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Full Length Article

Effects of Aluminium on *Clarias gariepinus* Physiology, Gills Histology and Cholinesterase *In vivo* and *In vitro*

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Abstract

The gill is the site of acid-base balance, ionic regulation, gas exchange, and nitrogenous waste excretion by fish. The physiological, histopathological and enzymatic alterations from *Clarias gariepinus* gills were examined to assess the effects induced in gill tissues within 96 h exposure to waterborne $Al_2(SO_4)_3$ with different concentrations (25 to 300 mg/L). The exposure of *C. gariepinus* to aluminium showed deleterious effects to normal fish behaviours and varying degrees of gill damage when visualised under inverted light microscopy (stained with haematoxylin–eosin), scanning electron microscopy and transmission electron microscopy. The mortality rate was observed increasing with increased Al concentration showing noticeably disrupted lamellae, irregular nucleus shape and increasing area of vacuolation in treated fish gills associated with $Al_2(SO_4)_3$ exposure concentration. Furthermore, electron microscopic X-ray microanalysis of fish gills exposed to sublethal Al revealed Al accumulated on the surface of the gill lamella. Al toxicity also gave inhibitory effects on cholinesterase (ChE) extracted from the fish gills with 90.0% inhibition at 300 mg/L Al highest concentration exposure (*in vivo*), whereas the Al metal ion inhibited 50% of ChE activity at 4.12 mg/L (IC_{50}). © 2019 Friends Science Publishers

Keywords: Aluminium toxicity; Gills; Physiology; Morphology; Cholinesterase; *Clarias gariepinus*

Introduction

Advancement has conveyed numerous great things to the mankind, which then again has additionally made suppressive impact towards nature with the amphibian condition being persistently subjected to various contaminants. Therefore, a few compound contaminants including overwhelming metals have fundamentally dirtied the water sources, which have ended up presenting significant impediments and extreme risk (Jasim *et al.*, 2016). Metals are naturally present in very low concentrations in aquatic environment. Howe ver, due to anthropogenic pressure, their load has alarmingly increased. Any change in the natural conditions of aquatic media causes several adjustments in fish with metals being the main culprit for these undesirable changes in the water quality (Garg *et al.*, 2009). Due to the possible dangers posed by various metals on aquatic organisms, few heavy metals such as aluminium (Al) has gained exclusive consideration since it is ubiquitous in the environment and considerable attention due to its potential human health hazards.

Al is the third most abundant element in the earth's crust that makes up approximately 8% of its rocks and minerals. Al and its compounds have a wide variety of usage including as structural materials in construction auto mobiles, air crafts and the production of metal alloy, glass, ceramic, rubber pharmaceuticals and water proofing textiles (Maharajan *et al.*, 2012). Al naturally enters the environment through the weathering of rocks and minerals. For a long time, it has been perceived that Al is poisonous to aquatic organisms. Besides, the aqueous Al has been recognised as the primary toxicant to fish. The component of Al danger to fish has been ascribed to the powerlessness of fish to keep up their osmoregulatory balance and respiratory issues related with precipitation of Al on the gill mucus (IPCS, 1997).

Fish, in comparison with invertebrates, is more delicate to numerous toxicants, which can be a helpful guinea pig for demonstrating the wellbeing of biological

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system. Plus, it is likewise broadly used to assess the soundness of aquatic biological community with its physiological changes that fill in as biomarkers of ecological contamination. Health conditions of the African catfish (*C. gariepinus*) has been reported for environmental contamination by trace metals (Ololade and Oginni, 2010) and has become among the most widely recognized freshwater angle utilized in toxicological investigations. It has the capability to rapidly respire and is very hardy since it tolerates well and poorly oxygenated waters to be applied as experimental test sample. Distinctive physiological changes every now and again saw in various fish species presented to Al are hematologic, cardiovascular, iono-regulatory, respiratory, conceptive, endocrine, metabolic, and gill harm. Gills are the principal focus of waterborne poisons because of their consistent contact with the outer condition. It is well known that changes in fish gills are among the most commonly recognised responses to environmental pollutants (Chitra and Sajitha, 2014). Sweidan *et al.* (2015) claimed that heavy metals are more concentrated in the gills than different parts of the fish in the water, in this manner showing that they were of good biomarkers to screen the contamination in the stream.

Gills play an important role in the capturing, accumulation and transfer of metal toward internal compartments via blood transport. The gills are the site of breath and transport framework engaged with osmoregulation and it has been affirmed that the collection of metal particles inside them may affect their capacities (Bhuvaneshwari *et al.,* 2015). Inferable from the immediate and consistent contact with the aquatic condition, fish gills, which are the organs for respiratory gas trade, osmoregulation, discharge of nitrogenous waste items and corrosive base control, are specifically influenced by contaminants. They are very sensitive to physical and chemical alterations in the aquatic media and to any adjustments in the structure of condition, or, in other words marker of water borne toxicants (Ogundiran *et al.,* 2009). In fish, Al might be related with gill harm because of its affidavit and changes in osmoregulation and with oxidative stress in lymphocytes (Galar-Martinez *et al.,* 2010).

Fish mostly accumulate many contaminants and toxicants including heavy metal directly through their gills and skin and indirectly *via* their food chain, which may cause diverse alternations in histopathology (Khatun *et al.,* 2016). The fish gills have been observed microscopically see the expanding degrees of harm in tissues associated to the nature of water that fills in as a biomarker for water quality (Sweidan *et al.,* 2015). Al-Ghais (2013) claimed that tissues cholinesterases protecting cell against oxidative damage and detoxicates xenobiotics or their metabolites have been likewise approved as contamination biomarkers in fish.

The full explanation on the detrimental impacts and roles of heavy metal toxicants in most important functions including metabolism, reproduction and respiration in aquatic animals can be obtained through histopathological study (Maharajan *et al.,* 2012). Besides, the histopathological biomarkers in environmental monitoring are advantageous by which they can allow the specific target organs like gills to be examined (Hadi and Alwan, 2012). Al accumulation, notwithstanding damage to the gill epithelium, causes apoptosis and putrefaction of gill particle transporting cells, which have been considered as the primary driver of particle administrative and osmoregulatory brokenness (Slaninova *et al.,* 2014). Moreover, other histopathological changes in gills such as hyperplasia and hypertrophy, epithelial lifting, aneurysm and increased mucus secretion have been reported after the exposure of fish to various noxious agents in the water including heavy metals (Ladipo, 2011). Moreover, a study has been recently conducted to study the activity of ChE in gill tissues as an early-warning biomarker and presented the sites and tissue-specific variations in acetylcholinesterase (AChE) responses. Furthermore, Hayat *et al.* (2016) in their examination outlined that with respect to the sensibility of different tissues, gills appeared to be the best tissues as a biomarker of pesticides and overwhelming metals introduction since the AChE from gills has been turned out to be to be the most defenceless to restraint by the toxins tried.

African catfish (*C. gariepinus*) is of extraordinary business significance as it is the most generally devoured freshwater angle in Malaysia. It is in this manner suitable to examine its reaction to ecological poisons, especially the substantial metals. With this in mind, this study aimed at evaluating gills histopathological changes and the possibility of using ChE from the local catfish *C. gariepinus* as a cheaper and more available source of ChE for the assay of heavy metals towards gills by *in vivo* and *in vitro* methods of Al exposure. Evaluation of the effects of Al on the histopathological and ChE activity in the gills of *C. gariepinus* could serve as a useful biomarker and help the aquaculturists to evaluate the substantial metals tainting level in the aquatic condition.

Materials and Methods

Fish

Fish known as *C. gariepinus* were obtained from a commercial supplier in Semenyih, Selangor (2.953003, 101.846004). Each has an average length of 20.0 ± 5.0 cm and average weight of 100.0 ± 20.0 g besides being in a good health condition. For maintaining an optimum health of the fish, the water condition at the supplier's sanctuary was often changed and monitored daily. An amount of 40 L dechlorinated water at room temperature (25°C) was utilised with full aeration for the acclimation of fish for three days under laboratory conditions in an aquarium (4"×3"×2"). Meanwhile, commercial pellet at 2% body weight was used to feed the fish daily.

To trap the remaining food waste, the water was

filtered using sponge layer filter. Excrement was carried out to make sure that the fish are clean. Moreover, the water in the aquarium was cleared to aid the monitoring process. The water in this study was changed twice per week to remove excretion by-products and unconsumed feeds as well as to maintain its cleanliness.

C. gariepinus Treatment and Behavioural Analysis

Each group of fish was separated into six animals in each aquarium treated with final aluminium sulphate ($Al_2(SO_4)_3$) concentrations i.e., 0, 25, 50, 100, 150, 200, 250 and 300 mg/L in triplicates. During 96 h treatment, behaviour analysis was completed by observing and recording the visual changes of *C. gariepinus* for 6 h interval in response to Al including respiratory distress and mortality. Along the test, dead fish was immediately removed to prevent water contamination (Anur *et al.*, 2011). After 96 h treatment, the fish were removed, placed in ice bath to be anesthetised and immediately decapitated. The dead fish were dissected, and the gross pathological changes of the gills tissue were observed via light microscopy, SEM and TEM and the inhibition of gills cholinesterase by Ellman assay.

Evaluation on Gross Histology, Ultrastructure and Cholinesterase Activity *C. gariepinus* Sample Preparation

Histology by light microscopy: After 96 h, all fish were killed with their gills taken and fixed in 10% formalin. The tissue samples were prepared using the standard methods for histological techniques and stained with haematoxylin and eosin (Culling, 1974). To inhibit the decaying or autolysis of the tissues in preserving the samples for future study, 10% formalin was used for 48 h. This was followed by placing the samples into the cassette, which was then fixed again using 10% formalin and subjected to tissue possessing by immersing it with a series of ascending concentration alcohol within the given period (80% alcohol for 2 h, 95% alcohol for 2 h, 100% alcohol for 3 h) and then chloroform and paraffin for 3 h, 5 h and 30 min, respectively. This was continued with embedding the samples in the wax and placing them in a cold plate for 15 min until a block was produced. After that, a microtome was used to cut the samples at 5 µM thickness, which were then stained using haematoxylin and eosin staining. At this stage, the samples were observed under light microscope with histopathological evaluated and described.

Ultrastructure for Electron Microscopy (SEM and TEM)

For SEM and TEM, the organs were cut into 1 cm^3 and 1 mm^3 sections and in this manner settled for 20 h utilising 4% glutaraldehyde. This process was repeated for setting up the examples before dehydration utilising increasing concentration of acetone pursued by washing and drenching them in 0.1 M sodium cacodylate buffer for 10 min in triplicates. After that post fixation was performed by absorbing them in 1% cool osmium tetroxide for 2 h and washing three times with 0.1 M sodium cacodylate cushion for 10 min each. To dry out the samples, they were drenched in a progression of increased acetone concentration from 35% (10 min), 50% (10 min), 75% (10 min), 95% (10 min) and lastly 100% (15 min) for three times. The readied tests were put to critical point drying in which they were moved into a basket with samples and put into critical dryer for 30 min before watching them by SEM microscopy technique. The examples were mounted by attaching them onto the SEM stub utilising a twofold sided tape and covered in a sputter coater. The prepared sample stubs were seen by SEM, while the design modifications of treated gills were contrasted with untreated gills and photographed. For TEM, the procedure proceeded by penetration process performed utilising a resin mixture and acetone with the proportion of 1:1 (1 h), 3:1 (2 h) and 100% resin (Overnight), individually. The 2 h incubation with 100% tar was done to guarantee an entire resin invasion on the following day. The examples were installed in the beam capsule filled with resin prior to the polymerisation procedure for 24 to 48 h at 60°C. This was trailed by separating the sliced samples utilising a rotatory microtome to ultrathin sections. Silver sections were grabbed utilising a network and after that dried utilising a filter paper. Uranyl acetic was utilised to stain the section samples preceding washing utilising refined water. Staining process was further carried out utilising lead for 10 min and washed again utilising twofold refined water. Produced sections were then imagined utilising TEM.

Determination ChE Activity

The gills of fish were cut with their total weight measured. Then, ChE was extracted by homogenising the fish gills using 0.1 M sodium phosphate buffer, pH 7.0 in the ratio of 1:4 (w/v) in cold condition containing final concentration of 0.5 mM phenylmethylsulfonyl fluoride (PMSF), which was added using an Ultra-Turrax T25 homogeniser. This addition was done to block the action of unwanted potentially destructive serine proteases and to stabilise the enzyme. The crude extract was continued to be centrifuged for 1 h at 10,000 x g and at 4°C (Son *et al.*, 2002) to remove large unwanted debris. After that, the supernatant was collected as crude and subjected to enzyme assay.

Enzyme Assay

In this study, the method of Ellman *et al.* (1961) was adopted to examine the activity of ChE extract with modification of microassay using the standard 96 well multimode detector (Fadzil *et al.*, 2019; Sabullah *et al.*, 2013). The assay utilised the synthetic substrates of acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC). The

microplate was pipetted with the mixture of 200 µL of 0.1 M sodium phosphate buffer (pH 7.0), 20 µL of 0.1 m*M* 5, 5-dithio-bis-2-nitrobenzoate (DTNB) and 10 µL ChE.

Before adding 20 µL of substrates, incubation was done to the mixture for 15 min. This was followed by further incubation for 10 min. Absorbance was recorded at 405 nm. This process was conducted in triplicate.

Half-inhibitory Effects (IC_{50}) of Al Metal Ionon ChE

The toxicant concentration that contributes to 50% inhibition of enzyme activity is known as IC_{50} values, which is suitable to be used in comparing the sensitivity with other types of assay. Al ion was separately incorporated into the screening assay at a final concentration ranging from 1.0 to 10.0 mg/L. The organs extracted from *C. gariepinus* (diseased and toxicant free) were included in IC_{50} determination and incubated with different concentrations of Al. The assay was conducted by replacing 50 µL of metal ion with various levels of Al ion from 1.0 to 10.0 mg/L. 0.1 M sodium phosphate buffer with pH 7 was used to replace the Al ion as the control of this study. Nonlinear regression analysis was employed to calculate the IC_{50} value using the exponential decay modelling type from the Graph Pad Prism Software.

Statistical Analysis

This study presents all the quantitative data as means with their standard deviation (SD). To compare the means between different concentrations of Al, Student t-test was utilised, whereas one-way ANOVA was used to examine the differences before conducting Tukey multiple comparison test using Graph pad prism 5.0 software at 5% of significance level (Miller and Miller, 2010). This study was carried out in triplicate with $P < 0.05$ considered as statistically significant.

Results

Physical and Behavioural Response of *C. gariepinus*

The study by Ezeonyejiaku *et al*. (2011) has been conducted studying the behavioural changes and emphasised that they are among the crucial media to assess the organisational level of the biomarker in fish. Behavioural changes can be referred to the behaviour of an organism that represents the final integrated results of a diversity of biochemical and physiological processes. The behavioural changes of *C. gariepinus*in this present study during a 96-h treatment with $Al_2(SO_4)_3$were identified according to respiratory distress by secretion of mucus, skin colour by white layer formation and mortality.

Respiratory Distress

The fish exhibited an ordinary reaction while dealing with 0 to 50 mg/L of Al. Fish expressed enormous opercula movements, which suggests breathing distress through excessive mucus secretion at concentration of 100 to 300 mg/L of Al (Table 1). The exposed fish demonstrated abnormalities of their behaviours in which they tend to swim vertically at the water surface with decreased swimming pattern as the concentration of Al increases together with the period of exposure.

Histopathological Determination

Santos *et al.* (2014) mentioned that the gills of fish are extraordinarily sensitive to chemical and physical modifications within the surroundings chiefly because of the massive surface of respiratory epithelium and high perfusion rate aiding the pollutants to enter this tissue. This has further suggested that the morphological changes within the gills can be potentially used as parameters in biomonitoring programmes since they are the defence mechanisms to potential stressors in the aquatic surroundings.

Light Microscopic Observation

The control fish used in this study demonstrated a normal pattern of gill filaments with no microscopic anatomy changes discovered (Fig. 1A). Within the sub-lethal exposure to Al, the gills of *C. gariepinus* displayed some histologic alterations. Considerable alterations were observed within the gills histology after treating them with 25 mg/L Al for 96-h including the lifting of epithelial cells and fusion between secondary lamellae (Fig. 1B). Severe damages were discovered when 100 and 200 mg/L of Al were applied in which dysplasia, multiple deformations, necrosis, cardiovascular disease and dropsy were discovered (Fig. 1C–D). Besides, the best concentration of Al at 300 mg/L presented forceful changes when respiratory epithelium was absent with a total loss of structure.

SEM Observation

From the control fish, it was seen that the SEM of gills indicated intact primary and secondary lamellae with uniform inter-lamellar spaces (Fig. 2A). Furthermore, the degeneration of lamellae and combination of auxiliary lamellae were recorded when exposed to Al, (Fig. 2B–C). Radical adjustments were seen where the greater part of the auxiliary lamellae was discovered cracked together with scattered lamellae at high Al exposure (Fig. 2D–E). At 300 mg/L of Al, SEM-EDX showed 15.2% aggregation of fish gills in the secondary lamellae (Fig. 3).

TEM Observation

The identification of a few cells; for example, column cells, asphalt cells and chloride cells were permitted through the histological of ultrastructure C. *gariepinus* gills observation

Table 1: Respiratory distress after exposure 96 h

Clinical sign	$Al_2(SO_4)_3$ concentration (mg/L)							
	control	25	50	100	150	200	250	300
Mucus secretion	-	-	-	+	++	++	+++	++++
Vertical Postures	-	-	-	-	-	++	+++	++++

Body colour: The fish skin colour changed by increasing formation of white layer as the concentration of Al increases (Fig. 1)
Mortality: Mortalities were recorded at the end of exposure period at concentrations from 150 to 300 mg/L (Fig. 2)

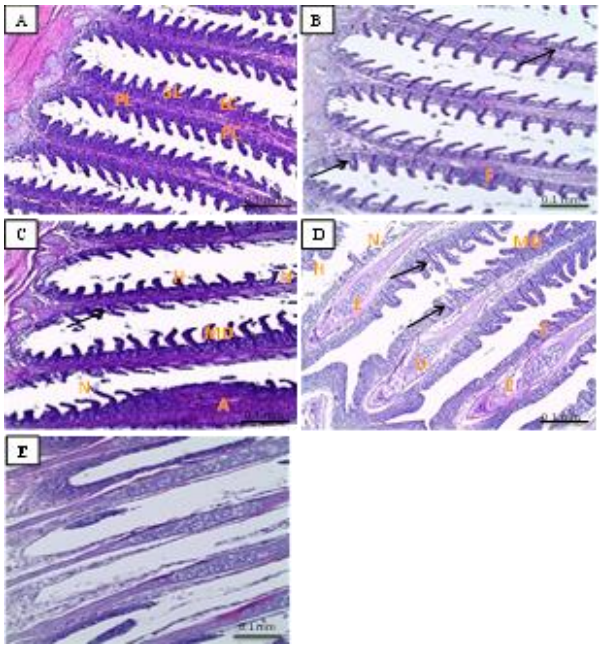

Fig. 1: Characteristic histopathological changes in the gill of *C. gariepinus* revealed by H&E staining (10X). (**A**) Control. (**B**) Exposure to 25 mg/L of Al. (**C**) Exposure to 100 mg/L of Al. (**D**) Exposure to 200 mg/L of Al. (**E**) Exposure to 300 mg/L of Al. Photomicrograph of gill showing the primary lamella (PL), secondary lamella (SL), pilar cells (PC), epithelial cell (EC), lifting of epithelial cells (arrow), fusion between secondary lamellae (F), hyperplasia of epithelial cells (H), multiple deformations (MD), necrosis (N), aneurysm (A), oedema formation (O), absence of respiratory epithelium and loss of structure

under TEM. The control fish of C. *gariepinus* in the present investigation demonstrated an ordinary ultrastructure of gill lamellae (Fig. 4A). The cells in Al-treated groups were in necrosis stage and the presentation to sub lethal fixation (25 mg/L) of Al gave denser cytoplasm with a nucleus surface that began to de-shaped in an unpredictable form. Besides, the presence of degenerative phenomenon by cytoplasm of pavement cells seemed vacuolated, deteriorated and apoptotic with decreasing number of column cells (Fig. 4B). In the interim, glycogen granules and degeneration of epithelium were seen at 100 mg/L of Al (Fig. 4C). Plus, deteriorated apoptotic asphalts cells and oedema was reported with the increasing of Al concentration to 200 and 300 mg/L (Fig. 4D–E).

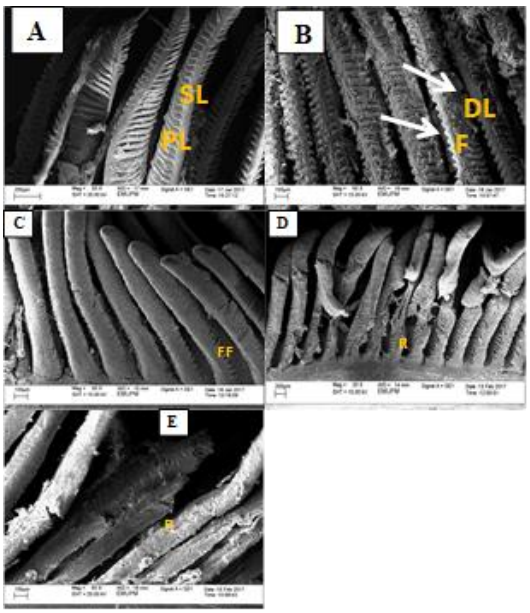

Fig. 2: Scanning electron microscope of *C. gariepinus* gills surface (50X). (A) Control revealing normal morphological features, (B) Exposure to 25 mg/L of Al. (C) Exposure to 100 mg/L of Al. (D) Exposure to 200 mg/L of Al. (E) Exposure to 300 mg/L of Al. General view of fish gill filaments and lamellae showing primary lamellae (PL), secondary lamellae (SL), degeneration of lamellae (DL/↑), fusion of secondary lamellae (F), fully fusion of secondary lamellae (FF), ruptured of secondary lamellae (R) and disorganised lamellae

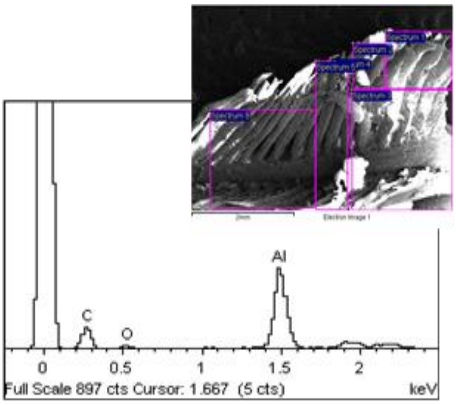

Fig. 3: SEM and EDX microanalysis of the gill lamellae from *C. gariepinus* after 96 h exposure to 300 mg/L of Al. Elemental analysis spectrum shows the appearance of Al in weight percentage (15.2%) in secondary lamellae area (Spectrum 4)

Cholinesterase Activity Study

In the gills, the most elevated ChE action was acquired with ATC. The study of ChE activity on the gills of treated fish was conducted by checking the cholinesterase activity of their enzymes as displayed in Fig. 5. The amassing of Al in the gills of C. *gariepinus* was due to intense exposure to Al for 96 h with a few fixations displayed. The control of this

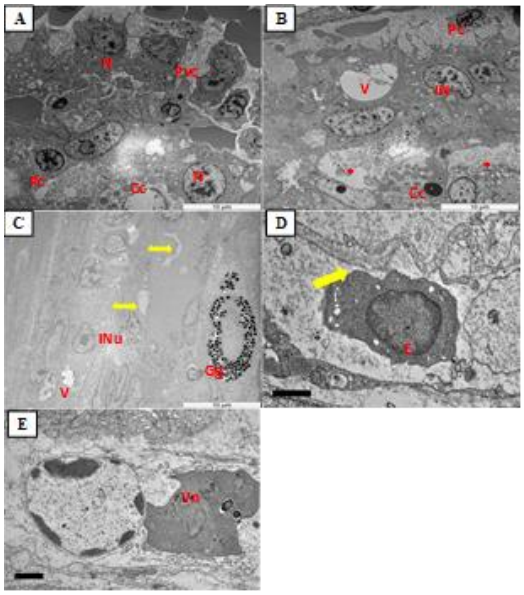

Fig. 4: Transmission electron microscope of epithelial cells of *C. gariepinus* gills (3000X). (A) Control. Normal cellular organisation of *C. gariepinus* epithelium cell. Nucleus (N), chloride cell (Cc), pillar cell (Pc) and pavement cell (Pvc). (B) Exposed to 25 mg/L Al illustrating epithelial detachment with oedema (*) and vacuolation (V), irregular nucleus shape (IN). (C) Exposure to 100 mg/L Al, presence of glycogen granule (Gg) and degeneration of epithelium (arrow). (D) Exposure to 200 mg/L Al showing the degenerated and apoptotic pavements cells (Pvc) and oedema formation in distal portion of lamellae (*). (E) Exposure to 300 mg/L Al and fully degraded epithelium cells with a vacuolated nuclear envelope (Vn)

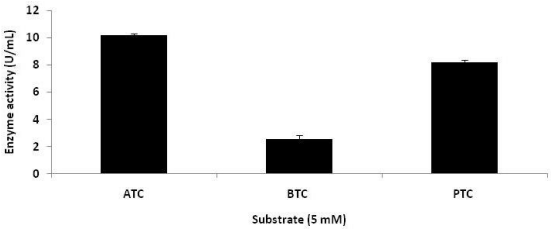

Fig. 5: Substrate specificity of ChE activity in the gills of *C. gariepinus*. Substrates used were acetylthiocholine iodide (ATC), butyrylcholine iodide (BTC) and propionylcholine iodide (PTC). Values are mean ± standard deviation (*n*=3)

investigation was alluded to as 100% since the rest of the activity as well as AChE activity in the gills were altogether diminished with the increment in Al concentration. Meanwhile, $Al_2(SO_4)_3$ at the most minimal concentration (25 mg/L) was seen to repress cholinesterase activity by 13% though at the most astounding concentration of Al (300 mg/L), 90% of the ChE activity was recorded to be hindered in contrast with the control (Fig. 6). Then, *in vitro* AChE restraint was identified by incubating extracted *C. gariepinus* gills AChE with 1 to 10 mg/L groupings of Al metal particles by reducing the activity to 50% inhibition concentration (IC_{50}) of 4.12 mg/L as in Fig. 7.

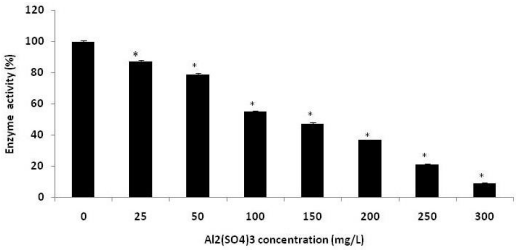

Fig. 6: Effects of various concentrations of $Al_2(SO_4)_3$ on acetylcholinesterase in *C. gariepinus* gills. Data were given as means ± SD (n = 3). Statistical significance of difference from control: $*P < 0.05$

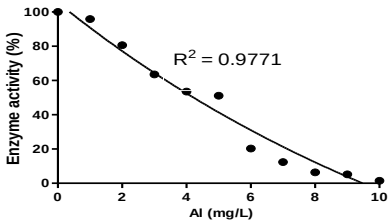

Fig. 7: Al metal ion inhibition profile studying the effects of Al in various concentrations on AChE from gills extract of *C. gariepinus*: IC_{50} value was 4.12 mg/L determined using GraphPad Prism

Discussion

The accumulation of heavy metals in the aquatic environment could cause toxicity to amphibian life and human. Nanda (2014) mentioned that eatable fish in amphibian bodies frame an essential group of creatures as the heavy metal could go about as a potential transporter of metal ion along the food chain once aggregated in fish tissues. Moreover, it has been stated by Galar-Martinez *et al*. (2010) that Al, being one of the heavy metals, might be related with the damage of gills in fish because of its deposition and changes in osmoregulation and oxidative stress in lymphocytes. It can be observed from the present examination that respiratory distresses including extreme mucus and vertical stances were reported from 100 mg/L to 300 mg/L Al (Table 1). Parra *et al*. (2013) considered mucus secretion as among the variables utilised to evaluate the toxicity of fish towards contamination using fish gills and skin, which are the mucosal surfaces of fish that produce a thin physical hindrance between the outer condition and the interior milieu. The measurement of mucous emitted was expanded when these tissues were tested as seen in the examination in Fig. 8B. Similar with the study by Anur *et al*. (2011), these motionless vertical stances in Fig. 8C were caused by the discharging of extreme mucus on the gills that decreased the respiratory action in fish, thus making them unfit to effectively complete gaseous exchange. Significant decrease in opercula recurrence made them to swim near the water surface to expand their oxygen intake on the water surface

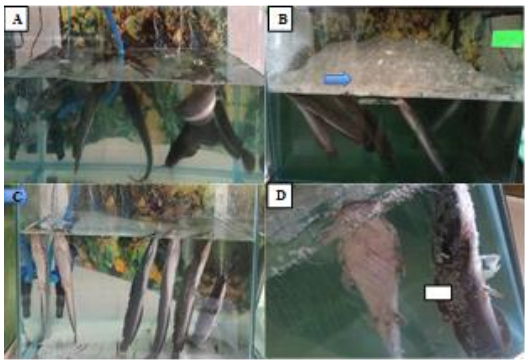

Fig. 8: Physiological changes of fish exposed to Al for 96 h. (A) Control fish showed no abnormal physical and behavioural changes. (B) Mucus secretion represented by the formation of bubbles on water surface (arrow). (C) Vertical motionless postures with exposed snouts. (D) Whitish layer formation (arrow)

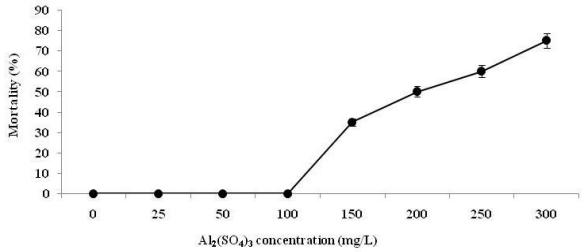

Fig. 9: The percentage mortality (%) of *C. gariepinus* after 96 h exposure ascending $Al_2(SO_4)_3$ concentration

(Hassan *et al*., 2013). Be that as it may, Singh and Banerjee (2008) argued that the defensive function of the expounded mucous may not last for too long because of the rapid exhaustion of the mucous cells with extensive loss and altered nature of the excreted mucous from prolonged exposure, which resulted in the disintegration of shallow cells especially on the skin of the exposed fish. This has further activated tear and wear, therefore prompting sloughing off the fish skin surface that goes about as a protective reaction by the exposed fish of high concentration Al. This was likewise upheld by Taghizadeh *et al*. (2013) exhibiting the increment in mucus emission and thickening or more striking changes in the bronchial epithelium combining with acidic water and Al towards Goldfish (*Carassius auratus gibelio*). Ibrahem (2012) conducted a study on C. *gariepinus* towards the exposure of phenol and additionally discovered more noticeable anomalous signs including skin disintegration and ulceration together with inordinate mucous emission following the post exposure along seven days period.

Alterations in body shading and descaling were seen in fish introduced to 200–300 mg/L amid 96 h of Al treatment (Fig. 8D). The external epithelia were the primary tissues presented to the water and presumably the first to be influenced by high concentration of Al. Generally, the skin or external epithelium in comprised living cells; for example, fibre cells, mucous cells, sensory cells and club cells that specifically exposed to water. Ahmad *et al*. (2017) has recorded comparative outcome on catfish (*Heteropneustes fossilis*) after acute and sub-acute introduction to cadmium in which the colour blurring was more conspicuous with much skin peeling off along with endoskeleton that was visibly discovered in caudal area in the greater part of the fish. El-sayyad *et al*. (2010) claimed that the *C. gariepinus* skin containing the external boundary tissue of the fish was not keratinised and secured by a layer of foul mucous. Furthermore, skin discolouration was activated by ecological factors including the toxicant presents in the water as well as colour of contaminated water as stated by Quifen *et al*. (2012).

The fish displayed a typical reaction with no mortality when treated with 0 to 100 mg/L of Al (Fig. 9). There was an increment in signs and death rate with increasing Al concentration. Further mortality rate was observed on fish exposed to high concentration (150–300 mg/L) of Al as appeared in Fig. 9. In 300 mg/L, mortality rate was read at 83%, showing the osmoregulatory failure created by the fish. The LC_{50} of 206.397 mg/L was produced utilising Probit analysis. This outcome was upheld by the investigation of Naskar *et al*. (2009) on *Clarias batrachus* towards $Al_2(SO_4)_3$ with LC_{50} value of 220 mg/L.

Gills of fish are hugely delicate to physical and chemical alterations in nature, for the most part due to the vast surface of respiratory epithelium and high perfusion rate that encourage the induction of toxins into this tissue (Santos *et al*., 2014). Along these lines, morphological changes in the gills are generally utilised as parameters in biomonitoring programmes since they are the protective components to potential stressors of the aquatic environment.

The gills of C. *gariepinus* are ordinarily made out of essential lamellae (epithelial tissue, ligament and vascular framework) and auxiliary lamellae, which jut along the whole length of the primary lamellae, each comprising blood arterioles through which oxygen exchange with the encompassing water occurs (Dyk *et al*., 2009). Histological modifications of gills surveyed in this examination on Al demonstrated a few changes with increased Al concentration (Fig. 1). The fish utilised as control showed an ordinary pattern of gill fibres with no histologic changes recorded (Fig. 1A). Diverse trials of Al concentration on histologic outcome caused morphological modification in gills epithelium and blood circulatory arrangement of *C. gariepinus* to happen in the present examination. For instance, the lifting of lamellar epithelial cells and combination between two optional lamellae at 25 mg/L Al were observed as in Fig. 1B. Winkaler *et al*. (2001) expressed that the proof of lifting of the respiratory epithelium is one of the earliest wounds found in fish described by the displacement of lining epithelium in the secondary lamellae by the development of a space called oedema. This has been

additionally identified with the nearness of chemical contaminants, decrease of the gills' surface, notwithstanding endangering the process of gas exchange. In the meantime, the combination of several secondary lamellae could be the example of protection system that lessen the bronchial shallow zone in contact with the outside condition (Fernandes *et al*., 2007). It is conceivable that the damage of gills could be an immediate after effect of Al entering the water. Comparative outcome has been also presented by Hadi and Ahwan (2012) on fresh water fish, *Tilapia zillii*, exposed to Al under acidic condition.

Fish gills introduced to 100 mg/L of Al indicated changes in hyperplasia of epithelial cell and numerous distortions of secondary lamellae as recorded in Fig. 1C. Hyperplasia of gill lamellae might be incited by the impact of the toxin that changes the glycoprotein in the mucus covering the cells, thus influencing the negative charge of the epithelium and favouring attachment to adjoining lamellae (Strzyżewska *et al*., 2016). Moreover, Dane and Şişman (2015) added that lamellar epithelium hyperplasia works by increasing the water – blood dispersion separates and hence reducing the ingestion of toxic agents. The event of aneurysms or telangiectasia in primary and secondary lamellae as in Fig. 1C demonstrated the breakdown of vascular integrity with the arrival of vast amount of blood that push the lamellar epithelium outward (Alazemi *et al*., 1996). There were hardly any necrosis areas in this examination, which suggest Al toxicity concomitant iono- and osmoregulatory dysfunction that can result in quickened cell necrosis, sloughing and death of fish (Exley *et al*., 1991). The consequences of this examination affirmed the outcome by Fernandes *et al*. (2007) on Nile tilapia, *Oreochromis niloticus* that was introduced to waterborne copper with 1.0 and 2.5 mg/L concentration.

Modifications in gill histological structure were seen more articulated than those of lower concentration at higher Al convergence of 200 mg/L (Fig. 1D). Hyperplasia of epithelial cells between secondary lamellae drove the combination and necrosis of lamellar epithelial cells to be expanded in arrangement. Furthermore, the oedematous lamellae of uncovered fish in this study might be because of enhanced capillary permeability of the veins of influenced gills (Olojo *et al*., 2004). Adeogun and Chukwuka (2012) revealed comparative oedematous gills in C. *gariepinus* presented to methanolic extract of Raphia hookeri at sub-lethal concentration. At 300 mg/L of Al exposure, the respiratory epithelium was seen absent with loss of gills structure (Fig. 1E). Nevertheless, all findings obtained in this examination may influence gas exchange and decrease the O2 uptake of *C. gariepinus* as mentioned by Fernandes *et al*. (2007). The whole histopathological modifications in this study were noticed to be similar to those in fish presented to some heavy metals (Jasim *et al.*, 2016).

SEM uncovered significant difference in morphological appearance between control gills and fish gills introduced to Al. It was apparent in Fig. 2 based on SEM result of gills from control fish that there was an intact primary and secondary lamella with uniform inter-lamellar spaces. The lamellae of the control fish were characterised, not melded and had a moderately vast surface territory for exchange with water. On the other hand, the lamellae of the fish introduced to 25 mg/L of Al seemed shorter with reduced surface territory (Fig. 2B). The gill lamellae showed wear and tear in numerous spots as well as combination of secondary lamellae with neighbouring and blebbing arrangement. The twisting and combination of secondary lamellae as noticed in the present investigation have been likewise revealed by Dey *et al*. (2016) studying the toxic impacts of blanched sulphite pulp mill effluents on the gills of *Heteropneustes fossilis*.

In the case of fish gills exposed to 100 mg/L of Al, the gills were more damaged that individual secondary lamellae were indistinguishable by fully fusion (Fig. 2C). Density in the secondary lamellae and formation of the inter-lamellar bridges reduced the total available respiration surface area of the gill and further reduced diffusing capacity, resulting in decreased gas exchange (Flores-Lopes and Thomaz, 2011). It was seen that the fish were asphyxiate due to the failure in getting enough oxygen. Fusion of secondary lamellae forming inter-lamellar bridge is common in polluted environment. The evidence of secondary lamellae fully fusion in gills of *C. gariepinus* observed in the present study has been also reported in some fish exposed to pesticides, industrial wastes and other organic wastes, which resulted in impaired functioning of gills (Venkataram *et al*., 2007). Drastic changes were noticed where breakages in primary lamellae, degeneration of secondary lamellae, necrosis and rupture of epithelium were recorded during the exposure of lethal concentrations of Al from 200 and 300 mg/L (Fig. 2D–E). Necrosis observed in the gills of *C. gariepinus* in this study was considered a direct response of the gills to environmental pollutants, which could interfere with respiration, ion secretion and reabsorption processes of the gills (Guite *et al*., 2015).

The percentage of the weight of the mineral content through the cross-section of gills was quantified by energy dispersive X-ray (EDX) spectroscopy to scan primary and secondary lamellae of gills in determining their metal composition. Al accumulated in organisms has been found to have various adverse effects on fish. An examination by EDX in SEM showed the increased spectrum in the Al element percentage to 15.2%, which suggests a cumulative susceptibility to the Al metal in the fish gills of *C. gariepinus* (Fig. 3).

Histological of ultrastructure *C. gariepinus* gills observation under TEM permitted the identification of several cells such as pillar cells, pavement cells and chloride cells. The control fish of *C. gariepinus* in the present study showed a normal ultrastructure of gill lamellae (Fig. 4A). The gill lamella is usually covered by a mucoid epidermis emerged from within its pale-staining saline, or salt

secreting chloride cells. These chloride cells are huge in number at the basal (proximal) part of the lamellae. They are surrounded by the pavement cells and accessory cells. Despite the well-maintained gross morphology, ultrastructural observation of the main epithelium demonstrated the alterations in the gills that can be found in the secondary lamellae.

The cells in Al-treated groups were in necrosis phase and the exposure to sublethal concentration (25 mg/L) of Al has displayed denser cytoplasm with a nucleus surface that started to de-shaping in an irregular form. Plus, the appearance of degenerative phenomenon by cytoplasm of pavement cells appeared vacuolated, degenerated and apoptotic with reduced number of pillar cells (Fig. 4B). The epithelium of filaments was altered by many intercellular spaces between these cells and some vacuolated pavements cells by exposing *C. gariepinus* to 100 mg/L of Al. Each pillar cell possessed a large nucleus with an irregular outline and cytoplasm containing fine particles known as glycogen granules (Fig. 4C).

Gills alterations in *C. gariepinus* were more conspicuous after 96 h Al exposure at 200–300 mg/L concentration. Profound degeneration of the gills apparatus was more evident by the massive degeneration of epithelial cells. Blood congestion with the formation of aneurysms in the distal portions of lamellae and noticeable degeneration often occurred under exposure to 200 mg/L of Al (Fig. 4D). Meanwhile, exposure to 300 mg/L of Al as the highest concentration revealed a total degeneration of epithelial cells along a vacuolated nuclear membrane (Fig. 4E), displaying a high toxicity of Al.

As stated in Fig. 4 for *C. gariepinus*, the intensity of cellular injury was increased in Al concentration as well as apoptotic and necrotic cells. Apoptosis and cell degeneration were testified in fish gills as a result of chemical and physical perturbations of aquatic medium (Daoust *et al*., 1984). Macirella and Brunelli (2017) stated that the necrosis of epithelium is frequently associated with heavy metals compared to other toxicants. The abnormal shapes of nuclei concerned in present study were related to impaired mechanotransduction and defective cytoskeletal integrity causing a decrease in mechanical stiffness in some cells (nucleus) (Lammerding *et al*., 2005). Hidouri *et al.* (2017) mentioned that abnormal nuclear shape is able to reflect mitotic instability linked to degenerating cells in some cases. Next, the alterations in pillar cells observed in Fig. 4C have been reported by exposure to heavy metals (Brunelli *et al*., 2011). Degeneration in pillar cells have been conveyed after exposure to 5 mg/L of copper by Basirun *et al.* (2019) in their study on *Oreochromis mossambicus*. The alteration of the pillar cells controlling the blood has contributed to the impairment of physiological exchange, thus leading to the appearance of aneurysms (Ribeiro *et al*., 2005) as observed in Fig. 4D.

ChE is a ubiquitous enzyme that can be utilised as a good biomarker for heavy metal detection as the response of inhibition towards a vast range of inhibitors is closely accompanied by the rise in mortality of aquatic organisms (Ahmad *et al*., 2016; Padrilah *et al*., 2017). Heavy metals including Al can influence the vital physiological functions of gills caused by the increased reactive oxygen species (ROS) upon exposure (Sevcikova *et al*., 2011; Sabullah *et al*., 2015). Since ChE is an abundant neurotransmitter hydrolysing enzyme in the CNS, massive generation of ROS can disrupt the impulse transmission and ultimately affect fish organs including gills (Choi *et al*., 2009). ChE from gills of untreated *C. gariepinus* was determined to be foremost with AChE when analysed with 5 mM of three synthetic substrates known as ATC, BTC and PTC by the assay method (Ellman *et al*., 1961) in this study. Fig. 5 demonstrates that ATC has catalysed much higher ChE compared to BTC and PTC. The result is in agreement with several previous studies in which a predominantly AChE was extracted from the gills of several fish species including *Pinna nobilis, Lates calcarifer, Danio rerio* and *Jenynsia multidentata* (Hayat *et al*., 2015; Natalotto *et al*., 2015; Bonansea *et al*., 2016) besides the recent study by Basirun *et al*. (2019) on *Oreochromis mossambicus*. The results of the preliminary screening of ChE activity by Jebali *et al*. (2013) on gills of *Cerastoderma glaucum*displayed the highest ChE activity obtained using ATC and PTC, whereas the hydrolysis of BTC was seen noticeably low (Jebali *et al*., 2011).

Furthermore, heavy metals are known by their capacity to inhibit *in vitro* or *in vivo* of AChE activity (Galloway *et al*., 2002; Banni *et al*., 2005) especially in fish. *In vivo* inhibition of AChE of *C. gariepinus* gills in Fig. 6 indicated that the percentage inhibition was inhibited by 13% at lowest $Al_2(SO_4)_3$ of 25 mg/L and continuously inhibited at the highest concentration of 300 mg/L. Similarly, decreased levels of AChE may be due to the attachment of Al to the SH-groups of enzymes at the active sites, which blocked their functions in certain chemical reactions as reported by Yellamma *et al.* (2010) on Al toxicity. It has been generally accepted that the main target of Al are the gills of fish (Muthukumaravel, 2014).

Meanwhile, *in vitro* AChE inhibition was determined by incubating extracted *C. gariepinus* gills AChE with 1 to 10 mg/L concentrations of Al metal ions by decreasing the activity to 50% inhibition concentration (IC_{50}) of 4.12 mg/L (Fig. 7). Inhibition by metal ions is related to the binding affinity towards amino acid side chain (Sabullah *et al*., 2014). Proteins containing histidine residue are vulnerable to metal binding including the Al in this study. The mechanism of inhibition by Al tested in this work is inadequate but can be speculated to act upon the catalytic triad Ser-His-Glu commonly conserved in AChE (Armentrout *et al*., 2013). This present study has proved the capability of Al metal ion to inhibit the activity of *C. gariepinus* ChE in fish gills.

Conclusion

In vivo and in vitro exposures of C. *gariepinus* to sub-lethal concentrations of Al have indicated toxicity impacts to the gills, which had also impeded gills functions as recorded physiologically and histologically through different microscopic perceptions. The different roles of conceivable damage mechanism from cellular to the atomic level suggest additional research to be carried out on these levels clarifying the components of Al toxicity in much detail. The actions of ChE of the gills were likewise reduced with the increment in Al concentration, proposing that this decrement has a potential biosensor application for checking Al contamination. Along these lines, this species may be reasonable to be utilised as a sentinel species for biomonitoring Al contamination.

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