



Comparative Assessment of Single and Joint Toxicity of Bulk and Nano Scale Heavy Metals (Al_2O_3 , CuO and SiO_2) Using Haematological Parameters as Biomarker in Juvenile African Catfish (*Clarias gariepinus*)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJFAR/2018/v2i330013

Editor(s):

(1) Dr. Pinar Oguzhan Yildiz, Assistant Professor, Department of Food Engineering, The Faculty of Engineering, Ardahan University, Turkey.

Reviewers:

(1) Idopiseabasi E. Asuquo, Akwa Ibom State University, Nigeria.

(2) Aysel Şahan, Cukurova University, Turkey.

Complete Peer review History: <http://www.sdiarticle3.com/review-history/45914>

Original Research Article

Received 29 October 2018
Accepted 24 January 2019
Published 28 February 2019

ABSTRACT

Global technological advancement has resulted in the gradual replacement of traditional bulk size compounds with nano-sized ones with diverse useful characteristics. This study is focused on the toxicological assessment of three nano-metallic oxides (SiO_2 , Al_2O_3 and CuO) in comparison with their equivalent bulk sizes using African Catfish (*Clarias gariepinus*). The study involved sub-lethal exposures to binary mixtures of selected ratios (1:1; 2:1 and 1:2) of the respective metallic bulk and nano oxides as well as their triple mixture (1:1:1) for 28 days. The catfishes after 28 days were subsequently subjected to haematological tests (Haemoglobin, Packed Cell Volume, Red blood cells, Platelets, Neutrophils, Lymphocytes, Mean cellular volume, Mean Cell haemoglobin, and Mean Cell haemoglobin Concentration). The results of the haematological assessment indicated that most of the nano-sized oxides showed no significant difference ($P>0.05$) compared to control whereas the bulk sizes either showed no difference or decrease in the count compared to control.

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In most of the binary mixtures, catfishes exposed to bulk sizes had lower levels of the haematological parameters but there was no overall trend. The introduction of nanoscale metallic oxides into production activities should be with caution since they may have a similar mode of toxicity to the existing bulk sizes.

Keywords: Nano-toxicology; haematology; biomarker; heavy metals; joint toxicity.

1. INTRODUCTION

The excessive contamination of aquatic ecosystems has evoked major environmental and health concerns worldwide [1]. These pollutants including nanoparticles could increase the levels of metals in natural water and seriously affect wetland habitats [2]. Researchers have focused on the sources, fate, transport and toxicity of engineered nanomaterials over the past decade [3]. The increasing use and production of nano-based materials have led to the contamination of the environment with nanomaterials [4]. Nanoparticle-based consumer products such as cosmetics, creams and detergents are an important source of nanomaterials in the environment, and discharges are assumed to increase with the development of nanotechnology [5].

Silica nanoparticles (SiO_2NPs) have been widely used in nanomedicine mainly in biomedical applications [6]. SiO_2 is the most abundant compound in the earth's crust and over one million tons of the compound are used annually in consumer products and much of those are washed down the drains into the aquatic environment [7]. SiO_2NPs could accumulate in liver, lung and spleen tissues, and potentially damage liver functions [8]. Copper nanoparticles (CuNPs) are used in textiles, food storage containers, home appliances, paints, and dietary supplements [9]. Because of their exclusive specifications, such as their small size, proportion of the surface to high volume ratio, and more activities, much attention has been paid to them [10]. For instance, Cu-NPs at 100 mg/L have been observed to inhibit the growth of juvenile carp [11]. Al_2O_3 NPs are the most abundantly produced nanomaterial [12]. Al_2O_3 NPs have been used in diverse fields for medical, military, and industrial purposes [13]. It has been demonstrated that the exposure to Al_2O_3 NPs causes cytotoxic effects [14]. One of the routes of NPs exposure to the environment is through effluents [15]. Additional routes of environmental exposure are spills from production, transport, and disposal of the nanomaterial or its products [16]. Nano-

remediation is one of the most promising environmental nanotechnologies, however, the use of nanoparticles in environmental remediation may lead to the release of nanoparticles into the environment and they may cause deleterious effects to the aquatic ecosystem [17]. For example, NPs have been found to induce oxidative stress in juvenile largemouth bass [18].

The physiological conditions arising from stress factor on fish health can be determined through analysis of blood samples [19]. Blood acts as an impressive tool for detection of alterations in organisms [20]. The most common haematological variables measured during stress include Red and White Blood Cells count, haemoglobin content, hematocrit value, and red blood cells indices [21].

Nigeria is positively affected by potential opportunities of nanotechnology as it helps address critical international developmental priorities which include water purification system, energy system, medicine, pharmaceuticals, food production and nutrition, information, and communication technology. As a result of the growing nanotechnology applications, many nano-materials are discharged into the aquatic habitats that affect their biota. This makes it imperative to assess the environmental risks associated with the applications of such materials [22]. The most prominent way nanoparticles may enter the Nigerian environment is as a by-product of industrial production, where they can be transferred as industrial waste, through either the air or fluid waste streams. Other sources of nano-pollution are consumer products, biological excretion, and destruction of nanoparticle-containing infrastructure. These nanoparticles have deleterious effects on aquatic life including fish even at minimal doses and finally affect or modulate the physiological processes. Therefore, it is pertinent to evaluate the various sub-lethal effects of these particles in terms of their potential to pose risks to wetland and the larger environment and make tangible comparisons with the bulk of such materials in

order to regulate the application of these materials.

The aim of this study was to determine the haematological effects in *Clarias gariepinus* exposed to single and mixtures (binary and triple mixtures) of three nano scale heavy metals and their bulk alternatives. The objectives were to determine the:

- Relative levels of White Blood Cell indices (WBC count, neutrophils and lymphocytes) in blood of *Clarias gariepinus* exposed to bulk and nano sizes as in a - e below and show different levels of significant difference when compared with the control group.
 - Relative levels of Red Blood Cell indices (Hbs, PCVs, RBC, MCVs and MCHs counts) in blood of *Clarias gariepinus* exposed to bulk and nano sizes as in a - e below and show different levels of significant difference when compared with the control group.
 - Relative levels of Platelet count in blood of *Clarias gariepinus* exposed to bulk and nano sizes as in a - e below and show different levels of significant difference when compared with the control group.
- (a) Single compounds (SiO_2 , Al_2O_3 and CuO)
 - (b) Double compounds of SiO_2 and Al_2O_3 in different ratios (1:1; 2:1 and 1:2)
 - (c) Double compounds of SiO_2 and CuO in different ratio (1:1; 2:1 and 1:2)
 - (d) Double compounds of Al_2O_3 and CuO in different ratio (1:1; 2:1 and 1:2)
 - (e) Triple compounds of SiO_2 , Al_2O_3 and CuO in ratio (1:1:1) for 28 days.

2. MATERIALS AND METHODS

2.1 Experimental Chemicals and Preparation of Test Solutions

The bulk and nano sizes of three salts (Al_2O_3 , CuO and SiO_2) were procured from Sigma Aldrich. They were stored at room temperatures in the laboratory before use.

Dilutions of the metallic oxides were made using dechlorinated tap water to obtain various concentrations of the test solution. Respective grams of the metallic oxides were dissolved in 1L of the dechlorinated tap water to obtain appropriate mixtures to obtain stock solutions.

Sub-lethal concentrations were selected based on the data from an initial acute toxicity evaluation. The concentrations of the test compounds were prepared as single solutions and mixtures of 1:1, 1:2, 2:1, 1:1:1 both for bulk and nano scale oxides.

2.2 Experimental Organisms: Collection and Acclimatization

The African sharp tooth Catfish/African Catfish, *Clarias gariepinus* was used for this study. It was selected on the basis of ease of culture and known responses to pollutants from existing literature. The fish were procured from a fish farm in Akoka, Lagos and transported in open drums to the Department of Zoology, Laboratory Annex at the Biological Garden. The fish were transported into large holding tanks (100 L capacity) in the laboratory which were half filled with dechlorinated tap water that was left open for 24 hours prior in order to allow for vaporization of excess chlorine. The juveniles fish (Mean Length: 12.3 ± 3.5 cm; Mean weight: 18.52 ± 6.41 g) were employed for the sublethal/chronic toxicity tests. Only fish of the same batches were used for the experiment.

The fish were acclimatized in the lab using the 100 L capacity holding tanks at a stocking density of 20 fish per litre for the fingerlings and 5 fish per litre for the juveniles. The acclimatization lasted for 7 days after which the bioassay commenced. The acclimatization was conducted under standard laboratory conditions (Temp $26.0 \pm 3.0^\circ\text{C}$; humidity $75 \pm 6\%$; Photoperiod, Light: Dark, 12:12 hours). The bioassay media (dechlorinated tap water) was also of suitable quality (pH- 6.8; DO- 8.5 mg/L; Salinity- 0.0 ppt).

2.3 Laboratory Bioassay

2.3.1 Chronic toxicity assay

The fish were employed in the chronic toxicity tests in which they were exposed to respective concentrations of bulk and nano size of the three metallic oxides either singly or in mixtures for 28 days. At the end of the exposure period, blood samples were collected from randomly selected set of three fish per concentration using caudal vein puncture and collected blood were kept in lithium heparin bottle containing anticoagulant before transport to the haematology lab. The fish were further immobilized by spinal puncture and dissected to collect respective organs and

samples were stored in universal bottles and appropriately preserved.

2.3.2 Assessment of haematological effects

To investigate the effect of exposure to the single and joint application of the metallic oxides on the haematological parameters of the fish their Platelet (PLT), Red blood cell (RBC), White blood cell (WBC), and Packed cell volume (PCV) indices were estimated using electronic counters from collected blood samples. Blood-filled heparinized micro-haematocrit capillary tubes were centrifuged at 12000 for 5 min using a micro-haematocrit centrifuge and the haematocrit (HCT) values were read directly. The haemoglobin concentration was measured by the cyan-methaemoglobin method at a wavelength of 540nm. Concurrently, the Total Red Blood Cell (RBC) was obtained by employing the methods described by Dacie [23]. Mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), and mean cell haemoglobin (MCH) were calculated using the following equations: $MCHC = (Hb / PCV) \cdot 100$, $MCH = (Hb/RBCC) \times 10$ and $MCV = (PCV / RBCC) \times 100$ [24].

2.4 Statistical Analysis

The readings obtained were presented as mean \pm SD while analysis of variance (ANOVA) was used to determine significant difference at $P < 0.05$. The significant means were separated using Least Squared Difference (LSD). All statistical analysis and graphical presentations were done using SPSS Version 20 (IBM).

3. RESULTS AND DISCUSSION

3.1 Haematological Effects Observed in African Catfish (*Clarias gariepinus*) Exposed to Single and Joint Action of Sub-lethal Concentrations of Three Metallic Oxides (Al_2O_3 , CuO and SiO_2)

3.1.1 White blood cell (WBC) indices

The results of the WBC counts are shown in Fig. 1(a-e). There was no significant ($P > 0.05$) difference across the particle size and the

compound in exception for bulk particles of SiO_2 and Al_2O_3 which showed significant decrease ($P < 0.05$) and increase in WBC count compared to control respectively. There was no significant ($P > 0.05$) difference across the compound and particles size but the bulk size of SiO_2/Al_2O_3 (1:2) showed clear decrease in the count to control. All the compounds showed clear increase in the WBC count in exposed fish compared to control except bulk size SiO_2/CuO (2:1). There was no significant difference ($P > 0.05$) across the particle size and the compound with the exception of bulk particles Al_2O_3/CuO (2:1) and (1:2) which both showed decrease to control. There was no significant difference ($P > 0.05$) in WBC counts between the two sizes and the control.

The results of the neutrophils counts are shown in Fig. 2(a-e). There was no significant difference ($P > 0.05$) in neutrophils count across the particle size and the compounds compared to control except the bulk size of SiO_2 which showed decreased count and the bulk size of CuO with showed increased count compared to control. There was no distinct significant difference ($P > 0.05$) across the compound and particles size except the nano sizes of SiO_2/Al_2O_3 (1:1 and 1:2) that showed clear significant increase ($P < 0.05$) and the bulk size (1:2) with decrease in the neutrophils count compared to control. They all showed clear significant difference ($P < 0.05$) compared to control with the nano sizes showing increase while the bulk sizes showed decrease except bulk size (2:1) with increase difference (Fig. 6c). No significant difference was observed in neutrophil except in catfishes exposed to bulk sizes of Al_2O_3/CuO (2:1 and 1:2) with decrease in neutrophils count to control. The bulk size showed significantly lower neutrophil count ($P < 0.05$) compared to control (Fig. 6e).

The results of the lymphocytes counts are shown in Fig. 3(a-e). Overall, there was also no clear trend of significant ($P > 0.05$) difference across the particle size and the compounds lymphocytes count to control. No distinct significant ($P > 0.05$) difference in lymphocyte counts was reported across the fish exposed to the three compounds and particles size except the nano sizes of SiO_2/CuO (1:1 and 1:2) that showed clear significant ($P < 0.05$) increase in lymphocytes count to control.

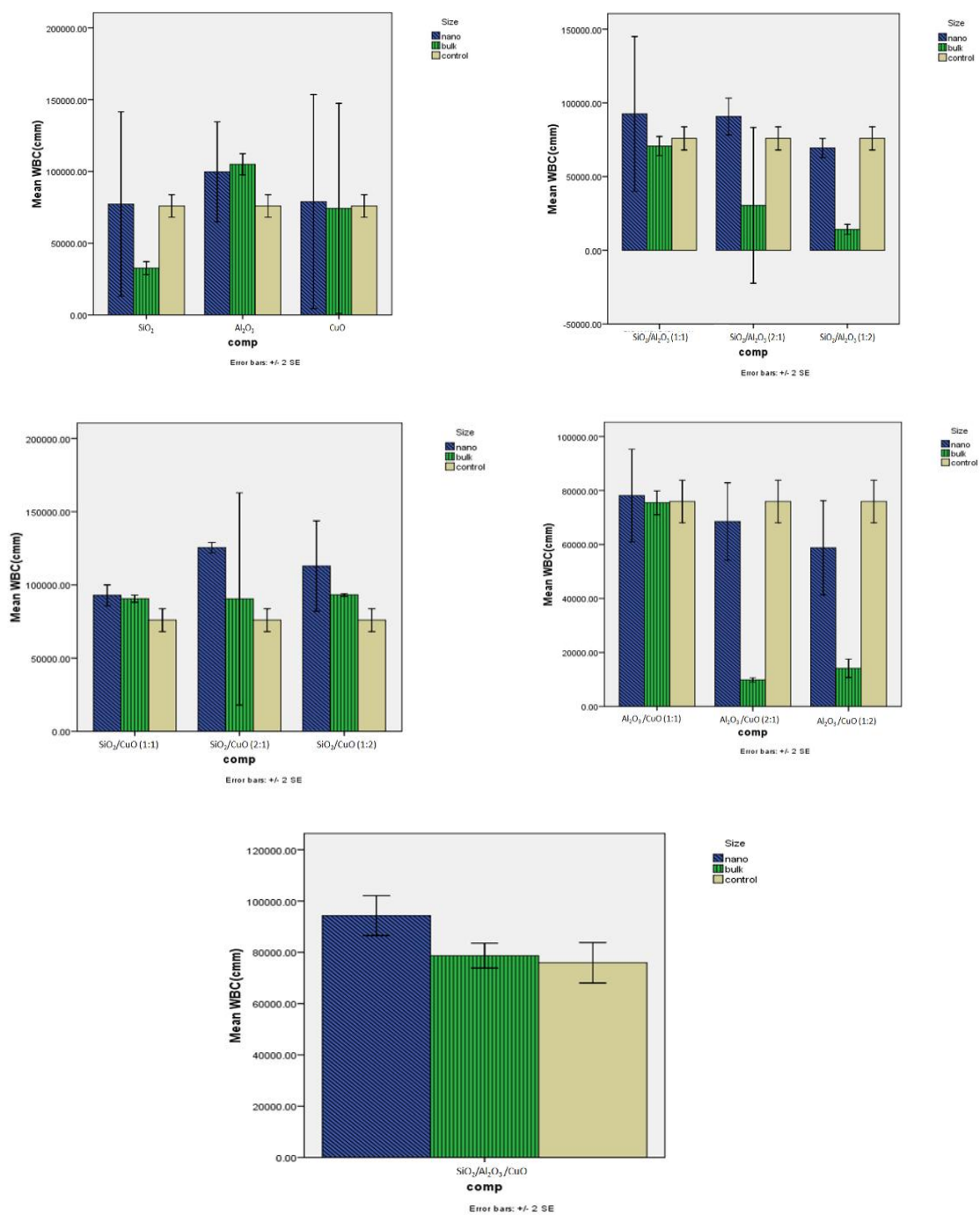


Fig. 1a-e. Relative levels of WBC count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

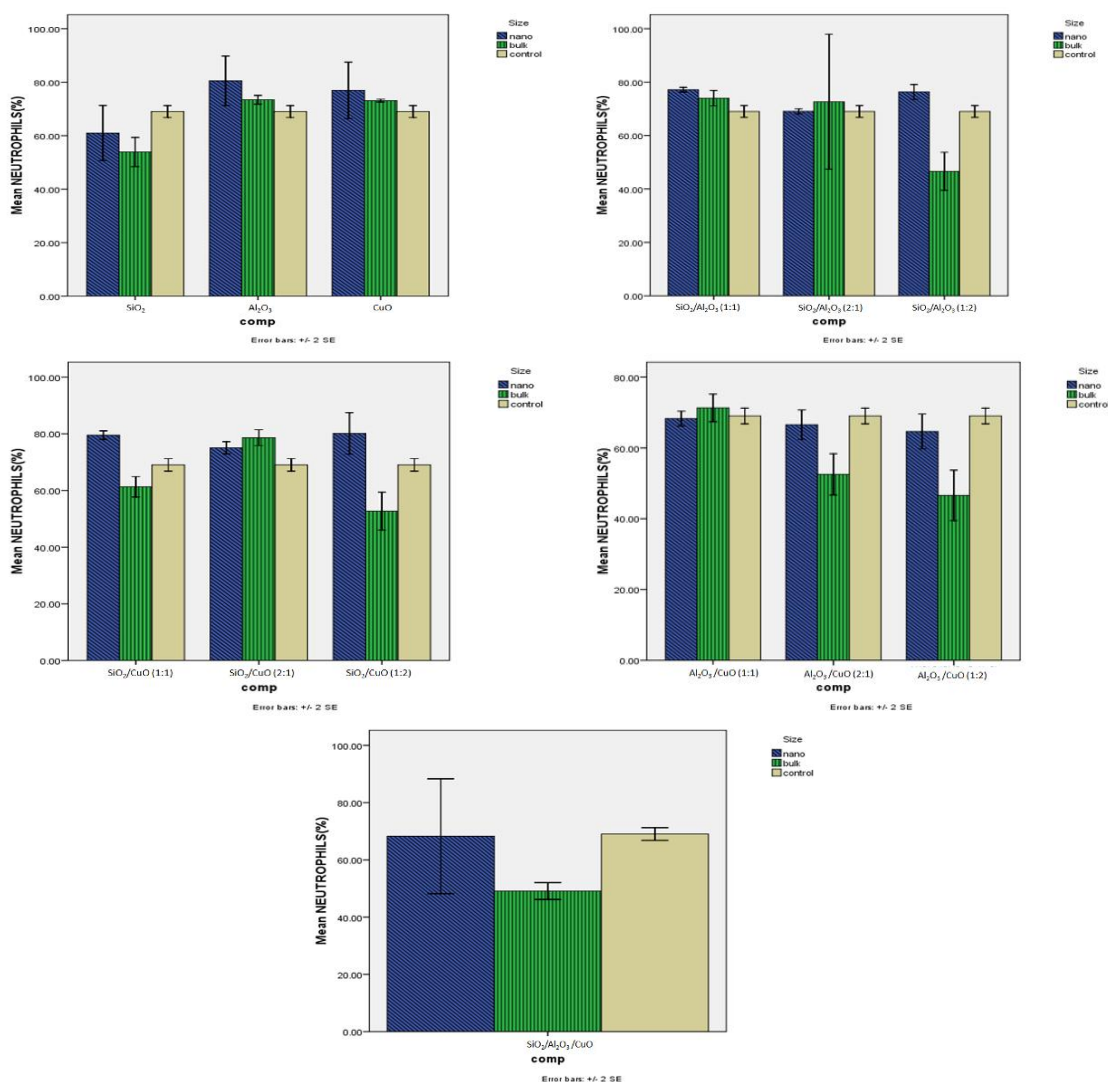


Fig. 2a-e. Relative levels of neutrophils count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

The white blood cell indices which this research focused include neutrophils, lymphocytes and white blood cell (WBC). Some of them showed decrease in the WBC counts as observed in the bulk sizes Al₂O₃/CuO (2:1 and 1:2) and SiO₂/Al₂O₃/CuO for neutrophils, SiO₂/Al₂O₃ (1:2) and Al₂O₃/CuO (2:1 and 1:2) for WBC. This is in accordance with what was reported in *Oreochromis mossambicus*, that the monocytes and neutrophils were reduced in circulation for the elevation of phagocytic activity in affected tissues such as gills, liver and kidneys which

were damaged by copper [25]. This is also corroborated by Remya et al. [26] that reported reduction in WBC level in *Labeo rohita* exposed to iron oxide nanoparticles. Gail et al. [27] opined that increased leucocytes (leucocytosis) count, one of the WBC indices, is a normal reaction of the fish body, against infections of foreign substances, which can alter the normal physiological processes in fish. Wepener [25] noted that white blood cells leave the circulating blood, to protect the body, by moving (ameboid movements) to the infected tissue which results

in reduced cell number in blood which was similar to this research.

3.1.2 Red blood cell (RBC) indices

The results of the Hb counts are shown in Fig. 4(a-e). There was no significant difference ($P>0.05$) in Hb levels across the particle size and the compounds in exposed fishes compared to

control. There was no distinct significant difference ($P>0.05$) across the compound and particles size except the bulk size of $\text{SiO}_2/\text{Al}_2\text{O}_3$ (1:2) that showed clear decrease in the Hb to control. No significant ($P>0.05$) difference except the bulk sizes SiO_2/CuO (1:1) and (1:2) with decrease in the Hb compared to control was observed. The bulk size showed clear significant decrease ($P<0.05$) compared to the control.

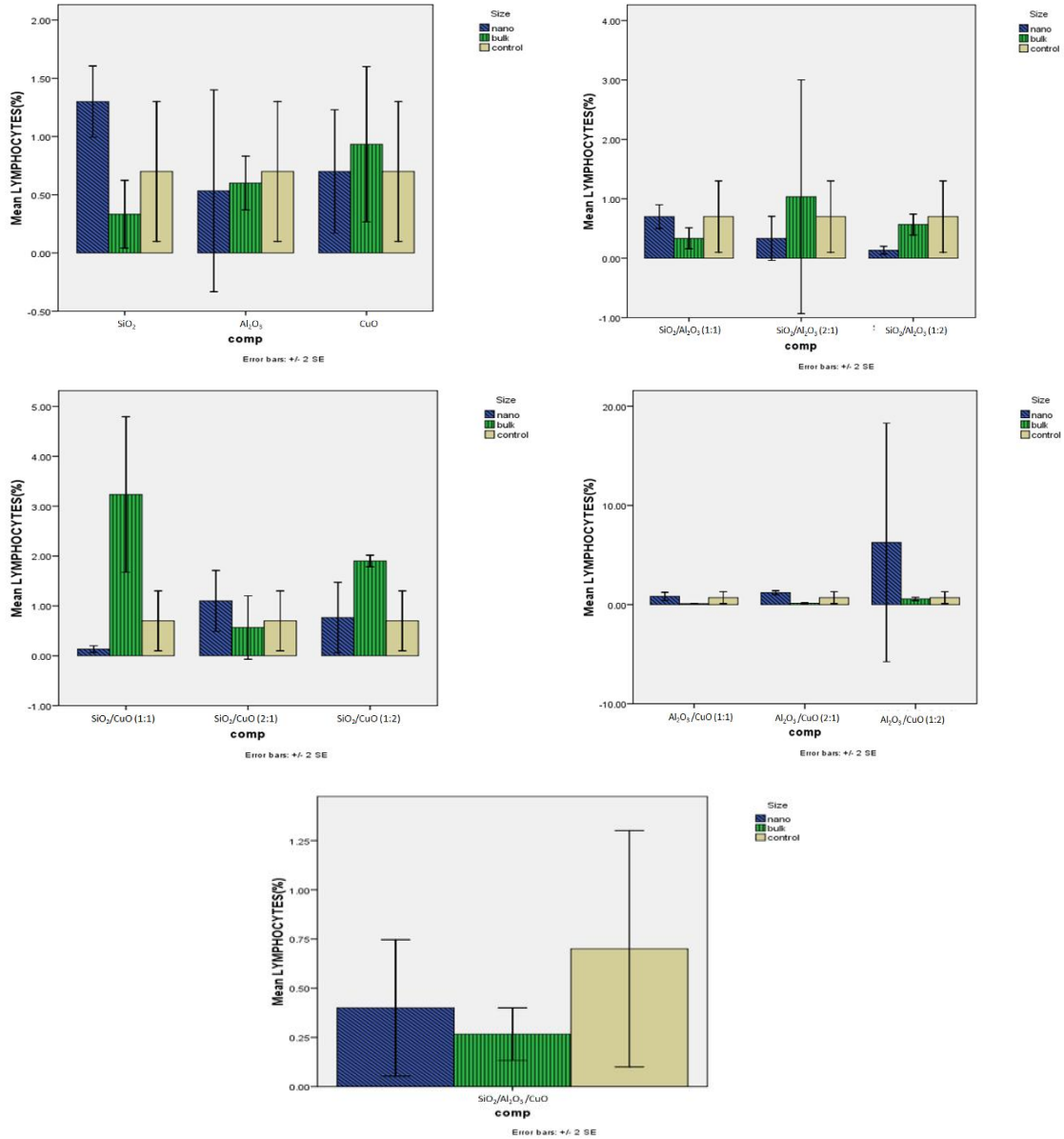


Fig. 3a-e. Relative levels of lymphocytes count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO_2 and Al_2O_3 in different ratio (c) double compounds of SiO_2 and CuO in different ratio (d) double compounds of Al_2O_3 and CuO in different ratio (e) triple compounds of SiO_2 , Al_2O_3 and CuO in different ratio for 28 days

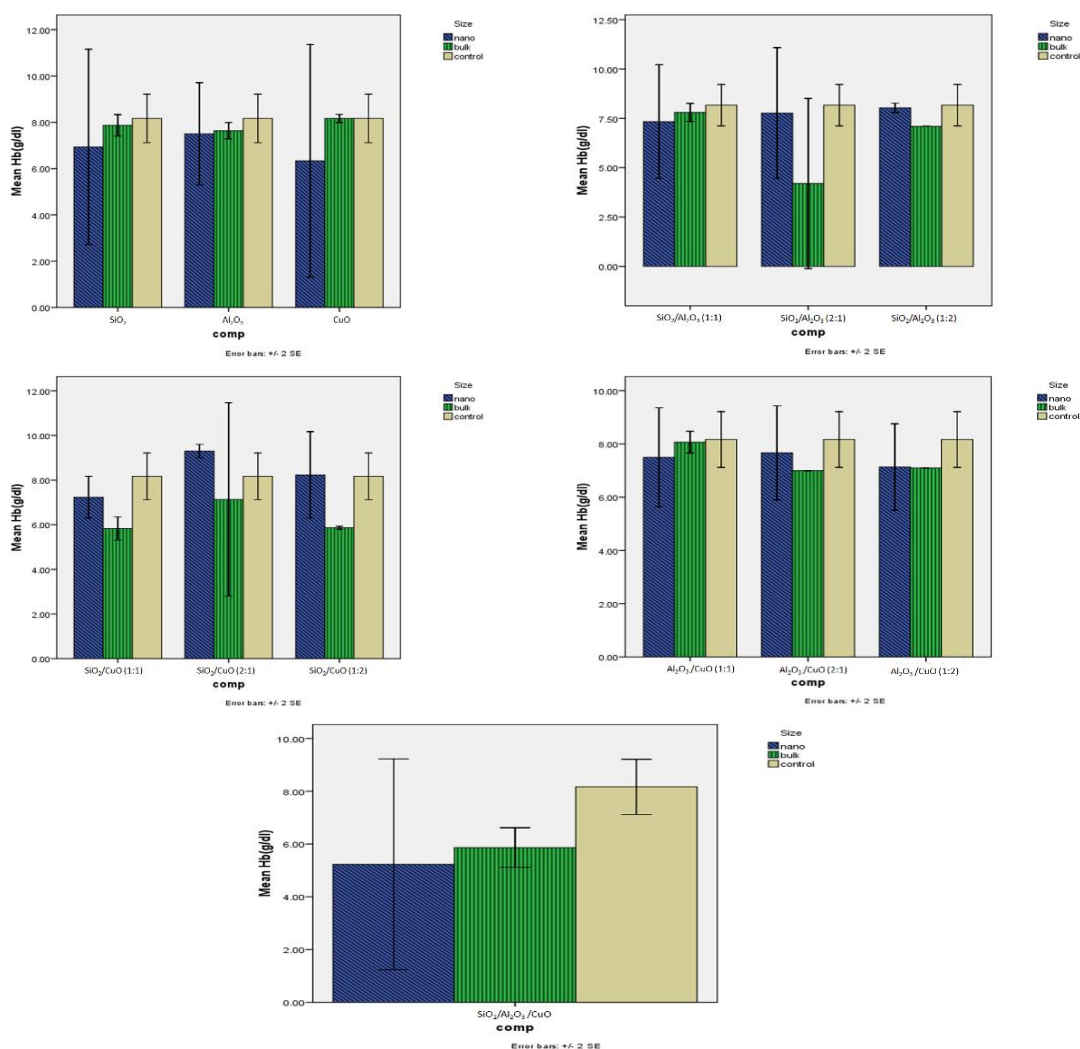


Fig. 4a-e. Relative levels of Hb count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

The results of the PCV counts are shown in Fig. 5(a-e). There was no significant difference ($P>0.05$) across the particle size and the compounds in PCV count to control. In the single action exposure, nano SiO₂ resulted in the lowest levels of PCV across all observed (Fig. 3a). Bulk concentrations of CuO and SiO₂ mixtures resulted in the lowest levels of PCV as well (Fig.3c) in another batch of the study.

The results of the RBC counts are shown in Fig. 6(a-e). There was no significant difference

($P>0.05$) across the particle size and the compounds in RBC count to control. There was no distinct significant ($P>0.05$) difference across the compound and particles size except the bulk size of SiO₂/Al₂O₃ (1:2) that showed clear significant increase ($P<0.05$) in the count to control. There was no significant difference ($P>0.05$) across the particle size and the compounds in RBC count compared to control except bulk size (1:2) with significant decrease to control. There is no clear significant ($P>0.05$) difference in the two sizes to control.

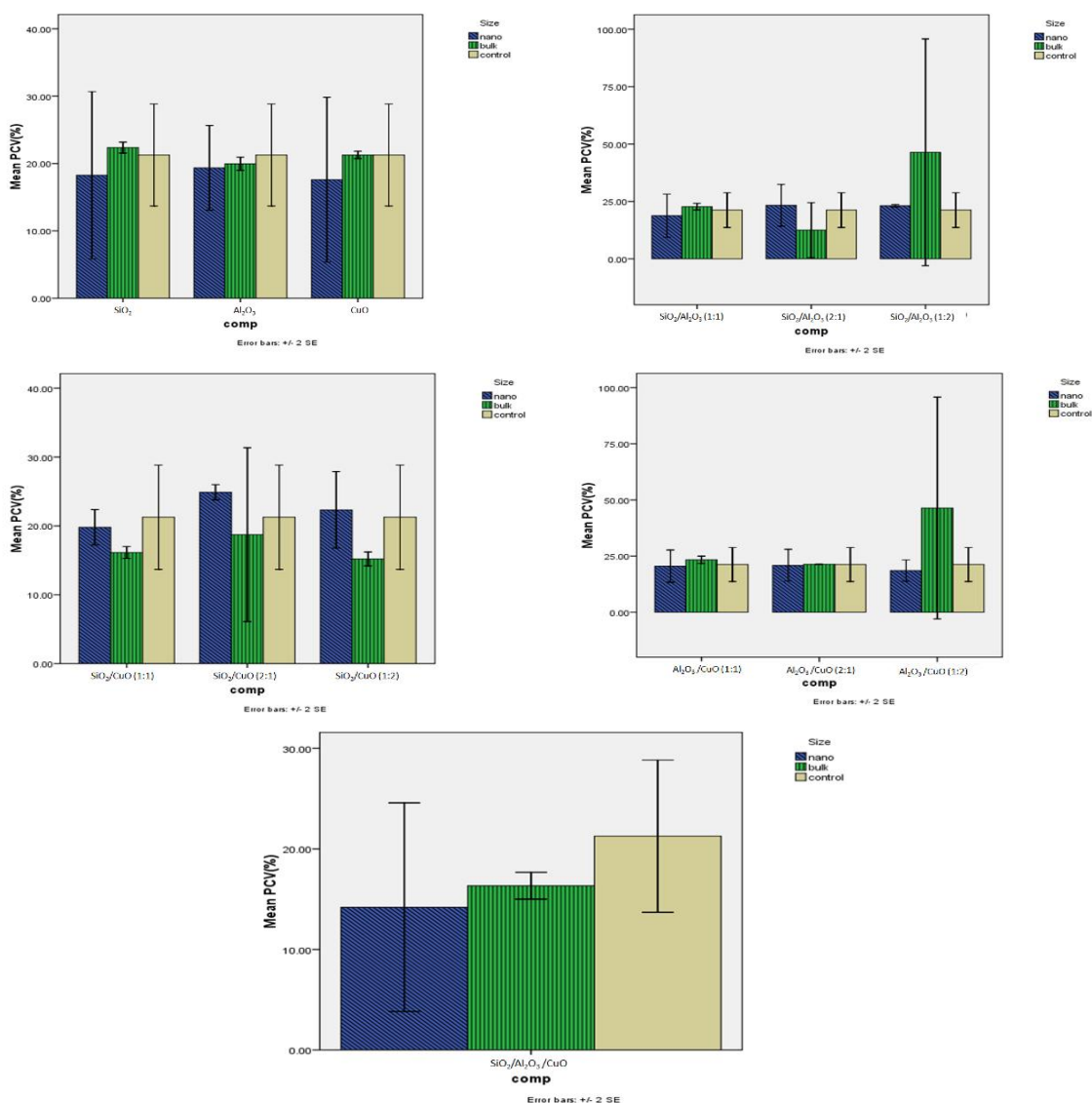


Fig. 5a-e. Relative levels of PCV count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

The results of the MCV counts are shown in Fig. 7(a-e). There was no clear significant difference (P>0.05) in the MCV for all the nano sizes while the bulk sizes of Al₂O₃ and CuO showed significant increase. There was significant (P < 0.05) decrease in the MCV in the blood of the catfishes exposed to bulk of SiO₂ compared to control. No clear significant (P>0.05) difference was observed except in the bulk of SiO₂/Al₂O₃ (1:2), with an evident decrease in the MCV

compared to control. No clear significant (P>0.05) difference was observed except in the bulk of SiO₂/CuO (1:1) with an obvious increase in the MCV to control. No significant (P>0.05) difference was observed except in those exposed to the bulk of SiO₂/CuO (1:2 and 2:1) with an evident decrease in the MCV to control. No significant difference (P>0.05) was observed in MCV recorded in those exposed to the triple mixtures compared to control.

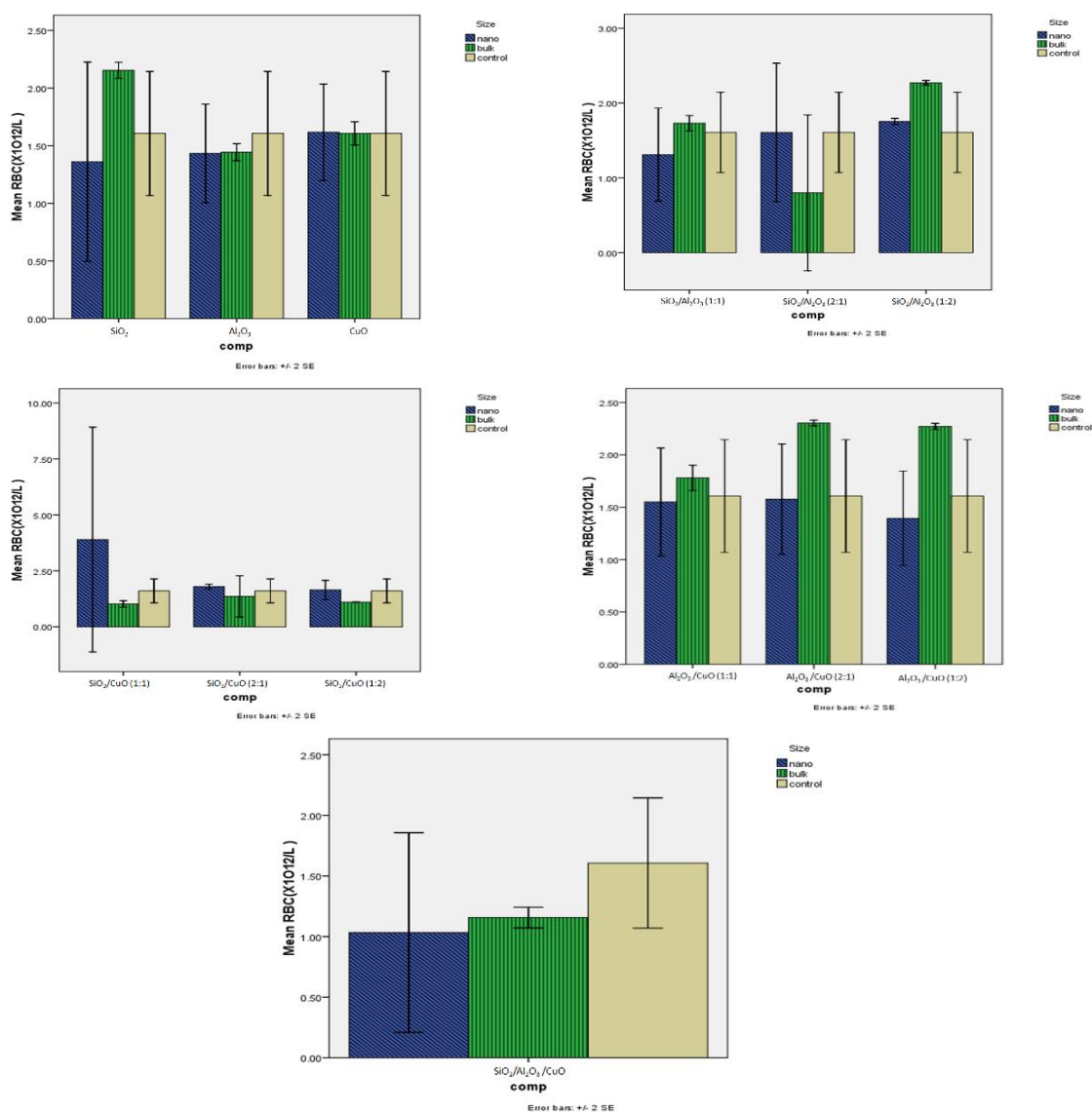


Fig. 6a-e. Relative levels of Red Blood Cell (RBC) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

The results of the MCH counts are shown in Fig. 8(a-e). There was no clear overall significant ($P>0.05$) difference ($P>0.05$) in the MCH levels compared to control. In the binary mixture group, there was no significant difference ($P>0.05$) observed except in those exposed to the bulk of SiO₂/Al₂O₃ (1:2) with an evident decrease in the level of MCH compared to control. No significant ($P>0.05$) difference was also observed except in the bulk of SiO₂/CuO (1:2 and (2:1) with an

evident decrease in the count of MCV to control (Fig 10d). The triple mixtures also showed no significant ($P>0.05$) difference in the blood MCH.

The results of the MCHC levels are shown in Fig. 9(a-e). Overall, there was no clear trend of significant ($P>0.05$) difference in the MCHC of fish exposed to the various ratios of bulk and nano-metallic oxides compared to control in all the tests. The MCHC was mostly lower in the

blood of fish exposed to bulk concentrations of the ratios.

The results from this study showed that most of the red blood cell indices (all PCVs, MCHCs, most Hbs and MCVs) in fish exposed to bulk and nano sizes of the mixtures of ratios of various metallic oxides did not show significant ($P > 0.05$) difference in their counts compared to the control group. Haematological studies give an index of physiological changes in fish [19,20]. The most common haematological variables measured

during stress include red and white blood cells count, haemoglobin content, and haematocrit value and red blood cells indices [21]. Barcellos et al. [28] and Kavitha et al. [29] also confirmed that the changes in red blood indices (RBC, Hb, Hct, MCV, MCH and MCHC) were greatly used to evaluate the toxic stress of the fishes. The results of haematological assessment of the bulk sizes of SiO_2/CuO (1:1 and 1:2), $\text{SiO}_2/\text{Al}_2\text{O}_3$ (2:1) for Hb, MCV and MCH showed decrease in the count in the exposed catfishes compared to control. Smith et al. [30] reported

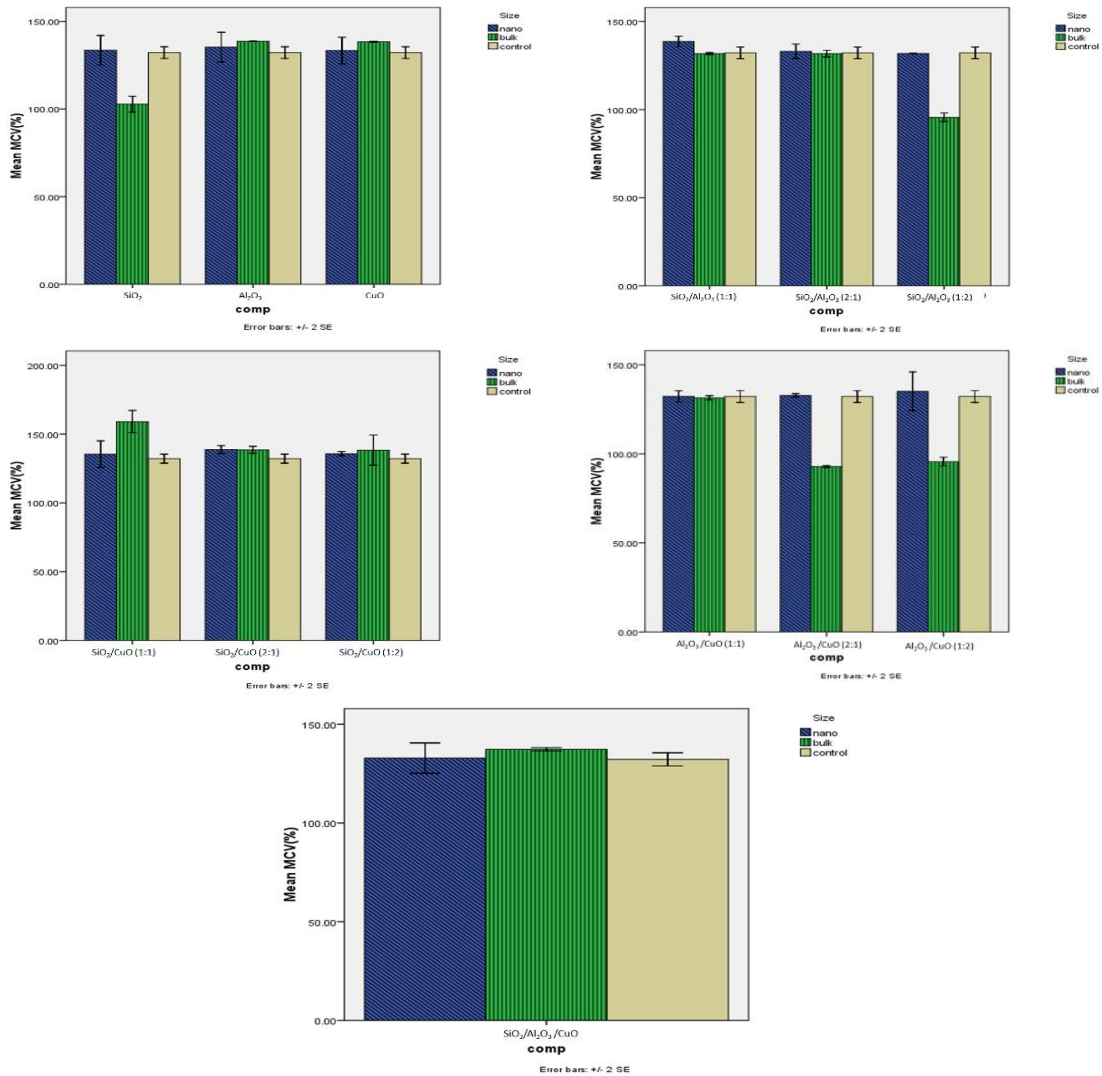


Fig. 7a-e. Relative levels of Mean cellular volume (MCV) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO_2 and Al_2O_3 in different ratio (c) double compounds of SiO_2 and CuO in different ratio (d) double compounds of Al_2O_3 and CuO in different ratio (e) triple compounds of SiO_2 , Al_2O_3 and CuO in different ratio for 28 days

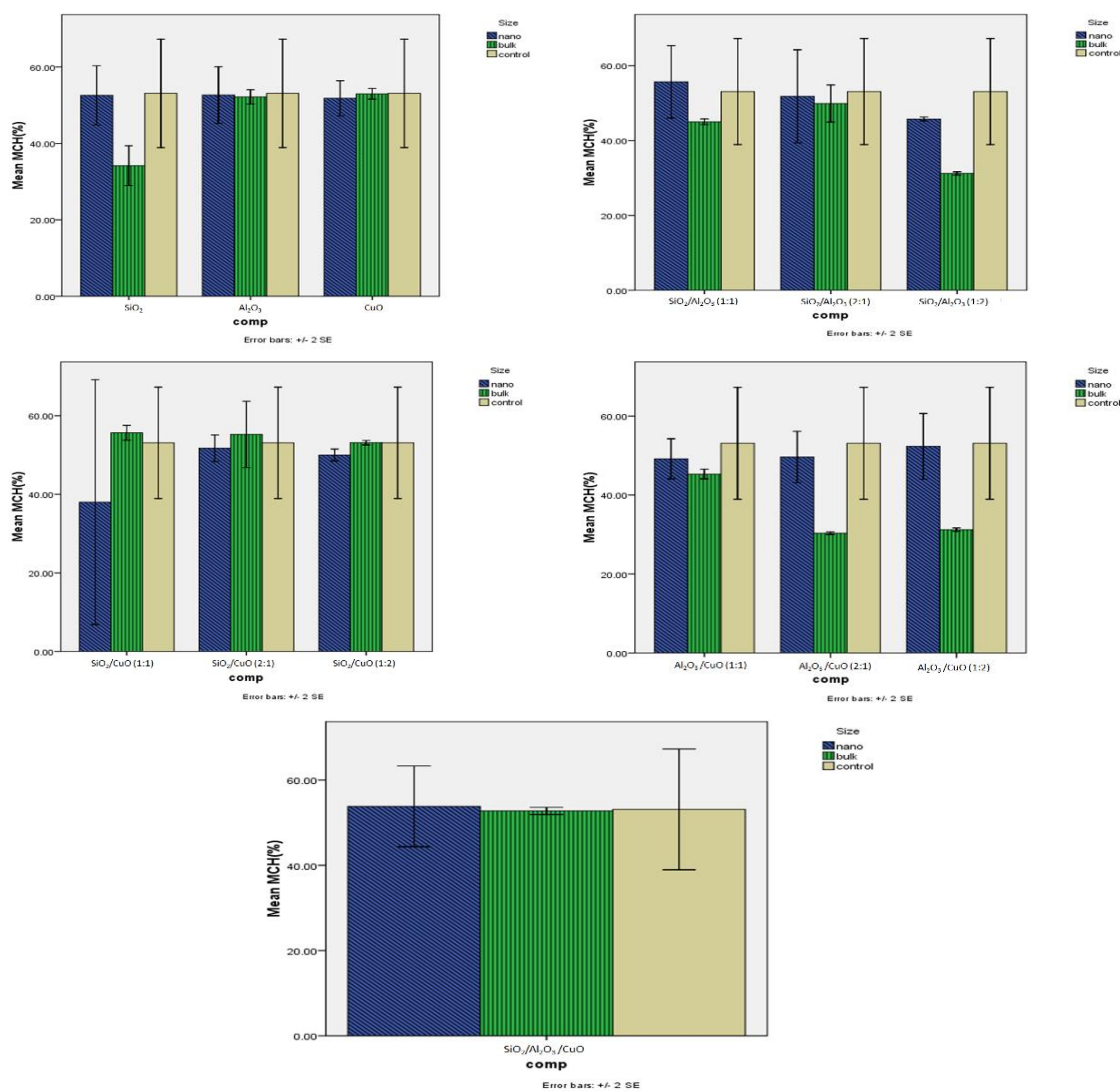


Fig. 8a-e. Relative levels of mean cell haemoglobin (MCH) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

That there was a significant decrease in the haematocrit (Hct) and blood haemoglobin in rainbow trout exposed to single-walled carbon nanotube (SWCNT). Remya et al. [26] reported a reduction in RBC level (Hb and Hct contents) when they exposed *Labeo rohita* to iron oxide nanoparticles. Panigrahi and Misra [31] also reported reduced haemoglobin and red blood cell count in fish *Anabas scandens* treated with mercury. Reduced RBC, Hct and Hb were also

reported in *Tinca tinca* exposed to lead and mercuric chloride [32]. The observed reduction in Hb and Hct contents might have resulted from structural changes in gill structure due to NP accumulation and toxicity as it has been reported by Griffith et al. [33] that nanoparticles may accumulate in gills and damage the organ resulting in respiratory disturbances. However, [34] opined that the reduction in the RBC level is due to severe anaemia.

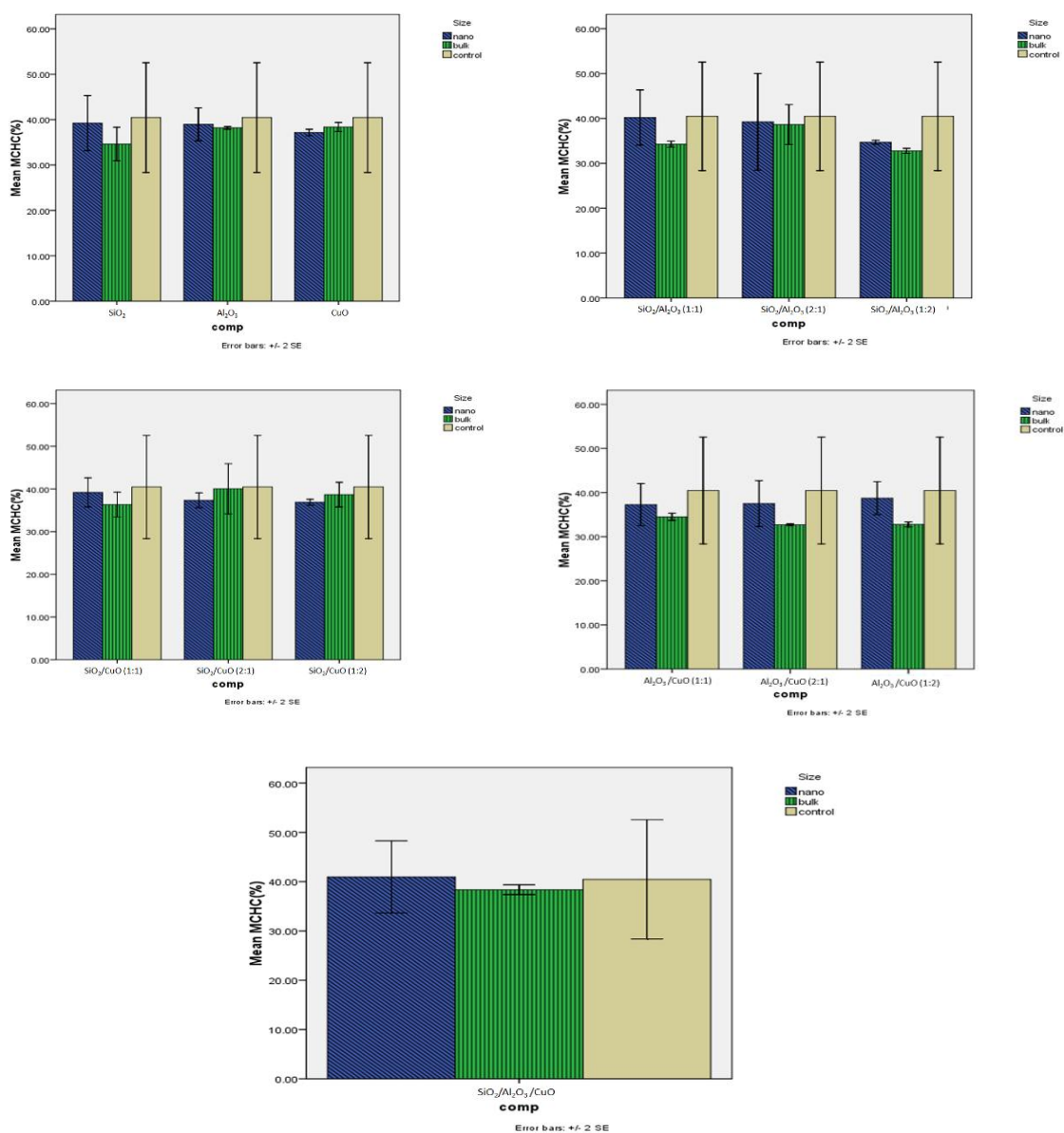


Fig. 9a-e. Relative levels of mean cell haemoglobin (MCH) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

3.1.3 Platelet counts

The results of the platelet counts are shown in Fig. 10(a-e). There was no overall significant difference ($P > 0.05$) across the particle size and the compounds in platelet count compared to control except the bulk size of SiO₂ which showed significant decrease compared to the

control. There was a significant difference ($P < 0.05$) between the bulk and nano concentrations of Al₂O₃ and CuO. There was no distinct significant difference ($P > 0.05$) across the compound and particles size except the bulk sizes of SiO₂/Al₂O₃ (1:1 and 1:2) that showed clear significant increase and decrease respectively in the platelet count to control. Both

nano and bulk particles SiO₂/CuO (1:2) showed decrease and increase respectively in count to control (Fig. 5c). There was no significant difference in platelet count across the particle size and the compounds to control except bulk sizes (1:2 and 2:1), with evidence of significant decrease ($P < 0.05$) compared to control. There is

no clear significant ($P > 0.05$) difference in the two sizes to control.

Simeonova and Erdely [35] observed that ultrafine carbon black induced accumulation of platelets in the hepatic microvasculature of mice.

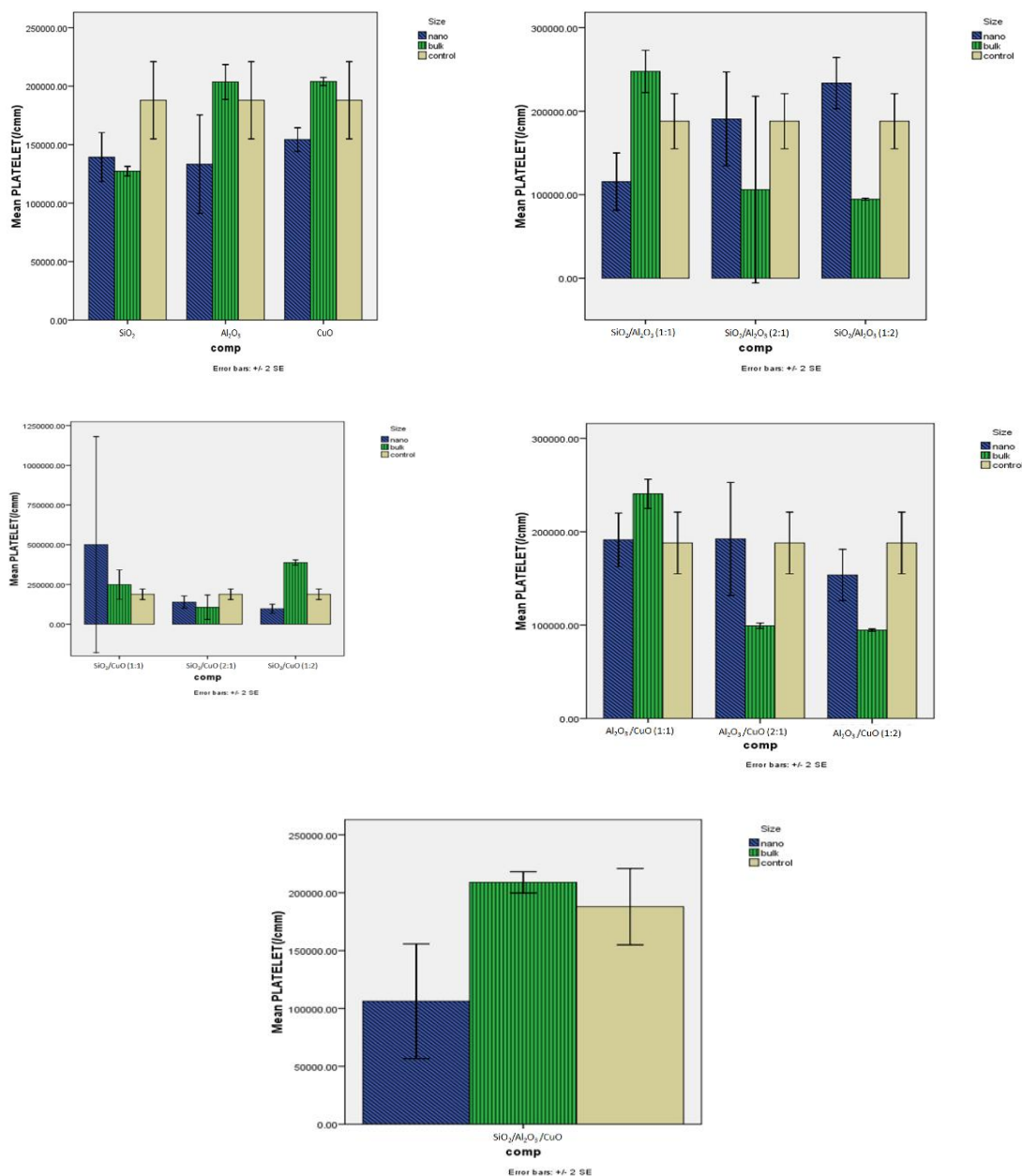


Fig. 10a-e. Relative levels of Platelet count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO₂ and Al₂O₃ in different ratio (c) double compounds of SiO₂ and CuO in different ratio (d) double compounds of Al₂O₃ and CuO in different ratio (e) triple compounds of SiO₂, Al₂O₃ and CuO in different ratio for 28 days

4. CONCLUSION

The findings of the study showed that exposure to nano scale metallic oxides (SiO₂, Al₂O₃ and CuO) did not cause significant haematological effects in experimental juvenile catfishes after 28 days of exposure. However, some of the mixtures were found to have significant effects on some of the assessed haematological parameters. The fact that there was no overall significantly higher toxicity to the fish caused by the nano sizes compared to the classical bulk sizes indicates that they both are likely to have similar mode of toxicity to the fishes. Concerted efforts must be in place to address potential health and environmental effects of nanoparticles as our society inevitably embraces the new technology.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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