Susceptibility of *Lymnaea natalensis* and *Oreochromis niloticus juveniles* to the Root-Extract of *Petiveria alliacea* As a Risk Barometer in Aquaculture

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Abstract

The application of organic and inorganic pesticides in environmental sanitation is associated with a degree of residual risk in the immediate environment and within tissues of animals in concern habitat. This research, therefore, investigated the lethal and sub-lethal effects and histopathological influence of a botanical (P. alliacea) on a commercial fish (Oreochromis niloticus) and an unwanted snail (L. natalensis) in ponds of Western-Nigeria. One hundred and eighty organisms of each species were randomly allotted in a Completely Randomized Design (CRD) into eighteen rectangular plastic tanks, measuring 0.55x 0.33 x 0.33 m^3 each. Phase one of the experiment involved an acute toxicity of 96-hr where L. natalensis was exposed to root-extract concentrations of 0.00, 0.22, 0.48, 1.06, 2.34 and 5.15 g/L while O niloticus received 0.00, 1.90, 4.30, 9.40, 21.0 and 45.0 mg/L in triplicates. Second phase involved a 56-day chronic toxicity test using 0.0021, 0.0031 and 0.0042 g/L from 40%, 60% and 80% of median concentration (Lc_{50}) in phase 1 before a withdrawal period of seven (7) days for O. niloticus. Acetylcholine esterase activity was then measured at 56th day, 7th day of withdrawal and fish with zero treatment using the brain of O. niloticus as tissue for investigation through colorimetric spectrophotometry. Probit analyses and descriptive statistics were applied to the data of mortality recorded during exposure to plant extract. Behavioural changes in the fish include imbalance, loss of co-ordination, reduction in operculum movement and partial or zero movement. The water snail displayed irritation, restlessness, attempt by visceral mass to vacate the shell and excessive secretion of mucous, production of bubbles and escape of body mass from shell before death. The Lc_{50} equals 0.159g/L and 0.0053g/L for L. natalensis and O. niloticus, respectively. Histopathological changes in the organs of fish were diverse: brain (spongiosis, gliosis, necrosis), gill (congestion of capillaries, sloughing-off of epithelium), and liver (softening and occasional bursting). Water snail recorded loss of muscle fibres, and reduced spermatozoon in the seminiferous tubules. Recovery in acetylcholinesterase activity was less than 10% after withdrawal. Root-extract of P. alliacea could be used in eradicating L. natalensis in earthen fish ponds but fish should be harvested or transferred due lower resistance to extract.

Keywords: Susceptibility, *Oreochromis niloticus*, *Petiveria alliacea*, Histopathology

Running Title: The use of root-extract of *P. alliacea* in sanitizing fish ponds

Susceptibilité des Juvéniles de *Lymnaea natalensis* et *Oreochromis niloticus* au Préparation à Base d'Extrait de Racine de *Petiveria alliacea* Comme Baromètre de Risque en Aquaculture

Résumé

L'utilisation de pesticides organiques et inorganiques dans l'assainissement environnemental est associée à un certain degré de risque résiduel dans l'environnement immédiat et au sein des tissus des animaux

concernés dans leur habitat. Cette recherche a donc examiné les effets létaux et sub-létaux ainsi que l'influence histopathologie d'une plante (*P. alliacea*) sur un poisson commercial (*Oreochromis niloticus*) et un escargot indésirable (*L. natalensis*) dans des étangs de l'Ouest du Nigeria. Cent quatre-vingts organismes de chaque espèce ont été répartis aléatoirement dans un Plan Expérimental Complet (PEC) en dix-huit réservoirs plastiques rectangulaires, mesurant chacun 0,55 x 0,33 x 0,33 m³. La première phase de l'expérience a impliqué une toxicité aiguë de 96 heures où *L. natalensis* a été exposé à des concentrations d'extrait de racine de 0,00, 0,22, 0,48, 1,06, 2,34 et 5,15 g/L tandis qu'*O. niloticus* a reçu 0,00, 1,90, 4,30, 9,40, 21,0 et 45,0 mg/L en triplicats. La deuxième phase a impliqué un test de toxicité chronique de 56 jours utilisant 0,0021, 0,0031 et 0,0042 g/L provenant de 40 %, 60 % et 80 % de la concentration médiane (Lc50) de la phase 1 avant une période de retrait de sept (7) jours pour *O. niloticus*. L'activité de l'acétylcholinestérase a ensuite été mesurée au 56e jour, au 7e jour après le retrait et chez les poissons sans traitement, en utilisant le cerveau d'*O. niloticus* comme tissu pour l'investigation par spectrophotométrie colorimétrique. Les analyses probit et les statistiques descriptives ont été appliquées aux données de mortalité enregistrées pendant l'exposition à l'extrait de plante. Les changements comportementaux chez les poissons incluent un déséquilibre, une perte de coordination, une réduction du mouvement des opercules et un mouvement partiel ou nul. L'escargot d'eau a montré une irritation, une agitation, une tentative de la masse viscérale de quitter la coquille et une sécrétion excessive de mucus, la production de bulles et l'évasion de la masse corporelle de la coquille avant la mort. Le Lc50 est égal à 0,159 g/L et 0,0053 g/L pour *L. natalensis* et *O. niloticus*, respectivement. Les changements histopathologies dans les organes des poissons étaient divers : cerveau (spongiose, gliose, nécrose), branchies (congestion des capillaires, desquamation de l'épithélium) et foie (ramollissement et éclatement occasionnel). L'escargot d'eau a enregistré une perte de fibres musculaires et une diminution des spermatozoïdes dans les tubules séminifères. La récupération de l'activité de l'acétylcholinestérase était inférieure à 10 % après le retrait. L'extrait de racine de *P. alliacea* pourrait être utilisé pour éradiquer *L. natalensis* dans les étangs en terre, mais les poissons devraient être récoltés ou transférés en raison de leur faible résistance à l'extrait.

Mots-clés : Susceptibilité, *Oreochromis niloticus, Petiveria alliacea*, Histopathologie

Introduction

The menace of invasive biota in controllable aquatic systems reduces the production efficiency of commercial fish species during culture. The influence is further strengthened under a polyculture system of commercial aquaculture. Therefore, the sanitation of the micro-environment of the pond before stocking focuses the elimination of diverse organisms like predators (hibernating mud-fish, frogs, and alligators), excessive phyto-plankton and a multiplication of water snails that serve as intermediate hosts to the larvae of parasitic trematodes that infect fish tissues (Singh and Singh, 2005; Adesina, 2008; Ajibade and Omitoyin, 2011). Consequently, the use of organic extract as an alternative to synthetic chemicals in biological management of aquatic system is becoming popular in Nigeria. The organic method of eradicating the invasive species reduces the accumulation and persistence of impacts on tissues/organs of water organisms and the environment unlike the commonly available synthetic chemicals; evident through insignificant changes in most haematological parameters of African catfish exposed to latex of *Calotropis procera* (Omitoyin *and* Ajibade*,* 2014).

Nigeria and other tropical regions of the world are blessed with multiple flora- resources that are exploited for various utilizations. One of such suitable uses is an effective sanitation of the pond micro-environment. A typical example of such plants with high toxic ability but underutilized in South- Western Nigeria is *Petiveria alliacea* (Skunk weed). It is usually planted around houses

to repel snakes, scorpions and insects (Olaifa and Akingbohungbe, 1987; Adebayo *et al*., 2004). It invades any land area such that complete expulsion of other plants (Allelopathic potential) in the habitat is enforced. The main compound in this plant is a sulphur compound called S-benzyl phenylmethane thiosulfinate (called Petivericine). The other isolates include S-benzyl (2-hydroxyl)ethane thiosulfinate, S-(2-hydroxylethyl) phenylmethane thiosulfinate and S-(2-hydroxylethyl) 2-(hydroxylethane) thiosulfinate (Roman Kubec, *et al*., 2002; 2003). The plant is antirheumatic, anti-carcinogenic, anti-flu, anti-tussive, analgesic and anti-inflammatory (Perez-Leal *et al*., 2005), and insecticidal (Adebayo *et al*., 2007). The root is more pungent or richer in active components than any other the part of the plant (Roman-Kubec, 2003).

Aquatic toxicology can be defined as the influence of a toxic agent on any aquatic organism at a cellular, individual, population and community level (Odiete, 1991). It can be grouped into three categories: acute toxicity (≤ 96 hours), sub-lethal toxicity (about 28 days of exposure) and chronic toxicity (≥ 56 days of exposure).

Lymnaea natalensis are species of snail found in all types of freshwater in which molluscs are able to live (Russel-Hunter, 1961 a and b).They are found in Africa and Eastern Mediterranean in ditches, streams and lakes. Aquatic snails have been linked with initiation of ill-health in organisms in their habitats. Examples include Lymnaea species (*Lymnaea natalensis* and *L. acuminata*), *Bulinus globosus*, *Indoplanorbis exustus, Bulinus bulinus* and *Biomphlaria pfeiferri* (Tiwari *et al*., 2003; Singh *et al.,* 2004). They serve as vectors for the larvae of trematodes which are harmful to fish, man and livestock; especially fascioliasis and schistosomiasis (Adenowo, 2015).

Oreochromis niloticus is not among the invasive species but may become an indirect recipient of botanical action; especially in a polyculture system of commercial aquaculture. That is an abuse of botanical application needs to be evaluated towards non-target organisms like commercial fish species as a pro-active approach in effective management of water resources. Also, limited information exists on the toxicological effect of botanicals on the different stages of fish development. This study, therefore, measured the effect of toxicity from the root-extract of *P. alliacea* in 3 organs (gills, brain, and liver) of *O. niloticus* juveniles; a precaution for farmers not to abuse the use of botanicals. It ranked tolerance via Lc_{50} value plus degree of recovery after one-week- withdrawal of biocide through acetylcholinesterase activity.

Materials and Methods

The root of *P. alliacea* was isolated from the plant after harvest from the rangeland (pasture-land) behind the University farm and Faculty of Veterinary Medicine in the University of Ibadan. The root of the plant was freshly cleaned off sand and homogenised by grinding with the aid of pistle and mortal before been weighed (500g) and soaked in distilled water in a stoppered flask for 48hrs ; using a minimum ratio of 1:3 (W/V) at 30g / 100ml. This was followed by the filteration of the aqueous solution and the evaporation (40^{o}C) of the solvent at reduced pressure using a rotary evaporator to obtain a semi-solid residue. A dry-freezing at -20^{0}C was achieved for a latter use with the aid of a lyotrap for a complete drying to a constant weight at the Central Laboratory of the University of Ibadan. This served as stocked extract and was diluted in subsequent bioassays ; a modification of method from Adebayo *et al.,* 2004).

A total of four hundred healthy juveniles with an average weight 230 ± 5.0 (g) and body length 12.0 ±2.1 (cm) were purchased from the Oyo State Fishery Department of the Ministry of Agriculture, Natural Resources and Rural Development, Agodi, Ibadan. Each species was

washed in 0.1% KMNO4 solution and acclimated for 21 days in large plastic aquaria. *O. niloticus* was fed on commercial pelletized feed (45.0 crude protein; 2-3mm) at 5% body weight (Reish and Oshida, 1986). *Lymnaea natalensis* were captured from earthen ponds at the fish farm in the Department of Aquaculture and Fisheries Management, University of Ibadan and fed with boiled water lettuce (Osundara, 2006) and associated with an average body weight of 0.25g ± 0.01. Prior to the toxicity test fish and snails were starved for 48-h.

The toxicity test involved two stages followed by a third stage of withdrawal period of 7 days associated with enzyme inhibition assessment. Phase one (1) involved a 96-hr acute toxicity test using a static method as described by Solbe (1995) and FAO (1977). Eighteen (18) rectangular tanks (five litres capacity) with dimensions of 0.55 x 0.33 x 0.33 m^3 were employed during experimental set-up. Six (6) concentrations of plant extracts in three (3) replications were used for each species of organisms: 0.001, 0.22, 0.48, 1.06, 2.34 and 5.15g/L for *Lymnaea natalensis*; 0.00, 1.90, 4.30, 21.0 and 45.0 mg/L for *Oreochromis niloticus* juveniles. A spacing factor of 2.2 was used. Ten organisms of each species were randomly allotted per tank in triplicate per treatment. The Median Lethal Concentrations (Lc_{50}) that kill 50% of sampled population was then determined by recording the mortality observed at a regular time interval of six hours. The 96-h median lethal concentration (Lc_{50}) value was then determined by a probit (regression) analysis and Finney computer programme at 95% confidence interval as described by Finney (1971; 1978). This was verified with a linear (y = mx + c) plots of the probit curve for the fish; plotting the probit mortality of the fish against log-concentrations as described by Sprague (1970; 1971).

The second stage of experiment involved the exposure of the chosen commercial fish (*O. niloticus*) to three (3) fractions (40%, 60% and 80%) of median lethal concentration (Lc_{50}) obtained from the 96-hr exposure of fish to root-extract of *P. alliacea.* The applied concentrations of extract were equivalent to 0.0021, 0.0031 and 0.0042 g/L. This is a chronic test because of the longer time of exposure but lower concentrations of extract to disallow death. The acetylcholinesterase activity was then determined on the 56th day in addition to an initial determination of enzyme activities in fish without exposure (0.00g/L) to root-extract as control; using the serum and brain tissues of fish and an Atomic Absorption Spectrophotometer (AAS) as appropriate machine for investigation of enzyme activity. This phase was terminated by the removal of botanical through the introduction of freshwater and feeding for seven days with Coppens feed containing 45% crude protein at 3% body weight due to stress of exposure to root-extract. Water was refreshed daily by siphoning and replacement (Oyelese, 1994). The enzyme rate of activity was then re-determined after withdrawal period of seven (7) days. The rate of activity of acetyl-cholinesterase enzyme was determined using the Grating spectrophotometer (752w uv-vis) on the 56th day of exposure (control) and after seven days of withdrawal of extract across the sub-levels. Weighing and homogenization of tissues in 0.1 M phosphate (0.1 M, pH 7.4) were the initial steps to determine enzyme activity. 0.4 ml aliquot of the homogenate was put into a curvette containing 2.6mls phosphate buffer and 100µlof DTNB (5, 5-doithio-bis nitrobenzoate). A proper mixing of the contents in curvette was followed by measurement of absorbance at 405 nm before taking the final readings(Ellman *et al*., 1961).

Calculations:

The enzyme activity was calculated using the formula:

$R = 5.74 \times 10^{-4} \times A/CO$ (Where R = Rate in moles of substrate hydrolyzed/min/gm tissue, A=

Change in absorbance per minute, Co = Original concentration of the tissue (mg/ml).

Histopathological investigation involved the selection, removal and dissection of organs (brain, gill and liver) of dead fish every 6 hours during acute test while the muscle of snail (*L. natalensis* was employed. Each organ was preserved in Bouin's fluid in sample bottles for 5h and then transferred into 10% formalin. The dehydration of tissues were carried out in a descending grade of ethanol, cleared in xylene and embedded in paraffin wax. A rotary microtome was then used for serial sectioning. The deparafinised sections were then stained with haematoxylin and eosin (Clayden, 1971). Photomicrographs of the slides, showing the changes, were taken using a digital photographic microscope at the histopathological laboratory of the Department of Microbiology and Parasitology, Faculty of Veterinary Medicine in the University of Ibadan.

Result and Discussion

The choice of water as the most suitable solvent of extraction is similar to the findings of Musa *et al*. (2011) that reported water or ethanol (interchangeably) as a better solvent for extracting six popular medicinal plants in Igala land of Kogi State in Nigeria in terms of antibacterial activity. Every 500.0 g of the homogenized fresh root yielded 12.41g as water extract after drying in a single extraction in the current study.

Behavioral attributes displayed by the juveniles of *O. niloticus* during exposure to water-root extract include zero / partial movement which later led to staggering, imbalance and loss of co-ordination; higher display was observed in the bigger fish per plastic aquarium. These were followed by reduction in operculum-movement, gulping for air at the water-air interface (15-30 degrees inclination) and sudden attempt to jump out of solution, convulsion, permanent opening of the mouth and immobility (death). Mortality increases with increasing concentration of root-extract of *P. alliacea* in present study (Table 1 and Table 2). This trend agree with the findings of Ajibade and Omitoyin (2011) that applied latex of *Calotropis procera* on *Clarias gariepinus* and Xavier and Kripasana (2020) that studied the acute toxicity of *Enydra fluctuans* leaf extracts to Zebra fish.

The 96-h Lc_{50} was first determined by SPSSx-Probit Software as 0.005341g/l; upper and lower boundaries were 0.00647 and 0.00441, respectively. Also, exposure of *L. natalensis* to root extract of skunk weed recorded a 96-h Lc_{50} of 0.159g/L . Similarly, a log concentration equivalent of probit 5 equals 0.728 from the graph, and a corresponding anti-log of 53.46×10^{-4} (since each concentration was multiplied by 10^4 before transformation into logarithm) was obtained. The regression analysis of the probit-curve gave a linear equation $y = 5.567 x + 0.857$ and correlation coefficient (r) of 0.972 for fish and $y = 3.117x + 3.5$ (where r = 0.46) for snail.

The results of the haematoxylin and eosin staining (H & E Method) reaction revealed departures from photomicrographs of tissues in the control that served as the normal (basal information). The brain tissues exhibited progressive increase in spongiosis, multiple foci of gliosis, congestion of blood vessels, necrotic neurons, spongiform vacuoles and extensive meningitis. The gills recorded mild to marked congestion of its capillaries, marked sloughing off and loss of the epithelium of secondary gill lamellae. Additionally, liver samples (tissues) were not available for sectioning because of softening-effect and complete damage (bursting) by root-extract during treatments in *O. niloticus (Plates)*. These abnormalities in the architecture of the organs agreed with the findings of Xavier and Kripasana (2020) and Hassan *et al*.(2021).

Table 1: Mortality pattern of *Oreochromis niloticus* exposed to varying concentrations of water extract of *Petiveria alliacea* root at 96-hr

Sample Size	Treatment (mg/L)	Mortality (%)			
		24-hr	48-hr	72-hr	96-hr
30.0	0.00	00.0	00.0	00.0	00.0
30.0	1.90	00.0	00.0	06.7	06.7
30.0	4.30	00.0	03.3	23.3	30.0
30.0	9.40	00.0	56.7	76.7	83.3
30.0	21.0	10.0	66.7	96.7	100.0
30.0	45.0	63.3	86.7	100.0	100.0

Table 2: Mortality pattern of *Lymnaea natalensis* exposed to varying concentrations of water root-extract of *Petiveria alliacea* at 96-hr

Sample Size	Treatment (mg/L)	Mortality (%)			
		24-hr	48-hr	72hr	96-hr
30.0	0.00	00.0	00.0	00.0	00.0
30.0	0.22	00.0	13.3	23.3	90.0
30.0	0.48	00.0	26.7	86.7	100.0
30.0	1.06	23.3	66.7	100.0	100.0
30.0	2.34	30.0	100.0	100.0	100.0
30.0	5.15	40.0	100.0	100.0	100.0

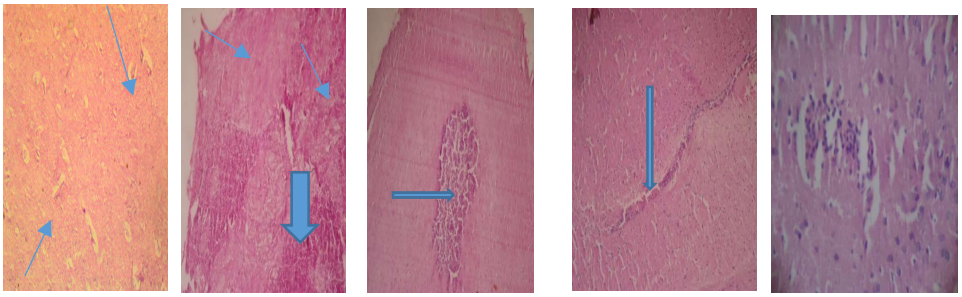

Plate 1 Plate 2 Plate 3 Plate 4 Plate 5:

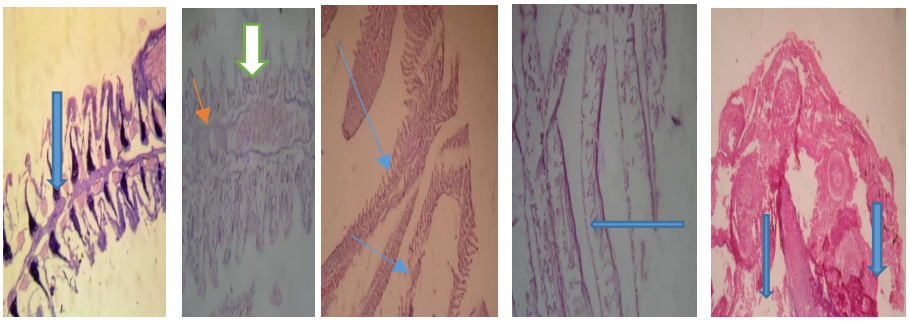

Plate 6 Plate 7 Plate 8 Plate 9 Plate 10

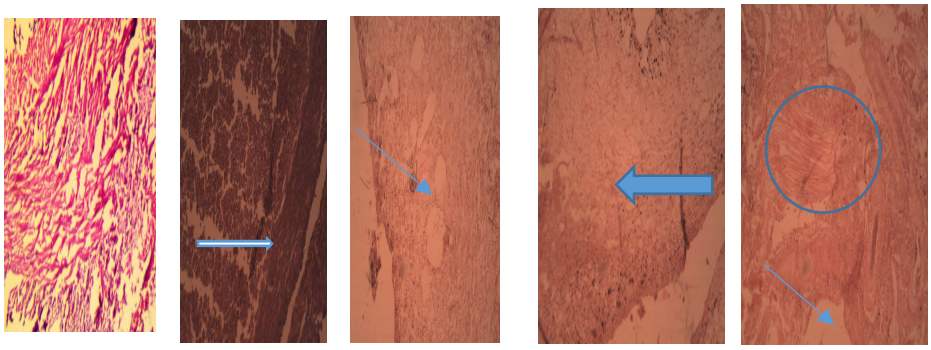

Plate 11 Plate 12 Plate 13 Plate 14 Plate 15

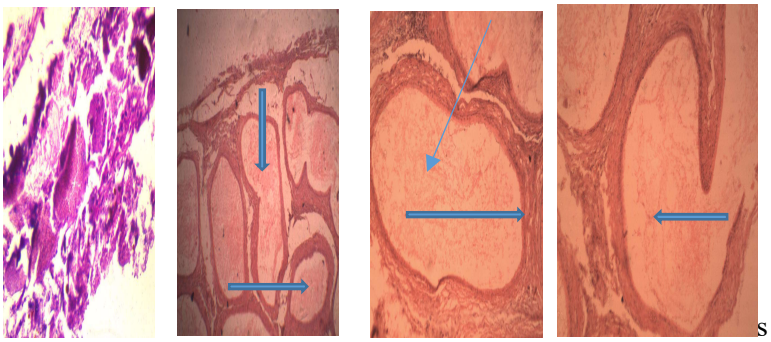

Plate 16Plate 17Plate 18Plate 19

Plate 1: Brain of *O. niloticus* exposed to 0.00 g/l of root-extract of *P. alliacea* showing normal array of brain capillaries (arrows) x 400

Plate 2: Brain of *O. niloticus* exposed to 0.0019 g/l of root- extract of *P. alliacea* showing moderate spongiosis (thin arrow) of the white matter and aggregates of glial cells (thick arrow) x 400

Plate 3: Brain of *O. niloticus* exposed to 0.0043 g/l of root- extract of *P. alliacea* exhibiting multiple foci of gliosis and a congestion of blood vessels x 100

Plate 4: Brain of *O. niloticus* exposed to 0.0094 g/l of root-extract of *P. alliacea* root exhibiting a few degenerates and congestion of blood vessels x 100

Plate 5: Brain of *O. niloticus* exposed to 0.021 g/l of root-extract of *P. alliacea* exhibiting moderate spongiosis (thin arrow) and meningitis x 400.

Plate 6: Gill of *O. niloticus* exposed to 0.00 g/l of root-extract of *P. alliacea*
root showing normal appearance x 100.

Plate 7: Gill of *O. niloticus* exposed to 0.0019 g/l of root- extract of *P. alliacea* exhibiting a mild congestion of the gill lamellae x 100

Plate 8: Gill of *O. niloticus* exposed to 0.0043 g/l of root-extract of *P. alliacea* exhibiting a slight distortion of gill lamellae x 100.

Plate 9: Gill of *O. niloticus* exposed to 0.0094 g/l of root-extract of *P. alliacea* showing marked sloughing- off and loss of epithelium of the secondary lamellae x 100.

Plate 10: Gill of *O. niloticus* exposed to 0.0094 g/l of root- extract of *P. alliacea* with marked sloughing-off (thick arrow), thickening and loss of the epithelium of the secondary gill lamellae (thin arrow) x 100

Plate 11: Muscle fibres in the foot of water snail (*L. natalensis*) exposed to 0.00 g/l of root-extract of *P. alliacea* showing rich meshwork of muscle fibres x 100

Plate 12: Foot of *L. natalensis* exposed to 0.22 g/l of root- extract of *P. alliacea* with a mild loss of the muscle fibres and moderate vacuolar degeneration in 0.22 g/l of root extract x 100

Plate 13: Plate 4.3. Foot of *L. natalensis* exposed to 0.48 g/l of root- extract of *P. alliacea* with slight depletion of the muscle fibres that form the muscular foot x 100

Plate 14: Foot of *L. natalensis* exposed to 1.06 g/l root -extract of *P. alliacea* with moderate sparseness of the muscle fibres x 100

Plate 15: Foot of *L. natalensis* exposed to 2.34 g/l of root-extract of *P. alliacea* showing moderate loss of the covering epithelium (arrow) and sparseness of the underlying connective tissue (Circle) x 100

Plate 16: Ovotestis of the gonad of *L. natalensis* exposed to 0.00 g/l of root-extract of *P. alliacea* showing various compartments in Ovotestis x 400

Plate 17: Gonad of *L. natalensis* exposed to 1.06 g/l of root- extract of *P. alliacea* with moderate amounts of spermatozoa x 100

Plate 18: Gonad of *L. natalensis* exposed to 2.34 g/l of root- extract of *P. alliacea* with seminiferous tubule (thick arrow) containing very few amounts of spermatozoa (thin arrow) in its lumen x 400

Plate 19: Gonad of *L. natalensis* exposed to 5.15 g/l of root- extract of *P. alliacea* with seminiferous tubules containing very sparse amount of spermatozoa x 400

Plant extract have been used to control many unwanted organisms to protect fish from different water bodies and aquaculture by many scientists. These findings range from control of infestation in fish by micro-organisms (Mishra *et al*., 2019), control of pests in aquaculture (Ajibade *et al*. 2021), and checkmating fish diseases (Stratev, 2018). Consequently, a neurotoxicity test is applied as an effective and rapid method to evaluate the influence of toxicants or pollutants in tissues of fish using the changes in activity of acetylcholinesterase (Dizer *et al*., 2001; Ajibade, 2017). Cholinesterase (ChE) is an enzyme to be considered when the interest is to study neural functions where a significantly low ChE activity indicates high level of disturbance by pollutants or toxicants. The three (3) sub-levels of Lc_{50} from acute toxicity (0.0021, 0.0031 and 0.0041 g/L) resulted in significant reductions in acetylcholine esterase activity from the brain of O. niloticus compared to the control (0.000 g/L) at the end of 56 days of chronic exposure to root-extract of *P. alliacea* (Table 3a). The two higher concentrations (0.0031 and 0.0041 g/L) exhibited similar level of reductions in enzyme activity

compared to the first exposure (0.0021 g/L). Recovery after seven (7) days of withdrawal was also similar and lower in the higher concentrations than that of the first degree of exposure in the current study. These general reductions in acetylcholinesterase activity increased with increase in extract concentrations as similarly reported by Johnson *et.al*. (2016) and Xavier and Kripasana (2020). The resultant effect on fish physiological activities include loss of co-ordination and poor orientation in water as first phase of behavioral displays at the exposure of fish to root-extract as signs of neurotoxicity. However, after seven (7) days of withdrawal of biocide, changes were observed as increase in enzyme activity revealed improvement in fish condition; although the rate of improvement was low and less than 10% across the three sub-levels of exposure (Table 3b) in the current study.

Table 3a: Comparisons of mean acetylcholinesterase activities in the brain of *Oreochromis niloticus* exposed to chronic levels of root-extract of *P. alliacea*

	Concentration of extract(mg/L)			
	0.00	2.10	3.10	4.20
56th day of exposure	$7.30^{a} \pm 0.52$	$4.96^{b} \pm 0.81$	$4.20^{c} \pm 0.44$	$3.71^{dc} \pm 0.50$
7th day of withdrawal	$7.30^{a} \pm 0.52$	$5.55^{b} \pm 0.55$	$4.45^{c} \pm 0.37$	$4.01^{dc} \pm 0.33$

Means with the same superscript along each row are not significantly different at p > 5%.

Table 3b: Variations in mean acetylcholinesterase activity in the brain of *Oreochromis niloticus* exposed to chronic levels of root-extract of *P. alliacea*

	Concentration of extract (mg/L)		
	2.1	3.1	4.2
Reduction at 56th day of exposure (%)	32.07	42.44	49.37
Reduction at 7th day of withdrawal (%)	23.97	39.04	45.07
Degree of recovery (%)	08.10	03.40	04.30

The initiation of a recovery process, as indicated by higher values of acetylcholinesterase activity after withdrawal, could serve as an indicator for the preference for natural biocides unlike synthetic chemicals such as fungicides (Strivastava and Singh, 2014) in agricultural/environmental management with zero change after 7 days of withdrawal during application to *Clarias batrachus*. Nevertheless, a need for caution on the part of aqua-culturists or fish-hunters, in tropical Africa, during usage by avoiding excesses in the applications of natural plant-extracts from medicinal plants because of piscicidal action and slow rate of recovery. Consequently, every farmer intending to apply the root of this plant as a tool in sanitizing the hatchery or nursery pond for effective result should be mindful of the Lethal Threshold Concentration (LC_{50}) of 0.0053 g/l. Also, the fresh root loses its potency after 48 hours of exposure to air, if crushed, and the crushed and air-dried root was ineffective owing to the escape (volatility) of some active components; especially the naturally unstable sulphides.

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