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Diagnosis of Toxic Stress Induced *Pseudomonas aeruginosa* Infection in Juvenile *Clarias gariepinus*; Not as Easy as it Seems

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ABSTRACT

The unknown cause of mass kills in juvenile *Clarias gariepinus* was investigated using different markers as a prerequisite to provide a solution for disease outbreaks. Thus, the following analyses were conducted; namely, hematology and serum biochemical analyses, stress biomarkers detection and quantification, water quality test, elemental quantification from pond soil sampled, quantitative and qualitative phytochemical analyses of stem bark of the *Mangifera indica* tree in the farm vicinity, gas chromatography-mass spectroscopy (GC-MS), identification of chemical constituents of essential oils in the latex, toxicological and microbiological analyses of the pond water, feed samples and visceral from necropsied juveniles were also conducted. The isolate obtained was identified using 16S rDNA gene sequence. There were significant changes in hematological and biochemical parameters. The water quality parameters showed significantly higher (P<0.05) turbidity, ammonia, and dissolved salts. The water also showed significantly higher (P<0.05) heavy metals and evidence of organic pollutants from the *Mangifera indica* latex that might have contaminated the pond water. The isolated bacterium was identified as *Pseudomonas aeruginosa*. It was concluded that the high mortality observed might be due to *Pseudomonas aeruginosa* infection sequel to compromised immunity due to toxicants exposure. It is suggested that sites of fish farms should be properly surveyed .

INTRODUCTION

One of the major roles of veterinarians is to identify the causes of diseases in animals as part of the requirements for instituting appropriate treatment or preventive measures **(Zautner *et al.*, 2017; Adamek *et al.*, 2019)**. The causes of diseases in aquatic lives are vast and identifying them may be thought-provoking because the cause of observed pathology in animals may be multifactorial **(Adamek *et al.*, 2019)**. Manifestation of pathology or clinical diseases in aquaculture like other habitats may be due to pathogens, an imbalance of the microbiome or

stress **(Zautner *et al*., 2017)**. It has been reported that a positive correlation exists between stress and disease in fish **(LeaMaster *et al*., 1997)**. The exposure to the sources of external stress could culminate in immune compromise in fish, resulting from various diseases manifestation in the stock **(Pandey *et al*., 2012)**. The younger naïve juvenile fish stocks are more susceptible to adverse detrimental effects of the environment unlike the older stocks, which could relatively withstand environmental adversity **(Emere & Dibal, 2014)**. Aquatic environmental contamination may result from industrial wastes, which are directly discharged into the rivers or chemicals used on the farms washed by rainwater to the rivers. This is common when earthen ponds are located at the same level as plane of water table, and/or are in close proximity to crop farms, which are at higher plane allowing used chemicals to trickle down into the pond **(Becerril-García *et al*., 2019)**. Diseases and common externally interfering factors such as contamination causing toxicity threaten fish health and productivity **(Alexander *et al*., 2013)**. These might have adverse effects on the morphology of the gills and skin **(Abalaka, 2013; Alexander *et al*., 2013)**. High percentage mortality occurrence is termed kills, which is a major problem this study tried to address.

Fish kills could affect the agricultural economy of a nation since fish farming has been identified as the fastest growing food production sector in the world **(FAO, 2012)**. Fish production increased from 29.9 million ton in 2007 to 41.9 million ton in 2012 **(FAO, 2010)**. Fish is the cheapest source of animal protein to all at different strata of society **(Haruna, 2006)**. Nigeria, despite its large freshwater bodies, has not succeeded in reaching its full capacity in terms of fish food sufficiency **(FAO, 2010)**. Contaminants and stress inducing factors resulting from diseases could be a major hinderance to the fish agrarian development for freshwater fish production. In Nigeria, it was estimated that freshwater fish occupy over 40% of the total species population of fish. Slight change in the environment of the fish habitat could easily predispose the fish to stress and hence, results in diseases **(Rafet & İlhan, 2014)**.

Nigeria with population estimation of 200 million in 2019 needed 3.32 million metric ton of fish to get a balance diet. The production of fish in Nigeria for the year 2018 was estimated to be 1.123 million metric ton. This estimated production is less than half of what will be enough for the population **(FAO, 2018)**. Remarkably, there is an alarming geometric increase in population, which urgently needs to be met by an increase in food production, especially protein. There is no option but to embark on various methods of aquaculture. This study addressed a typical problem confronted when earthen pond is used as an aquacultural system. In addition, it reflected the appropriate management of fish production in the sub-Saharan regions of Nigeria in which the earthen pond system is one of the commonest means of fish production **(FAO, 2010)**.

The commonest fish, which is commercially cultured in sub-Saharan Africa, particularly in Nigeria, is the *Clarias gariepinus* **(FAO, 2012)**. It is noteworthy that, the production of fish is controlled by environment, and this enables the producer to predetermine the management system and production process and predict yield **(FAO, 2012)**. There are many challenges in the external environment that could upset the homeostasis of fish and affect production. Lack of orientation and difficulty to assess capital and land for fish farming leaves the farmer with no

alternative than to resort to the establishment of their farm at any available piece of land. There are challenges of standard enforcement of environmental laws in Nigeria **(Abatan, 2012; Ola-Davies *et al*., 2017)**. The establishment of fish farms at inappropriate locations could be detrimental to fish and could predispose to stress and diseases.

Bacterial diseases of fish are of great importance among the challenges facing aquaculture industry and fish production in the world **(Kitao *et al*., 1983; Thomas *et al*., 2014)**. *Pseudomonas aeruginosa* causes fish diseases which is responsible for appreciable economic losses in aquaculture industry. *Pseudomonas* species is one of the most common causes of bacterial disease of fish and appears to be a stress related **(Thomas *et al*., 2014)**. The disease caused by *Pseudomonas* species is characterized by a development of red patches on the skin and hence, it is called Redskin disease. It occurs throughout the year **(Mastan, 2013; Thomas *et al*., 2014)**. This is a circumstantial study to determine the cause of kills in juvenile *Clarias gariepinus* in an earthen pond and proffering solutions to the researched problem.

MATERIALS AND METHODS

Ethics

All works in this study were conducted in accordance with University of Ilorin, Faculty of Veterinary Medicine Ethical Review Committee (FVERC) standards, and the research was approved with FVER/010/2018.

Veterinary clinical approach and observations

The owner of four $50m^3$ earthen ponds, each with capacity of 4,000 juvenile *Clarias gariepinus* presented a major complaint of the death of over four thousand juveniles to the Veterinary Teaching Hospital, University of Ilorin. The fish farm was located at Sango area (Lat: 8.4796; Long: 4.5320) in Ilorin metropolis, Kwara State, Nigeria. Clinical history further reveals that only the adversity of juveniles was affected on the farm. Sand and loamy bags were used on the walls of the pond. The farmer also disclosed that few days before lodging the complaint of the incident, the pond was limed by a non-professional. The fish were then treated with antibiotics florfenicol® in water by the attendants after noticing a lower feed intake sequel to liming. The principle of epizootiology was the clinical outlook in this case; on arrival, the pond had fetid odor due to autolysis of the dead fish. For a critical observation on the environment, there was a PMS filling station close to the farm. In addition, there was a mango tree (*Mangifera indica*) with stem bark latex trickling into the pond where the kills occurred. The pond water was highly turbid with the latex and oils spills from the environment on the surface of the water, which showed an evidence of contamination. On close observation of other closer ponds where older fish were bred, it was observed that they had good appetite, active and were within average weights. The dead fish were floating; the moribund life fish were inclined perpendicular to the plane of water surface struggling for breath, and there was an obvious mouth opening and a

forceful irregular movement of opercula. This was an evidence of fish inability to utilize dissolved oxygen in water and signs of dyspnoea as presented in Fig. (1).

Fig. 1. Juvenile *Clarias* species showing signs of dyspnoea due to low dissolved oxygen

Sample collection

Fresh dead and moribund juveniles *Clarias gariepinus* (n= 10) were collected in a container for the diagnosis. These were collected for necropsy purposes. Water, feed and visceral samples (during necropsy) were asceptically collected for microbiological analysis, while blood and liver samples were collected for haematological and biochemical investigations. Additionally, pond water, pond bed soil and *Magnifera indica* latex samples were collected for quality and toxicological analyses.

Haematological and biochemical analyses

Blood samples were subject to haematological analysis using a veterinary autohaemoanalyzer. The serum and homogenized liver samples were subjected to biochemical analysis using commercial test kits Randox® (United Kingdom) in adherence to standard spectrophotometric procedures to assay the following: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), uric acid, urea, potassium ion, sodium ion, glucose-6-phosphate dehydrogenase, Lactate dehdyrogenase (LDH), gamma-glutamyl transferase (GGT), total cholesterol. Other biochemical analysis conducted are the evaluation of stress biomarkers such as malondialdehyde (MDA), glutathione peroxidase (GP_x), glutathione–S-transferase (GST), Superoxide dismuthase (SOD) and catalase (CAT).

Water quality, Soil and *Mangifera indica* analyses

Three hundred milliliter (300 mL) of water was collected from the fishpond to assess water quality. The water parameters were: temperature, pH, turbidity, dissolved oxygen,

conductivity and total dissolved salts. The pond's bed soil and *Mangifera indica* bark latex were also collected for soil elemental analysis using atomic adsorption spectrophotometric procedure, while the chemical constituents of essential oils in *Magnifera indica* latex were profiled, and organic compounds were identified using gas chromatography-mass spectroscopy (GC-MS) **(Rohloff, 2015)**. Phytochemical quantitative and qualitative analyses were conducted using established protocol **(Ola-Davies *et al*., 2017)**.

Necropsy Procedure

Five dead and five moribund fishes were collected from the pond site, and post- mortem procedure was conducted on them. The external integument was cranio-caudally observed. The head, fins, opercula & gills were closely observed for lesions. A mid- line incision was made at the ventral region, craniocaudally extending to expose the viscera.

Microbiological analysis

Samples of skin swab and visceral from the fish, water and feed were aseptically collected, and 1.0g of each solid sample and 1mL of water sample were pre-enriched in 9.0ml of buffered peptone water and incubated at 37^0 C for 18-24h **(Ahmed *et al*., 2017)**. Pre-enriched samples were sub-cultured on sabouraud dextrose (SDA) agar (Oxoid, UK) and 5 % sheep blood agar (Oxoid, UK) for fungal and bacterial isolation, respectively. The blood agar plates were incubated at 37^0C for 24h, whereas the SDA plates were incubated at 25^0C for 7 days **(Cowan & Steel, 2002)**. Bacterial growth on blood agar was sub-cultured on nutrient agar (Oxoid, UK), the growth which was subjected to biochemical reactions, including Gram staining, catalase and oxidase tests. No growth was detected on SDA at the end of the 7th day, and the samples were considered negative for fungi. The isolates had similar colonial morphology; they were shipped in a cool box containing ice packs to the International Institute for Tropical Agriculture (IITA), Ibadan for molecular detection and identification.

Molecular detection and identification of the isolate using 16S rDNA sequence

DNA extraction was carried out in accordance with manufacturer's instruction using ZR fungal/bacterial DNA miniPrep^{TM} as previously described **(Ahmed *et al*., 2017)**. The PCR assay was done according to the standard protocol as described by **Wagner *et al*. (2008)** using the following set of primers: 16SF: GTGCCAGCAGCCGCGCTAA, 16SR: AGACCCGGGAACGTATTCAC. The PCR reaction contained a final volume of 50µL which included 1 X PCR buffer (Inqaba Biotech, S. Africa), 5U Taq DNA polymerase, 2.5mM each of dATP, dCTP, dTTP and dGTP, 2.5mM $MgCl_2$ and 10ng/µl PCR buffer, 1 μL (5p*M*) each of forward and reverse primers and 2.0μL of template DNA (10ng/ µL DNA) and 12μL of deionized water. The reaction was performed in a thermocycler (Perkin- Elmer, USA), with an initial denaturation at 94^0C for 5mins, followed by 40 cycles at 94^0C for 30secs, annealing at 56^0C for 30secs and an initial extension at 72^0C for 45secs. A final extension phase of 72^0C for 7mins followed the previous step. The amplicon from the reaction was loaded on 1.5 % agarose

gel, containing 0.5µl/ ml of ethidium bromide (Pharmacia, Sweden) at 100V for 1hour and visualized under UV light in a Gel doc 2000 documentation system (Pharmacia, Sweden). The amplified fragment of PCR was purified from the agarose gel by adding 20µL of absolute ethanol to the PCR product and incubating at room temperature for 15mins. The mixture was then centrifuged at 10000 rpm for 15mins after which the supernatant was decanted. The sediment was centrifuged at 10000 rpm for 15mins after which 40μL of 70 % ethanol was added. The supernatant was decanted, and the sediment was air- dried, after which about 10μL of ultrapure water was added. Amplicon aliquat was checked on 1.5 % agarose. Partial genome sequencing of the amplified product was done at Bioscience laboratory of IITA, Ibadan. The obtained 16S rDNA sequences were compared to sequences available in GenBank database of the National Center for Biotechnology Information (NCBI), using the algorithm BLASTn program **(Wagner *et al*., 2008; Thomas *et al*., 2014)**.

MEGA version X was used to conduct phylogenetic analyses of the isolate and those obtained from NCBI **(Wagner *et al*., 2008)**. Consensus sequence from the test isolate was generated using seqtrace (version 9.0), and sequences of nine *Pseudomonas* and five *Aeromonas* (outgroup) strains used in constructing the phylogenetic tree were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). ClustalW from MEGA-X was used to align the isolate sequence with the sequences of *Pseudomonas* type strains and outgroup (*Aeromonas* species), and then a phylogenetic tree was constructed. The tree was constructed using the maximum likelihood program with the Tamura-Nei model and Boot strapping 500 times **(Wang *et al*., 2014)**.

RESULTS

Table (1) shows the biochemical parameters of juvenile *Clarias gariepinus* exposed to *Mangifera indica* bark latex and other environmental factors in an earthen pond. The ALP, MDA, GPx, CAT, K and Na were significantly higher (P<0.05) in the liver than in the serum. The haematological parameters of juvenile *Clarias gariepinus* exposed to *Mangifera indica* bark latex and oil spills in an earthen pond showed significantly (P<0.05) lower thrombocytes (thrombocytopenia), lymphocytes (lymphocytopenia) and MCV, compared to reference value (Table 2).

Water quality analysis in earthen pond incriminated in the kills of juvenile *Clarias gariepinus* showed significantly (P< 0.05) higher turbidity, total ammonia nitrogen, conductivity, total dissolved salt, and the water was foul smelling (Table 3). Table (4) displays minerals' concentration in water and soil bed of the earthen pond incriminated in kills of juvenile *Clarias gariepinus*. The soil, water, latex had significantly (P< 0.05) higher concentration of mineral ions than the standard.

Table 1. Biochemical parameters of juvenile *Clarias gariepinus* exposed to *Mangifera indica* bark latex and other environmental factors in an earthen pond

Parameter	Serum level	**Liver level**
ALT (IU/l)	15.27±5.07	13.40±5.31
AST (IU/l)	12.57±5.70	15.77±5.85
ALP (IU/l)	13.07±1.16	72.94±7.50*
Urea (mmol/L)	4.63±0.55	5.13±0.42
Uric acid (mg/dl)	19.45±6.92	17.12±3.27
LDH (IU/l)	97.09±26.89	118.1±9.41
GGT(U/l)	116.70±23.33	236.70±16.00
Total Cholesterol (mg/dl)	487.30±13.68	6570.00±25.27*
MDA (mol/l)	1.70±0.15	5.87±2.01*
GP_X (IU/l)	23.33 ±3.33	90.00±23.09*
SOD (U/l)	107.80 ± 29.90	281.90±29.90
CAT (IU/l)	680.0±159.00	4733.00±32.70*
G6PDH (U/l)	52.34±20.81	18.69±13.73
K (ppm)	2.340 ± 0.08	4.32 ± 0.05*
Na(ppm)	71.64 ± 5.07	95.68 ± 6.02*

*Significant (P<0.05). ALT: alanineransferase, AST: aspartateaminotransferase, ALP: alanineaminotransferase, LDH: lactatedehydrogenase, GGT: gamma –glutamyl transferase, MDA: malondialdehyde, GPx: glutathione peroxidase, SOD: superoxide dismutase, CAT: catalase, G6PDH: glucose -6-phosphate dehydrogenase.

In Table (5), qualitative and quantitative analyses of some phytochemicals in *Mangifera indica* bark latex incriminated in juvenile *Clarias gariepinus* kills are presented. The latex of *Mangifera indica* bark was positive for alkaloids, phenolics, cardiac glycosides, saponins, tannins, flavonoids, cardenolides, amino acid, lipids and phlobatannins. Analysis of organic chemicals in essentials oils of *Mangifera indica* bark latex revealed the presence of cyclohexane, alloaromadendrene, octahydronaphthalene, naphthalene, oleic acid and epimers of phenol and p-cresol (Table 6).

Table 2. Haematological parameters of juvenile *Clarias gariepinus* exposed to *Mangifera indica* bark latex and other environmental factors in an earthen pond

Parameter	*Clarias gariepinus* (from expose Pond)	Normal value for *Clarias* species
PCV (%)	32.52±3.38	49.87 + 3.11
HB (mg/dl)	10.84±1.13	2.33 + 2.23
Platelets (10^3µl)	3.33±0.33*	133.00±1.10
Heterocytes (%)	32.50±0.33	25.00±2.00
Lymphocytes (%)	15.23±1.44*	69.00±1.00
Granulocytes (%)	19.71±2.52	16.00±0.58
RBC x (10^6/mm^3)	2.74±0.40	1.32 ± 0.02
WBC (10^3µl)	14.12±3.54	10.93± 0.002
MCV (F/L)	16.63 +1.03*	326.24 ± 0.28
MCH (pg)	41.85 ±8.55	48.41 ± 0.07

*Significant (P<0.05).

Table 3. Water quality analysis in earthen pond incriminated in kills of juvenile *Clarias gariepinus*

Parameter	**Result**	♀**FEPA, 1991 Specifications**
Odour	Foul smelling	ND
Ph	7.90±0.00	6.5-8.5
Turbidity (NTU)	5.18 ±0.00*	ND
Temperature (^{0}C)	27.00±0.00	26-32
Dissolved oxygen (mg/L)	3.80±0.00	5.0
Total ammonia nitrogen (mg/l)	7.0±0.00*	≤ 0.3-2.0
Conductivity(µs/cm)	540.00±.0.00*	ND
Total dissolved salt (mg/dl)	280.00±0.00*	200.0

*Significant (P<0.05) ND= non detectable

♀**:** The standard values from FEPA were used as control value.

FEPA: Federal Environmental Protection Agency

Table 4. Minerals' concentration in water, soil bed of earthen pond incriminated in kills of juvenile *Clarias gariepinus*

Minerals (ppm)	Soil	Water	Latex	♀FEPA, 1991 Specification (ppm)
Cu^{2+}	0.35	1.99	2.80	0.0058-0.006
Na^{2+}	12.36	2.44	143.23	Ns
K^{+}	13.54	4.32	1038.34	Ns
Mg^{2+}	608.29	29.05	407.99	Ns
Ca^{2+}	975.35	64.24	704.03	Ns
P	111.76	12.39	104.43	Ns
Si	2.093	0.005	0.63	Ns
AS	0.98	0.43	ND	Ns
Pb	23.55	5.58	4.76	<0.001
Cr	13.44	0.44	3.21	<0.001
Cd	0.33	0.01	6.32	0.005
Zn	1.25	0.08	3.83	<0.001
Mn	0.24	ND	0.80	<0.001

Ns: Not specified
♀**:** The standard values from FEPA were used as control value.
FEPA: Federal Environmental Protection Agency

Table 5. Qualitative and quantitative analyses of phytoconstituents of *Mangifera indica* bark latex incriminated in in juvenile *Clarias gariepinus* kills

Phytochemical	Qualitative	Quantitative
Alkaloids (mg/100g)	+	602.03
Phenolics (mg/kg) QAE	+	55.44
Cardiac glycosides (mg/100g)	+	453.64
Saponins (mg/kg)	+	1590.56
Tannins (mg/kg)	+	22.87
Flavonoids (mg/kg)	+	425.44
Cardenolides	+	NAQ
Amino acids	+	NAQ
Lipids	+	NAQ
Phlobatannins	+	NAQ
Triterpenes	-	ND
Phytosterols	-	ND

+ = present, - = absent, NAQ =Not quantitatively analyzed, ND =Not detected

Table 6. Detected organic chemicals in *Mangifera indica* bark latex incriminated in kills of juvenile *Clarias gariepinus*

S/N	Detected organic chemicals
1.	1-ethenyl-1-methyl-2, 4-bis (1-methylethenyl) -, [1S-(1. alpha, 2. beta.,4. beta.)]- Cyclohexane
2.	Alloaromadendrene
3.	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene
4.	Decarhydro-4a-methy-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7 alpha.,8a.beta.)]-Naphthalene
5.	Oleic acid
6.	3-pentadecyl- Phenol
7.	2-methyl-phenol
8.	3-methyl-phenol
9.	p- cresol

The gross pathological lesions showed haemorrhagic gills (8/10), dicolored caudal fins with evident degeneration (7/10), skin discolorations with ecchymotic haemorrhage (6/10) and engorged hepatomegaly (8/10) and hydroperitoneum (8/10) (Fig. 2).

Microbiological analysis revealed isolates with similar colonial morphology. The colonies on blood agar had medium size, producing bluish green coloration on nutrient agar. They were Gram-negative rods, catalase and oxidase positive. On phylogenetic analysis, the test isolate associated genetically with different strains of *Pseudomonas aeruginosa*, especially the strain SF with the accession number of KP799012.2. It does not show association with the outgroup (*Aeromonas* species) (Fig. 3).

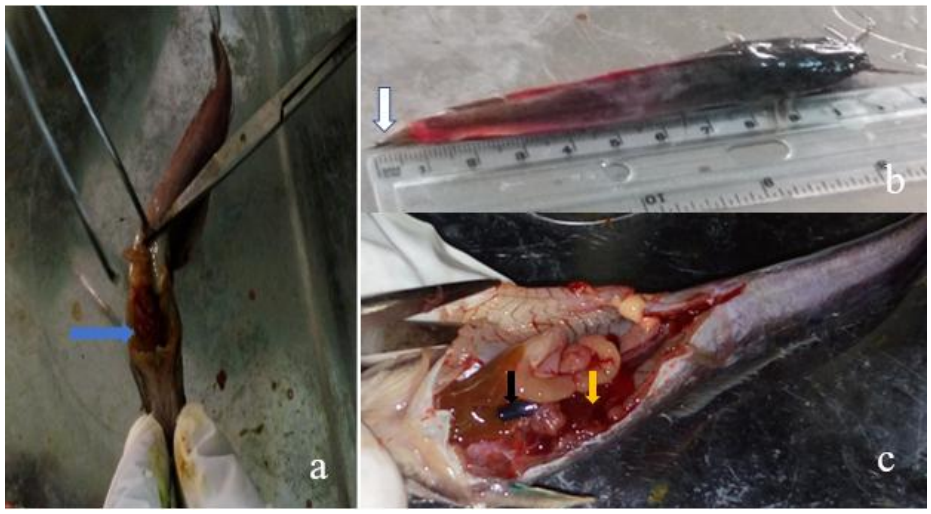

Fig. 2. The gross pathological lesions of juvenile *Clarias gariepinus* that died after *Pseudomonas* species infection: haemorrhagic gills (a, blue arrow), dicolored caudal fins with evident degeneration (b, white arrow), skin discoloration with ecchymotic haemorrhage (b) and engorged hepatomegaly (c, black arrow) and hydroperitoneum (c, yellow arrow).

Fig. 3. Phylogenetic tree generated from *Pseudomonas* and *Aeromonas* (outgroup) type strains and consensus sequences from fish isolate at sequence similarity threshold of 97.2 % to *Pseudomonas* species. Bootstrap values are based on 500 replications. The scale bar represents the nucleotide substitution per site.

DISCUSSION

The cause of kills in juvenile *Clarias gariepinus* was investigated using different diagnostic techniques. This gave credence to the fact that an array of organic compounds from phytotoxins, heavy metals and other water pollutants resulted in stress and disease susceptibility to a bacterial agent identified to be *Pseudomonas aeruginosa*. The exposure to various organic and inorganic compounds in the pond might be the reason for the peroxidation of phospholipids in membrane, which resulted to compromise ALP as one of the liver enzymes associated with other organs since liver enzymes often leak during stress **(Adeyemi *et al*., 2014)**. This was evident by the increase in the MDA level in the liver tissue. The GP_X in the cells might have responded to minimize the stress by an increase in this anti-oxidative enzyme. Moreover, the CAT increased as this could be an attempt to mitigate the adverse effect of the reactive oxygen and nitrogen species generated. This was due to the xenobiotics caused by pond contamination by the *Mangifera indica* bark latex and heavy metals such as lead, chromium, cadmium and zinc, which were detected to be higher than the optimum standard for water used for *Clarias species* aquaculture in the latex and soil contaminated pond **(FEPA, 1991)**. Additionally, the liver sodium and potassium ions increased probably due to the high concentration of these ions in the soil, the latex and the pond water. Since the liver is one of the principal detoxification organs, it might have had these high concentrations of the ions to maintain homeostasis **(Çoğun & Şahin, 2013)**. The finding in this study agrees with that of **Charan *et al*. (2015)** who reported that the activities of most antioxidant enzymes such as SOD, CAT, GP_x, glutathione-S-Transferase and glutathione were significantly increased in *Clarias batrachus* exposed to phenolic compounds. The effects of contaminants of *Mangifera indica* bark latex, heavy metal and other water pollutants are likely the cause of thrombocytopenia, lymphocytopenia and decreased MCV. Generally, the WBC count is higher than the normal value in the juvenile *Clarias gariepinus* probably due to immune response to toxicant exposure. The present results of the haematological parameters of the toxicant exposure agree with those of previous studies reporting that toxicants' exposure cause many physiological changes in fish which may be reflected in the values of one or more of the haematological parameters **(Musa *et al*., 2013; Adi *et al*., 2017)**. The naphthalene which was detected in *Magnifera indica* latex is haemotoxic **(Varadarajan *et al*., 2014)**, and this coupled with target organ toxicity could be responsible for the thrombocytopaenia in fish. This was evident in this study by the haemorrhages and hyperaemia found on the integument and gills of the fish, respectively. Cresol which was also component of the *Magnifera indica* latex has been reported to cause destruction of lymphocytes **(Li *et al*., 2005)** and subsequently immunoglobulin decrease. The increase in cortisol due to the exposure to cresol could probably be responsible for the lymphocytopaenia. The heavy metals which were also found at a higher level in the pond soil and the latex have haemotoxic effects. Lead is specifically known for its detrimental effect on the metabolism and maintenance of porphyrines, haemoglobin and haemopoetic centers **(Adeyemi *et al*., 2014)**.

Phytochemical principles found in the latex include cardiac glycosides excess which could predispose the fish to cardiotoxicity **(Ola-Davies *et al*., 2017)**. This, alongside the target organ

toxicity of cresol, phenol naphthalene and other compounds might have been the factors that affected the haemogram and metabolism, which in turn, resulted in high mortality. The concentration of saponins was relatively high which is another constituent that has adverse effect on the membranes, causing instability in one or various cellular components of blood and could cause haemolysis **(Böttger & Melzig, 2013)**.

Cyclohexane was one of the organic compounds in the *Mangifera indica* latex, which could affect the aquatic ecosystem, specifically causing ecotoxicity of the pond. In a previous toxicological finding by **Varadarajan *et al*. (2014)**, the concentration of 30- 45µg/ L considered the LC_{50} markedly caused mortality in the aquatic habitat. The exposure through oral and gills routes from the latex might have probably caused covalent bond formation with the macromolecules of the organelles of the fish. This subsequently causes adverse metabolic effects that might have interfered with the oxygen utilization and cellular metabolic process occurring in the matrix of mitochondria, causing deficit in tissue oxygen utilization. The evidence of this was manifested as dyspnea and resulting in the low partial pressure in the blood-air in the respiratory membrane inter-phase of fish, which probably resulted in the kills. The phenol and phenolic compounds are another group of xenobiotics from the *Mangifera indica* latex contamination. These are immiscible compounds and organic solvents which interfere with the diffusion of oxygen. This could also affect the percentage solubility of oxygen in the pond's water **(Pandey *et al*., 2012)**. The phenolic compounds could cause degenerative changes to the gills, hepatotoxicity, anemia and increases cortisol level which would result in immunosuppression and subsequent susceptibility to infection **(Pandey *et al*., 2012)**.

Pseudomonas septicemia has been reported as one of the deadly bacterial infection affecting fish in many parts of the world **(Oisson *et al*., 2006; Tripathy *et al*., 2007; Eissa *et al*., 2010; El-deen & Rawway, 2014; Rafet & İlhan, 2014)**. *Pseudomonas* species have been reported from both freshwater and marine fish throughout the world causing severe economic losses and decrease in fish farm efficiencies **(Eissa *et al*., 2010; Thomas *et al*., 2014)**. The isolated organism from this study associated closely with *Pseudomonas aeruginosa* and dissociated itself from the outgroup (*Aeromonas* species) during phylogenetic analysis, which showed that it is most likely to be *Pseudomonas aeruginosa*. The occurrence of *Pseudomonas aeruginosa* in the present investigation may be responsible for the mortality recorded in the fish farms studied since *Pseudomonas* has been reported as the most common agents of bacterial diseases of fish **(Oisson *et al*., 2006; Tripathy *et al*., 2007; Eissa *et al*., 2010; El-deen & Rawway, 2014; Rafet & İlhan, 2014; Thomas *et al*., 2014)**. *Pseudomonas* species causes ulcerative diseases including tail and fin rot, ulcerative syndrome, bacteria gill rot, dropsy and haemorrhagic septicaemia **(Thomas *et al*., 2014)**. The environmental toxicants reported in this study could have predisposed the fish to depressed immunity and subsequent susceptibility to *Pseudomonas aeruginosa* infection, which might be the cause of mass kills as observed in this study.

CONCLUSION

The death of fish in this study could be due to cumulative effects of phytotoxins from the *Mangifera indica* stem bark latex, heavy metal toxicity and poor water quality which caused *Pseudomonas aeruginosa* infection. Intending farmers and stakeholders in the fish production should ensure appropriate consultation when selecting a site for ponds, making use of professionals in aquaculture, soil scientists, environmental scientists, toxicologists, veterinary practitioners and extension service workers. The site should have water parameters monitored to ensure ecological stability of the intended fish. The higher plants within the vicinity of the pond should be removed to prevent interference with water parameters and likelihood of contamination. It is also advisable for environmental regulatory bodies to enforce environmental laws to ensure a healthy environment for the maintenance of ecosystem for aquatic life and man.

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