**Effect of Dietary Nanoselenium on Biochemical Profile and Histopathology of *Clarias gariepinus*.**

**ABSTRACT**

Research was conducted for 60 days to determine the effects of dietary nanoselenium formulated diets on biochemical profile and histopathology of *Clarias gariepinus*. Fish acclimatization was done for 14 days, randomly selected 10 fish were stocked in triplicate per dietary treatment. Two diets supplemented with nanoselenium at 2 mg/kg, 4 mg/kg, and the control with absence of nanoselenium were fed to the fish. The proximate composition of the feeds were analysed at the end of the feeding trial likewise the biochemical profile of the blood, gill, liver as well as the histopathology of the gill and liver of *Clarias gariepinus* fed varying inclusion level of nanoselenium were analysed. There was no significant difference (*P>0.05*) in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, uric acid, urea, albumin, and total cholesterol in fish fed 2 mg/kg nanoselenium formulated diet compared to those fed 4 mg/kg nanoselenium formulated diet and the control. Glutathione peroxidase (GPx) and malondialdehyde in the gill and liver of fish fed 2 mg/kg nanoselenium formulated diet, however, increased significantly as compared to those fed 4 mg/kg formulated diet and the control, while the superoxidase dismutase (SOD) in the gill and liver of fish significantly increased as the concentration of dietary nanoselenium increased. There were histopathology alterations in the gill, and liver of the fish fed the two dietary treatment diets but with less impacts on fish health fed 2 mg/kg. The study indicated that the dietary inclusion of nanoselenium at 2 mg/kg had less effects in the hepatocytes and limited stress on the health of *Clarias gariepinus* as compared to 4 mg/kg nanoselenium formulated diets.

Keywords: Clarias gariepinus, nanoselenium, biochemical, histopathology, proximate.

**INTRODUCTION**

Fish is an important animal protein source to the world because of its high protein, vitamins, minerals and polyunsaturated fatty acids. Nutritionally, fish is best for human consumption as it is low in fat, calories, and cholesterol. Fish contribute more than 60% of the world protein supply, most especially in the developing countries of the world [1]. Globally, fish products are indispensable to one billion individuals for protein security and particularly vital for juvenile and pregnant women [2]. As a result, the demand for fish from consumers around the world is increasing due to growing population, rising average incomes and greater awareness of fish as part of a healthy diet. The yield from the wild catch cannot be increased sustainably, however, aquaculture can fill the gap. Therefore, to fill the gap there is need to improve technologically to overcome these challenges.

*Clarias gariepinus* belong to the family Clariidae, genus Clarias [3]. They are popularly known as African catfish because of its hardness to withstand adverse environmental condition, omnivorous in nature and efficient in feed utilization. It is commonly known as African catfish because of its hardness and ability to withstand stress to a tolerable range. Catfish is the most cultured fish in Nigeria and are widely distributed throughout the country. It is an economic important fish species with increase growth rate and global market demands [4].

Nanoselenium has being playing important functional roles in the biological system of fish. Biological systems of fish contain chemical composition which can be altered by any chemical reaction in the body. Biological systems of fish and its constituent macromolecules are susceptible to oxidative damages which disrupt their structure by distorting native chemical composition. Apart from these, oxidative stress (OS) leads to the generation of reactive oxygen species (ROS) like superoxide ion, hydroxide radicals, hydrogen peroxide etc. which trigger the apoptosis in tissues. To neutralize these adverse effects of ROS, the living system uses several antioxidative defense systems including various enzymes like catalase, superoxide dismutase, glutathione-S-transferase, and peroxidase, etc. Likewise, nanoselenium is also an important antioxidant located at the catalytic site of thioredoxin reductase and glutathione peroxidase enzymes [5].

Cells naturally contain enzymes for their functions but damages to cellular membrane led to their escape into the blood where their presence or activities can be measured as an index of cell integrity [6]. Some chemical composition of fish could be used to identify tissue damage such as Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) which are normally found within the cells of the liver, heart, gills, kidney, muscles, and organs but their increase in the plasma indicate tissue injury or organ dysfunction [7, 8, and 9].

However, changes in plasma glucose, total proteins and cholesterol concentrations can be indicative response to stress in fishes exposed to pollutions [10], this is because fish is very sensitive to pollution- induced stress [11]. As good as nanoparticles are in enhancing growth performance, their effects as stressors in fish has been monitored by the assessment of biochemical and physiological changes in both acute and chronic toxicity tests. Changes in enzymes profile are important biotoxicity indices (biomarkers) and have been used to assess the biochemical physiological health of vital organs (tissues) in fishes [12; 13].

Earlier research revealed the role that nanoselenium played on growth performance in animal and various fish species [14, 15, 16 and 4] but little literature on the role that dietary nanoselenium supplementation played on biochemical profile and histopathology of *Clarias gariepinus* which led to this study. The research, therefore, aimed to determine the effect of dietary nanoselenium at different inclusions level on biochemical profile and histopathology of *Clarias gariepinus*.

**2. EXPERIMENTAL DETAILS**

**2.1 Experimental design**.

Sizeable healthy uniform fish (average weight of 12.85+0.01g and average length of 6.65±1.0) were procured from a commercial fish farm and were transferred to the Aquaculture center, University of Ilorin. The fish were acclimatized to the environmental conditions for 14 days and fed with a conventional feed. After acclimatizing the fish, 10 fish were randomly selected per treatment in triplicate.

**2.2 Experimental Diets**

Three diets, consisting of control diet (basal diet, without nanoselenium) and two treatment diets at 2 mg/kg and 4 mg/kg nanoselenium inclusion level were formulated. The diets were fed to the fish twice daily (08:00hr and 18:00hr) for 60 days at 3% body weight [17].

**2.3 Proximate analysis of the feed composition.**

The proximate analysis of the formulated diets of nanoselenium supplemented at different dietary inclusion levels were determined using the methods described by [18]

Table 1: Percentage composition (%) of the basal diet

|  |  |
| --- | --- |
| **Ingredient\*** | **Quantity (%)** |
| Fish meal | 220 |
| Toasted soya bean | 220 |
| Groundnut cake (GNC) | 220 |
| Maize | 200 |
| Wheat offal | 90 |
| Salt | 5 |
| Fish premix | 10 |
| Vitamin | 10 |
| Lysine | 10 |
| Methionine | 10 |
| Bone meal | 5 |
| **Total** | **1000** |

\*All the ingredients were measured in gram (Source:[4].)

**2.4 Water quality parameters**

The water quality parameters were monitored and measured weekly during the feeding trial. The water temperature was measured using mercury-in-glass thermometer (1000C), pH was measured using Jenway pH meter (model E 512) and the dissolved oxygen (DO2) were measured using Milwaukee DO2 meter (model MW600).

**2.5 Collection of serum and biochemical analysis**

The blood samples of fish fed varying inclusions level of dietary nanoselenium were collected in replicate at the end of the feeding trial into sampling bottles without anticoagulant [14, 16] and labeled for proper identification. The tubes were inserted in a slanting rack and centrifuged for 15 minutes at 3500 revolution per minutes (rpm). A clear fluid which is the serum was pipetted out into a clean and sterilized sampling bottle for further analytical process.

Alkaline phosphate (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was conducted according to the methods described by [19] and [20]. And the Uric acid and Urea were conducted with the Randox machine analyzer (Model RX MONXA UA 230).

**2.6 Collection of *Clarias gariepinus* organs for biochemical analysis**

The liver and gill of *Clarias gariepinus* fish fed with different nanoselenium formulated diet in each rearing troughs were removed, then pooled in 0.25ml of sucrose solution before taken to the laboratory for further analysis. The parameter analysed are superoxide dismutase (SOD), glutathione peroxidase (GPx), total protein and malondialdehyde (MDA). Liver and gill samples were homogenized with ice cold 0.86% NaCl and centrifuged at 8000 rpm at 40C for 10 min. The resulting supernatants were used for the determination of GPx, SOD and protein. GPx activity was assessed using Hydrogen peroxide as substrate [21]. This method is based on the oxidation of glutathione by Hydrogen peroxide via glutathione peroxidase. SOD activity was measured using xanthine-xanthine oxidase and Nitro blue tetrazolium (NBT). Protein contents were assayed by the Bradford dye-binding assay with bovine serum albumin as standard [22]. The absorbances of GPx, SOD and protein were respectively measured at 412, 550 and 590nm using a Microtiter plate reader (RANDOX, Rx Monxa Tp 245).

**2.7 Collection of organs and histological analysis**

The liver and gill of *Clarias gariepinu*s fed with different inclusion level of dietary nanoselenium and control were removed, then pooled in 10% formalin solution before further histological analysis. Fixed tissues (liver and the gill) were put through slides to obtain micro thin slides for photomicrography. The tissues were dehydrated by passing through ascending grades of alcohol from 50%, 70%, 90%, absolute 1 and absolute 2 alcohols, with the tissues spending one hour in each of the alcohol. Then the dehydrated tissues were cleared in two changes of xylene for one hour each. The cleared tissues were then infiltrated with two changes of wax at 600c for one hour each. The infiltrated tissues were embedded in the infiltrating medium and then cooled at room temperature to solidify, after which the embedded tissues were trimmed to reveal the tissue surface for microtomy which led to trimming of the blocks. The trimmed blocks were section at 7 microns to obtain tissue ribbon, which were later floated in the water bath at 400c. The tissue ribbons were picked with glass slides and then dried on a hot plate, the slides obtained were cleared in 2 changes of xylene for one minute each then the slides were allowed to be passed to descending grades of alcohol and then rinsed in water. After which the slides were stained in hematoxylin for 20minutes and then rinsed in tap water. The slides were differentiated by dipping in 1% acid alcohol for 5 seconds and then rinsed in running tap water which were later stained in eosin for two minutes and then rinsed in tap water. The slides were then upped through ascending grades of alcohol and cleared in xylene, the obtained slides were mounted in DPX which were later captured by a camera attached to the microscope and then analyzed for histopathology.

**2.8 Statistical analysis**

The experimental data were subjected to statistical data using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) and expressed as mean ± SE [23]. Statistical significance for all the tests were set at *p* < 0.05. All statistical analyses were performed using Microsoft Excel 2010 and IBM SPSS statistics 20.0.

**3. RESULTS**

**3.1 Proximate composition**

The proximate composition of the formulated diets supplemented with different dietary nanoselenium is shown in Table 2. The proximate composition of the formulated diets showed that there was significant difference (*p* < 0.05) in the crude protein and crude fibre in the two nanoselenium formulated diets and the control diets while there was no significance difference (*p* < 0.05) in the ash content, nitrogen free extract (NFE), moisture content and crude fat.

**3.2 Water quality parameters**

The water temperature in the rearing media ranged from 25.6 to 28.20C, the pH ranged from 7.1 to 8.0 while the dissolved oxygen ranged between 6.02 and 6.48 mg/l. All the water parameters measured were within the tolerable range.

**3.3 Biochemical profile of serum**

Biochemical profile of serum of *C. gariepinus* fed different inclusions levels of nanoselenium formulated diets is available in Table 3. The results of the biochemical profile of the fish fed different inclusion level of dietary nanoselenium revealed that fish fed with dietary supplementation of 2 mg/kg nanoselenium formulated diets were significantly higher in alkaline phosphate (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) while fish fed with 4 mg/kg dietary nanoselenium formulated diets were significantly lower in value for the same biochemical parameters.

**3.4. Biochemical analysis of the organs**

The biochemical profile of the organs (gill and liver) of *Cl gariepinus* fed different inclusions levels of dietary nanoselenium formulated diets is shown in Table 4. The results showed variations in the parameters of the liver and the gill. There was no significance difference (*p* < 0.05) in all the dietary treatments and control for all the biochemical parameters of the gills except SOD which significantly (*p <* 0.05) increased as the dietary nanoselenium increases.

For the biochemical indices of the liver such as superoxidase dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), total protein and malondialdehyde (MDA) there were no significance difference (*p <* 0.05) between fish fed with 2 mg/kg nanoselenium formulated diets and 4 mg/kg nanoselenium formulated diets and the control.

Table 2: Proximate composition (%) of the experimental diets at different dietary inclusion level of nanoselenium.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Dietary Nanoselenium (mg/kg)** | | |
| **Parameter** | **0 (Control)** | **2** | **4** |
| Moisture content | 7.65±0.28a | 8.00±0.06a | 8.53±0.24a |
| Ash content | 9.23±0.10a | 8.81±0.31a | 9.39±0.08a |
| Crude fibre | 5.65±0.38a | 4.89±0.05a | 4.34±0.16b |
| Crude protein | 33.67±0.65a | 36.83±0.34b | 39.65±0.36c |
| Crude fat | 8.75±0.12a | 8.45±0.01a | 8.52±0.10a |
| Nitrogen free extract (NFE) | 35.06±1.33a | 33.02±0.08a | 29.58±0.26a |

*Within rows, means with different alphabets are significantly different (P<.05).*

Table 3: Biochemical profile of the serum of *C. gariepinus* fed varying inclusion levels of nanoselenium formulated diets**.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters** | **0 (Control)** | **2mg/kg** | **4mg/kg** |
| ALP (µ/I) | 279.22±14.96a | 262.66±11.06a | 332.12±49.44b |
| ALT (µ /l) | 59.87±11.42a | 59.77±0.41a | 65.35±5.85a |
| AST (µ /l) | 213.95±0.37a | 198.16±5.58a | 220.53±17.12a |
| Creatinine mg/l | 0.06±0.06a | 0.13±0.02a | 0.01±0.04a |
| Total protein (mg/l) | 24.33±0.74a | 22.53±0.38a | 27.86±2.65a |
| Albumin (g/dl) | 2.88±0.11a | 3.29±0.86a | 4.37±2.54a |
| Urea (mm/l) | 1.47±0.16a | 1.33±1.02a | 1.09±0.70a |
| Uric acid (mm/l) | 0.15±0.08a | 0.27±0.05a | 0.09±0.00a |
| T CHOL (mg/dl) | 235.01±14.53a | 220.11±6.83a | 223.61±11.18a |
| Triglycerides (mg/dl) | 358.08±4.52a | 348.12±1.06a | 380.83±35.09b |
| HDL-C | 132.34±4.23a | 121.16±1.41a | 129.15±11.01a |
| LDL-C | 31.05±11.20a | 29.33±5.20a | 18.29±6.84a |

Means within rows with different superscript are significantly different (*p* < 0.05).

ALP- Alkaline phosphate AST- Aspartate aminotransferase

ALT- Alanine aminotransferase HDL-C - High density lipoprotein cholesterol

LDL-C - Low density lipoprotein cholesterol T.CHOL- Total Cholesterol

Table 4: Biochemical profile of the organs of *Clarias gariepinus* fed varying inclusion levels of nanoselenium formulated diets**.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Inclusion level of dietary Nanoselenium (mg/kg)**   |  |  |  |  | | --- | --- | --- | --- | | **Parameters** | **0 (Control)** | **2** | **4** |   **Gills** | | | |
| SOD (U/mg protein) | 323.38±70.36a | 410.45±334.20b | 1057.21±263.85c |
| GPX µ/l | 125.00±0.00a | 229.17±147.31a | 104.17±29.46a |
| CAT µ /l | 20.83±5.89a | 12.50±5.89a | 19.44±3.93a |
| MDA (µ /mg protein) | 1.67±0.51a | 2.02±0.91a | 1.32±0.04a |
| Total Protein (mg/l) | 12.72±0.18a | 10.06±0.08a | 9.56±0.18a |
| **Livers** | | | |
| SOD (µ/mg protein) | 223.88±35.18a | 435.32±334.20a | 447.76±175.90a |
| GPX (µ /l) | 125.00±58.93a | 145.83±29.46a | 104.17±29.46a |
| CAT (µ /l) | 45.83±17.68a | 12.50±0.00a | 25.00±11.79a |
| MDA (µ/mg protein) | 1.34±0.40a | 1.48±0.23a | 0.94±0.43a |
|  |  |  |  |
| Total Protein (mg/l) | 11.25±1.26a | 12.95±0.74a | 11.25±0.37a |

Means within rows with different superscripts are significantly different (*p* < 0.05).

SOD - Superoxidase Dismutase GPX - Glutathione Peroxidase

MDA- Malondialdehyde CAT- Caalase.

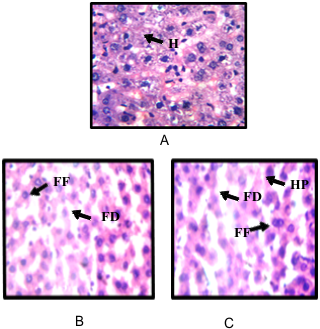


Fig.1: Histopathology of the livers of C. gariepinus fed different inclusion levels of nanoselenium formulated diets.

**3.5 Histopathological analysis**

Histopathology of the livers of C. gariepinus fed different inclusions of dietary nanoselenium formulated diets were shown in Figure 1 (A-C) while that of the gills of C. gariepinus fed different inclusions of dietary nanoselenium formulated diets is shown in Figure 2 (A-C).

[A] Nuclei of the hepatocytes (H) of the liver fed the control diets

[B] Slight focal fibrosis (FF), gradual fatty degeneration (FD) of the liver of fish fed 2 mg/kg of nanoselenium formulated diets.

[C] High focal fibrosis (FF), high fatty degeneration (FD) and Hyplasia (HP) of the liver of fish fed 4 mg/kg of nanoselenium formulated diets.

Fig. 1A, showed the histomorphology of liver tissue presented with typical cellular density and cellular distribution of fish fed the control diets. The nuclei of hepatocytes were distinctively stained and properly disposed within their respective cytoplasm. There were no histopathological alterations in the histological presentation of these tissues.

Fig. 1B, showed the panoramic view of the fish fed 2 mg/kg nanoselenium formulated diets. It revealed histomorphology of liver tissue such as slight focal fibrosis (FF), gradual fatty degeneration (FD) presented with typical cellular density and cellular distribution. The nuclei of hepatocytes were distinctively stained and properly disposed within their respective cytoplasm. There were slight histopathological alterations in the histological presentation of these tissues.

Fig. 1C, showed the panoramic view of the hepatocytes (black arrow) of the fish fed 4 mg/kg nanoselenium formulated diets`. It showed high focal fibrosis (FF), high fatty degeneration (FD) and hyplasia (HP) of fish liver tissue presented with typical cellular density and cellular distribution. There were obstructions of bile flow because of fat deposition within the central vein. This suggest that there were pathological changes in the liver.

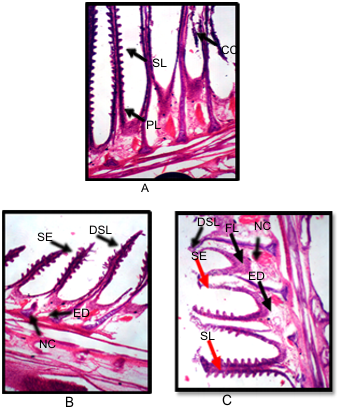


Fig. 2: Histopathology of the gill of C. gariepinus fed different inclusion levels of nanoselenium formulated diets.

[A] Normal cellular arch of the gill. Primary lamella (PL), secondary lamella (SL) and cartilaginous core (CC) in fish gill fed the control diets.

[B]Hyperplasia (HP), degeneration of the secondary lamella (DSL), necrosis (NC), oedematous changes (ED) and sloughing of epithelia tissue (SE) of fish fed 2 mg/kg nanoselenium formulated diets.

[C] Degeneration of the secondary lamella (DSL), fusion of the lamella (FL), necrosis (NC), oedematous changes (ED) and sloughing of the epithelia tissue (SE).

Fig. 2A, showed typical histomorphological presentation of the gill filament of the fish fed the control diets with intact supporting cartilage surrounding the well-placed vasculature of the primary lamella from which the secondary lamella is jotting out. The secondary lamella appears to have begun to undergo slight degenerative changes, also slight changes in the cartilaginous core (CC) in fish gill fed the control diets. This suggesting possible slight histopathological alterations.

Fig. 2B, showed typical histomorphological presentation of the gill filament of *Clarias gariepinus* fed with 2 mg/kg nanoselenium formulated diets with intact supporting cartilage surrounding the well-placed vasculature of the primary lamella from which the secondary lamella is jotting out. There was hyperplasia (HP), degeneration of the secondary lamella (DSL), necrosis (NC), oedematous changes (ED) and sloughing of epithelia tissue (SE) in the gill of fish fed 2 mg/kg nanoselenium formulated diet. Also, the secondary lamella appeared to have begun to undergo degenerative changes suggesting possible histopathological alterations.

Fig. 2C, typical histomorphology presentation of the gill filament of *Clarias gariepinus* fed with 4 mg/kg nanoselenium formulated diets. It revealed the supporting cartilage surrounding the well-placed vasculature of the primary lamella from which the secondary lamella is jotting out. Degeneration of the secondary lamella (DSL), fusion of the lamella (FL), necrosis (NC), oedematous changes (ED) and sloughing of the epithelia tissue (SE) were seen in the gill of fish fed 4 mg/kg of nanoselenium formulated diets. There were possible histopathological alterations in this organ.

**4. DISCUSSION**

Physicochemical parameters are crucial factors put into consideration in the rearing of fish. They are significant parameters that restrict survival, growth, and distribution of fish [24]. As a result of the effect of physicochemical parameters, optimal tolerable range in the rearing media were maintained throughout the rearing period which reduce adverse effects on survival and the blood composition of the fish.

The inclusion of nanoselenium in the fish diets might be reason for the increase in crude protein as the concentration of the micronutrients increases, this is because nanoselenium contain some essential amino acids which has the tendency of increasing the protein content of the feed. The nutritive status of fish can be linked to the health status of animal and potential way they deal with stress [25, 26], resulting from their surrounding environment.

Biochemical indices such as AST, ALP, ALT, urea, uric acid, albumin etc. can be used to estimate the functionality of the fish organs such as the liver, gill, kidney, and heart. The high increase of AST, ALP and ALT activities of fish fed 4 mg/kg of nanoselenium in the fish diets are suggestive of hepatic cellular damage leading to their leakage in circulation [27, 28]. This equally means that the lowest value obtained for the same biochemical parameters of fish fed 2 mg/kg dietary nanoselenium are likely to be saved from hepatic cellular damage of the vital organs. As a result of this, there is possibility that 2 mg/kg nanoselenium in the fish diets of C. gariepinus can help in hepatic cellular activity stability.

The high-density lipoproteins cholesterol (HDL-C) helps in removing other form of cholesterol in the body systems of fish. The high value of HDL-Cholesterol in control revealed that the heart organs of fish are at lower risk of heart diseases as compared to fish fed 4 mg/kg of nanoselenium formulated diets which has the lowest value. The low-density lipoproteins (LDL-C) is of high value in the fish fed the control diets, this simply mean that as the inclusion level of nanoselenium increase the LDL-C significantly decrease.

Antioxidant which are known to be oxidative stress biomarker are playing important roles in fish growth, preventing oxidative reaction in fish. Superoxide dismutase is an enzyme that helps break down potentially harmful oxygen molecules in cells, which prevent damage to tissues and vital body organs and GPx biological role is to protect the organism from oxidative damage that might arise from enzymatic reaction. These two important enzymes play important roles in the build-up of the fish body organs. The GPx and the malondialdehyde (MDA) of gills and livers of *C. gariepinus* fed 2 mg/kg of nanoselenium increase significantly as compared to those fed 4 mg/kg nanoselenium formulated diet. As a result of this increase in the two important oxidative indices, the normal activities of the gill function which serve as a vital respiratory organ might increase in fish fed 2 mg/kg nanoselenium formulated diet.

The alteration in the liver of fish fed 4 mg/kg nanoselenium formulated diets revealed some histopathological changes such as obstruction of the bile and fatty degeneration which might be because of increased concentration of the nanoselenium. This simply mean that, concentration at 4 mg/kg nanoselenium formulated diets and above will possibly alter normal function of the liver which is a vital fish organ known to be the main target organ of selenium toxicity [29], this agreed with the statement of [30], which reported that liver is the target organ of different xenobiotic substances.

Also, the changes observed in the gills of all the dietary treatments and the control can be due to stress in the management process and not only from dietary nanoselenium intake. But the degeneration of the lamella, oedematous change, and necrosis of the gills in fish fed 4 mg/kg of nanoselenium formulated diet were more pronounced as compared to the gill of fish fed 2 mg/kg of nanoselenium formulated diets which are indications of environmental stress. The reason could be that concentration of nanoselenium beyond a tolerable range for fish might be a contributing factor to stress which could be a factor contributing to the alterations of the gills with tendency to increase the mortality rate of fish fed 4 mg/kg nanoselenium formulated diets.

Conclusion : the study indicated that dietary inclusion at 2 mg/kg nanoselenium formulated diets had better effect on biochemical profile and histopathology of the fish in vital organs with less stress on the fish health as compared to 4 mg/kg nanoselenium formulated diets. More research work needs to be conducted on the two dietary concentrations on *Clarias gariepinus* in other vital organs such as heart and kidney as well as fish muscle for a prolong period to reveal their impacts.

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