

## **Original Research Article**

### **Comparative Assessment of Single and Joint Toxicity of Bulk and Nano Scale Heavy Metals (Al<sub>2</sub>O<sub>3</sub>, CuO and SiO<sub>2</sub>) using Haematological Parameters as Biomarker in Juvenile African Catfish (*Clarias gariepinus*)**

**Abstract** Global technological advancement has resulted in the gradual replacement of traditional bulk size compound with nano-sized ones with diverse useful characteristics. This study is focused on the toxicological assessment of three nano metallic oxides (SiO<sub>2</sub>, Al<sub>2</sub>O<sub>3</sub> 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 assay (Haemoglobin, Packed Cell Volume, Red blood cells, Platelets, Neutrophils, Lymphocytes, Mean cellular volume, Mean Cell haemoglobin, Mean Cell haemoglobin Concentration). The results of the range finding/ acute toxicity test indicated that the single action of the oxides were not acutely toxic at environmentally relevant concentrations. 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. 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 nano scale metallic oxides into production activities should be with caution since they have been found to have similar negative health effects as the existing bulk sizes.

**Keywords:** nano-toxicology, haematology, biomarker, heavy metals, joint toxicity, environmental relevance

**Comment [WK1]:** Where is the "White blood cell"

#### **1. Introduction**

The excessive contamination of aquatic ecosystems has evoked major environmental and health concerns worldwide [1]. These pollutants including nanoparticles could increase the level of metals in natural water and seriously affect wetland habitats [2].

Blood serves as a transport medium in living things carrying along nutrients and wastes in its flow. Changes in blood cells can usually be traced to environmental pollution in the ecosystem. This could be expressed as an increase in white blood cells (the organism compensatory mechanism to fight against stressors), irregularities in cell morphology, variation in haemoglobin values and blood counts, etc. The physiological conditions arising from stress factor on fish health can be determined through analysis of blood samples [3]. Haematological studies furnish an index of physiological changes in fish [4] and the fish blood acts an impressive tool for detection of alterations in the tested organism [3,5]. The most common haematological variables measured during stress include Red and White Blood Cells count, haemoglobin content, hematocrit value, and red blood cells indices [6].

As a result of the growing nanotechnology applications, many nano-materials are discharged into the aquatic habitats that affect their biota. These nanoparticles cause 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 sublethal 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.

## **2. Materials and Methods**

### **2.1. Experimental Chemicals and Preparation of Test Solutions**

The bulk and nano sizes of three salts ( $\text{Al}_2\text{O}_3$ , CuO and  $\text{SiO}_2$ ) 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. Literature search was used to obtain the test concentrations. 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

### **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 left open 24 hours prior to allow for vapourization of excess chlorine. The juveniles fish (Mean Length:  $12.3 \pm 3.5$  cm; Mean weight:  $18.52 \pm 6.41$  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; D.O 8.5 mg/L; Salinity 0.0ppt).

### 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 bulk and nano size of the three metallic oxides either singly or in mixtures for 30 days. The chronic toxicity assays were conducted in larger plastics (11cm x9cm x7.5cm) using 5L of water because of the larger sizes of the fish compared to the acute toxicity test. 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.

Comment [WK2]: haematology

#### 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 [7]. Mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), and mean cell haemoglobin (MCH) were calculated using the following equations:  $\text{MCHC} = (\text{Hb} / \text{PCV}) \cdot 100$ ,  $\text{MCH} = (\text{Hb} / \text{RBCC}) \times 10$  and  $\text{MCV} = (\text{PCV} / \text{RBCC}) \times 100$  [8].

## 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 ( $\text{Al}_2\text{O}_3$ , CuO and $\text{SiO}_2$ )

The various haematological parameters analysed showed different levels of significant difference when compared with the control group. Most of the nano sized metallic oxides showed no difference to control whereas the bulk sizes either showed no difference or decrease in the count compared to control. Technological advancement despite its positive impacts has a major drawback of compounding environmental pollution problems as have been observed from the results of this study.

#### 3.1.1. White Blood Cell (WBC) Indices

The results of the WBC counts are shown in figure 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 and  $\text{Al}_2\text{O}_3$  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/ $\text{Al}_2\text{O}_3$  (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/CuO (2:1). There was no significant difference ( $P > 0.05$ ) across the particle size and the compound with the exception of bulk particles  $\text{Al}_2\text{O}_3$ /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 figure 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 which showed decrease count and the bulk size of CuO with increase 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/ $\text{Al}_2\text{O}_3$  (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 neutrophils except in catfishes exposed to bulk sizes of  $\text{Al}_2\text{O}_3/\text{CuO}$  (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).

**Comment [WK3]:** count compared to

**Comment [WK4]:** neutrophils except

The results of the lymphocytes counts are shown in figure 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 were reported across the fish exposed to the three compounds and particles size except the nano sizes of  $\text{SiO}/\text{CuO}$  (1:1 and 1:2) that showed clear significant ( $P < 0.05$ ) increase in lymphocytes count to control.

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  $\text{Al}_2\text{O}_3/\text{CuO}$  (2:1 and 1:2) and  $\text{SiO}/\text{Al}_2\text{O}_3/\text{CuO}$  for neutrophils,  $\text{SiO}/\text{Al}_2\text{O}_3$  (1:2) and  $\text{Al}_2\text{O}_3/\text{CuO}$  (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 [9]. This is also corroborated by [10] that reported reduction in WBC level in *Labeo rohita* exposed to iron oxide nanoparticles. [11] 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. [9] noted that white blood cells leave the circulating blood, to protect the body, by moving (ameboid movements) to the infected tissue resulted in reduced cell number in blood which was similar to this research.

**Comment [WK5]:** You can now use abbreviations of white blood cell. You don't have to write WBC's expansion every time.

**Comment [WK6]:** rohita exposed

**Comment [WK7]:** leukocyte (leukocytosis)

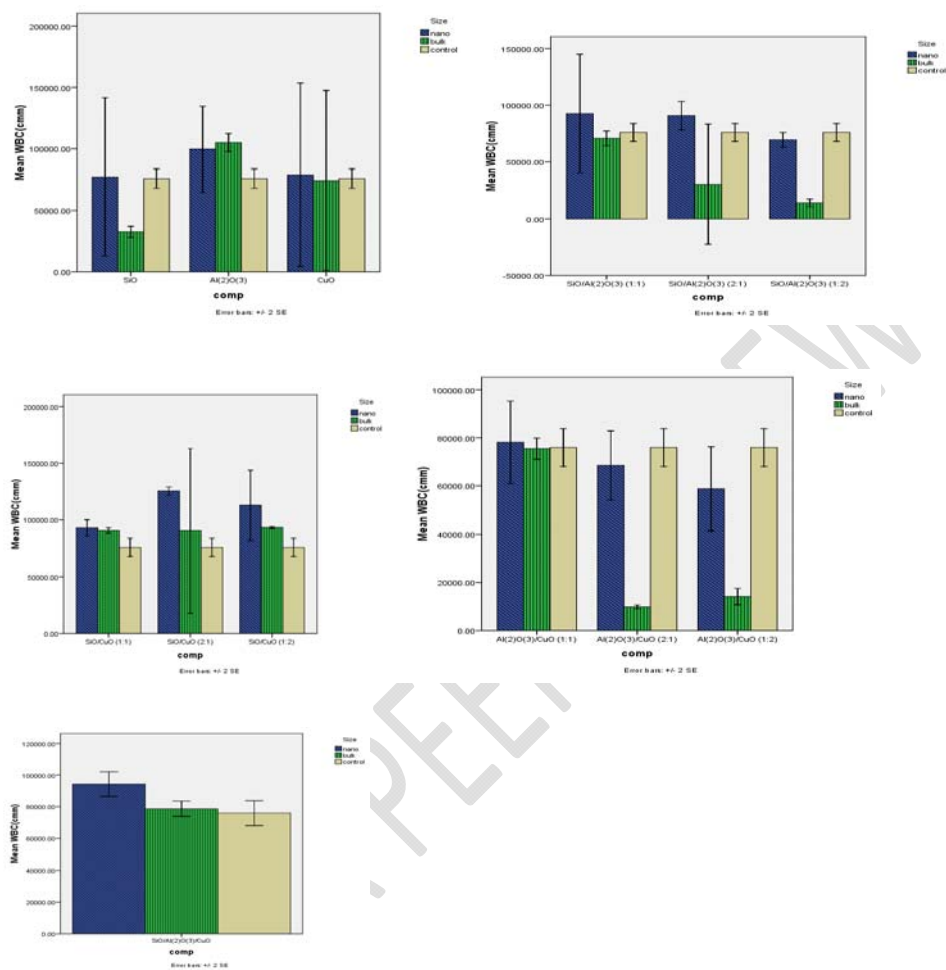


Figure 1a-e: Relative levels of WBC count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

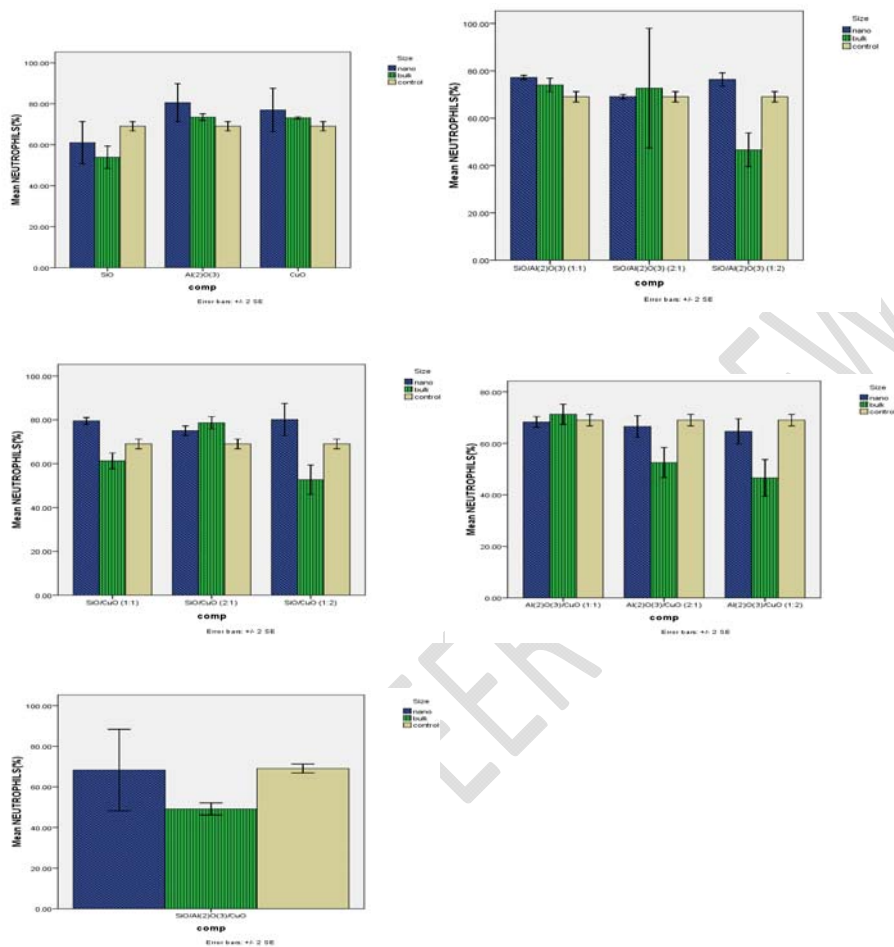


Figure 2a-e: Relative levels of neutrophils count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

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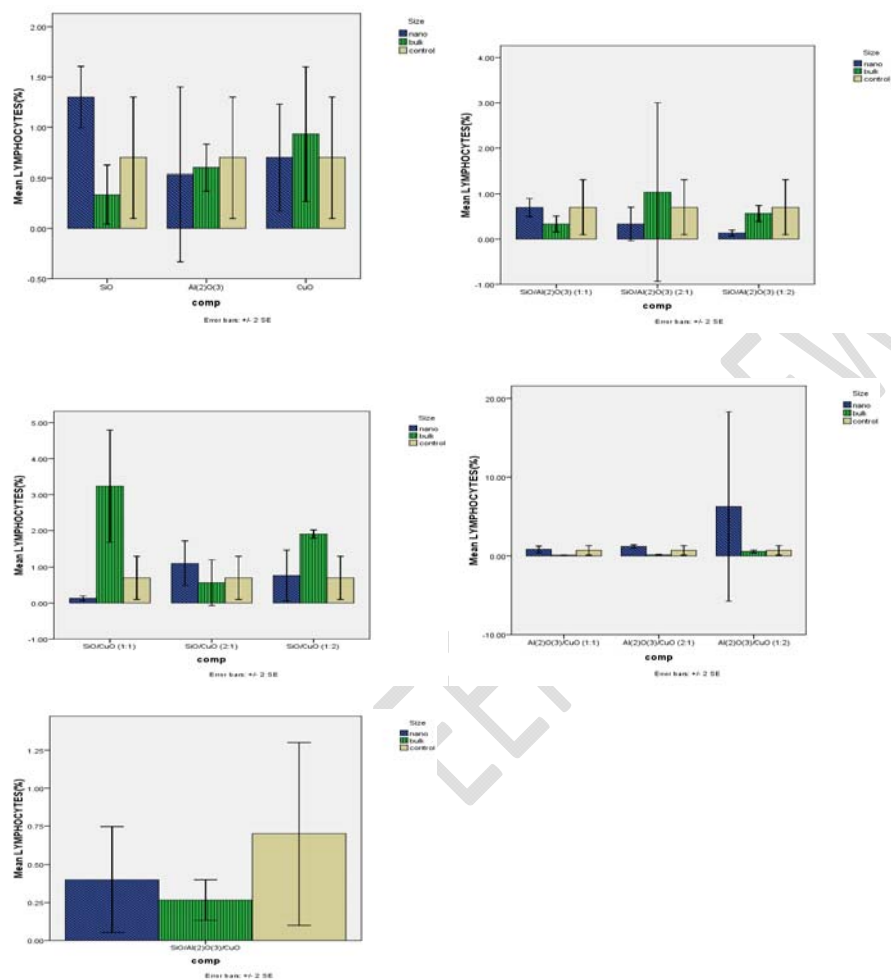


Figure 3a-e: Relative levels of lymphocytes count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

### 3.1.2. Red Blood Cell (RBC) Indices

The results of the Hb counts are shown in figures 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  $\text{SiO}/\text{Al}_2\text{O}_3$  (1:2) that showed clear decrease in the Hb to control. No significant ( $P>0.05$ ) difference except the bulk sizes  $\text{SiO}/\text{CuO}$  (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.

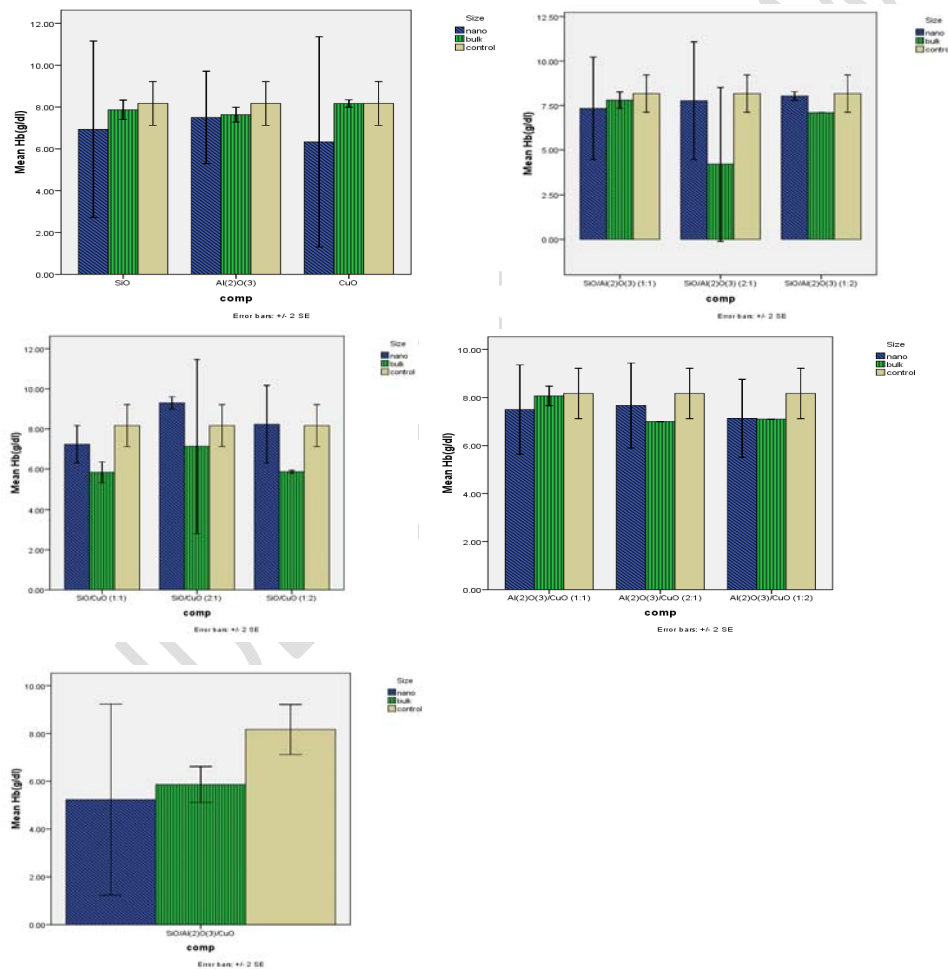
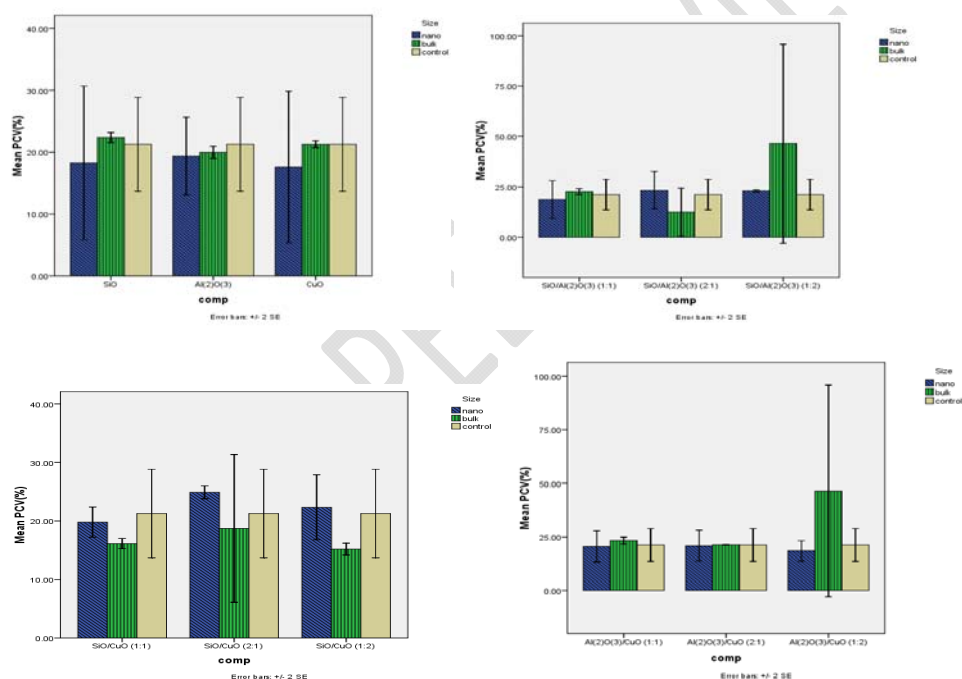


Figure 4a-e: Relative levels of Hb count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

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The results of the PCV counts are shown in figures 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 SiO resulted in the lowest levels of PCV across all observed (Fig. 3a). Bulk concentrations of CuO and SiO mixtures resulted in the lowest levels of PCV as well (Fig. 3c) in another batch of the study.

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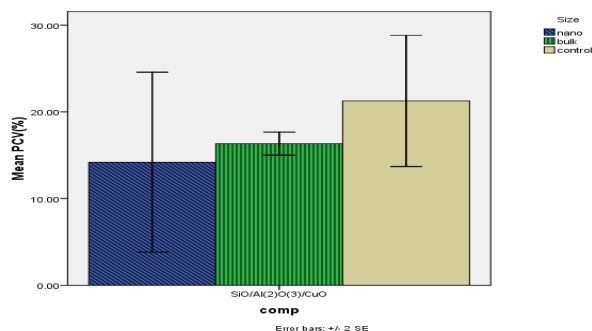
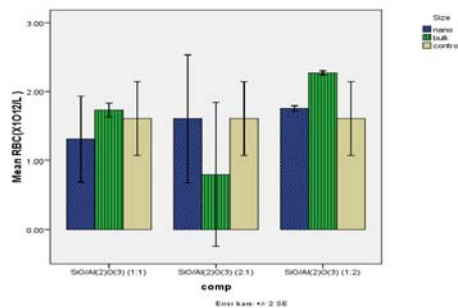
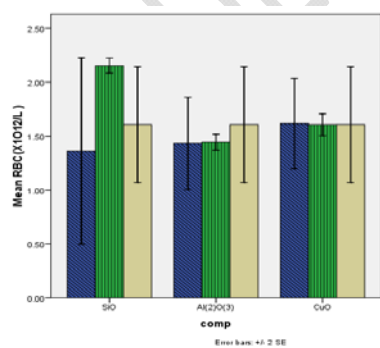


Figure 5a-e: Relative levels of PCV count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

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The results of the RBC counts are shown in figure 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 SiO/Al<sub>2</sub>O<sub>3</sub> (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.



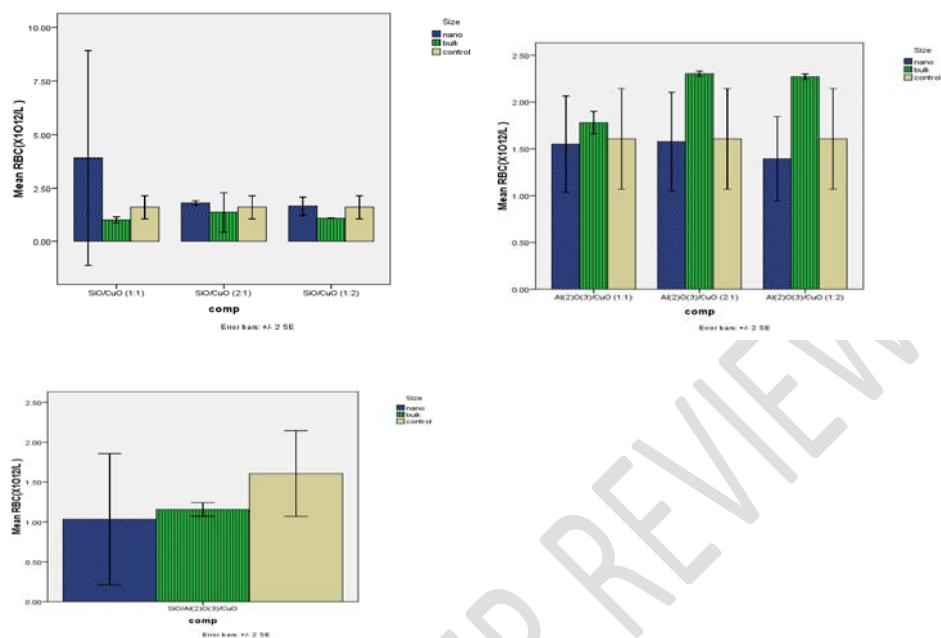


Figure 6a-e: Relative levels of Red Blood Cell (RBC) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

The results of the MCV counts are shown in figure 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 Al<sub>2</sub>O<sub>3</sub> 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 SiO compared to control. No clear significant ( $P > 0.05$ ) difference was observed except in the bulk of SiO/Al<sub>2</sub>O<sub>3</sub> (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 SiO/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 SiO/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 was recorded in those exposed to the Prible mixtures compared to control.

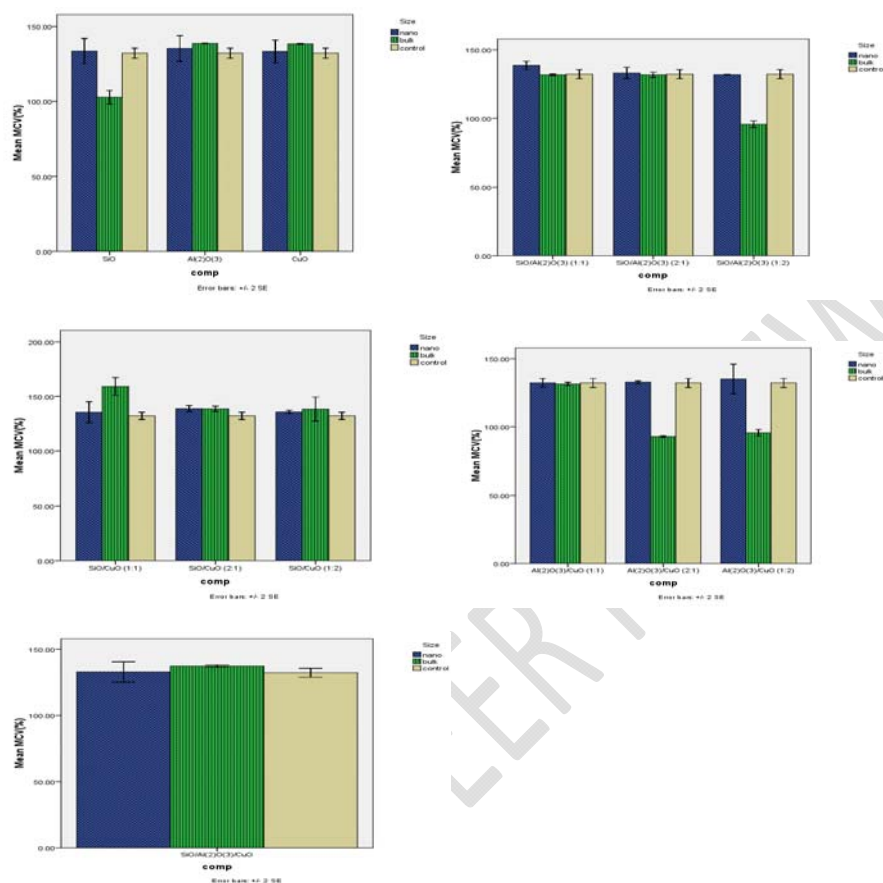


Figure 7a-e: Relative levels of Mean cellular volume (MCV) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

The results of the MCH counts are shown in figure 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 SiO/Al<sub>2</sub>O<sub>3</sub> (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 SiO/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.

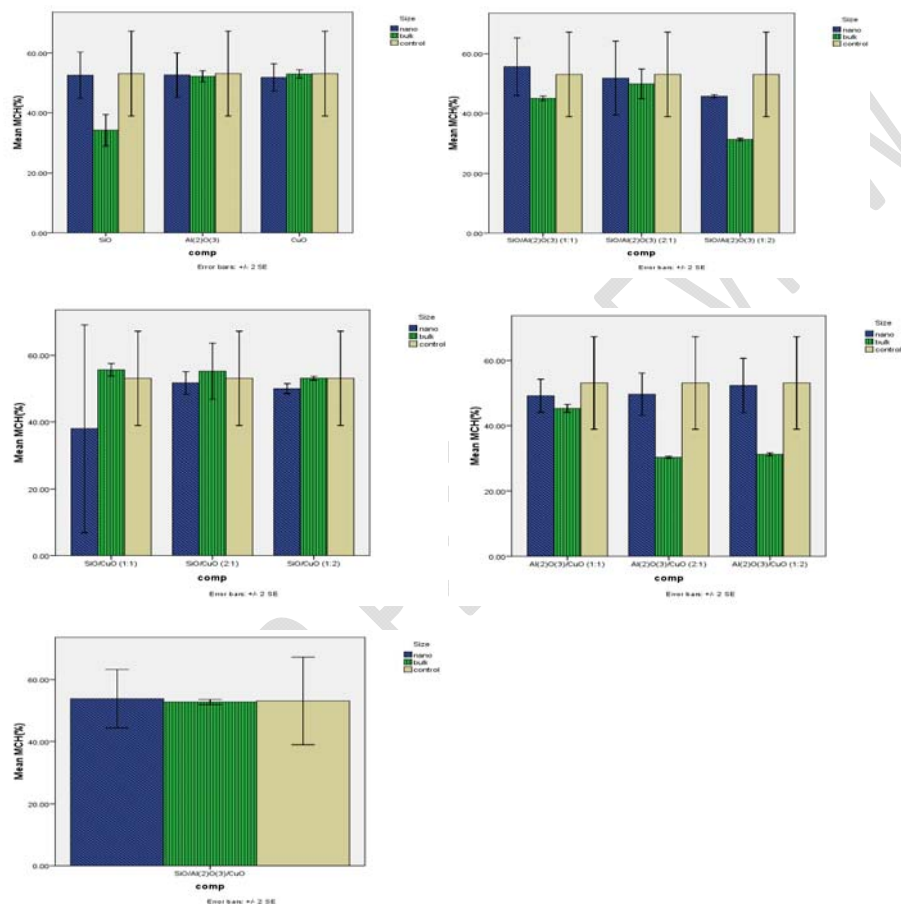


Figure 8a-e: Relative levels of Mean Cell haemoglobin (MCH) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

The results of the MCHC levels are shown in figure 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.

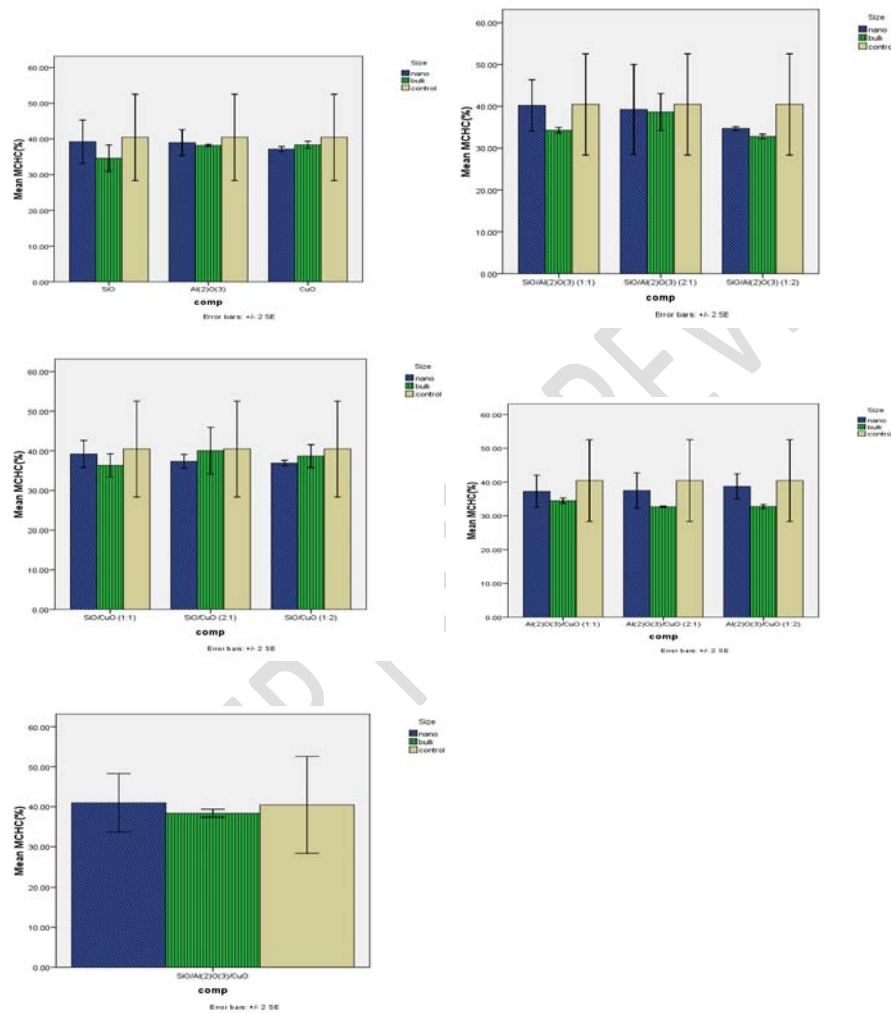


Figure 9a-e: Relative levels of Mean Cell haemoglobin (MCH) count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO<sub>2</sub> and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double

compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

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 [3,4,5]. 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 [6]. [12] and [13] 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/CuO (1:1 and 1:2), SiO/Al<sub>2</sub>O<sub>3</sub> (2:1) for Hb, MCV and MCH showed decrease in the count in the exposed catfishes compared to control. This is in conformation with the findings of [14] that reported a significant decrease in the haematocrit (Ht) and blood haemoglobin in rainbow trout exposed to single-walled carbon nanotube (SWCNT). This is also corroborated by [10] that reported a reduction in RBC level (Hb and Ht contents) when they exposed *Labeo rohita* to iron oxide nanoparticles. [15] also reported reduced haemoglobin and red blood cell count in fish *Anabas scandens* treated with mercury. Reduced RBC, Ht and Hb were also reported in *Tinca tinca* exposed to lead and mercuric chloride [16]. The observed reduction in Hb and Hct contents might have resulted from structural changes in gill structure due to NP accumulation and toxicity [10] as it has been reported by [17] that nanoparticles may accumulate in gills and damage the organ resulting in respiratory disturbances. However, [18] opined that the reduction in the RBC level is due to severe anaemia.

### 3.1.3. Platelet Counts

The results of the platelet counts are shown in figures 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 SiO which showed significant decrease compared to the control. There was a significant difference ( $P<0.05$ ) between the bulk and nano concentrations of Al<sub>2</sub>O<sub>3</sub> and CuO. There was no distinct significant difference ( $P>0.05$ ) across the compound and particles size except the bulk sizes of SiO/Al<sub>2</sub>O<sub>3</sub> (1:1 and 1:2) that showed clear significant increase and decrease respectively in the platelet count to control. Both nano and bulk particles SiO/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. [19] observed that ultrafine carbon black induced accumulation of platelets in the hepatic microvasculature of mice

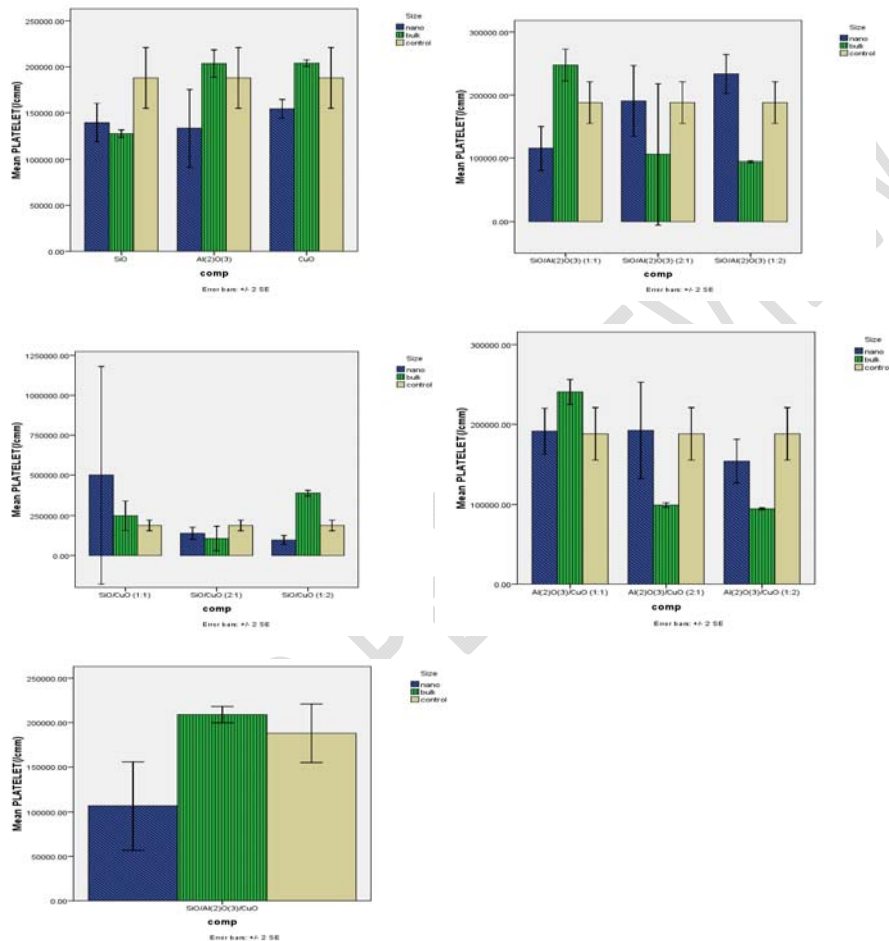


Figure 10a-e: Relative levels of Platelet count in blood of *Clarias gariepinus* exposed to bulk and nano sizes of (a) single compound, (b) double compounds of SiO and Al<sub>2</sub>O<sub>3</sub> in different ratio (c) double compounds of SiO and CuO in different ratio (d) double compounds of Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio (e) triple compounds of SiO, Al<sub>2</sub>O<sub>3</sub> and CuO in different ratio for 28 days.

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#### 4. Conclusion

The findings of the study clearly show that exposure to nano scale metallic oxides (SiO, Al<sub>2</sub>O<sub>3</sub> and CuO) caused pathological effects in experimental juvenile catfishes after 28 days of exposure. The fact that there was no overall significantly higher toxicity to the fish caused by the nano sizes compared to the classical bulk sizes indicated 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 nano particles as our society inevitably embraces the new technology.

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