±0.79	6.15±0.85	2.07	6±0.82	-0.41
	14day	5.33±0.78	6.73±0.78	26.29	7.40±1.09	38.97
	21day	5.03±0.73	7.05±1.17	40.3	7.00±1.02	39.3

Results are represented as Mean ± SD (n=4)
'+' denotes per cent increase over control
'-' denotes per cent decrease over control

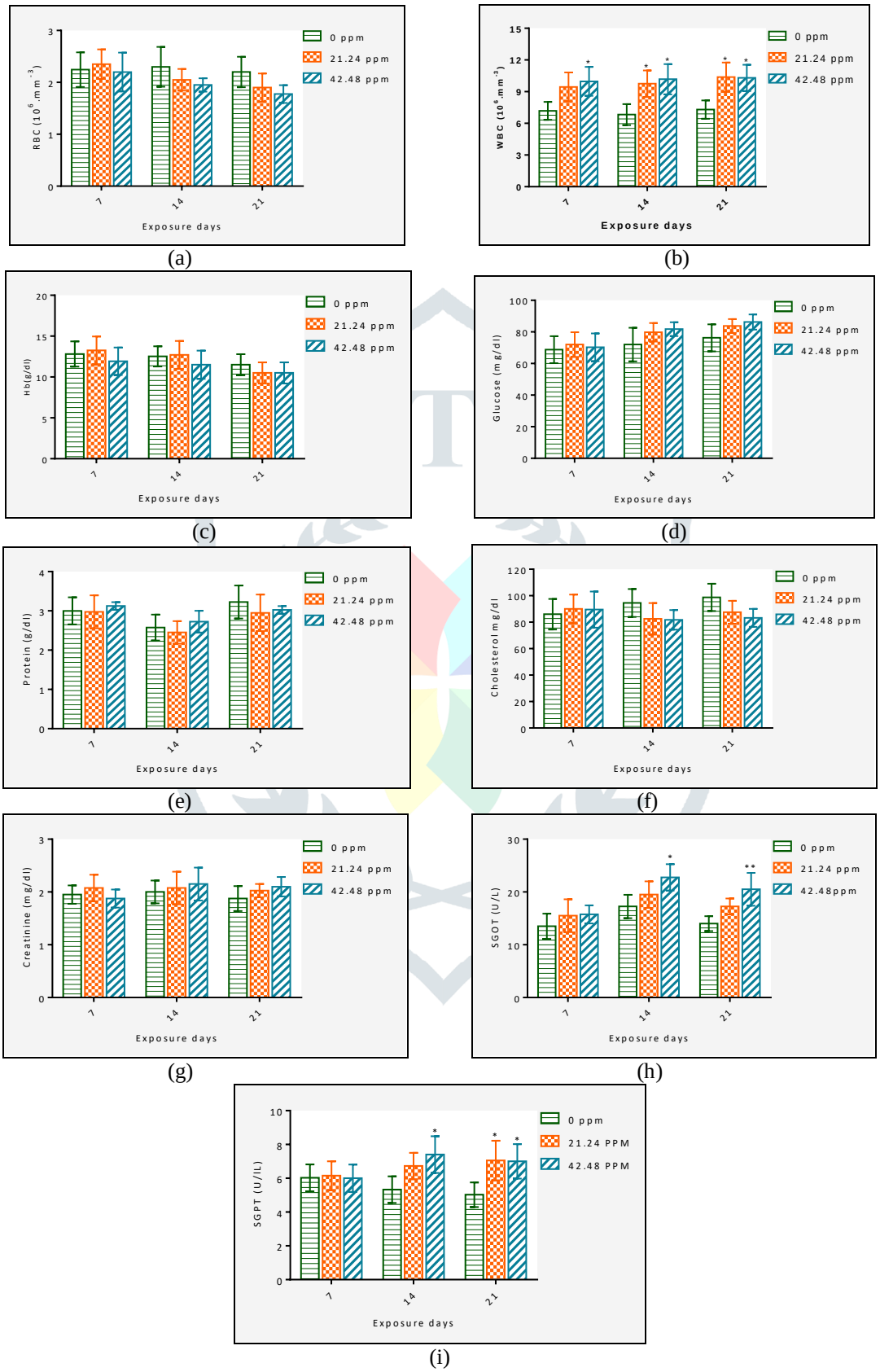

Figure 2: Haematology and biochemical parameters *C. carpio* exposed to sublethal concentrations of $CaMgO_2$ NP for varying periods [(a) RBC, (b) WBC, (c) Hb, (d) Glucose, (e) Protein, (f) Cholesterol, (g) Creatinine, (h) SGOT and (i) SGPT] **P<0.01, *P<0.05

V. Discussion

Calcium magnesiate is a novel nanoparticle and hence no literature is available for calcium magnesiate toxicity. Some studies have investigated the toxic effects of MgO NPs using fish as model organisms.Thomas *et al.*, 2014 studied the toxic effects of MgO NPs (50nm) in tilapia and zebrafish. The results showed that MgO bulk particles were more toxic than nanoparticles. In another study, the acute toxicities of 31 different nanoparticles were evaluated in zebrafish (Kovriznych *et al.*, 2013). It was demonstrated that MgO NPs caused cumulative mortality in zebrafish.*Griffitt et al.*, 2008, examined the toxic effects of MgO NP on zebrafish embryos as a model organism. The 96-h LC_{50} value in this experiment was 428 mg/L. The toxicity of magnesium oxide nanoparticle and magnesium oxide bulk against two fishes Tilapia (*Oreochromis mossambicus*) and zebrafish (*Danio rerio*) for toxicity and found the 66% Mortality at 100ppm of MgO NPs exposed to fish tilapia (John Thomas *et al.*, 2014).

The present investigation showed that the RBC and haemoglobin values are significantly decreased in nanoparticle treated fish when compared to control (Fig 2a&2c). 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 (Vutukuru, 2005; Shalaby *et al.*, 2001). 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 decreased in RBC might be due to the effect of nanoparticle on blood-forming organs (Bone marrow and liver) and inhibition of many steps of biosynthesis of fish, as the results of nanoparticle exposure. The increased WBC count in the present study indicates the stress condition of the fish caused by nanoparticle which might have produced hypoxia and gill damage.

Cholesterol level decreased as the concentration of the $CaMgO_2$ NP increased which is in accordance with the findings of Samson *et al.*, 2011. Alaa *et al.*, 2010, asserted that cholesterol is the most important sterol occurring in plasma and red blood cells. From this, we can infer that the decrease in RBC content due to increased sublethal concentrations of nanoparticle resulted in decrease cholesterol level in the blood of the exposed fish.

The present results showed a general increase in serum creatinine in *C. carpio* (Fig. 2g). This is in accordance with Al-Zahaby *et al.*, 1998, who found that the exposure of fish to high concentrations of heavy metals led to the disintegration of the renal epithelium, displacement of nuclei, shrinkage of glomeruli, breakdown of Bowman's capsule and heavy infiltration by inflammatory cells. Elghobashy *et al.*, 2001, showed an elevation in serum creatinine and uric acid in fish and they attributed this increase to the action of metals on the glomerular filtration rate which causes pathological changes of the kidney.

The obtained results showed a general significant increase in liver enzyme (SGOT and SGPT) activities in *C. carpio* exposed to sublethal concentrations of $CaMgO_2$ after 7, 14 & 21 days when compared with the control group. This is in agreement with Nemcsok and Hughes, 1988, who observed an increase in liver enzyme activities of fish *Oncorhynchus mykiss* exposed to Cu and Vaglio and Landriscina, 1999, in the case of *Sparus aurata* exposed to Cd metal. Wu *et al.*, 2003, recorded an increase of liver enzyme activities in stressed *Epinephelus areolatus* fish due to hepatic cells injury or increased synthesis of these enzymes by the liver.

VI. Conclusions

The median lethal concentration of $CaMgO_2$ nanoparticle for freshwater fish *Cyprinus carpio* was found to be 212.409 mg/L. Exposure to sublethal concentrations of $CaMgO_2$ NP resulted in no significant haematological alterations in the fish except for WBC count, SGOT and SGPT levels in serum. The alterations of these parameters may provide a better understanding of the toxic level of nanoparticles and their effects in the aquatic environment.

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