ACETYLCHOLINESTERASE INHIBITORY EFFECT OF *FICUS PLATYPHYLLA* ROOT METHANOLIC EXTRACT ON A CATFISH MODEL

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ABSTRACT

The aim of this study was to evaluate the acetylcholinesterase inhibitory effect of Ficus platyphylla metabolic root extract in Clarias gariepinus. The root was extracted with 80% methanol. A toxicity study was carried out on adult catfish. Cholinesterase inhibitory activities were also evaluated on the same fishes using Ellman's method. Result revealed high cholinesterase inhibitory activities of the crude extract with significant differences at p<0.05 between the group treated with crude extract only, group treated with crude extract and exposed to arsenic and group that were exposed to arsenic only as well as group that are maintained in complete media. It can be concluded that low toxicity and high cholinesterase inhibitory effect of the crude extract is responsible for it therapeutic effects of this crude extract. Toxicity screening of this crude extract on a mammal such as mice and rat to reaffirm their toxicity profile are recommended. Antioxidant screening as well as isolation of bioactive compounds present in this plant part is strongly recommended.

Keyword: Arsenic, Cholinesterase, Toxicity study, *Ficus platyphylla* metabolic root extract, *Clarias gariepinus*

INTRODUCTION

Neurodegenerative diseases are characterized by structural alterations resulting on changes that lead to atrophy and loss of neurons and glia in specific limbic regions and circuits. Stress and depression has played important role in the pathogenesis of these illnesses (Pitsillou *et al*., 2020). Some of the fundamental changes that caused diseases include dysregulation of neuroprotective and neurotrophic signaling mechanisms that are required for the maturation, growth and survival of neurons and neuroglia (Chang *et al*., 2019). Several studies showed that changes in behavioral and life styles as well as medicinal approached can reverse these structural alterations by stimulating neuroprotective and neurotrophic pathways and by blocking the damaging, excitotoxic and inflammatory effects of stress (Sinha *et al*., 2020; Chitimus *et al*., 2020; Sharma and Singh, 2020). Some of the predisposing factors to this condition include lifetime exposure to cellular and environmental stressors as well as interactions with genetic factors (Furman *et al*., 2019). Clinical therapies of these diseases mostly aim at slowing down the symptoms. There are no effective clinical treatments that enhance complete recovery or prevent the recurrence. Hence, none of the conventional drugs used so far were reported to cure or reverse the clinical syndrome of these

diseases. The treatments efficacies of these diseases are not satisfactory, most of the treatment involved high financial cost and prolong duration (Crippa *et al*., 2019). This discourage several patient affected by these disease from taken conventional drug in develop countries.

Traditional Chinese medicines (TCM) have been in practice since time immemorial and were reported to yield positive results on the management of several diseases. Scientific finding have reported bioactive compounds from herbal extract to be effective in managing neurodegenerative diseases (Ondua *et al*., 2019). Pathogenesis of neurodegenerative diseases are complex, therefore combination of bioactive compounds from different herbs could be effective therapeutic approach to these illnesses. Herbal decoctions may work on multiple targets to enhance the therapeutic effects synergistically in most disease conditions (Babazadeh *et al*., 2019).

To find solution to these problems, medicinal plant *Ficus platyphylla* Del. root was obtained, extracted and evaluated for its neuroprotective effect. *F. platyphylla* belongs to the Genus *Ficus* and the Family Moraceae consisting of not less than 850 species. More than 200 different types of *Ficus* are commonly use as woody trees, shrubs and vines in the forests of tropical and subtropical regions (Peguero-Pina *et al*., 2020). About 500 species of *Ficus* are found in Asian region such as Malaysia and Australia. Several species of Ficus are grown as indoor as well as outdoor ornamental plants in Asia (Xu *et al*., 2019). Species of this plant are very rich in nutritional components and are used as a source food in Egypt, India, south China, Turkey and Malaysia (Nawaz *et al*., 2019). Parts of this plant have been used as medicinal plant for the treatment of several ailments. Several literatures reported the presence of phenolic acid and flavonoids in high concentration in *Ficus* plants which highly contributed to it therapeutic power (Aruwa *et al*., 2019; Ashraf *et al*., 2020). Hence, *Ficus* plants have high potential to protect against disorders arising from oxidative stress. *Ficus* species are well known in the field of traditional medicine. Research on the medicinal importance of *Ficus* plant, revealed it effectiveness in the treatment of diabetes, stomach ache, piles, ulcer, dysentery, inflammation, oxidative stress and cancer (Iqbal *et al*., 2018). Anti-cancer, anti-inflammatory and anti-diabetic potential of this plant support it ethno-medicinal application, and thus made it popular among other medicinal plants and marketed worldwide (Soib *et al*., 2019). Therefore, the aim of this research was to evaluate the neuroprotective effects of *F. platyphylla* methanolic root extract by studying it effect on acetylcholinesterase enzyme of fish. To achieve this catfish (*Clarias gariepinus*), a non-mammalian vertebrate was used in this research.

Fish model (*Danio rerio*), has been recently used scientifically for pathological and pharmaceutical researches including examination of symptoms, diagnosis of several diseases, drug screening and disease initiation mechanisms (Babich *et al*., 2020). Unlimited advantages were accomplished with fish model as a result of their high fertility, easy maintenance, and short generation time.

MATERIALS AND METHODS

Ethical Approval: This study was scrutinized and approved by the University Committee on Medical and Scientific Research Ethics, in accordance with National Health Research Ethics Committee of Nigeria (NHREC) norms and standards for conducting research on humans and animals (NHREC, 2007).

Animal Model: A total of 230 adult catfish of having length and weight ranges of 8 – 10 cm and 1.8 – 2.5 kg respectively were purchase from a Fish Farm at More Ward, Sokoto South LGA, Sokoto State. Fishes were acclimatized for two weeks at Veterinary Physiology and Biochemistry Laboratory, Usmanu Danfodiyo University Sokoto in ten class aquaria (12 x 12 x 30 cm^3) with chlorine free tap water filled to it one third. The fishes were fed twice daily with 5 % body weight of feed in divided rations using Vital Fish Feed (catfish) (42.0 crude protein) (Grand Cereals Limited, Jos, Nigeria). The

aquaria were washed and the water changed every other day at 8 a.m.

Plants Collection and Identification: Leaves and root of the plant were collected from Department of Veterinary Pharmacology and Toxicology Garden at the City Campus of Usmanu Danfodiyo University, Sokoto (UDUS). A botanist at Biological Science Department, UDUS, identified and authenticated the plant leaf and root, and voucher specimen number UDUS-BSDH-FP/009 was kept in the Departmental Herbarium.

Plant Extraction: *F. platyphylla* root was cleaned, cut into tiny bits and allowed to dry for two weeks at room temperature (26 ± 1^{o} C). The dried root pieces were powdered using pistil and mortar, and latter sieved with Japanese Stainless Steel Tea Strainer (85 mm/40 very fine mesh). A total of 10 kg of the resulting root powder was soaked for three days in 1000 mL of 80 % methanol in a flat bottom flask (Sigma Aldrich, USA). The powdered root methanol mixture was shaken daily for three days at 26^{o} C to obtain rich crude extract. The extract obtained was filtered with Whatman Filter Paper Number 1 (Sigma Aldrich, USA) and concentrated to semi-solid form at 42° C with a rotary evaporator (IKA® RV 10, USA). The resultant semi-solid crude extract obtained was weighed, powdered and stored in sealed vials under refrigeration (4° C) pending use.

Percentage yield of the crude extract was calculated as the weight of the filtrate divided by the total weight of the grounded seed in semi powder multiply by 100 (Yield (%) = [wt of extract (g)/wt of plant material (g)] x 100).

Crude Extract Dilution and Dose Preparation: Stock solution was prepared by dissolving 1 g of *F. platyphylla* crude root extract in 10 mL of 100 % DMSO. DMSO was used to solubilize the crude extract, since the extract is not absolutely soluble in aqua solvent. Preparation of sub-stocks solution in microliter (g/mL) was done by diluting the stock solution to the concentration of interest using distilled water. Working solution was prepared from sub-stock solution using two-fold serial dilution with distilled water at eight concentrations ranging from 7.81 – 1000 g/mL.

Acute Toxicity Test of Arsenic on Adult Catfish (*Clarias gariepinus*): Acute toxicity test of arsenic was conducted using 15 catfishes per treatment for 2 days. Catfishes of both sexes were randomly chosen and exposed to prepared arsenic dose concentrations of 0.0, 0.5, 1.0 and 2.0 g in four three liter capacity rectangular glass aquaria. Mortality was monitored continuously (hourly) and the fishes were considered dead when they fail to respond to touch with glass rod, and there was no evidence of operculum respiration. Dead fishes were immediately recorded and removed from the tank. Using the number of dead, Probit analysis was done to determine the acute toxicity of arsenic (Finney, 1952). After 24 hours, non-dead exposed fishes were transferred to fresh aquaria containing chlorine free fresh tap water for recovery and the number of fishes recovered was used in calculation of percentage survival using the using log rank and modified Wilcoxon tests (Jones and Whitehead, 1979). The fish were fed prior to and during the experimental period. During the experiment, the fish were closely monitored.

Acute Toxicity Test of Plant Extract on Adult Catfish (*Clarias gariepinus*): Acute toxicity test of the crude extract was carried out on 60 adult catfish for 2 days in chlorine free tap water. Random selection of catfish of both sexes was carried out and maintained at the stocking density of ten fish per three liter aquaria. Six different concentrations (0.1 % DMSO (Control), 62.5, 125, 250, 500 and 1000 g/L crude methanolic extracts) were tested. Continuous monitoring of mortality was carried out and dead fishes were confirmed after the operculum movement was no longer detected and inability of the fishes to respond when touched with a glass rod. Using the number of dead, Probit analysis was done to determine the acute toxicity of arsenic (Finney, 1952). After 24 hours, non-dead exposed fishes were

transferred to freshwater aquaria for recovery and the number of fishes recovered was used in calculation of percentage survival using the using log rank and modified Wilcoxon tests (Jones and Whitehead, 1979).

Chronic Toxicity Test of the Arsenic and Crude Extract on Adult Catfish: Chronic toxicity test of the crude extract and arsenic was carried out on 120 adult catfish for 10 days in chlorine free tap water. This was done to determine the changes in total protein and acetylcholinesterase caused by prolong exposure to the crude extract and arsenic. Fishes were assigned to a completely randomized designed (CRD) experimental setup of four treatment groups, replicated thrice with 10 fishes per replicate. The fishes were maintained in separate aerated 14 liter tanks and water was changed after every 24 hours. The fishes were initially exposed to the safe dose of crude extract 250 g/L for 24 hours and later exposed to 0.5 M arsenic. These concentrations were chosen based on previous studies of acute toxicity test of crude extract and arsenic. Group I catfishes were exposed to 250 g/L of crude extract, Group II catfishes were exposed to 0.5 M of arsenic, Group III catfishes were exposed to 250 g/L of crude extract for 24 hours and then 0.5 M of arsenic for 9 days, and lastly Group IV catfishes were maintained in 0.1 % DMSO (control group) without arsenic and crude extract.

At the end of the experiment, the fishes were cryo-anesthetized by exposing them to ice for 60 seconds. The fish head was dissected and the brain was carefully removed without any damage. The sampled brain tissue was weighed, washed with 50 M Tris-HCl buffer and homogenized in a bottle with tissue homogenizer (Polytron PT-6100, USA). Tris-HCl buffer (1 % Triton X and 0.1 % PMSF) (Sigma Aldrich) was used as homogenizing solvent. Sample was centrifuged at 12,000 x g for 20 minutes with (GRACE High Speed Refrigerated Centrifuge, India). The supernatant was transferred into separate tube and used as enzyme source (Hassan *et al*., 2020).

Total Protein Estimation: The protein content was determined on homogenized fish brain using Bradford method (Hassan *et al*., 2020). Bovine Serum Albumin (BSA) 7.81 – 1000 µg/mL was prepared and used as a standard. A 1 ml stock solution of 1000 µg BSA/200 ul Tris-HCl (10 mg/200 mL) pH 7.4 was diluted with Bradford reagent and fed into 96-well micro plate. Reading was taken at 590 nm using micro-Plate reader (Filter Berthold Technologies GmbH and Company, KG) and standard curve was plotted. The same was repeated for determination of total protein in the brain. Tris–HCl pH 7.4 was diluted with the brain sample at different concentration from 0 to 1000 µg/mL, 200 µL of sterile phosphate saline (PBS), Bradford reagent was later added and then maintained for 30 minutes. Reading was taken at 590 nm using micro-plate reader (Filter Berthold Technologies GmbH and Company, KG). After running the assay, the standard curve was used to extrapolate the protein concentration according to OD values.

Determination of Acetylcholinesterase Activity: A 50 M Tris-HCl pH 7.4 was used as a buffer throughout the experiment. Cholinesterase (Ache) used in the assay was from the homogenized brain of experimental fishes. 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), acetylcholine (ATC) was prepared in 50 M Tris-HCl in 96-well plates I. A 210 μL of Tris-HCl buffer (pH 7.4), 20 µL of 0.1 M DTNB, and 10 µL of acetylcholinesterase enzyme (AChE) from the brain (54 mg/mL) were transferred in to another 96-well plate II and incubated for 15 min at 28°C. Then, 10 μL of ATC (2.5 M) were then added to the mixture in 96-well plate II and incubated for 10 minutes. Based on Ellman's method, anti-cholinesterase (ChE) activity was measured using a modified 96-well microplate assay. The hydrolysis of the substrate acetylthiocholine by cholinesterase enzyme results in the production of thiocholine. Thiocholine reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine which was measured at 405 nm with micro plate reader. Reading was taken at 405 nm using a micro plate reader (Tecan Multimode Microplate,

United Kingdom) at fluorescent excitation of 485 nm and 535 nm emission (Hassan *et al.*, 2020).

Statistical Analysis: Data collected were processed and analyzed for their central tendencies using analysis of variance (ANOVA). Probit analysis was done to determine the LC_{50} (Finney, 1952). Log rank and modified Wilcoxon tests (Jones and Whitehead, 1979) were used to compare the survival curves. Significant means were separated using Dunnett's post hoc test at $p \leq 0.05$. The results were presented as mean ± standard error of the mean (SEM). All data were statistically analyzed using GraphPad Prism Version 5.0.

RESULTS

Crude Ficus Extract Yield: The result of *F. platyphylla* root percentage yield after extraction, evaporation and concentration with a rotary evaporator at 42 °C was 1988.07 g (10.06 %).

Acute Toxicity of Arsenic: The result of acute toxicity test of arsenic on catfish indicated LC_{50}, LC_{70}, LC_{90} of 0.53, 0.67 and 0.95 M respectively. There was high mortality in groups exposed to 1 M. Up to 80 % of the fishes survived at 24 hours post exposure in groups that were exposed to 0.5 M arsenic. Survival analysis of groups that were exposed to 1 M shows only 20 % of the fishes survived at day 1 post exposure and all fishes die at 48 hour post exposure. All fishes exposed to 2 M arsenic die at 24 hour post exposure. Comparison of survival curves using log rank and modified Wilcoxon tests showed significant difference ($p<0.05$) between the control group and the groups that were exposed to different concentration of the arsenic (Figure 1).

Acute Toxicity of Crude Extract: The result of acute toxicity test of *Ficus* methanolic extract on catfish had calculated LC_{50}, LC_{70}, LC_{90} of 346.7, 1148.2 and 6760 g/L. it was observed from the result that all fishes exposed to 62.5 g/L of the crude extract survived at day 2 post exposures.

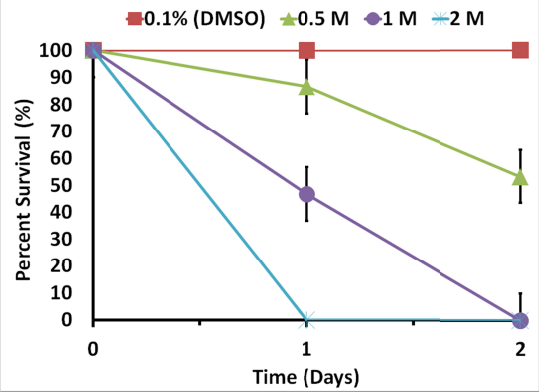

Figure 1: Acute toxicity of arsenic on the survival of adult *Clarias gariepinus* treated with different concentrations (0.5 – 2 M)

Up to 70 % of the fishes survived the 24 hours post exposure and only 20% survived the 48 hours post exposure in groups that were exposed to 125 g/L of the crude extract. Survival analysis of groups that were exposed to 250 g/L crude extract shows only 20 % survived at 24 hour post exposure and all the remaining fishes die at 48 hour post exposure. All fishes exposed to 500 and 1000 g/L of the crude extract die at 24 hour post exposure. Log rank and modified Wilcoxon tests to compare the survival curves at df 1 and 5 % showed significant difference ($p<0.05$) between the groups that were maintained in 0.1% DMSO and the groups that were exposed to different concentration of the extract (Figure 2).

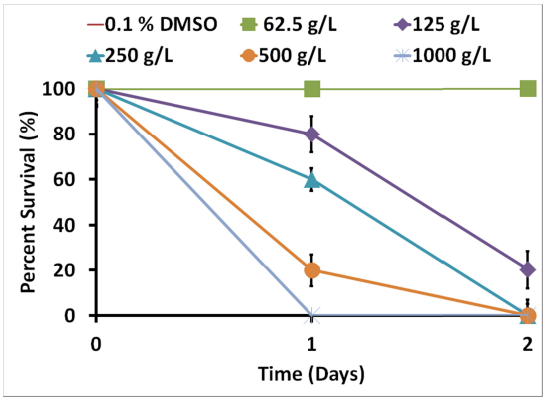

Figure 2: Acute toxicity effect of crude extract on the survival of adult *Clarias gariepinus* treated with different concentrations (62.5 – 1000 g/L)

Total Protein: The results on total protein content showed high protein content in groups exposed to the crude extract (250 g/L) only.

Increased protein content was also observed in groups that were maintained in 0.1 % DMSO as well as the groups that were treated with crude extract (250 g/L) and exposed to arsenic (0.5 M) compared to the groups that were exposed to arsenic only (0.5 M). There was significant difference at $p<0.05$ between fishes that were exposed to arsenic (0.5 M) compared to those that were maintained in 0.1% DMSO, treated with crude extract (250 g/L) and those that were treated with crude extract (250 g/L) and exposed to arsenic (0.5 M) (Figure 3).

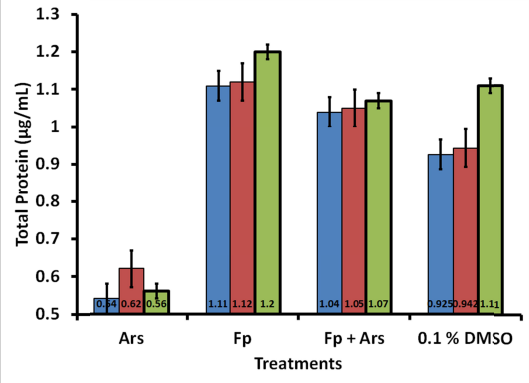

Figure 3: Effects of *Ficus platyphylla* (root) 250 g/mL extract and arsenic (0.5 M) on total protein content of the brain of adult Clarias gariepinus. *Key: Ars = arsenic and Fp = Ficus platyphylla (root) extract*

Acetylcholinesterase Inhibitory Activity: Result of acetylcholinesterase inhibition showed high activities in groups that were maintained in 0.1 % DMSO. Increased activities were also observed in groups that were treated with crude extract (250 g/L) only and groups that were treated with crude extract (250 g/L) and later exposed to arsenic (0.5 M) compared to the groups that were exposed to arsenic (0.5 M) only. There was significant difference ($p<0.05$) between the fishes that were treated with arsenic (0.5 M) when compared to those that were maintained in 0.1 % DMSO, those that were treated with the extract (250 g/L) only as well as those that were treated with the extract (250 g/L) and later exposed to arsenic (0.5 M) (Figure 4).

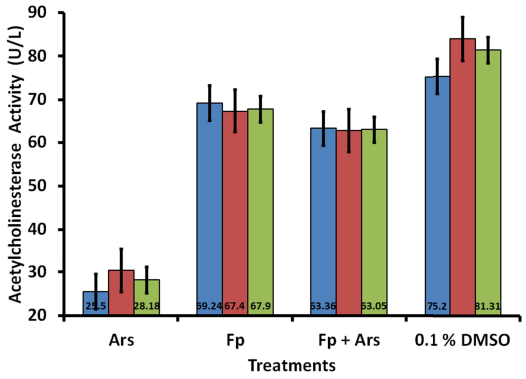

Figure 4: Acetylcholinesterase inhibitory effects of *Ficus platyphylla* root extracts against prolong arsenic exposure in *Clarias gariepinus* measured using DTNB, acetylcholine iodide substrate with a microplate reader at 405 nm. *Key: Ars = arsenic and Fp = Ficus platyphylla (root) extract*

DISCUSSION

Neurodegenerative diseases are incurable and debilitating conditions that result in progressive degeneration and / or death of nerve cells. This causes problems with movement (called ataxias), or mental functioning (called dementias) (Lalotra and Vaghela, 2019). Treatment of these diseases with several conventional drugs may yields no positive result (Ikhsan *et al.*, 2019). To overcome these problems, *F. platyphylla* root methanolic extract was evaluated for toxicity and neuroprotective effects through anticholinesterase inhibitory assay on adult catfish. The solvent methanol is used widely for the extraction of several bioactive compounds in both plant and animals (Yahia *et al.*, 2020). Other solvent used for the extraction of the bioactive compound from living organisms include hexane, ethanol, ethyl acetate, acetone and water (Mogashoa *et al.*, 2019). Methanol and water are the safest polar solvents commonly used by traditional herbalist for the constitution of the traditional medicine (Mohotti *et al.*, 2020). Therefore, 80 % methanol was chosen as extraction solvent in this research. Polarity of solvent plays very important role on the types and concentration of the bioactive compound recovered following extraction.

Increase in phenolic compounds as well as its antioxidant effects was high in solvents with high polarity (Lakka *et al*., 2019). High extract yields in fruit and vegetable following extraction were reported in solvents with high polarities; acetone and methanol (Ali *et al*., 2019). Effects of acetone in separating large portion of nonpolar part of the herbs have also been reported (Manjare and Dhingra, 2019). High solubility of the nonpolar compounds in acetone might be the major reason for the high recovery of these compounds in the aforementioned solvent (Knez Hrnčič *et al.,* 2019). Several studies also documented high yield of phenolic compounds from the roots of horseradish plant with ethanol as well as ethanol/water solvents and honey (Tomsone *et al*., 2012; Ullah *et al.,* 2019; Borges *et al.,* 2020; Phaisan *et al.,* 2020).

Similarly, result of *Moringa oleifera* leaf extraction revealed high extract yield with methanol solvent compared to acetone, hot water and chloroform (Ihegboro *et al.,* 2020). Time and techniques used during extraction also play important role in types and amounts of bioactive constituents recovered following the experimental extraction (Gómez-Mejía *et al*., 2019).

It has been observed that causes of increased mortality of catfish exposed to crude extracts may be associated with types and concentration of bioactive compound present in different part of the plant. Most of the active compound that are used as medicinal agent have side effect especially if given at high concentration (Kuruppu *et al*., 2019). Increased mortality rate in catfish may be among the major side effect of this extract recorded at higher concentrations. Some medicinal plants such as *Digitalis purpurea*, *Hyoscyamus niger*, *Atropa belladonna*, *Physostigma venenosum*, *Podophyllum peltatum* and *Solanum nigrum* are reported to have high therapeutic effects due to the present of high phenolic compounds (Alamgir, 2017). Compounds that are of therapeutic important in the aforementioned plant also turn to be toxic to the animals if taken at high concentration. Researchers have reported alkaloid which is among the bioactive compounds with high therapeutic values to impair respiration in fish models (Babich *et al*., 2020; Bilal and Iqbal, 2020). The same compound was also reported to affect osmoregulation in fish by increased stimulation of opercula beat on gills (Dobolyi *et al.,* 2020). Nerve malfunctions and or neurotransmitter denaturation by several phytochemical constituents that are of medicinal important were also reported especially following high dose administration (Furtado *et al.,* 2018). Most of the abnormal changes observed following the used of these compounds in fish include sluggish movements, atypical heart beat and death.

On the effect of crude extracts on survival rate, it was shown that the extracts at higher concentration significantly affected the survivability of the fishes. This finding was similar to the result reported on *C. gariepinus* which showed increased death rate of juveniles fish with increased concentration of *Carica papaya* seeds extract (Ayotunde *et al*., 2010; 2011). Even though, plant bioactivities compound such as saponin, flavonoid and terpenoid may be responsible for most of it therapeutic potential, it is also implicated as the major causes of death and teratogenic defect of embryos, larvae and adult fishes following high doses exposure (Bajalan*et al*., 2016).

The effect of plant extract on acetylcholinesterase enzymes have been well researched (Ghribia *et al.,* 2014; Ranjan and Kumari, 2017; Nwidu *et al*., 2018). Generally, esterases are the biochemical catalyst that neutralizes the choline base esters. They are group of enzymes referring to as cholinesterase or choline esterase (Cao *et al.,* 2020). Major functions of these enzymes are catalysis and transformation of neurotransmitters acetylcholine or acetyl choline-like substances to choline and acetic acid. The changes involved in this pathway are very essentials to allow the cholinergic neuron to come back to its resting state after excitation (Mateo *et al.,* 2017). Muscle contractions trigger the release of acetylcholine at neuromuscular junctions to enhance contraction, and this support the locomotive movement of the body organs. Neutralization of acetylcholine by the enzyme cholinesterase hence enhances relaxation of the muscle from it contractile state for a while

(Fogarty *et al.,* 2019). Plasma cholinesterase or pseudocholinesterase such as butyryl-cholinesterase (BChE, BuChE), is one of the nonspecific cholinesterase enzyme that neutralizes acetylcholine to choline-based ester. This neurotransmitter is formed in the liver and transported mainly through blood plasma to different body tissues and organs (Zlatković *et al*., 2017). It has high similarity with the neuronal acetylcholinesterase; they are also called erythrocyte cholinesterase (Alizadeh *et al.,* 2020). Another pseudo-cholinesterase available in the different body organs and tissues is in the plasma that is propionyl-cholinesterase. It is less common compared to Acetylcholinesterase and butyryl-cholinesterase but have almost the same biochemical effects (Ramsay and Tipton, 2017).

Most of the abnormal changes observed in these fishes exposed to arsenic may be due to the alteration in either production of acetylcholine, alteration of it function or over production of acetylcholinesterase followed by rapid metabolism of acetylcholine caused by arsenic. Preventive effect of arsenic in groups that were treated with crude extract before exposure to the arsenic can be clearly stated since there are increase acetylcholine activities in the groups.

Conclusion: It can be concluded that low toxicity and high cholinesterase inhibitory effect of the crude extract is responsible for it therapeutic effects of this crude extract. Toxicity screening of this crude extract on mammal such as mice and rat to reaffirm their toxicity profile are recommended. Antioxidant screening as well as isolation of bioactive compounds present in this plant part is strongly recommended.

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