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Toxicity of aqueous extracts of bitter leaf (*Vernonia amygdalina)* on haematological profile of African catfish (*Clarias gariepinus*) juveniles

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Abstract
The toxic effects of the aqueous extracts of *Vernonia amygdalina* on *Clarias gariepenus* were investigated under laboratory conditions a 96h exposure period using blood profile and behavioral responses as biomarkers of environmental quality in toxicity testing. One hundred and eighty (180) healthy juveniles of *C. gariepinus* (17.05±0.25g) were acclimatized for a week, weighed and randomly distributed into eighteen bioassay tanks (20L) with stocking density of ten (10) fish per tank. A range finding test was conducted prior to the experiment. The fish were later exposed to 0ml (contol), 40ml, 45ml, 50ml, 55ml, and 60ml per litre concentrations of aqueous extracts of *V. amygdalina* for 96hrs in a static bioassay procedure. The erratic swimming led to loss of equilibrium, respiratory disturbance, lethargies and mortality of the text organisms which then increased significantly with increasing concentrations of plant extract. Differences observed in the mortalities of *C. gariepenus* at varying concentrations were significant ($P < 0.05$). Hematological indices indicated a significant ($P < 0.05$) decreasing trend in the red blood cell, haemoglobin and the packed cell volume with increasing concentrations of *V. amygdalina* while the leucocyte counts increased with increasing concentrations of *V. amydgalina*. The 96h LC50 was determined as 53.52ml/l.

Keywords: toxicity, *Vernonia Amygdalina*, aqueous extract, haematology, *Clarias Gariepinus*

Introduction
The aquatic environment is affected negatively by daily human activities. These activities which include the discharges of various pollutants such as agricultural pesticides, insecticides into the water body and alter ecological balance [1] Due to the residual effects of these contaminants, important organs like kidney, liver, gills, brain and genital organs are damaged in exposed fish [2] The residual effect of agrochemical and their high toxicity has necessitated the needs to explore other environment and health friendly fish toxicants such as botanical plants with piscicidal activity. Unlike synthetic chemical pesticides which leave harmful residues in the aquatic environment, botanical biocides are believed to be more ecologically friendly because of their easy biodegradation, availability and less expensive nature [3] Plants have been traditionally used to harvest fish in small water bodies due to their potency against fish [4] According to [5] the toxic parts of plants employed as fish poisons include the roots, seeds, fruits, latex, bark and leaves. These plants have been persistently and indiscriminately abused through the use of concentrations higher than necessary, thereby causing mass mortality of fish in water bodies and contaminating the environment [6, 7]. Reported an adverse effect of tobacco leaf dust on the blood profile of *O. niloticus* while histological examination of *O. niloticus* by [8, 9, 10] indicated high toxicity of *Morinda lucida* to the fish. *Vernonia amygdalina* popularly known as bitter leaf in Nigeria is a perennial shrub of 1- 3m in height that grows throughout tropical Africa [11, 12] It is a highly appreciated vegetable in west and central Africa where it is commonly used in traditional medicine. It performs both medicinal and nutritive functions [13] Fish farmers have resorted to the use of nonconventional and unregistered fish toxicants such as agrochemicals because they are fast to kill and readily available in the market without any consideration on the negative impacts of these substances on aquatic organisms, and other users of the water bodies. Hence, there is need to explore other toxicants (e.g. botanical biocides) that are environment and fish-health friendly, biodegradable and cheap. Therefore this present study aims to assess the toxicological effect of

bitter leaf extract on the behavioral response and haematological parameters of *Clarias gariepinus* juveniles

Materials and Methods

Experimental plant materials

Fresh leaves of *Vernonia amygdalina* were collected from the Botanical garden of the Federal University of Technology Akure, Ondo State. The plant was identified and authenticated with the aid of Odugbemi (2008). The fresh leaves of 1kg were collected, washed to remove any soil material and air-dried at room temperature (25±2) °C for 2 weeks. After drying, the leaves were pulverized with a sterile manual grinding machine (Binatone UK) and then sieved with 100-micron sieve to obtain a fine powder. Three hundred grams (300g) of the dry fine powder was later soaked in 1 liter of deionized extracted (agitated in an orbital shaker at 185r/min for 48 h) with 1000mL of deionized water for 48hrs. The solution was filtered using muslin cloth to separate extract from residue. The aqueous solution was then kept in a plastic container at room temperature (25±2) °C until the time of use.

Experimental fish

One hundred and eighty (180) healthy juveniles of *Clarias gariepinus* (wt, 17±0.5g) were used as test organisms for the toxicity test because of its suitability. The Clarias Juveniles were bought from Teaching and Research farm of the department of Fisheries and Aquaculture, Federal University of Technology, Akure. The test fish were transported in aerated polythene bags to the laboratory. The fish were acclimatized to laboratory conditions for forty eight hours (48 h) in 48L plastic tanks of (60cm x 45cm x 45cm). The fish were fed using coppen commercial feed. The water was changed daily to prevent accumulation of toxic waste metabolite. Feeding of experimental fish stopped a day before the bioassay test. The *Clarias gariepinus* juveniles were weighed with a top Metler Balance (Metler Toledo, PB8001 London) and randomly distributed into the bioassay tanks at ten fish per tank with each treatment replicated.

Bioassay study

A preliminary test was carried out at first to determine suitable range of concentration for the bioassay experiment. Behavioural activities of the fish were properly monitored and recorded. Results from the range finding tests provided a guide for the concentration level used for the definitive test. The concentration ranges chosen after the preliminary test were: 0ml/L, 40ml/L, 45ml/L, 50ml/L, 55ml/L and 60ml/L. These concentrations were carefully measured out to make up 10L of solution in twelve bioassay tanks. Another bioassay tank with 10L of water, free of extract served as the control. Ten *Clarias gariepinus* juveniles (17±0.5g) were introduced into each tank and monitored for mortality at 24, 48, 72 and 96 hours. The LC_{50} concentration of *V. amygdalina* extract was determined after 96 hour of exposure of test fish using Probit analysis. The test media were monitored for their pH, dissolved oxygen and temperature levels using the method described by [14].

Fish blood collection and analysis

At the end of the 96h exposure, test organisms from each of the experimental tanks were taken out immediately for blood analysis. Five milliliters (5ml) of blood was collected from the fish by cardiac puncture using different 5ml disposable heparinized syringes, with ethylene diamine tetracetic acid (10ml EDTA) as anticoagulant. The blood analyses followed the method described by [15].

Statistical analysis

Accumulative mortalities of exposed fish were used to estimate the LC_{50}. This was determined from the graph of percentage mortality against concentration using Probit Analysis. All biological data resulting from the experiments were subjected to one-way analyses of variance (ANOVA) using SPSS 18.0 software. Differences were considered significant at P= 0.05.

Results

Water quality parameters measured in all the treatments were marginally similar statistically apart from the dissolved oxygen levels which reduced drastically in the exposure concentrations from 40-60ml. The lowest dissolved oxygen was observed in the 55ml and 60ml bioassay tanks which were significantly the same (P>0.05).

Behavioral response of *C. gariepinus* exposed to aqueous extract

The test organism showed distress in behavior immediately they were introduced into the bioassay tanks. Abnormal behaviours displayed by the fish increased with increasing concentration of the test extract and with the time of exposure. There were no obvious changes in fish behaviour at concentrations less than 50ml/L for the first 24 h of exposure. However, the control (0.0ml) fish did not exhibit any abnormal behaviour (Table 1). Behavioral responses noted were frequent jumping, restlessness, skin discolouration, opercula movement, hyperactivities, gulping of air and erratic swimming before death (Table 2). At very low concentration (0ml, 40ml), test organisms tolerated the test solution up to 24hours of exposure, while at high doses (45, 50, 55, 50 and 65mls), they lost their swimming patterns. As the experiment progressed, the test organisms were seen to get weaker, with ventral surface turned upward and those that couldn't tolerate the concentrations any longer went into comatose. Normal behaviour was however observed in the control 0.0ml/L.

Table 1: Behavioural Response of *Clarias Gariepinus* Juveniles Exposed To Different Concentrations of Aqueous Extracts of Bitter Leaf

	24 Hours						48 Hours						72 Hours						96 Hours					
Concentration (ml)	0.0	40	45	50	55	60	0.0	40	45	50	55	60	0	40	45	50	55	60	0.0	40	45	50	55	60
Frequent jumping	-	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
Erratic swimming	-	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
Loss of reflex	-	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
Hyper ventilation	-	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+
Discolouration	-	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+

KEY

+ = Indicates the presence of a particular observation

- = Indicates absence

Mean mortality of *C. gariepinus* exposed to aqueous extract of *V. amygdalina*

The mean mortality of the test fish exposed to various concentrations of *V. amygdalina* is as presented in Fig. 1. The results reveals that the varying concentration of the test media caused significant (P<0.05) but variable death rate in the exposed fish. Mortality rate was concentration dependent with the highest death (87%) observed in the highest concentration (60ml/l) (Table 2). No mortality was observed in the control (0.0ml) group of fish. The lethal effect of *V. amygdalina* on *Clarias gariepinus* expressed as LC_{50} at 96 h was 53.517ml (Fig. 1)

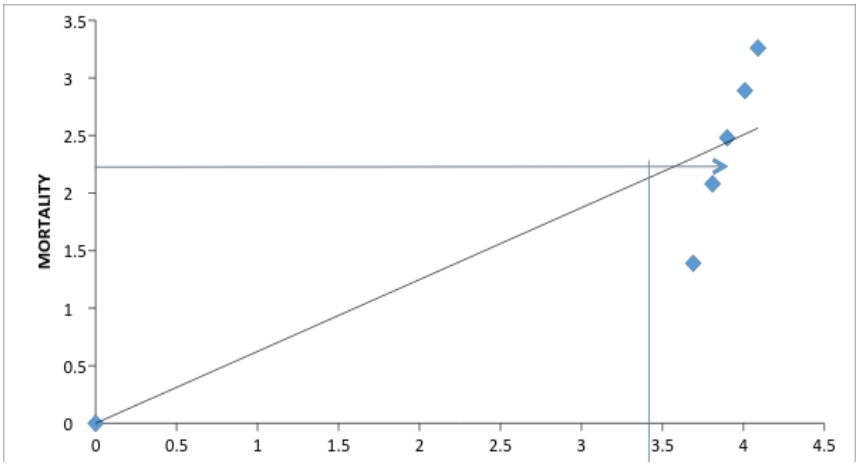

Fig 1: 96-h LC_{50} of *C. gariepinus* juveniles exposed to different concentrations of aqueous extract of *Vernonia amygdalina*

Table 2: Mean mortality response of *C. gariepinus* exposed to different concentrations of aqueous extract of *V. amygdalina* leaf for 96 hours

		Number of deaths					
Concentration (mgl^{-1})	**No. of fish exposed**	**24 h**	**48 h**	**72 h**	**96 h**	**Mortality (%)**	**Survival (%)**
0	30	0	0	0	0	0	100
40	30	0	0	1	4	13	87
45	30	0	0	2	8	27	73
50	30	6	7	9	12	40	60
55	30	9	10	14	18	60	40
60	30	6	14	22	26	87	13.3

Physico-chemical Parameters obtained during exposure of *Clarias gariepinus* juveniles to aqueous extract of *V. amygdalina* for 96 h.

The result showed a significant reduction in dissolved oxygen of the test concentrations when compared with the control (0.0ml) (Table 3). The highest value of dissolved oxygen (7.40±0.06) was observed in the control (0.0ml) and was significantly higher (P<0.05) than the test groups. The least value of DO was observed in group of fish exposed to 60ml of *V. amygdalina*. There were fluctuations in the values of the pH and temperature. The pH value also decreased with an increasing concentrations of *V. amygdalina* across the treatment. The highest pH value (7.49±0.02) was observed in the control (0.0ml), while the lowest value was observed in concentration (60ml) with a value of (6.41±0.03). There were no significant differences in temperatures in the highest concentration (50-60 ml/L). (Table 3).

Table 3: Physico-chemical Parameters Obtained during Exposure of *C. gariepinus* to *V. amygdalina* Concentration (ml/L)

Parameters	**0.0 (control)**	**40**	**45**	**50**	**55**	**60**
DO_2	7.40 ± 0.05^{a}	3.86 ± 0.06^{b}	3.66 ± 0.06^{c}	3.06 ± 0.06^{d}	3.05 ± 0.11^{d}	3.05 ± 0.05^{d}
pH	7.50 ± 0.10^{a}	7.44 ± 0.04^{ab}	7.38 ± 0.08^{b}	7.01 ± 0.01^{c}	7.10 ± 0.06^{c}	7.10 ± 0.40^{c}
Temp	27.75 ± 0.05^{a}	26.72 ± 0.02^{a}	27.72 ± 0.03^{a}	27.73 ± 0.06^{a}	27.74 ± 0.03^{a}	27.73 ± 0.01^{a}

Values of 3 replicates on the same row with the same superscript are not significantly different (P>0.05).

Haematological Observations of *C. gariepinus* subjected to Bitter leaf extract

Result of the blood profile reveals that *Clarias gariepinus* exposure to varying concentrations of bitter leaf affected the blood profile as there were significant differences (P<0.05) in all the parameters tested, except for the mean corpuscular haemoglobin which was the same for all the treatments (Table 4). Highest value (6.10±1.00) of the leucocytes count was observed in the concentration of 50ml followed by 55ml (5.60±0.92) while the lower value (4.90±0.70) of leucocytes was observed in 40ml. Haemoglobin values ranged between 9.05±0.25 and 12.40±0.30. Highest Hb (12.40±0.30) was observed in the control (0.0ml) which was significantly the same (P>0.05) with Hb of fish exposed to 40ml (11.43±0.96) and 45ml (11.20±0.90). The control (0.0ml) had the highest RBC counts (4.58±0.45), followed 40ml (4.48±0.45) while the least count (3.38±0.45) of red blood cell was observed in 55ml which was significantly the same (P>0.05) with 60ml (3.63±0.42). The Packed Cell Volume (PCV) varied marginally with varying concentrations of aqueous extract of the test plant. Highest PCV (37.0±1.00) was observed in the control (0.0ml) which was significantly the same with 40ml (36.67±1.53). However the lowest PCV (28.00±4.0) was obtained in fish exposed to 50ml *V. amygdalina*.

The mean values of the haematological parameters of the *C. gariepinus* exposed to varying concentrations of *V.*

amygdalina leaf extract are shown in Table 4. Result of haematocrit, haemogloin values, erythrocyte and leucocyte count of the fish exposed to different concentration of *V. amygdalina* leaf extract revealed significant haematological alteration and changed (Table 4).

Hb counts decreases from 12.40 g/dl to 9.05 ±0.25g/dl with increase in concentrations of *V. amygdalina*. The decrease in values were dependant on time (96h) and concentrations of the plant extract. There was increase in the WBC with increase in concentration of the plant extract. The PCV decreased from 37.0 ± 1.00 to 26.00 ± 2.00 as concentration increased.

Table 4: Haematological parameters of *C. gariepinus* subjected to varying levels of *V. amygdalina* Concentrations (ml/L)

PARAMETER	0.0ml	40ml	45ml	50ml	55ml	60ml
HB (g/dl)	12.40±0.30^{a}	11.43±0.65^{ab}	11.20±0.90^{ab}	10.65±0.96^{bc}	9.20±1.32^{cd}	9.05±0.25^{d}
PCV (%)	37.0±1.00^{a}	36.67±1.53^{a}	33.67±2.52^{ab}	28.07±4.0^{c}	28.00±2.03^{c}	26.00±2.00^{c}
RBC(g/dl)	4.58±0.45^{a}	4.48±0.23^{ab}	4.10±0.28^{abc}	3.73±0.55^{bc}	3.38±0.45^{c}	3.63±0.42^{c}
WBC(mm^3)	3.07±0.20^{b}	4.90±0.70^{a}	5.25±0.65^{a}	6.08±1.00^{a}	6.10±0.92^{a}	6.13±1.21^{a}
MCV(fl)	89.18±0.82^{a}	89.47±0.08^{a}	90.25±0.56^{a}	90.34±0.22^{a}	90.58±0.81^{a}	88.66±0.85^{a}
MCH (%)	2.99±0.04^{a}	2.98±0.01^{a}	3.00±0.02^{a}	3.00±0.02^{a}	3.01±0.01^{a}	2.80±0.19^{b}
MCHC(pg)	33.65±0.31^{ab}	33.28±0.05^{b}	33.26±0.34^{b}	33.22±0.10^{b}	33.27±0.26^{b}	34.48±1.26^{a}

Values of 3 replicates on the same row with same superscript are not different (P>0.05) (mean values ± SD) HBC: Haemoglobin concentration, PCV: Packed Cell Volume, WBC: White Blood Cell, RBC: Red Blood Cell; MCV: Mean Corpuscular Volume, MCHC: Mean Corpuscular Haemoglobin Concentration, MCH: Mean Corpuscular Haemoglobin.

Discussion

This study agrees with the report of [16] who documented that the introduction of a toxicant into an aquatic system might decrease the dissolved oxygen concentration which will in turn impair respiration thereby leading to asphyxiation. The reduction in the dissolved oxygen level across the treatments in this study also agreed with the findings of [17] on exposure of *C. gariepinus* to aqueous extracts of *Ipomea aquatica* leaf. The dissolved oxygen levels observed for in this study were below 5mg/L as reported by [18] and [19] as the essential level of dissolved oxygen for good fish production. The pH range recorded in this study falls within the physiological range of 7.0 to 8.5 which is ideal for biological productivity of fishes as reported by [20, 21] Behavioural response of the fish to aqueous extract of *V. amygdaline* shows a toxic effect of the plant on *C. gariepinus* juveniles and the effect of the toxicity increased with the concentration and time of exposure. *C. gariepinus* exposed to increasing concentration of *V. amygdalina* exhibited erratic swimming and aggressiveness when placed in the bioassay tanks. Increased physical activity, excess mucus production, distress, operculum ventilation, respiratory stress, gulping of air and skin whitening prior to death were associated with *V. amygdalina* toxicity in this study. Similar result was reported by [22] who worked on exposure of *Sarotherodon galilaeus* to extract of *Tetrapleura tetraptera.* Exhibition of stressful behaviour by the fish could have arisen due to respiratory impairment caused by the components of the phytochemicals of bitter leaf. *C. gariepinus* became inactive at higher concentrations (50-60ml) of the toxicants. Haematological parameters like haemoglobin, haematocrit, blood cell count, glycemia, and ion concentration can be used to find physiological response of contaminated environment [23]. Past investigations have also identified changes in several haematological parameters as indicators of pollutant [24] In the present study, significant reduction (P<0.05) of some blood parameters (haemoglobin, PCV, RBC) were noted across the group. The control fish (0.0ml) had the highest concentration of haemoglobin, packed cell volume, and erythrocyte counts which were significantly (P<0.05) better than other groups of fish exposed to *V. amygdalina*. Reduction in different blood parameters might have been as a result of malfunctioning of the fish haematopoietic system caused by bitter leaf exposure. A decreasing trend in the values of red blood cell count, haemoglobin and haematocrit agreed with the report on rainbow trout exposed to diazinon by [25]. They attributed this to the destruction of hematopoietic organ due to the adverse effect of toxicant. Reduced haemoglobin, red blood cell count and packed cell volumes have also been reported for *C. gariepinus* exposed to lead nitrate [26] Contrary to the haematological indices observed in this study, Rao (2010) reported an enhanced blood profile of common carp exposed to carbaryl. In the present study, the white blood cells (WBC), increased with increasing dosage of *V. amygdalina*. This could be attributed to stress, due to the ability of white blood cells to act as defence mechanisms of the body. *V. amygdalina*, as a medicinal plant could also help in increasing the white blood cell counts [27] also reported increased leucocyte counts of *Channa punctatus* exposed to copper.

Conclusion

The vast growth in human population and the proliferation of industries in Nigeria has resulted in the discharged of large amount of toxic waste into water bodies where they pollute and degrade both the flora and fauna of the ecosystem resulting in habitat loss. This study has revealed that *C. gariepinus* juveniles exposure to extract from bitter leaf is enough to induce various toxic effect which make the fish vulnerable to diseases and eventually death.

From this study, it is evident that increasing concentrations of bitter leaf could lead to fish abnormal behavioural responses and dysfunction in the health of the fish. Haematological alterations observed demonstrated that examination of fish blood profile and other eco-toxicological bio-indicators can be used as an early warning for *C. gariepinus* survival and environment protection. Hence, preventive measures must be adopted to prevent the indiscriminate discharge of this effluent into our water bodies.

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