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Evaluation of using of some novel natural nano-pesticides on fish health and water physico-chemical parameters

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ABSTRACT

Nowadays there is a general demand for green chemistry to keep our environment. Chlorophyllin is widely used as a promising natural pesticide instead of a chemical one, which raises the concern for studying its eco-toxicological effect on the surrounding environment. In this study, both silver and graphene oxide nano-materials were grafted by porphyrin-based chlorophyllin to produce four novel natural nano-pesticides (Mg-Chl/Ag, Mg-Chl/GO, Cu-Chl/Ag, and Cu-Chl/GO), their effects on the physicochemical properties of water and *Clarias gariepinus* (*C. gariepinus*) health were evaluated. Each nano-composite was used in three dilutions; 10^{2}, 10^{-3}, and 10^{-4} ml/l. The obtained results indicated that such nano-composites maintain the ionic composition of the surrounding water within the international permissible limits, while high concentrations caused increased BOD, COD, NH_4, and NO_3 values. Using such nano-composites at High concentrations also alters the hematological and biochemical functions of *C. gariepinus*; they caused a decrease in RBCs count, Hb, Hct %, serum protein, and globulin concentrations. Also, there was an increase in total WBCs count, serum albumin, ALT, AST, glucose, cholesterol, creatinine, and uric acid concentrations. This study provides the environmental impact of some novel natural nano-pesticides and points to their possible toxic effects on fish health, especially at higher concentrations.

INTRODUCTION

The simultaneous increase in crop growing and chemical pesticide uses is an inevitable result of human overpopulation. Although chemical pesticides are used to protect crops from pests and diseases, they have affected various non-targeted organisms, mainly fish (**Aktar *et al*. 2009**; **Murthy *et al*. 2013**). These pesticides can find their way to water bodies through leaching of the irrigation water and persist for a long time in the environment (**Helfrich *et al*. 2009**). Many chemical insecticides are forbidden because of their toxicity, consequently, there is an urgent need for non-toxic, biodegradable, inexpensive, and highly effective natural insecticides.

Chlorophyllin is a water-soluble chlorophyll derivative, it is used as a green eco-friendly natural pesticide, and it has been reported as a safe and effective natural photosensitizer against several insect larvae. It has a very short lifetime and displays effective photodynamic properties in light by producing reactive oxygen species that can kill larvae of target organisms (**Shaapan *et al*.. 2012**; **Azizullah and Murad 2015**). It was effective against mosquito larvae, some fish parasites, and larval stages of freshwater snails (**Casida 2012**; **Azizullah *et al*.. 2014**; **Häder *et al*. 2016**). Moreover, it was used successfully to control malaria, dengue fever, and filarial by controlling their vectors (**Abdel-Kader and El-Tayeb 2012**).

Nanoscience is an interdisciplinary science, it is applied in many fields such as medicine, agriculture, paints, water filters, and nano-pesticides. The nano-pesticides of biological origin (mainly plant) are called bio-nano pesticides. Silver (Ag) and graphene oxide (GO) are widely applied in nanotechnology according to their unique properties and they have many biotechnological and biomedical applications (**Ghosal and Sarkar 2018**; **Khosravi-Katuli *et al*. 2018**). The widespread applications of nanomaterials resulted in their release in the environment, specifically the aquatic environment. Recently there was a necessity to study the nanomaterial as a double edged-weapon, and discover its possible toxic effects on living organisms besides its wide benefit (**De Marchi *et al*. 2018**; **Abbas 2021**).

To the best of our knowledge, there wasn't any information about the effect of applying natural nano-pesticides in the environment on water characteristics and fish health; as an important non-target organism. Concerning the mentioned scenario, the current paper calculates the lethal dose of four novel bio-nanopesticides, determines their effects on the physico-chemical constituents of the water and evaluates the health status of *Clarias gariepinus* after direct exposure to such composites at different dilutions.

MATERIALS AND METHODS

Preparation of chlorophyllin

Two types of photosensitizers were used; Magnesium Chlorophyllin (Mg-Chl) and Copper Chlorophyllin (Cu-Chl). Cu-Chl was prepared by extract leaves of *Stevia rebaudiana* and Mg-Chl was prepared by extract fresh spinach leaves with acetone (**Bobbio and Guedes 1990**; **Wohllebe *et al*. 2011**). Simply add 1 mg $CaCO_3$/g plant, filter the extract, add petroleum benzene, shake, separate the two formed phases using a separator funnel, add 1 ml of methanolic KOH/50 ml of benzene phase, then chlorophyll is found as water-soluble chlorophyllin in the KOH phase.

Grafting of nanomaterial with Chlorophyllin extract

Both silver (Ag) and graphene oxide (GO) nanomaterial were selected to be used in the formation of the natural extract porphyrin-based photosensitizer nanocomposite.

The electrostatic deposition method was used for grafting the two photosensitizers over the two nanomaterials to form the required nanocompsites. Tens of experimental trials were preceded with different concentrations and conditions to achieve the optimum and appropriate method needed to achieve the desired formula. At the end of each trial, the formula was fully characterized to select the best one. Finally, four natural extracts of prophyrin-based photosensitizer nanocomposites were prepared [Mg-Chl /Ag nanocomposite (MgAg), Mg-Chl/GO nanocomposite (MgGO), and Cu-Chl/Ag nanocomposite (CuAg) and Cu-Chl/GO nanocomposite (CuGO)]. Some insect attraction materials (Pheromones) were added to the composite, just to attract the insects to the formula deposition sites.

Experimental fish

A total of 390 freshwater catfish (*Clarias gariepinus*) weighing (80-100 gm) were purchased from a private fish farm in Kafr El-Sheikh and transported alive to the laboratory. Fish were acclimatized for the laboratory aquaria conditions with a continuous flow of de-chlorinated and aerated water for two weeks, then they were randomly divided into thirteen groups with ten fish per each (X three replicates). The first twelve groups were received four different insecticide compounds (MgAg, MgGO, CuAg, and CuGO) at three different concentrations per each of them ($1=10^{-2}$, $2=10^{-3}$, and $3=10^{-4}$ ml/L). The thirteenth group was the control one which received normal water without any composites. All groups were subjected to sunlight for four hours, then remained in the laboratory conditions for two weeks.

Determination of *96h-LC_{50}*

The number of dead fish per group was recorded for 4 successive days. LC_{50} was calculated for each composite according to **Busquet *et al*. (2014)**.

Water analysis

Some physicochemical parameters of the water were determined according to **APHA (1995)**: Chemical Oxygen Demands (COD), Biological Oxygen Demand (BOD), pH, Electric Conductivity (EC), Carbonate (CO_3), Bicarbonate (HCO_3), Chlorine (Cl), Sulphate (SO_4), Ammonium (NH_4), Nitrate (NO_3), Calcium (Ca^{+2}), Magnesium (Mg^{+2}), Sodium (Na^+) and Potassium (K^+).

Blood specimens

Blood samples were collected from the caudal vein of fish by a 3ml syringe and divided into two parts, one collected in anticoagulant EDTA tubes for the hematological measurements and the other part collected in clean glass tubes, centrifuged for 15 min. at 3000 rpm to collect the serum stored in -20^{o}C for biochemical assays.

Hematological assays

Red blood cells (RBCs) and white blood cells (WBCs) were counted manually using an improved Neubauer hemocytometer (**Natt and Herrick 1952**). Hemoglobin concentration (Hb) was recorded using a spectrophotometer according to the cyanmethemoglobin method (**Drabkin 1948**). Hematocrit (Hct %) was determined using heparinized capillary tubes that were centrifuged in a microhematocrit centrifuge. The red blood indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC)] were calculated using Hct, Hb, and RBC measurements.

Biochemical assays

Serum total proteins and albumin (**Elfadaly *et al*. 2012)**, globulin; by subtracting albumin concentration from total protein concentration, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (**Reitman and Frankel 1957**), uric acid and creatinine (**Tietz 1990**), cholesterol (**Abu El Ezz *et al*. 2011**) and glucose concentrations (**Caraway and Watts 1987**) were estimated using commercial biochemical kits (Spectrum-diagnostics, EGYPT). The endpoint of the biochemical reactions was detected according to the kit instructions using a spectrophotometer (AGILENT CARY 100/300 Series UV-Vis, UNITED STATE).

Statistical analysis

Results are presented as means ± SE. Significant differences in the measured values between the control and experimental groups were determined by a one-way ANOVA test (**Elfadaly *et al*. 2018**). All statistical analyses were performed using a computer program of MSTAT (version 17.0 for Windows) at $p<0.05$.

RESULTS

96h-LC_{50} of nano-composites

The highest LC_{50} value was recorded in MgAg nano-composite which was 9.2µl/l, followed by MgGO nano-composite; 0.9µl/l. While CuAg and CuGO nano-composites showed no fish died during the experiment (Table 1).

Table 1**:** 96-h LC_{50} of some new natural nano-pesticides on *Clarias gariepinus*.

Compound	96-h LC_{50} (µl/l)
MgAg	9.2
MgGO	0.9
CuAg	0.0
CuGO	0.0

MgAg: Mg-Chlorophyllin/Ag nanocomposite, MgGO: Mg-Chlorophyllin/GO nanocomposite, CuAg: Cu-Chlorophyllin/Ag nanocomposite and CuGO: Cu-Chlorophyllin/GO nanocomposite.

Physicochemical analysis of water

Significant increases (*P*<0.05) in both COD and BOD values were recorded in all groups when compared to the control group. The highest values were belonged to the higher concentrations of the four compounds, while their values in the third dilution groups of CuAg and CuGO are lower than the safe permissible levels estimated by **FAO (1985)** (Table 2). Although pH values of water exposed to the different used nano-composites were significantly increased in MgAg1, 2 and CuAg1, they were under the permissible limits estimated by **USEPA (1994)**. Generally, values of EC, Mg^{+2}, Na^{+}, K^{+}, HCO_3^{-}, Cl^{-} and P^{+3} were significantly increased (*P*<0.05) in all groups, compared to the control group, except for the values in CuGO compound that showed insignificant changes (*P*>0.05) in EC, K^{+}, HCO_3^{-}, Cl^{-} and P^{+3} values. On the other hand, Ca^{+2} and SO_4^{-2} values were significantly decreased (*P*<0.05) in almost all groups. It is worth here mentioning that all values of EC, Mg^{+2}, Na^{+}, K^{+}, HCO_3^{-}, Cl^{-}, P^{+3}, Ca^{+2}, and SO_4^{-2} are under the permissible limits of **FAO(1985)**, **USEPA (1994)**, and **WHO (1996)** (Table 2). Concerning NH_4 and NO_3^{-} values, there was a highly significant increase (*P*<0.05) in all groups, except for NH_4 values in CuGO compound, that were insignificantly affected (*P*>0.05). Moreover, the values of NH_4 and NO_3^{-} were also higher than the permissible limits of **FAO (1985)** (Table 2).

Haematological results

There was a slight significant decrease (*P*<0.05) in RBCs count, Hb, and Hct % in *C. gariepinus* that were exposed to MgAg1, 2, and MgGO1, compared to the control group (Table 3). Other groups showed insignificant changes (*P*>0.05) in the hematological parameters compared to the control. On the other hand, *C. gariepinus* showed a significant increase (*P*<0.05) in WBCs in groups that were exposed to MgGO and CuAg and only the highest concentration of CuGO (Table 3). While exposure to MgAg compound showed a contrary significant decrease (*P*<0.05) in WBCs.

Biochemical results

Concerning liver function enzymes of *C. gariepinus* after two weeks of exposure to four different natural nano-composites at three dilutions, ALT was significantly increased (*P*<0.05) by about 1.3 and 1.7 fold in the highest concentrations of MgGO and CuAg compounds respectively, compared to control group. While there were insignificant changes (*P*>0.05) in ALT values in MgAg and CuGO nano-composites (Table 4). AST was also significantly increased (*P*<0.05) after exposure to all compounds, especially at their highest concentrations. The highest AST increases were recorded in MgGO1 and 2 (about 2.5 fold), followed by 2.4, 2.2, and 1.8 folds of increase in CuGO, MgAg, and CuAg respectively compared to the control group. On the other hand, only the third dilution in both CuAg and CuGO nano-composites showed insignificant changes (*P*>0.05) in AST values (Table 4).

Table 2: Physicochemical analysis of water samples after exposure to four natural nano-pesticides (each at three concentrations).

Compound	COD (mg/L)	BOD (mg/L)	pH	EC (dSm-1)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)
MgAg1	411.8±4.3[b]	222.3±8.4[b]	8.7±0.3[a]	1.1±0.0[cde]	2.5±0.1[cd]	1.4±0.15[def]	4.3±0.16[c]	1.4±0.15[f]
MgAg2	509.5±3.2[a]	287.5±3.2[a]	7.0±0.0[e]	1.7±0.1[ab]	4.3±0.1[a]	2.9±0.24[a]	7.1±0.05[a]	2.2±0.15[b]
MgAg3	104.0±0.4[e]	62.50±1.7[c]	7.4±0.1[cd]	1.4±0.1[bcd]	3.7±0.1[b]	2.0±0.20[b]	4.6±0.21[b]	2.6±0.10[a]
MgGO1	115.9±0.8[d]	57.5±0.6[c]	7.3±0.2[cde]	1.5±0.3[bc]	1.8±0.1[fg]	1.7±0.07[bcd]	2.3±0.08[gh]	1.6±0.09[ef]
MgGO2	116.6±1.0[d]	61.5±0.8[c]	7.2±0.0[cde]	1.5±0.3[bc]	2.5±0.1[cd]	1.9±0.03[b]	2.7±0.04[ef]	1.7±0.1[de]
MgGO3	86.0±0.5[g]	43.3±0.6[de]	7.5±0.1[bc]	1.3±0.2[bcde]	2.2±0.2[def]	1.9±0.03[b]	2.4±0.04[fg]	1.6±0.08[de]
CuAg1	96.9±1.1[f]	48.5±0.5[d]	7.8±0.1[b]	1.4±0.2[bc]	2.3±0.1[de]	1.7±0.05[bcd]	2.2±0.11[gh]	1.7±0.04[de]
CuAg2	100.0±0.4[ef]	62.0±2.5[c]	7.3±0.1[cde]	2.1±0.2[a]	2.0±0.2[efg]	1.5±0.1[cde]	3.3±0.12[d]	1.8±0.11[cd]
CuAg3	56.9±0.5[i]	28.3±0.8[f]	7.3±0.1[cde]	1.8±0.2[ab]	2.3±0.0[de]	1.9±0.05[b]	3.0±0.03[e]	2.0±0.07[bc]
CuGO1	116.2±0.8[d]	61.0±0.8[c]	7.4±0.1[cde]	1.0±0.2[cde]	2.4±0.2[cde]	0.7±0.03[g]	1.6±0.08[i]	0.3±0.01[h]
CuGO2	160.7±0.3[c]	64.5±1.0[c]	7.1±0.1[de]	0.8±0.1[e]	1.7±0.1[g]	1.8±0.1[bc]	1.8±0.06[i]	0.4±0.02[h]
CuGO3	74.13±0.4[h]	38.3±3.3[e]	7.1±0.0[de]	0.9±0.1[de]	2.7±0.2[c]	1.1±0.08[f]	2.1±0.03[h]	0.7±0.04[g]
Control	27.6±0.6[j]	19.0±1.3[g]	7.4±0.1[cd]	0.8±0.1[e]	3.4±0.2[b]	1.3±0.1[ef]	1.2±0.13[j]	0.2±0.02[h]
Permissible concentration	80	40	6.5-8.5	0.7-3.0	100	30	200	20

Compound	CO_3 (mg/L)	HCO_3 (mg/L)	Cl (mg/L)	SO_4 (mg/L)	NH_4 (mg/L)	NO_3 (mg/L)	P (mg/L)
MgAg1	nd	6.00±0.20[cd]	3.25±0.14[b]	0.60±0.08[gh]	208.5±2.90[b]	39.92±1.49[e]	30.50±0.65[d]
MgAg2	nd	7.20±0.12[b]	4.25±0.14[ab]	4.65±0.25[b]	146.0±16.99[ef]	39.25±1.11[e]	30.13±1.01[d]
MgAg3	nd	6.83±0.12[bc]	3.40±0.14[b]	2.53±0.19[c]	96.5±2.25[g]	41.0±1.58[e]	30.50±0.65[d]
MgGO1	nd	5.50±0.35[d]	1.70±0.12[gh]	0.95±0.03[f]	135.68±5.1[f]	262.75±3.3[a]	32.35±0.46[d]
MgGO2	nd	5.23±0.13[d]	2.43±0.15[cd]	1.50±0.04[e]	188.23±4.71[c]	176.30±2.19[b]	31.73±0.76[d]
MgGO3	nd	6.00±0.20[cd]	2.15±0.06[de]	0.24±0.02[i]	172.65±4.36[cd]	164.98±2.04[c]	22.98±2.05[e]
CuAg1	nd	5.72±0.08[cd]	2.04±0.02[ef]	0.53±0.17[ghi]	156.15±7.45[de]	139.33±0.92[d]	36.00±0.82[c]
CuAg2	nd	14.0±1.29[a]	2.53±0.09[c]	7.35±0.12[a]	280.90±4.46[a]	264.33±1.73[a]	93.33±1.16[a]
CuAg3	nd	6.05±0.18[cd]	2.38±0.11[cd]	0.86±0.02[fg]	176.15±1.66[c]	164.73±1.84[c]	39.55±1.74[b]
CuGO1	nd	3.15±0.09[e]	1.50±0.08[h]	0.50±0.08[hi]	38.55±1.69[h]	23.48±1.18[g]	11.98±0.41[f]
CuGO2	nd	3.37±0.12[e]	1.73±0.11[fgh]	0.68±0.05[fgh]	35.23±0.85[h]	30.03±1.61[f]	13.00±0.82[f]
CuGO3	nd	3.23±0.09[e]	2.39±0.12[cd]	0.72±0.01[fgh]	30.23±0.11[h]	31.75±0.64[f]	11.93±0.40[f]
Control	nd	2.58±0.22[e]	2.00±0.08[efg]	1.85±0.12[d]	25.08±2.33[h]	3.40±0.64[h]	11.78±0.24[f]
Permissible concentration			250	250	0-5	5-30	

MgAg: Mg-Chlorophyllin/Ag nanocomposite, MgGO: Mg-Chlorophyllin/GO nanocomposite, CuAg: Cu-Chlorophyllin/Ag nanocomposite and CuGO: Cu-Chlorophyllin/GO nanocomposite
1: 10^{-2} ml/l, 2:10^{-3} ml/l, 3:10^{-4} ml/l. nd= not detected
Means with the same letter within the same column are not significantly different *at P*>0.05. Data are represented as mean ± SE (standard error).

Table 3: Haematological analysis of *Clarias gariepinus* after exposure to four natural nano-pesticides (each at three concentrations) for two weeks.

Compound	**RBCs X 10^6/µl**	**Hb (g/dl)**	**Hct (%)**	**MCV (fl)**	**MCH (pg)**	**MCHC (%)**	**WBCs X 10^3/µl**
MgAg1	1.01 ± 0.05^{d}	11.73 ± 0.30^{e}	31.07 ± 0.32^{f}	410.2 ± 17.6^{a}	116.48 ± 2.6^{ab}	28.56 ± 0.7^{d}	32.29 ± 0.34^{ef}
MgAg2	1.31 ± 0.03^{bc}	11.73 ± 0.30^{de}	32.86 ± 0.71^{def}	289.8 ± 06.4^{cde}	89.92 ± 3.3^{de}	31.01 ± 0.7^{cd}	23.79 ± 0.45^{h}
MgAg3	1.20 ± 0.02^{cd}	12.66 ± 0.39^{ab}	31.30 ± 0.67^{f}	345.0 ± 05.7^{abc}	105.78 ± 3.6^{bcd}	30.76 ± 1.4^{cd}	18.86 ± 0.34^{i}
MgGO1	1.07 ± 0.09^{d}	11.77 ± 0.11^{de}	32.01 ± 1.71^{abc}	363.7 ± 37.6^{ab}	119.00 ± 9.2^{ab}	33.50 ± 1.8^{bcd}	38.29 ± 0.42^{c}
MgGO2	1.22 ± 0.10^{cd}	12.10 ± 0.17^{bcde}	33.27 ± 1.13^{bcd}	377.9 ± 41.7^{ab}	125.72 ± 12.9^{a}	33.53 ± 1.1^{bcd}	34.43 ± 0.48^{de}
MgGO3	1.20 ± 0.08^{cd}	11.97 ± 0.26^{bcde}	31.81 ± 2.37^{def}	325.5 ± 27.8^{bcd}	124.93 ± 6.8^{ab}	38.84 ± 2.8^{ab}	35.29 ± 0.78^{d}
CuAg1	1.28 ± 0.02^{bc}	11.79 ± 0.13^{cde}	31.07 ± 2.72^{ef}	244.3 ± 23.3^{efg}	92.92 ± 2.6^{de}	40.04 ± 3.7^{a}	38.86 ± 1.16^{c}
CuAg2	1.43 ± 0.07^{ab}	12.34 ± 0.27^{bcde}	34.65 ± 2.50^{bcdef}	248.1 ± 26.3^{efg}	88.76 ± 5.2^{de}	37.13 ± 3.5^{ab}	53.57 ± 1.38^{a}
CuAg3	1.49 ± 0.08^{ab}	12.14 ± 0.19^{bcde}	31.00 ± 1.79^{ef}	205.5 ± 17.0^{g}	83.05 ± 4.7^{e}	41.47 ± 2.9^{a}	46.86 ± 1.28^{b}
CuGO1	1.17 ± 0.07^{cd}	12.99 ± 0.18^{a}	32.31 ± 2.27^{cdef}	278.5 ± 20.8^{cdef}	112.41 ± 5.8^{abc}	41.29 ± 2.7^{a}	40.57 ± 2.31^{c}
CuGO2	1.38 ± 0.07^{abc}	12.16 ± 0.25^{bcde}	32.16 ± 1.71^{def}	235.0 ± 11.9^{efg}	89.32 ± 4.2^{de}	38.36 ± 1.9^{ab}	27.86 ± 0.96^{g}
CuGO3	1.56 ± 0.02^{a}	12.41 ± 0.14^{abcd}	34.03 ± 1.97^{bcdef}	217.7 ± 12.1^{fg}	79.55 ± 1.5^{e}	37.24 ± 2.2^{ab}	29.71 ± 0.75^{fg}
Control	1.42 ± 0.15^{abc}	12.49 ± 0.39^{abc}	34.91 ± 0.69^{abcde}	265.3 ± 29.7^{defg}	94.97 ± 11.3^{cde}	35.84 ± 1.3^{abc}	31.00 ± 0.84^{f}

MgAg: Mg-Chlorophyllin/Ag nanocomposite, MgGO: Mg-Chlorophyllin/GO nanocomposite, CuAg: Cu-Chlorophyllin/Ag nanocomposite and CuGO: Cu-Chlorophyllin/GO nanocomposite

1: 10^{-2} ml/l, 2:10^{-3} ml/l, 3:10^{-4} ml/l. Means with the same letter within the same column are not significantly different *at* $P>0.05$.

Data are represented as mean ± SE (standard error).

RBCs: total red blood corpuscular count, Hb: haemoglobin content, Hct%: haematocrit %, MCV: Mean corpuscular volume, MCH: mean corpuscular haemoglobin and MCHC: mean corpuscular haemoglobin concentration, WBCs: total white blood corpuscular count.

Regarding the total protein, albumin, and globulin concentrations in *C. gariepinus* serum, protein, and globulin were significantly decreased ($P<0.05$) in the groups with the highest concentrations of MgAg, MgGO, and CuGO. The lowest protein and globulin concentrations were in MgAg1 group (0.67 and 0.41 fold decrease, respectively). Contrary, serum albumin concentration showed significant increases ($P<0.05$) in all groups except of the third dilution of CuAg and CuGO nano-composites, and the highest albumin concentrations were recorded in the highest concentrations of the used nano-composites (Table 4). Serum glucose level was significantly increased ($P<0.05$) in the highest concentrations of all compounds. The highest fold increase was 2.6 in CuAg1 group, followed by 1.7, 1.3, and 1.1 in MgGO1, MgAg1 and CuGO1 respectively. While cholesterol level was significantly increased ($P<0.05$) in all groups (Table 4). Concerning kidney function analysis, both creatinine and uric acid were significantly increased ($P<0.05$) in MgGO1 and CuAg1 compared to the control group, while the third dilution in all used nano-composites showed a decrease in creatinine and uric acid values or insignificantly affected (Table 4).

Table 4: Biochemical analysis of *Clarias gariepienus* serum after exposure to four natural nano-pesticides (each at three concentrations) for two weeks:

Compound	ALT U/L	AST U/L	Protein g/dl	Albumin g/dl	Globulin g/dl	Glucose mg/dl	Cholesterol mg/dl	Creatinine mg/dl	Uric acid mg/dl
MgAg1	21.87±0.16^{a}	160.01±1.78^{c}	36.94±2.76^{c}	18.60±0.34^{b}	18.34±3.14^{c}	96.98±0.62^{c}	292.22±1.79^{e}	1.85±0.09^{cd}	2.71±0.19^{e}
MgAg2	15.93±0.08^{bc}	115.44±2.81^{e}	49.22±2.73^{ab}	13.51±0.16^{e}	35.71±2.54^{ab}	38.34±0.32^{ij}	274.82±2.71^{e}	1.69±0.05^{d}	3.27±0.16^{c}
MgAg3	14.05±0.26^{cd}	113.39±2.53^{e}	45.19±3.04^{bc}	12.72±0.19^{f}	32.48±2.89^{bc}	54.75±1.16^{gh}	157.08±1.68^{h}	0.78±0.06^{e}	2.85±0.14^{de}
MgGO1	17.81±1.40^{b}	182.89±1.65^{a}	45.62±3.06^{bc}	16.22±0.24^{d}	29.40±2.76^{bc}	125.78±3.18^{b}	186.80±4.45^{g}	2.06±0.17^{bc}	5.01±0.18^{b}
MgGO2	17.68±1.86^{b}	183.43±1.20^{a}	45.88±5.56^{bc}	17.61±0.14^{c}	28.27±3.33^{bc}	81.00±2.38^{d}	228.62±10.8^{f}	0.52±0.04^{f}	3.10±0.15^{cde}
MgGO3	15.33±1.38^{bc}	162.36±1.96^{c}	45.26±4.67^{bc}	12.40±0.18^{f}	32.86±4.03^{bc}	35.98±3.50^{j}	231.21±10.3^{f}	0.65±0.04^{ef}	3.40±0.14^{c}
CuAg1	23.14±1.42^{a}	128.08±1.15^{d}	48.61±2.30^{ab}	16.74±0.26^{d}	31.87±3.86^{ab}	185.64±6.21^{a}	504.96±15.5^{b}	2.51±0.11^{a}	5.80±0.11^{a}
CuAg2	22.86±1.17^{a}	96.74±1.24^{f}	37.20±3.89^{c}	11.33±0.35^{g}	25.87±2.65^{c}	55.11±7.03^{hi}	377.59±8.99^{d}	1.62±0.11^{d}	3.21±0.18^{cd}
CuAg3	12.40±0.94^{cd}	70.88±1.77^{g}	50.13±1.49^{ab}	10.80±0.50^{h}	37.33±2.42^{ab}	70.16±2.54^{ef}	449.28±14.0^{c}	1.67±0.05^{d}	3.41±0.14^{c}
CuGO1	15.14±3.47^{bc}	175.48±2.26^{b}	46.00±2.98^{bc}	21.38±0.38^{a}	24.62±1.99^{bc}	79.51±1.89^{de}	375.99±15.3^{d}	2.27±0.09^{b}	3.40±0.25^{c}
CuGO2	12.97±1.26^{cd}	128.32±1.96^{d}	46.19±1.52^{b}	12.25±0.26^{f}	33.94±1.89^{b}	63.65±2.53^{fg}	537.57±13.3^{a}	2.01±0.05^{c}	3.32±0.14^{c}
CuGO3	9.48±0.88^{de}	76.84±1.99^{g}	56.17±2.57^{a}	10.76±0.20^{h}	37.41±3.09^{a}	53.62±2.77^{gh}	239.07±5.57^{f}	0.61±0.02^{ef}	3.16±0.13^{cd}
Control	13.32±1.68^{cd}	71.77±1.04^{g}	55.16±3.54^{a}	10.55±0.24^{h}	44.60±3.28^{a}	72.64±1.64^{def}	151.55±10.2^{h}	1.71±0.09^{d}	3.10±0.12^{cde}

MgAg: Mg-Chlorophyllin/Ag nanocomposite, MgGO: Mg-Chlorophyllin/GO nanocomposite, CuAg: Cu-Chlorophyllin/Ag nanocomposite and CuGO: Cu-Chlorophyllin/GO nanocomposite
1: 10^{-2} ml/l, 2:10^{-3} ml/l, 3:10^{-4} ml/l.
Means with the same letter within the same column are not significantly different *at P*>0.05.
Data are represented as mean ± SE (standard error).

DISCUSSION

The application of nano-pesticides as a novel technology is growing fast, it enhances pest control through increasing permeability, solubility, and stability (**Abd El-Rahman *et al.,* 2020**). The aquatic environment represents the final fate of such natural nano-pesticides, so there is a necessity to evaluate its use and effect on both water characters and the life of aquatic non-targeted living organisms, especially fish. To the best of our knowledge, there weren't any previous literature discussing the effect of natural nano-pesticides on water characteristics and its ionic composition.

The ionic composition of water is the main contributor to total hardness and is very important for fish life. The concentration of divalent cations; $Ca^{2+}$and Mg^{2+}, affect many vital functions in fish; branchial permeability, ionic regulation, and growth. Also, numerous studies have reported that water hardness has significant influences on fish fertilization, hatching, and growth (**Luo *et al.* 2016**). Although the present study revealed significant increases in the electric conductivity (EC) of water in some groups that were subjected to natural nano-pesticides, this was under the permissible limits of water. **Bhattacharyya *et al*.. (2010)** proved that nanoparticles exhibited novel properties such as high EC and increased chemical reactivity. Also, the study recorded that all values of EC, Mg^{+2}, Na^{+}, K^{+}, HCO_3^{-}, Cl^{-}, P^{+3}, Ca^{+2}, and SO_4^{-2} are under the permissible limits of **FAO (1985)**, **USEPA (1994)**, and **WHO (1996)**. This may enhance using of such novel natural pesticides since they don't affect the ionic composition of water. On the other hand, biological oxygen demand and chemical oxygen demand (BOD and COD) were increased in water in the present study. Generally, adding chlorophyllin to water bodies participate in increasing the concentration of suspended phytoplankton and organic matter, which in turn increases both BOD and COD values. Also, the basic chemical structure of chlorophyll is a porphyrin ring which composed of four pyrrole groups (C_4H_5N) (**Chen *et al*. 2016**), which may explain the augmentation of NO_3 and NH_4 concentrations of water. Furthermore, the elevation in BOD, COD, $NO_{3,}$ and NH_4 values in the present work was found to be dependent on nano-composites concentrations, so the study recommends using such novel natural nano-pesticides in diluted concentrations.

Hematological and biochemical parameters represent good biomarkers that reflect the effect of any external stress factor on the health status of fish. Some of the nanomaterials have toxic adverse effects on fish health that is more or less comparable to the effects of metals in their ionic form (**Shaw and Handy 2011**). The slight decrease in RBCs count, Hb and Hct % and increase in WBCs count in *C. gariepinus* after exposure to chlorophyllin/GO nanocomposites (MgGO and CuGO) especially at the highest concentrations in the present study was compatible with **Paital *et al.* (2019)** who also revealed a reduction in both total RBCs count and hemoglobin concentration and an increase in WBCs count of *Anabas testudineus* fish after injected with different doses of nano GO. The negative charge of the GO surface may represent a good explanation of RBCs and Hb reduction because of the positive charge of Fe which is the main structural unit of Hb (**Paital *et al*. 2019**), also the reduction in serum protein level in the present study in MgGO and CuGO groups may be returned to the same theory since protein has a positive charge too. Concerning the significant increase in ALT and AST activities in *C. gariepinus* subjected to MgGO and CuGO nano-composites, especially at higher concentrations, it may be returned to the toxic effect of nano-GO on liver cells. This conformed with a previous study that reported cell rupture, vacuolation, and necrosis in zebrafish (*Danio rerio*) hepatocytes after exposure to nano-GO (**Souza *et al*. 2017**). Regarding the exposure to chlorophyllin/Ag nanocomposites (MgAg) in the present study, a significant decrease in RBCs count, Hb, Hct% and WBCs count, especially at high concentration may be returned to the toxic effect of Ag nanoparticles. This result was in accordance with **Khosravi-Katuli *et al.* (2018)** who reported adverse effects of nano Ag on the health status of juvenile common carp (*Cyprinus carpio*). Ag nanoparticles easily penetrate and bio-accumulate in different tissues and cells causing damage, biochemical and histological alterations, and destroying many physiological functions (**Ale *et al.* 2018**). Moreover, Ag Nanoparticles (NPs) can enter through the cell membrane causing mitochondrial dysfunction and impairing liver and gill functions (**Bacchetta *et al*. 2017**). Also, hepatotoxicity has been demonstrated in zebrafish after exposure to Ag-NPs that induced oxidative stress, apoptosis, and DNA damage (**Choi and An, 2008**).

Regarding using of chlorophyllin as a natural pesticide, chlorophyllin represented a promising new safe strategy to control many pests and parasites, for example, *Fasciola gigantica* larvae in different wavelengths of visible light (**Singh and Singh 2015**). While the natural pesticide may alter the function of some fish enzymes and excessive doses of such natural pesticides have toxic effects on aquatic organisms as the chemical pesticides (**Alak *et al*. 2017**). In a previous laboratory study, chlorophyllin didn't cause any adverse effects on adult fish (**Wohllebe *et al*. 2009**). While advanced studies they were reported adverse effects of chlorophyllin on daphnia and fish larvae (**Wohllebe *et al*. 2011**). This may be attributed to the chemical structure of chlorophyll derivatives and their ability to penetrate the cell and cause toxicity. Also, **Azizullah and Murad (2015)** highlighted the possible risk of chlorophyll derivatives to non-target organisms. So application of new technologies to introduce an effective novel natural nano-pesticide in the environment needs more and more studies to ensure its safety in the surrounding environment.

CONCLUSION

The results of the present study represent a guide of the eco-toxicological studies to the application of some novel natural nano-pesticides in the environment. The study reported that using these nano-composites doesn't affect the ionic composition of the surrounding water, while they increase BOD, COD, NH_4, and NO_3 values in the high concentrations of such nano-composites. Also, the study stated that the third diluted concentration of both CuAg and CuGO nano-composites was the safest on *C. gariepinus* health. Hence, the study recommends the careful use of Cu chlorophyllin nano-composites by applying them in their diluting concentrations.

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