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Original Research Article

Methanol Extract of *Ficus platyphylla* Stem Bark Inhibits Cholinesterase Enzyme on Catfish

Ibrahim M. Hassan and Abdullahi I. Ja'afaru

Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto.Sokoto State, Nigeria

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ABSTRACT

Traditional medicinal plants have been used for many years by different cultures around the world for the management of different diseases. Current treatments of neurodegenerative diseases are not effective and result in several side effects. Thus, the search for alternative medicines is in high demand. Therefore, this study aim to evaluate the cholinesterase inhibitory effect of *Ficus platyphylla* 80% methanol (stem bark) extracts. The stem bark 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 activity of the crude extract with high significant differences at $P < 0.001$ between the group that was 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 the group that were maintained in complete media. It can be concluded that the low toxicity and high cholinesterase inhibitory effect of the crude extract could be responsible for its therapeutic effects. Toxicity screening of this crude extract on a mammal such as mice and rat to reaffirm their toxicity and antioxidant screening as well as isolation of bioactive compounds present in this plant part is strongly recommended.

***Keyword**:* Arsenic, Cholinesterase, Extracts, Catfish, Toxicity Study.

Introduction

Free radicals are exceptionally toxic substances that cause damages to several tissues by reacting with several molecules such as glycoproteins or amino acids. The effect of these reactions may result in protein denaturation, cellular membrane destabilization, and eventually cell death.[1] Several literatures implicated free radical as the major causes of neuropsychiatric disorder such as aging of the central nervous system (CNS), schizophrenia, and the development of tardive dyskinesia during chronic use of neuroleptics.[2] Pathogenesis of several neurodegenerative diseases also reported free radical as the major substrates or intermediaries that affect several tissues and body fluids. The major targets of free radicals are nervous tissues and nerve depending organs.[3] The effect of free radical in nerve sensitive organs may affect the impulse transmission leading to a severe effect that results in the malfunction or complete damages of the affected tissues or organs.[4]

The plant *Ficus platyphylla* belonging to the family Moraceae, locally known as "gamji in Hausa" is a new potential medicinal plant in savannah region.[5] There was no enough literature on the chemical constituents of *F. platyphylla* but some of the compounds claimed to be present in *F. platyphylla* are the flavonoids: isovitexin, vitexin, proanthocyanidins, flavan-3-ol monomers, and flavones glycosides.[6] It was reported that the aqueous leaf extract of *F. platyphylla* contains water-soluble insulin-secreting constituents with better insulin secretion activity than a well-known hypoglycaemic agent, glibenclamide.[7] Toxicology study on *F. platyphylla* aqueous extract shows no toxic compounds present in all parts of this plant and treatment with the same extract resulted in no adverse effects in rat model.[8] Leaf extract from this plant was reported to exhibit high blood glucose-lowering effects, anti-nociceptive effect, ulcer healing effect, antioxidant effect, anti-inflammatory effect and anti-melanogenic effect.[9] However, to date, there is no available report on the anticholinesterase effect of the stem bark extract of this plant on the animal model. Therefore, the present study attempts to investigate the anticholinesterase effect of the stem bark extract of this plant on Catfish.

Catfish also referred to as *Pterygoplichthys* is a large bony fish that is commonly found in Africa including Nigeria.[10] They are abundant in brackish water, freshwater, and saltwater and have been widely used in experimental research as the vertebrate animals for many years.[11] They are characterized by a moderate life cycle and life span with wide geographical range and availability.[12] The fish have been used as a suitable bio-indicator animal for the diagnosis of water pollutants caused by heavy metals due to their long-lived, reasonablly large size, and ability to adapt well to laboratory environment.[13] Moreover, this species is easy to be identified; they are known to have a fast growth rate and are easy to culture in the laboratory which make them an excellent research model. They are sensitive to toxicants and endocrine-disrupting chemicals at an early life stage.[14]

Materials and Methods

Plants collection and identification

Ficus platyphylla stem bark was collected at Pharmacology garden city campus Usmanu Danfodiyo University Sokoto. Sokoto State Nigeria. Identification of the stem bark was carried out by a botanist at the Department of Biological Science Usmanu Danfodiyo University Sokoto and voucher number of 007 was given.

Plant extraction

Ficus platyphylla stem bark was initially washed and chopped into small pieces with the aid of an anvil pruner, (UK). It was then dried by

*Corresponding author. E mail: ibrahim.hassan@udusok.edu.ng
Tel: +2349034990736

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exposing it to air for two weeks at room temperature (26 ± 1°C) in the Biochemistry laboratory, Usmanu Danfodiyo University Sokoto, and later ground to reduce its size (40–60 mesh). A 200 g of the stem bark sample was soaked in 1000 mL of 80% methanol and allowed to stand for three (3) days with daily shaking in a flat bottom flask (Sigma Aldrich, USA). The mixture was then filtered with Whatman filter paper (1.5 Sigma Aldrich, USA) and solidified with a rotary evaporator (IKA® RV 10, USA) at 42°C. The crude extract obtained was then weighed and kept at -4°C in sample bottles until required.
The percentage yield was calculated as the weight of the dried extract divided by the total weight of the ground powder.

Yield (%) = [wt of extract (g)/wt of plant material (g)] x 100.

Crude extract dilution
The stock solution from the crude extract was prepared by dissolving 100 mg of *Ficus platyphylla* stem bark extract in 1 mL of 100% Dimethyl sulfoxide (DMSO). DMSO was used to dissolve the crude extract. A sub-stock was constituted in microliter (mg/L) by diluting the stock solution with distilled water to the concentration of interest using two-fold serial dilution.

Evaluation of toxicity effect of arsenic
A 2-day sub-acute toxicity test of arsenic and 7-day sub-chronic toxicity test was carried out on adult catfish (8 months, 2 kg body weights). Five fishes were treated with arsenic 0.5–4 M in 15 L aquarium tank filled to 1/2 level with de-chlorinated water for the sub-acute toxicity test[15]. Five doses were prepared and used (0.1, 1, 2, and 4 M) using 2 fold dilution method alongside the control groups in this experiment. The following doses; 0.05, 0.15, 0.75, 1.75, 2.75, 3.75 M along with control groups that were maintained in tap water were used for the sub-chronic toxicity test of arsenic. The choice of the doses for the sub-acute toxicity test was based on available literature on the neurologic effect of arsenic doses on other aquatic animals. For the sub-chronic toxicity test, the choice of the doses was based on the outcome of the sub-acute toxicity test. Survival rate was monitored throughout the experimental period; signs of mortality included the inability to move the operculum covering the gills and lack of response to touch. Dead fishes were removed from the tank immediately it was noticed, and water was replaced every 24 h. The fishes were fed before the commencement of the experiment for the sub-acute toxicity test. Fishes were fed before and during the experimental study for the sub-chronic toxicity test.
Behaviors of the experimental fishes were monitored regularly by taking note of any abnormal movement or response during the study.

Evaluation of the toxicity effect of crude extract
A 2-day sub-acute toxicity test and 2 weeks sub-chronic toxicity test of crude extract were carried out on adult catfish (8 months 2 kg body weights) in de-chlorinated water[16]. Fishes were exposed to 62.5–1000 mg/L doses of the crude extract for the sub-acute toxicity test in 5 L aquarium. For the sub-chronic toxicity test of crude extract, fishes were exposed to 35, 45, 55, 65, 75, 85, and 95 mg/L in 5 L aquarium. Each group contains five fishes per aquarium, 3 replicates were used in this experiment alongside with the control groups. Continuous monitoring for dead fishes, removal, and replacement of the water was also carried out.

Experimental design
Evaluation of anticholinesterase effects of the crude extract on adult catfish was accomplished. The experimental group was divided into 4 with 10 fishes in each group[17]. Fishes were acclimatized for 2 weeks at the Biochemistry Laboratory, UDUS. Aerated 20-liter tanks were used for this experiment, and water was changed every 24 h. Fishes were initially exposed to the safe concentration of the crude extract (50 mg/L) for 24 h, and later arsenic (0.15 M) was added to the experimental tank. These concentrations were chosen from the results of the toxicity studies. Untreated fishes were kept under similar conditions in tap water. The experiment lasted for ten days.

Sample preparation
At the end of the treatment, fishes were cryo-anesthetized by exposing them to ice for 60 seconds[18]. Whole fishes were then washed with 50 mM Tris–HCl buffer weighed and homogenized with the aid of tissue homogenizer (Polytron PT-6100, USA) in a scope bottle [19]. The buffer was constituted with 1% Triton X and 0.1% phenylmethylsulfonyl fluoride (Sigma Aldrich) at pH 7.4; and were used as the homogenizing solvent [20]. The homogenized sample was then centrifuged at 12,000x g for 20 min with a high-speed refrigerated centrifuge (GRACE India). The supernatant was transferred into a separate tube and used as a source of enzyme.

Total protein estimation
Evaluation of the total protein content from homogenized catfish was carried out following treatment with the crude extracts and exposure to arsenic with Bradford reagent. Bovine serum albumin (BSA) was used as a standard in this experiment [21]. A prepared 1 mL stock solution consisting of 1000 µg BSA, 200 uL Tris–HCl (10 mg/200 mL) was constituted and stored in the refrigerator until needed. The supernatant from the homogenized sample was later thawed and diluted with Tris–HCl at different concentration (0 to 1000 µg/mL). A 200 uL of sterile phosphate buffered saline (PBS) was added to each well and diluted with the homogenized sample, blank wells have no sample added but only PBS. After running the assay, the standard curve was plotted. The same procedure was repeated with 200 uL supernatant from the homogenized fish brain. The plotted standard curve was used to determine the concentration of samples according to the optical density (OD) values.

Evaluation of enzyme inhibitory effect of crude extract
Enzyme inhibitory activity was estimated using Ellman's method [22]. Acetylcholinesterase (ChE) was harvested by homogenizing the brain of experimentally exposed fish. Prepared Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholine, butyrylcholine, and propionylcholine each was individually constituted in Tris–HCl buffer (50 mM) at pH 7.4. A 210 μL Tris–HCl, 20 µL of 0.1 mM Ellman's reagent and 10 µL acetylcholinesterase (AChE) was mixed in 96-well plates and incubated for 15 min at 28°C. Acetylcholine, butyrylcholine, and propionylcholine (10 μL of 2.5 mM) was then added in separately prepared test sample containing cholinesterase (AChE) and kept in an incubator at 28°C for ten minutes. Biochemical neutralization of the substrate acetyl-thiocholine by the enzyme ChE results in the formation of thio-choline. Thio-choline combined with DTNB to form 2-nitrobenzoate-5-mercaptothiocholine. The fluorescent measurement was taken with a microplate reader (Tecan multimode microplate UK) at 485 nm and 535 nm (excitation and emission).

Statistical analysis
Each study in this research was carried out three times. Results were gathered, processed and interpreted as mean ± standard error of the mean (mean ± S.E.M). The survival rate and anticholinesterase effect of the crude extract on Catfish were analyzed with statistical software GraphPad Prism version 5.0. Statistical analysis using a one-way analysis of variance and Dunnett's post hoc test was carried out to calculate the p-value.

Results and Discussion

Crude extract yield
The result of *Ficus platyphylla* leaf percentage yield after extraction, evaporation, and concentration 42°C was 12.79%.

Sub-acute effect of arsenic
The result of the 2-day sub-acute toxicity test of arsenic on catfish showed high mortality at a concentration of 0.5 M and above. Only 20% of the fish survived the first day and all the fishes died on day two of the experiment. Almost all fishes at a concentration above 0.5 M died at day one of exposure (Figure 1).

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Sub-chronic effect of arsenic

The result of the sub-chronic effect of arsenic on Catfish shows almost all fishes exposed to doses above 0.75 M died on day four of the experiment. There was a significant difference at $p < 0.5$ between the groups that were maintained in tap water and the groups that were exposed to 0.075 M of arsenic. Significant difference at $p < 0.001$ was also observed between the control groups and the groups that were exposed to 1.75, 2.75, and 3.75 M arsenic (Figure 2).

Sub-acute toxicity effect of crude extract

The result of the sub-acute effect of the crude extract on the fishes revealed high mortality at a concentration above 500 mg/L. At the concentration of 500 mg/L, less than 48.9% of the fishes survived day 1 of the experiment and all died on day 2. All the fishes exposed to 1000 mg/L died on day 1 of the experiment (Figure 3).

Sub-chronic effect of crude extract

The result of the sub-chronic toxicity effect of crude extract on the fishes revealed high mortality at 55 mg/L with a survival rate of more than 50%. There was a significant difference at $p < 0.05$ between the group that was exposed to 50 mg/L and the control group. Significant difference at $p < 0.001$ was also observed between the fishes that were maintained in tap water and those that were exposed to 55, 65,75, 85, and 95 mg/L extract compared to the control (Figure 4).

Total protein content

The result of the total protein assay showed a high protein content in groups that were exposed to the crude extract only. Increased protein content was also observed in groups that were maintained in tap water as well as the groups that were treated with crude extract and exposed to arsenic compared to the groups that were exposed to arsenic only. There was a significant difference at $p < 0.001$ between control group (maintained in tap water) and the remaining groups (Figure 5).

Acetyl-cholinesterase inhibitory assay

The result of the acetylcholinesterase assay showed a high activity in groups that were maintained in tap water. Increased activity were also observed in groups that were treated with crude extract only and groups that were treated with crude extract and exposed to arsenic compared to the groups that were exposed to arsenic only. There was a significant difference at $p < 0.001$ between the fishes that were treated with arsenic and the remaining groups (Figure 6).

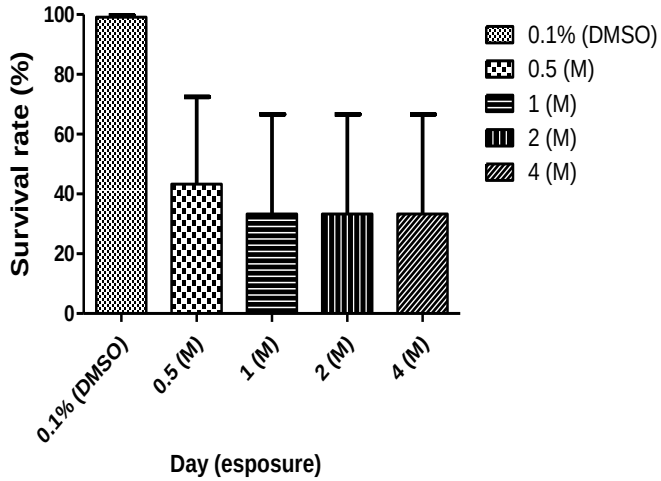

Figure 1: Sub-acute toxicity effect of arsenic on the survival rate of adult catfish.
The percentage of survival rate is shown versus the concentration of the tested sample. The values represent mean ± SEM from three independent experiments.

Butyrylcholinesterase inhibitory assay

The result of the butyryl-cholinesterase inhibitory assay showed a high activity in groups that were maintained in tap water. Similarly, increase activity were also observed in groups that were treated with crude extract only as well as the groups that were treated with crude extract and exposed to arsenic. There was a significant difference at $p < 0.001$ between the fishes that were maintained in tap water and the remaining groups (Figure 7).

Propionyl-cholinesterase inhibitory assay

The result of the propionic cholinesterase inhibitory assay showed a high activity in groups that were maintained in tap water only. Increase activity were also observed in groups that were treated with crude extract only, followed by the groups that were treated with crude extract and exposed to arsenic. There was a significant difference at $p < 0.001$ between the fishes that were maintained in tap water and the remaining groups (Figure 8).

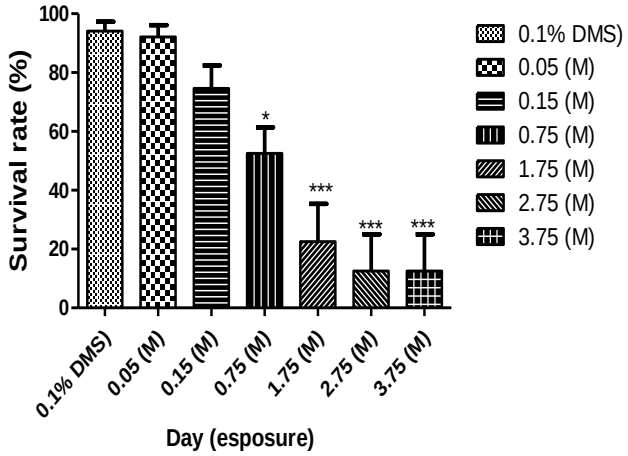

Figure 2: Sub-chronic toxicity effect of arsenic at different concentrations (0.05-1.75 M) tested on the survival rate of adult catfish. Percentage of survival is shown versus the concentration of the tested sample. *P < 0.05 and ***P < 0.001 represented significantly different values of survival rate. The values represent mean ± SEM from three independent experiments.

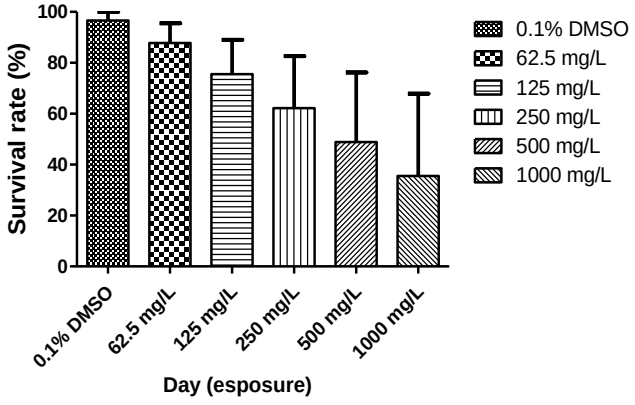

Figure 3: Sub-acute toxicity effect of *Ficus platyphylla* leaf extract on the survival rate of adult catfish treated with different concentrations (62.5 – 1000 mg/L) of the plant crude extract.
Percentage of survival is showed versus the concentration of the tested sample. The values represent mean ± SEM from three independent experiments.

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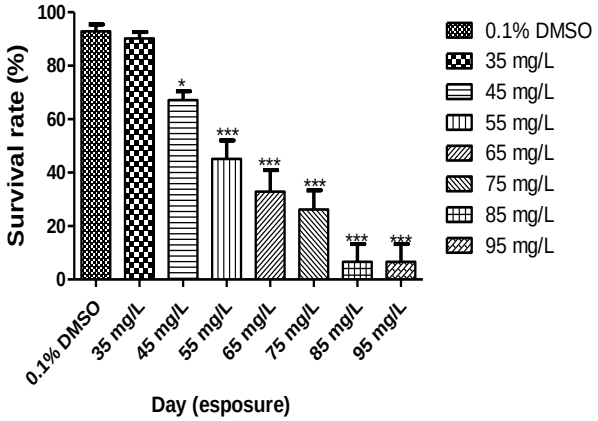

Figure 4: Chronic toxicity effect of *Ficus platyphylla* leaf extract on the survival rate of adult catfish treated with different concentrations (35 – 95 mg/L) of the plant crude extract. Percentage of survival is shown versus the concentration of the tested sample. *P < 0.05 and ***P < 0.001 represented significantly different values of survival rate. The values represent mean ± SEM from three independent experiments.

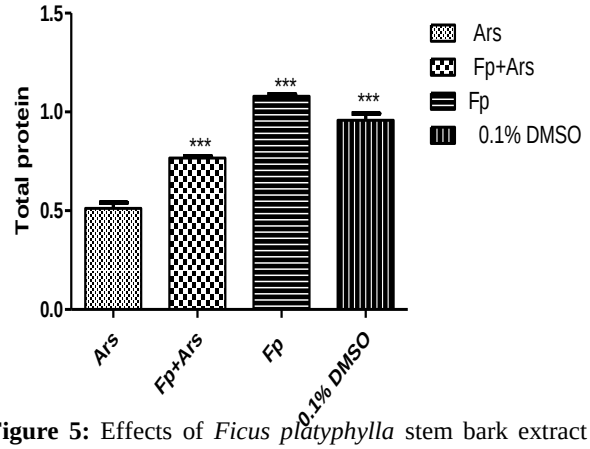

Figure 5: Effects of *Ficus platyphylla* stem bark extract (50 µg/mL) and arsenic (0.15 M) on total protein content extracted from the brain of adult catfish. ***P < 0.001 represented significantly different values from an arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = arsenic and Fp = *Ficus platyphylla* stem bark extract.

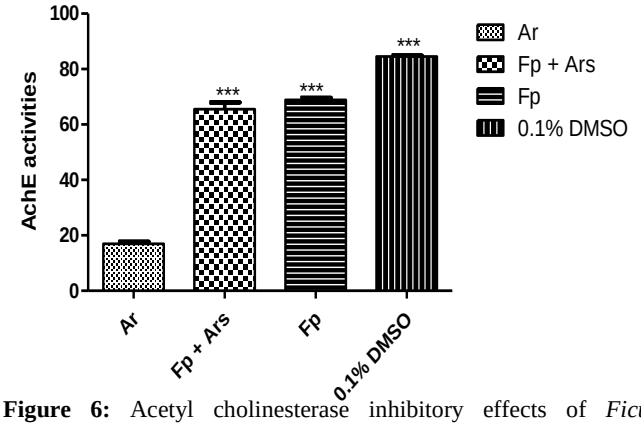

Figure 6: Acetyl cholinesterase inhibitory effects of *Ficus platyphylla* stem bark extracts against arsenic. ***P < 0.001 represented significantly different values from an arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = arsenic and Fp = *Ficus platyphylla* stem bark extract.

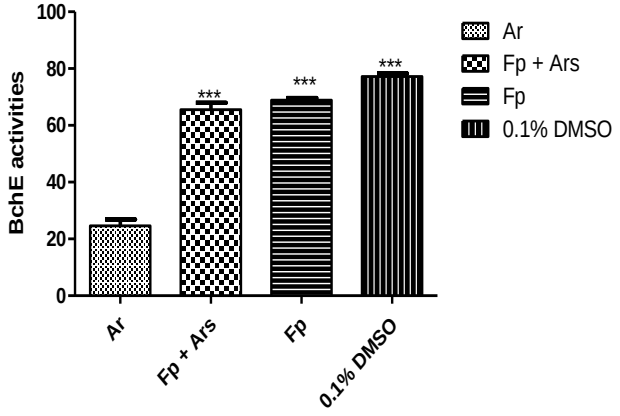

Figure 7: Butyrylcholinesterase inhibitory effects of *Ficus platyphylla* stem bark extracts against arsenic.
***P < 0.01 represented significantly different values from an arsenic-treated group. The values represent mean ± SEM from two independent experiments. Ars = arsenic and Fp = *Ficus platyphylla* stem bark extract.

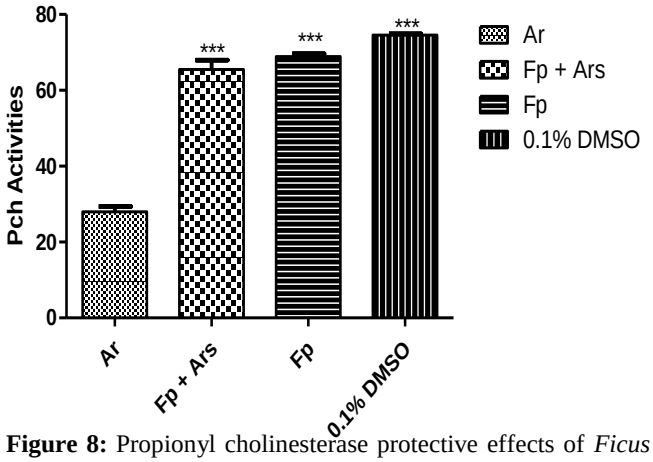

Figure 8: Propionyl cholinesterase protective effects of *Ficus platyphylla* stem bark extracts against arsenic. ***P < 0.001 represented significantly different values from an arsenic treated group. The values represent mean ± SEM from two independent experiments. Ars arsenic and Fd = *Ficus platyphylla* stem bark extract.

Degeneration of the nervous system is one of the major problems especially in developing countries where nutrition and or metabolic disorders are the common predisposing causes of this condition.[23] Problems associated with norms and cultures as well as scarcity of quality healthcare in most rural communities elevated the incidence of this disease in developing countries.[24] To find a lasting solution to neurodegeneration caused by exposure to free radicals and or formation of free radicals in the body system, *Ficus platyphylla* 80% stem bark was extracted and evaluated for toxicity and anticholinesterase effects on Catfish. Methanol is one of the commonly used solvent for the extraction of plant phytochemical constituents and was reported to be safe compared to the hexane and ethanol. Hence, 80% of methanol was chosen as an extraction solvent in this research.

Extracting solvents that are mostly used in phytochemical studies are methanol, ethanol, acetone, and ethyl acetate. They produce high crude extract yields on extraction and several phytochemical compounds were reported to be recovered after extraction with these solvents.[25] The polarity of the solvent plays a vital role in the types

and concentration of the bioactive compound recovered following extraction. An increase in phenolic compounds as well as its antioxidant effects was recorded high in solvents with high polarity.[26] High extract yields in fruit and vegetable following extraction were reported in solvents with high polarities; acetone and methanol.[27] The effects of acetone in separating a large portion of the nonpolar part of the herbs have also been reported. The high solubility of the nonpolar compounds in acetone might be the major reason for the high recovery of these compounds in the aforementioned solvent.[28] Several findings also documented a high yield of phenolic compounds from the roots of horseradish plant with ethanol as well as ethanol/water solvents.[29] Similarly, the result of *Moringa oleifera* leaf extraction revealed high extract yield with methanol solvent compared to acetone, hot water, and chloroform.[30] Time and techniques used during extraction also play an important role in types and amounts of bioactive constituents recovered following the experimental extraction.[31]

It has been observed that causes of increased mortality of crude extracts on catfish are associated with types and concentration of bioactive compounds present in different parts of the plant. Most of the active compounds that are used as medicinal agents have side effects especially if given at high concentrations. The increased mortality rate in catfish may be among the major side effect of this extract as recorded at higher concentrations.[32] 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 presence of high phenolic compounds.[33] Compounds that are of therapeutic importance in the aforementioned plant also turn to be toxic to the animals if taken at high concentrations. Researchers have reported alkaloid which is among the bioactive compounds with high therapeutic values to impair respiration in the fish model.[34] The same compound was also reported to affect osmoregulation in fish by increased stimulation of opercula beat on gills.[35] Nerve malfunctions and or neurotransmitter denaturation by several phytochemical constituents that are of medicinal importance were also reported especially following high dose administration.[36] Most of the abnormal changes observed following the use of these compounds in fish include sluggish movements, atypical heartbeat, and death.

Esterases are the biochemical catalyst that neutralizes the choline-based esters. They are a group of enzymes referred to as cholinesterase or choline esterase.[37] Their major function is to catalyse the transformation of the 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.[38] Muscle contractions trigger the release of acetylcholine at neuromuscular junctions to enhance contraction, and this supports the locomotive movement of the body organs. Neutralization of acetylcholine by the enzyme cholinesterase enhances the relaxation of muscle from its contractile state for a while.[39] Plasma cholinesterase or pseudocholinesterase such as Butyryl-cholinesterase (BChE, BuChE), is one of the nonspecific cholinesterase enzymes 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.[40] It has high similarity with the neuronal acetyl-cholinesterase, they are also called RBC or erythrocyte cholinesterase.[41] Another pseudo-cholinesterase available in the different body organs and tissues and in the plasma is Propionyl-cholinesterase. It is less common compared to Acetyl-cholinesterase and Butyryl-cholinesterase but has almost the same biochemical effects.[42]

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 its function, or overproduction of acetyl-cholinesterase followed by rapid metabolism of acetylcholine. The preventive effects of arsenic in groups that were treated with crude extract before exposure to the arsenic can be clearly stated since there are absent of abnormal movements and mortality as well as increase acetylcholine, butyrylcholine, and propionylcholine activities.

Conclusion

It was concluded that the low toxicity and high cholinesterase inhibitory effect of the crude extract of *Ficus platyphylla* was responsible for the therapeutic effects of the crude extract. Toxicity screening of this crude extract on mammals such as mice and rat to reaffirm their toxicity profile is recommended. Further studies to evaluate the antioxidant effects as well as isolation of bioactive compounds present in this plant part is also strongly recommended.

Conflict of interest

The authors declared no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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