

Sublethal Effects of *Vernonia amygdalina* (Bitter leaf) Extract on Liver Oxidative Stress and Histopathology of *Clarias gariepinus* Juvenile

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Abstract: This study investigated the effects of sublethal concentrations of aqueous crude leaf extract of *Vernonia amygdalina* on oxidative stress biomarkers, liver histopathology, and histomorphometry in juvenile *Clarias gariepinus*. Juvenile African catfish were exposed to 0.00, 0.40, 0.80, and 1.60 g/L of the extract for 56 days in a static-renewal bioassay system. Liver superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA) were determined using standard biochemical procedures. Liver tissues were also examined using routine paraffin-wax processing and haematoxylin and eosin staining, while histomorphometric parameters, including hepatocyte diameter, hepatic cytosurface area, hepatic nuclear surface area, and hepatic surface area, were measured using image analysis software. The results showed a concentration-related reduction in SOD activity, with the lowest value recorded at 1.60 g/L. GPx activity varied significantly among the treatment groups ($p < 0.05$), whereas the variation in MDA was not statistically significant ($p > 0.05$). Histological examination revealed normal hepatic architecture in the control fish, mild central venous and sinusoidal congestion at lower concentrations, and moderate sinusoidal congestion with diffuse hepatocellular degeneration at 1.60 g/L. Significant alterations ($p < 0.05$) were also observed in the measured liver histomorphometric parameters. The findings demonstrate that prolonged exposure to sublethal concentrations of *V. amygdalina* leaf extract can induce oxidative imbalance and structural alterations in the liver of juvenile *C. gariepinus*. Therefore, the use of *V. amygdalina* as a piscicide or pesticide near aquatic ecosystems should be carefully regulated to minimise potential adverse effects on fish health and aquatic biodiversity.

Keywords: Bitter leaf; Catfish, Liver; Oxidative Stress; Histopathology; Histomorphometry.

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Introduction

Anthropogenic activities have continued to exert negative effects on aquatic environment.

Organic and inorganic pollutants (pesticides, insecticides, herbicides, fertilizers) from agricultural activities ends in to water bodies, which disrupts the natural ecological balance. The accumulation of these substances can cause damage to key organs of fish, including the liver (Adamu, Audu and Audu 2008). Because agrochemicals are highly toxic and tend to persist in the environment, there is growing interest in safer alternatives, particularly plant-based materials with piscicidal properties. In contrast to synthetic chemical pesticides, plant-derived biocides are thought to be more environmentally acceptable since they decompose easily, availability, relatively inexpensive and productiveness (Sulaiman et al., 2022).

Vernonia amygdalina commonly called bitter leaf is cosmopolitan plants in most States of Nigeria including Nasarawa and Plateau State. *Vernonia amygdalina* is the most widely cultivated species of the genus *Vernonia* which has about 1,000 species of shrubs (Munaya 2013). It belongs to the family *Asteraceae* and it is vegetatively cultivated by stem cutting (Abdurrazzaq, Mallam, and Muhammad 2019). Bitter leaf is called Onugbo, Ewuro and Shiwaka in Igbo-Eastern, Yoruba-Southern, and Hausa- Northern Nigeria respectively.

Leave of the *V. amygdalina* is mainly used as vegetable in the preparation of soups. It has a bitter taste which is attributed to antinutritional factors and phytochemicals such as alkaloids, saponins, tannins and glycosides (Sobukola, et al., 2006). Research has also shown that *V. amygdalina* have some beneficial effects in disease management of poultry (Ekeocha, 2011) and anti-oxidant (Erasto, Grierson, and Afolayan, 2006). Dried *C. gariepinus* sample treated with 1.5% solution of the *V. amygdalina* extract exhibited the highest antibacterial effect (Abdurrazzaq, et al 2019). The authors reported that bitter leaf extract solution could be used in protecting stored smoked-dried catfish from bacterial spoilage. The non-significant effects of the *V. amygdalina* leaves on semen parameters such as motility, liveability percentage and morphology makes the plant relatively safe at tested inclusion levels for use as a feed supplement in the diet of male *C. gariepinus* (Ajala & Owoyemi 2016).

The inclusion up to the 400g/1 50litres of water can be used to replace synthetic growth promoter in fish culturing without any deleterious effect (Alagoa and Osakwe 2021). However, the authors reported that higher inclusion of bitter leaf causes elevation of blood sugar level in *C. gariepinus* juveniles. The authors also recommended more studies on the mechanism of bitter leaf and of its effect in the fish body. Sublethal concentrations (0.40, 0.80g/L) of *V.*

amygdalina extract has no effects on daily weight change except in the heighest concentrations (1.60 g/L) of Catfish juveniles (Sulaiman, Audu and Shehu 2025). The authors also reported that the plant extract induced fluctuations oxidative stress biomarker in kidney of the exposed fish.

Histological alterations are used as indicators of aquatic pollutants and overall health of the entire population of organisms in an ecosystem (Damshit, Audu, And Wade, 2025). These histological parameters are related to other biomarker of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular changes in the affected organism (Damshit etal 2025). Liver is a detoxification organ and it is essential for both, the metabolism and the excretion of toxic substances in the fish body (Salamat and Zarie 2012).

There is scarcity of information on oxidative stress, histology and histomorphometrical changes in the liver of *C. gariepinus* fish exposed to sublethal concentrations of *V. amygdalina*. Thus, this study seeks to investigate the possibility.

Materials and Methods

Procurement and Processing of Experimental Plant *V. amygdalina*

Fresh leave of *V. amygdalina* were collected from Keffi, Local Government Area of Nasarawa State, Nigeria. The leave was air dried at room temperature ($27 \pm 2^\circ\text{C}$) in the laboratory of Zoology Department, Faculty of Natural and Applied Sciences, Nasarawa State University Keffi. The dried leave was pounded with laboratory mortar and pestle into powder, sieved with a 30 μm mesh size sieve.

Collection, Transportation and Acclimatization of Experimental Animal

Clarias gariepinus was purchased from Rayuwa Fish Farm, Karu Local Government Area, Nasarawa State, Nigeria. Experimental animals were transported in 50 liters Jericans containing water from the pond to the laboratory unit of Zoology department, Nasarawa State University, Keffi. Fish was transferred into aquaria containing dechlorinated municipal tap-water and allowed to acclimatize to the laboratory conditions for a period of two weeks. During this period, the fish was fed to satiation at 8:00am and 6:00pm with commercial fish feed (Vital feed®).

Sublethal Concentrations of Aqueous Crude Leaves Extract of *V. amygdalina* and Test Procedure

Mortality (0, 1, 2, 5, 7 and 10) of *C. gariepinus* juveniles exposed to acute concentrations of 0.0, 0.19, 0.38, 0.75, 1.50 and 3.00g/L of crude leaf extract of *A. amydalina* was reported by (Audu et al., 2018). The LC_{50} was calculated as 0.82g/L by using Finney calculator. *V. amydalina* crude leaves extracts was administered at sublethal concentrations

1/5, 1/10, 1/20 of the LC₅₀ of (Abubaka, Stanley and Emmanuel 2018) in a static renewal bioassay system.

The weighed samples (1.6, 0.8 and 0.4g) were macerated in 1L of distilled water for 24 hours at room temperature (27.0 °C) to obtain the stock solution. The obtained stock solution was filtered through a funnel choked with non-absorbent cotton wool and each filtrate was transferred into tanks A1(1.6 g/L), B1 (0.8 g/L), C1 (0.40 g/L) and D1 (0.0g/L) which was not inoculated with the test materials which served as the controls. Each of the filtrates was diluted by adding 9L of dechlorinated municipal tap water. The same procedure was repeated in replicate tanks A2, B2, C2 and D2. Sublethal toxicity test lasts for two months (56 days or 8 weeks). The test fish was fed 3% body weight at 08.00 and 18.00hrs with photoperiod being natural.

The experiments consist of four circular rubber tanks in duplicate and eighty *C. gariepinus* juveniles of mean weight 32.24±1.51g and mean total length 15.26±0.23cm. The fish were distributed in a non-randomized block design. Each of the six rubber tanks were filled with 9L borehole water, three of the filled tanks were inoculated with various concentrations (powder earlier macerated in 1L of water) of aqueous crude leaves extract of *V. amygdalina* and *C. gariepinus* juveniles were introduced into each.

Assay for Biomarkers of Oxidative Stress of *Clarias Gariepinus* Juveniles Exposed to Sub Lethal Concentrations of Aqueous Crude Leaves Extract of *V. amygdalina*

Preparation of the Tissue Samples for the Study for Oxidative Stress Biomakers

At the end of the experiment, the fish were carefully removed by using scooping nets to minimize stress and sacrificed. Tissues such as gills, liver, testes and ovaries were carefully removed. Tissues were washed in chilled phosphate buffer saline. Sample homogenates were made of individual tissues with chilled phosphate buffer (0.1 M, pH 7.4) and centrifuged in a centrifuge at 9,000rpm for 20 minutes at 4°C (Charity *et al.*, 2018). The supernatant obtained were used for further analysis. Samples were transported to Biochemistry Unit of National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Superoxide dismutase (SOD), Malondialdehyde (MDA), reduced Glutathione (GSH), Catalase Activity and Glutathione peroxidase (GPx) were determined in liver of the exposed fish by using standard procedures adopted by (Sulaiman *et al.*, 2025)

Procedure for Histological Examination of Organs of *C. Gariepinus* Juveniles Exposed to Sublethal Concentrations of Aqueous Crude Leaves Extract of *V. Amygdalina*

The routine Paraffin wax method and haematoxylin-eosin staining techniques of tissue processing described by Drury and Wallington (1967) and Avwioro (2011) were adopted for the examination of the liver of *C. gariepinus* exposed to aqueous crude leaves extract of *V. amygdalina*. The harvested tissues exposed sublethal concentrations of bitter leaf of *C. gariepinus* were fixed in 10% formalin for 3 days, cut into thin slices of 5 x 2 x 1mm thick and then processed with the SPIN Tissue Processor (STP) 120 (Thermoscientific model). The tissues were buffered in 10% formalin before passing through the following levels of hydrocarbons for two hours each: 70% Alcohol, 80% Alcohol, 90% Alcohol, 95% Alcohol, Absolute Alcohol I, Absolute Alcohol II, Absolute Alcohol III, Xylene I, Xylene II, Paraffin Wax Oven I, Paraffin Wax Oven II.

Tissues were embedded in molten paraffin wax using embedding moulds. The tissues were embedded using embedding cassettes on a tissue Tek Embedding Centre (SLEE MPS/P2), and cooled rapidly on the cooling component. Tissues were sectioned using a rotary microtome (MICROM HM340E ThermoScientific)) set at 4 micromes, picked on slides and ready for staining.

Haematoxylin and eosin staining technique was used for the staining of the tissues. Tissue sections were dewaxed and hydrated by passing through two changes of xylene and through descending levels of alcohol (100%, 80%, 70%) for three min each and then into water before staining in Harris' haematoxylin solution for 5 min and washed in running water. They were differentiated in 1% Acid alcohol and then washed thoroughly in water, blued in Scott's tap water substitute for 5 min and rinsed briefly in distilled water. Each tissue was then counterstained in 1% aqueous eosin for 2 min and then washed in water, dehydrated in descending grades of alcohol before clearing in xylene and mounted in Destrene, Plasticiser and Xylene (DPX). Sections were then placed in slide carriers and placed in a 40°C oven to dry overnight. Each tissue was read microscopically. Photographs of the prepared sections were taken using a mounted photo-microscopic camera.

Histomorphometrics of Liver of *C. gariepinus* juveniles Exposed to sublethal Concentrations of Aqueous Crude Leaves Extract of *V. amygdalina*

Hepatic histomorphometric measurements like hepatocyte diameter (HD) and hepatic cytosurface area (HCA), hepatic nuclear surface area (HNSA) and hepatic surface area (HSA) were determined by using Motic image plus 2.0 (Motic Asia, Hong Kong) software (Audu, Omirinde, Gosomji and Wazhi 2017; Sulaiman *et al.*, 2022).

Statistical Analyses

Data collected were subjected to analysis of variance (ANOVA) using SPSS version 16. Significant means were set at p<0.05 and separated using Duncan new multiple

range test. Results were presented as means ± standard errors of mean.

Results

Liver Antioxidant Responses

Activities of liver superoxide dismutase (SOD), glutathione peroxidase (GSH), and lipid peroxidation (MDA) are shown in Table 1. The highest SOD activity (29.18 ± 4.27 μmole SOD/min) was observed in the control, while the lowest (26.98 ± 0.45 μmole SOD/min) occurred at liver of fish

exposed to 1.60 g/L (highest) concentration of *V. amygdalina*. Glutathione peroxidase (GSH) activity was lowest (9.25 ± 0.25 μg/ml) at 0.80 g/L and highest (12.00 ± 0.00 μg/ml) at 1.60 g/L. Lipid peroxidation (MDA) peaking at 1.92 ± 0.36 mmol/mg protein (0.40 g/L) and reaching the lowest level at 1.32 ± 0.18 mmol/mg protein (0.80 g/L). Compared with the control, exposed groups demonstrated clear alterations in antioxidant profiles, indicating that *V. amygdalina* extract induces oxidative imbalance in fish liver tissues.

Table 1: Mean activities of liver Superoxidase dismutase, Glutathione peroxidase and Lipid peroxidation of *C. gariepinus* exposed to sub-lethal concentrations of crude leave extract of *Vernonia amygdalina*

Conc. (g/L)	SOD (μmoleSOD/min)	GSH (μg/ml)	MDA (mmol/mg protein)
0.00	29.18 ± 4.27	11.25 ± 0.50^A	1.46 ± 0.01
0.40	28.32 ± 0.43	11.63 ± 0.13^A	1.92 ± 0.36
0.80	27.93 ± 1.62	9.25 ± 0.25^B	1.32 ± 0.19
1.60	26.98 ± 0.46	12.00 ± 0.00^A	1.50 ± 0.36
pvalue	0.920	0.008	0.497

Conc.=Concentration SOD=Superoxidase dismutase GSH= Glutathione peroxidase MDA= Lipid peroxidation

Histopathology of liver of *Clarias gariepinus* Juveniles Exposed to subLethal Concentrations of crude leaf extract of *V. amydalina*

The liver of control fish bears normal hepatic histo-architecture as shown by hepatocytes with characteristic polyhedral outline, roundish nucleaus and normal sinusoidal space (plate A). B. 0.4 g/L: With the exception of mild central veinous (CV) congestion, histo-architecture was similar to the control (arrowhead and white arrow) (plate B). C. 0.8 g/L: No visible hepatic parenchyma lesion except for mild sinusoidal congestion (plate C). D. 1.6 g/L: Presence of moderate sinusoidal congestion (arrowhead) and diffuse hepatocellular degeneration (oval outline) (plate D).

Histomorphometry of Liver of *Clarias gariepinus* Juveniles Exposed to Sub Lethal Concentrations of crude leaf extract of *V. Amydalina*

There is reduction in histomorphometry parameters with the increase in the sub lethal concentrations of crude of crude leaf extract of *V. Amydalina*. The least hepatocyte diameter and hepatic cytosurface area of 23.32 ± 0.70 um and 343.35 squ were recorded in liver of fish from the control tank and 0.40g/L while the highest values of 44.90 ± 1.40 and 1387.04 ± 105.52 was recorded in highest sub lethal concentrations (1.6g/L) and 0.40 g/L respectively. The least hepatic nuclear and surface area values of 70.33 ± 7.21 and 6.59 ± 0.40 (Squ) were recorded in 0.00g/L while the highest mean values of 386.20 ± 34.08 and 20.19 ± 1.08 (Squ) were recorded in 0.40 and 1.6g/L respectively (figure 6 -7). There is significant different ($P<0.05$) in liver of fish exposed to sub lethal concentrations of crude leaf extracts of bitter leaf compared with the control in HD, HCA, HNSA and HAS.

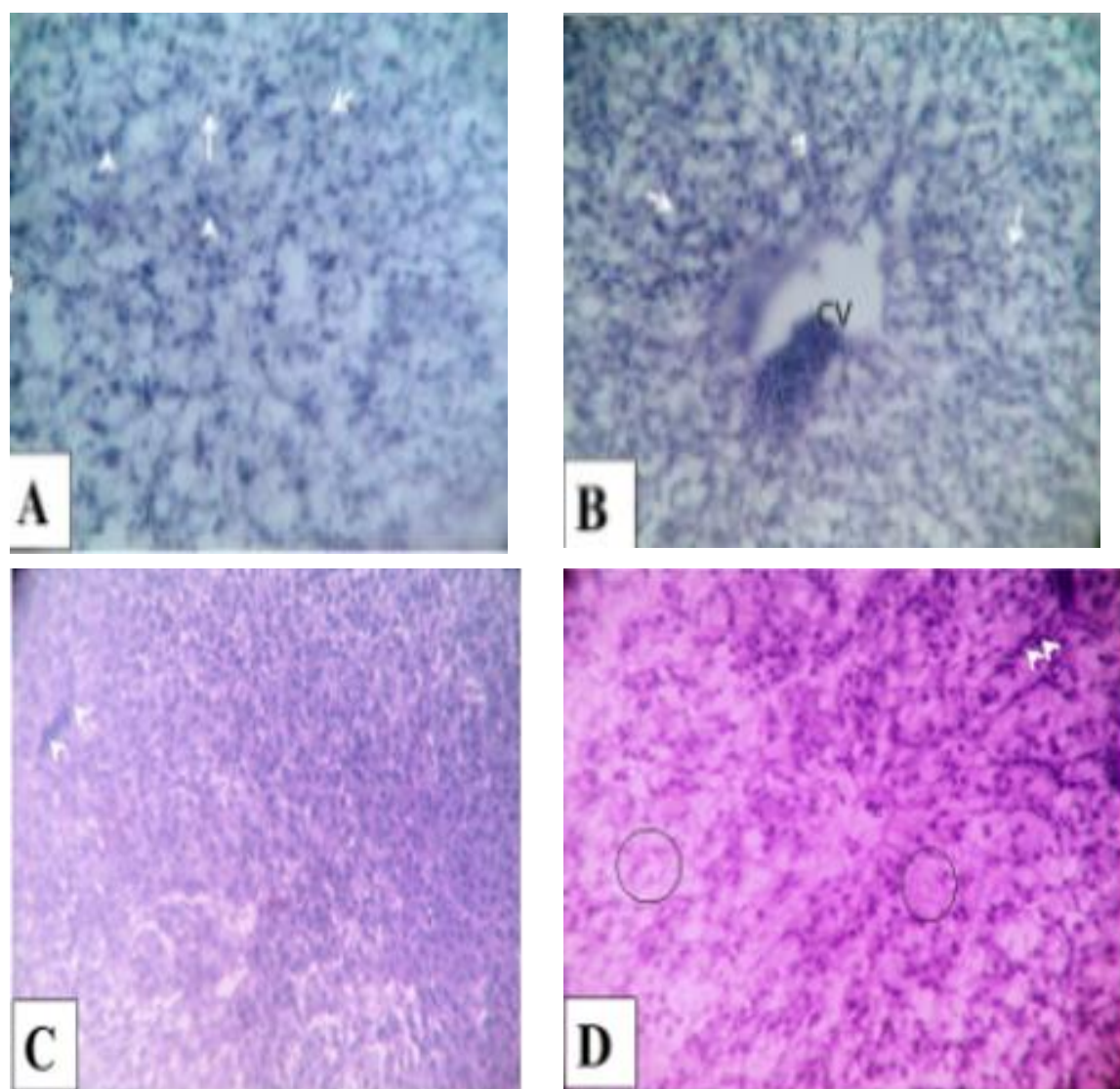


Plate 1: Photomicrographs of the liver of *Clarias gariepinus* exposed to sub lethal concentrations of *Vernonia amygdalina*. A. Control (0.00 g/L): normal hepatic histoarchitecture as shown by hepatocytes with characteristic polyhedral outline, roundish nucleus (white arrow) and normal sinusoidal space (arrowhead). B. 0.4 g/L: With the exception of mild central veinous (cv) congestion,

histoarchitecture was similar to the control (arrowhead and white arrow). C. 0.8 g/L: No visible hepatic parenchyma lesion except for mild sinusoidal congestion. D: Presence of moderate sinusoidal congestion (arrowhead) and diffuse hepatocellular degeneration (oval outline). Magnification: x400; Stain: Haematoxylin-eosin.

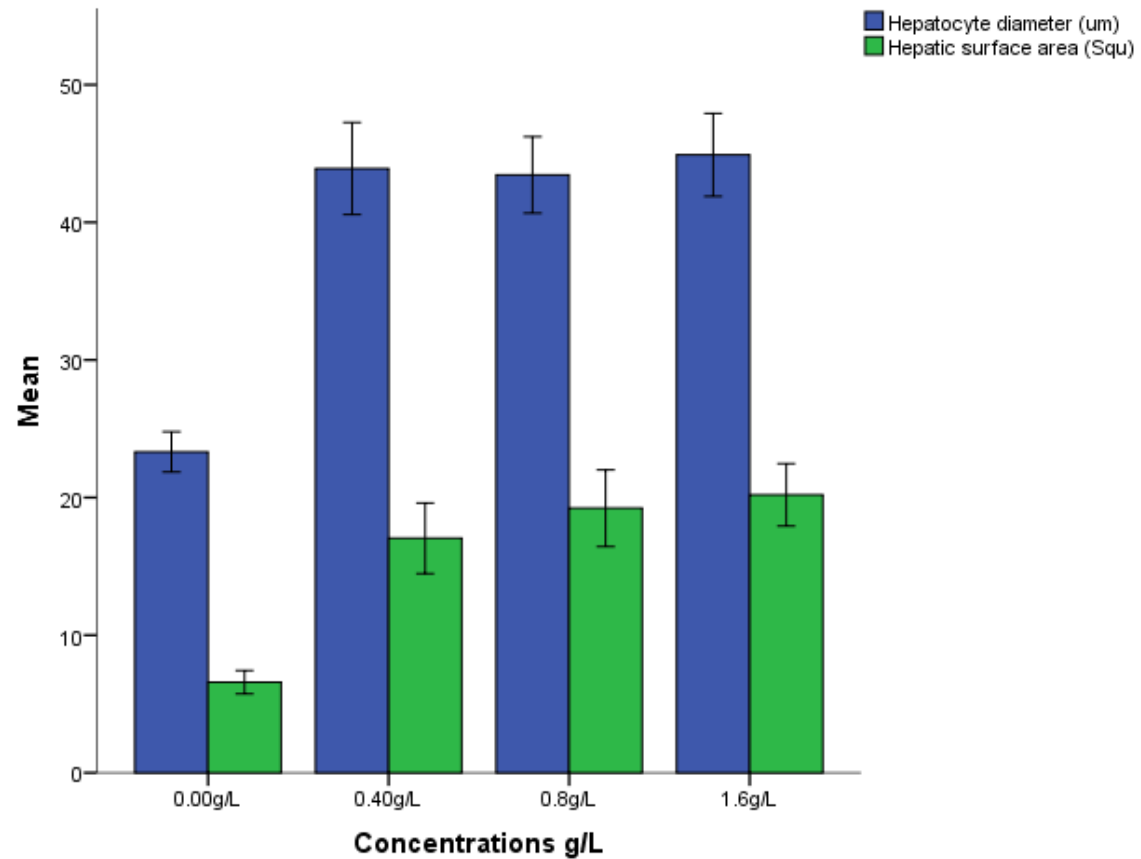


Figure 1: Hepatocyte diameter (um) and hepatic surface area (Squ) of liver of *C. gariepinus* juveniles exposed to sublethal concentration of *V.amygdalina*.

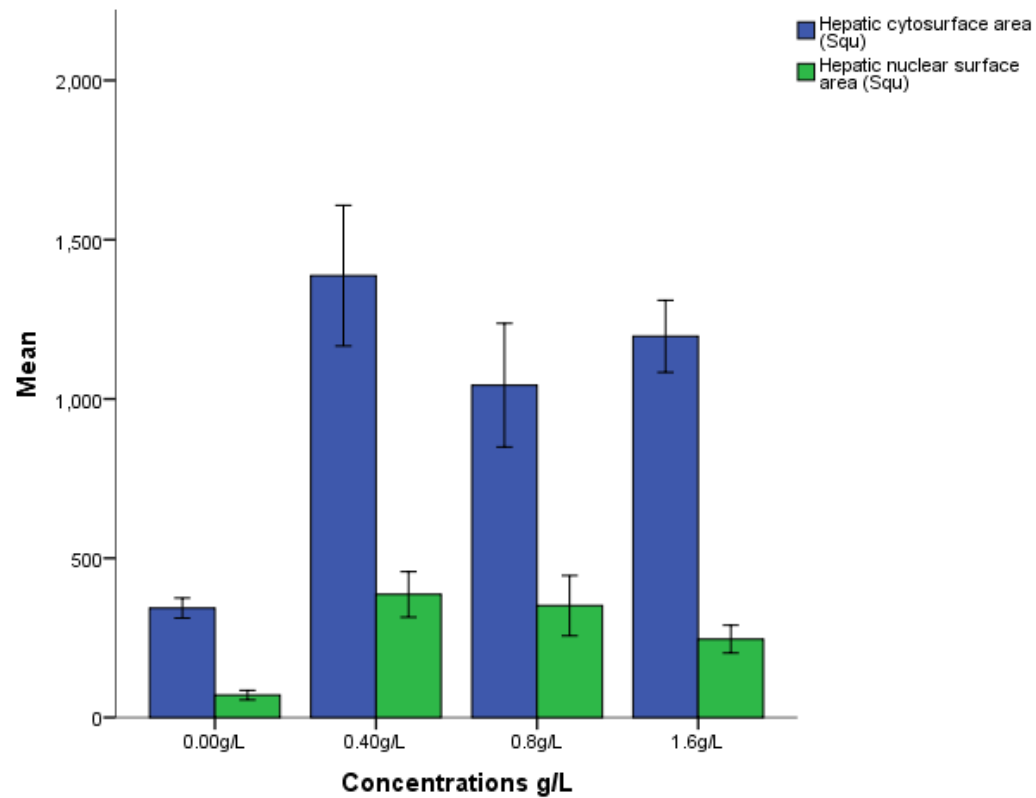


Figure 2: Hepatic cytosurface area (Squ) and hepatic nuclear surface area (Squ) of liver of *C. gariepinus* juveniles exposed to sublethal concentration of *V. amygdalina*.

Discussion

Antioxidant Biomarkers

Alteration in antioxidants biomarkers have been used as indicator of toxicity in Pisces (Jhamtani, Shukla, Dahiya & Agarwal, 2017). Reduction in SOD, GSH, and MDA in liver of catfish exposed to sub lethal concentrations of crude leaf extracts of *V. amygdalina* indicate oxidative stress in liver of the exposed fish. Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. Increase or decrease level of malondialdehyde (MDA) as lipid peroxidation (LPO) level in different organs of various fish species and aquatic organisms, is an indication of oxidative stress (Charity et al., 2018). There was reduction in level of MDA in liver of the exposed fish in this study. It disagrees with findings of (Necati, et al., 2017) who reported an increment in liver MDA levels of fish *Alburnus tarichi* (abnormal) and normal fish (*A. tarichi*) caught from polluted and unpolluted sites of river Van lake. Similarly, (Naglaa & Mohammed, 2019) reported increased in LPO levels in both Cd and Pb in liver of *C. gariepinus* exposed to sub lethal concentrations of $CdCl_2$ and $PbCl_2$ along with the control for 60 days. Conversely, there was reduction in MDA levels in juvenile *C. gariepinus* exposed to SLCs of Pb for 28 days (Joseph & Khafilat, 2012) which agrees with the finding of this research.

The enzymes SOD, GPx and CAT are recognized as the first defensive antioxidants of xenobiotic induced oxidative stress (Stanley et al., 2021), whose function includes the prevention of free radical formation in cells and tissues, dismutase superoxide to hydrogen peroxide and water, catalyze direct decomposition of hydrogen peroxide to water and molecular oxygen and remove hydrogen peroxide into harmless molecule by reducing it to water while oxidizing glutathione (Mikko, 2014). Reduction in SOD, CAT and GPx were reported by researchers, which is in line to the results of this research. Stanley et al., (2021) reported reduction in liver SOD, CAT and GPx activities of *C. gariepinus* exposed to WSFs of burnt tire ash for 28 days. Similarly, according to Juliane et al., (2020), there was decreased in three days exposure of used, and new lubricating oil CAT in gills of the *O. niloticus*. A reduction in hepatic SOD activity was also reported by (Francine et al., 2015) after exposing *Prochilodus lineatus* juveniles to Ni for 4 days. This finding also collaborates with a previous investigation made by (Joseph & Kafilat, 2012) in which SOD and CAT activities in gill and liver were reduced after 28 days of *C. gariepinus* exposed to chronic concentrations of $Pb(NO_3)_2$ and $ZnCl_2$ when compared to the control. There was a decrease SOD, CAT, GST and GSR in blood of *C. gariepinus* juveniles exposed to aqueous extract of the stem-bark of *Albizia chevalieri* (Ibiam et al., 2015) which is similar to the results this research.

Histopathology of Liver of *Clarias gariepinus* Juveniles exposed to Sub lethal Concentrations

Mild central venous congestion, sinusoidal congestion and diffuse hepatocellular degeneration were observed in liver of fishes exposed to sublethal concentrations of bitter leave, which is in line with the work of (Audu et al., 2017) who reported similar alterations (central venous congestion and hepatocellular degeneration) in liver of *C. gariepinus* exposed to acute (4 days) concentrations of bitter leave. There is alteration in the normal architecture, parenchymal and fibrosis with fat deposit the liver of *C. gariepinus* exposed to Moringa (*Oleifera saponins*) extract as reported by (Obaroh et al., 2020) which is not in line with the findings of this research. This research work shows that there is hepatic sinusoidal congestion in the histology of the liver of exposed fishes which commensurate with the findings of Shalaka and Pragna (2013) after treating (*Oreochromis mossambicus*) to plant nutrient. There is expansion in the central vein (CV), blood congestion and nuclear degeneration in the liver of Nile tilapia (*Oreochromis niloticus*) after been exposed to BenzalkoniumBhloride mixture with Treated Produced water as reported by Ikisa et al., (2019) which is not the same with the findings of this research. Liver of *C. gariepinus* juveniles exposed to acute concentrations of aqueous crude fruit extract of *A. nilotica* showed histological distortions such as hypertrophy, hemorrhage and cellular degeneration (necrosis) which increased as the toxicant concentration increased which further substantiates the toxic potential of this plant (Damshit et al 2025) which is similar to the result of this research.

This research work shows that there is significant difference compared with the control in hepatocyte diameter (um), hepatic cytosurface area (Squ), hepatic nuclear surface area (Squ) and hepatic surface area (Squ) which agreed with findings of (Audu et al., 2017) that reported alterations in liver (hepatocyte nuclear diameter and surface area) morphometrics of *C. gariepinus* after exposure to acute concentrations of *V. amygdalina*.

It is concluded that this study confirmed that juveniles of catfish exposed to two months sub lethal concentrations of crude leaf *V. amygdalina* extracts suffered from great pollution that affected the oxidative stress biomarkers of liver, histology and histomorphometry of liver of *C. gariepinus* juveniles which may have effects on the fish population.

Recommendation

Farmers should stop using *V. amygdalina* as pesticide close to water bodies. Further studies on reproductive hormones, oxidative stress biomarkers, reproductive organs biochemistry and effects of the plants extracts on growth parameters of Aquatic organisms should be conducted.

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