PRINT ISSN 1119-8362
Electronic ISSN 2659-1502

Full-text Available Online at
https://www.ajol.info/index.php/jasem
https://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 27 (10) 2213-2219 October 2023

Assessment of Ackee Apple (*Blighia Sapida*) on Cholinergic and Antioxidant Enzymes; Possible Use of the Plant Stem-Bark Extract as a Biological Pest Controlling Agent

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ABSTRACT: Environmental protection practices include minimizing the level of synthetic chemicals as pesticides in agricultural activities. Reduction of cholinergic and metabolizing enzymes by natural products are safer pest-controlling alternatives in food security. Hence, the objective of this study is to assess the ability of Ackee apple (*Blighia sapid)* stem-bark extract as a potential biological pest control agent to interfere with acetylcholinesterase (AChE), glutathione S-transferase (GST), and biomarkers in brain, liver, and blood of Wistar rat using standard methods. The Wistar rat brain and liver were excised and blood was collected into heparinized tubes at the end of a 28-day experiment for biochemical investigations. Data obtained revealed that the activities of AChE and GST decreased at a dose-dependent rate ($P < 0.05$). A non-significant difference in alkaline phosphatase (ALP) for all the treated groups and a dose-dependent increase in total protein concentration were detected. The extract did not significantly alter ALT, AST, and ALP, particularly at repeated doses of 50 and 100 mg/kg. The extract of *Blighia sapida* reduced AChE and GST activity; a property that could be exploited in the formulation of pest control agents.

DOI: https://dx.doi.org/10.4314/jasem.v27i10.11

Cite this paper as: ADEKOLA, M. B; TAIWO, A. M; ORIYOMI, V. O; OGUNLEYE, O. S; OYEBAMIJI, A. T; RAHEEM, A. O; KASALI, S. O; BELLO, I. R (2023). Assessment of Ackee Apple (*Blighia Sapida*) on Cholinergic and Antioxidant Enzymes; Possible Use of the Plant Stem-Bark Extract as a Biological Pest Controlling Agent. *J. Appl. Sci. Environ. Manage.* 27 (10) 2213-2219

Dates: Received: 03 August 2023; Revised: 25 September 2023; Accepted: 04 October 2023 Published: 30 October 2023

Keywords: *Blighia sapida*; stem-bark; pest control; Wistar rats, biomarkers.

Since ancient times, man has engaged in agriculture to meet his food needs and used pesticide to prevent pre- and post-harvest losses of the crop from pests (Saroj *et al.*, 2020). Pest attack on crops is a threat to global food security and its control with synthetic pesticides is associated with health and environmental problems (Wilson and Huber, 2021). Pesticides are chemicals formulated to destroy, control, or prevent pests but because their mode of action is not specific, they also affect non-target organisms including humans (Kaur *et al.*, 2019; Nartop *et al.*, 2020). Synthetic pesticides exhibit harmful effects such as neurological, behavioral dysfunctions, hormonal imbalances, kidney and liver disorders, genotoxicity, and others due to their persistence in the environment and absorption by humans/animals thereby constituting major causes of environmental and health problems (Ravindran *et al.*, 2016; Malhat *et al.*, 2018). The effects of synthetic pesticides on the environment and organisms have led to the search for safer alternatives from natural sources (Chowdhary *et al.*, 2018). According to Yang *et al.* (2008), plants exhibit pesticide activity by interfering with cholinergic and antioxidant enzymes such as acetylcholinesterase and glutathione S-transferase. Acetylcholinesterase (AChE) terminates nerve impulses by catalyzing the hydrolysis of a neurotransmitter, acetylcholine into acetic acid and choline in the nervous system of various organisms. Also, glutathione S-transferases (GST) play an important role in pesticide resistance and detoxification (Gullner *et al.*, 2018). GSTs are the main cytosolic enzymes that catalyze the conjugation of electrophile molecules with reduced glutathione (GSH), making potentially toxic substances more

water-soluble and less toxic (Dobritzsch *et al*., 2020). Inhibitors of AChE and GST are good pesticidal agents (Umar *et al*., 2015). Culturally, *Blighia sapida* stem-bark powder is usually mixed with seeds of African finger (*Abelmoschus esculentus*) and pearl millet (*Pennisetum glaucum*) before sowing to avoid insect attacks. Presently, there is no scientific support for this practice. Hence, the objective of this study is to assess the ability of Ackee apple (*Blighia sapida*) stem-bark extract as a potential biological pest control agent for African finger (*Abelmoschus esculentus*) and pearl millet (*Pennisetum glaucum*) by evaluating its interference with acetylcholinesterase (AChE) and glutathione S-transferase (GST), and biomarkers in brain, liver, and blood of Wistar rat.

MATERIAL AND METHODS

Sample collection: Fresh stem-barks of *Blighia sapida* were obtained from a farm, at the Federal University of Agriculture Abeokuta, Ogun State Nigeria. The plant had been earlier identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. The specimen copy was deposited at the Herbarium and specimen voucher number 17623 was given.

Preparation of Hydro-alcohol extract of plant materials: Blighia sapida stem bark was air-dried for two weeks at 25 ± 2 ºC and ground into powder by an electrical Grinding Machine (SR-14733, Marlex, Daman). The powder material (1066.71 g) was macerated in 70 % (v/v) ethanol/water for 72 hours at room temperature using the method of (Handa *et al*., 2008). The resulting suspension was filtered and strained with a muslin cloth. The filtrates were pooled together and concentrated with a rotary evaporator at 40 ºC to yield a residue termed ethanol extract (EE). The resulting extract was weighed, labeled, and stored in the desiccator until required for further analysis.

Experimental animals: Thirty Wistar rats, weighing between 150 and 220 g were obtained from the Department of Anatomy, University of Ibadan, Ibadan, Nigeria. The rats were acclimatized to the laboratory conditions for two weeks in the Animal House. Rats were fed with standard pellets obtained from Ladokun Feeds, Ibadan, Oyo State, Nigeria, and were allowed free access to clean water. The principle of laboratory animal care (NIH publication No. 85–23) guidelines and procedures were followed for the study (NIH Publication Revised, 1996). Animal handling and care complied with international laboratory animal use and care guidelines. The approval of the departmental animal ethical committee, the Federal University of Agriculture Abeokuta Environmental Management and Toxicology (FUNAAB/EMT/20142094) was obtained prior to the experiment.

Determination of LD_{50} of the Blighia sapida extract: The lethal dose (LD_{50}) of the ethanol extract was designed in two phases (Lorke, 1983). Nine (9) Wistar rats were divided into three groups (3 Wistar rats per group) in phase one. The ethanol extract was given at 10, 100, and 1000 mg/kg body weight orally to all the groups (1, 2, and 3) respectively once before feeding. The Wistar rats were checked regularly for signs of toxicity, like withdrawal to a corner of the cage, rough fur, and salivation during the first 4 hours. Then, they were observed for the next 24 hours and every other day for 14 days, for effects of toxicity. In phase two, 3 Wistar rats were divided into 3 groups (1 rat per group), and 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg body weight of the extract were administered orally, this followed the procedure of the first phase.

Experimental design: Thirty (30) rats were randomly divided (Adekola *et al*., 2020) into five groups, and each group contained six rats; Group 1 –received 1.0 ml of distilled water; Group 2 –received Rambo pesticide 10% (w/v), Group 3 –received 50 mg/kg EE, Group 4 –received 100 mg/kg EE, Group 5 –received 150 mg/kg EE

Based on the result of the acute test (LD_{50}) conducted, three doses (50, 100, and 150 mg/kg) of *B. sapida* ethanol extract were chosen for the study. The rats were treated by gavage administration of the extract and locally produced insecticide 'Rambo' containing 0.6% permethrin (synthetic pesticide) every other day for 28 days. On day 28, the animals fasted overnight before being sacrificed under light chloroform anesthesia, their blood was obtained through the cardiac puncture, and organs of interest were excised. Collected blood samples were kept in separate heparinized tubes while the organs were stored in plain sample bottles. The samples were labeled and used for the estimation of biochemical parameters.

Preparation of blood plasma: Blood plasma was prepared according to the standard procedure of Chawla (1999). Typically, blood samples collected in heparinized tubes were centrifuged at 3000 rpm for 10 min in a Table Centrifuge (Model 90–2) at 25 °C. The plasma was collected into sterile tubes with sterile Pasteur pipettes and used for biochemical analyses.

Preparation of liver and brain homogenates: Liver and brain homogenates were prepared as described by (Babalola and Areola, 2010). One gram (1 g) of tissue was homogenized with 100 mM of phosphate buffer, pH 7.2, to produce 10% (w/v) homogenates with pestle

and mortar. The homogenates were carefully transferred into centrifuge tubes and volumes were adjusted to 10 ml. This was centrifuged at 4000 rpm for 30 min in a Table Centrifuge (Model 90–2) at 25 ^{o}C. The supernatants were collected into clean sample bottles, labeled, and kept in the freezer for the assay of biomarker enzymes.

Determination of biochemical parameters: The biochemical parameters were assayed according to the methods described by Klein and Kaufman (1967) for ALP, total protein by Lowry *et al*. (1951), Habig et al. (1974) for GST, and Voss and Sachsse (1970) for AChE.

Histopathological examination: Liver samples were cleared from adhering tissues, fixed in 10% formal saline, dehydrated through ascending grades of alcohol (50%, 70%, 80%, 90%, and 100%) - and cleared in xylene. The wax-infiltrated tissues were embedded in paraffin wax and 5μm thick sections were prepared from the tissues using a LEICA rotary microtome. These sections were floated in a water bath (45 ^{o}C) to allow spreading of the folded sections and mounted on glass slides Dewaxed slide sections were then rehydrated in descending grades of alcohol (100%, 90%, 80%,70%, and 50%) and stained with hematoxylin and eosin. Sections were differentiated in a mixture of (1% hydrochloric acid in 70% alcohol) to remove excess dye from the tissues. The stained tissues were observed under the microscope (LEICA DM750) interfaced with a LEICA (ICC50) camera.

Statistical analysis: The data obtained were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test using the software Graph pad Prism 5. The statistical significance was set at $p < 0.05$. Values were expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The acute toxicity test showed that the extract is nontoxic at the highest dose (5000 mg/kg) tested. No toxic symptoms or mortalities were observed in the two phases of the acute toxicity test (LD_{50} > 5000 mg/kg).

Biochemical parameters: The biochemical parameters of plasma, liver, and brain homogenates of Wistar rats administered with different concentrations of *B. Sapida* extract and Rambo are shown in Table 1. A significant decrease in AChE activity in the experimental animal that received a high dose (150 mg/kg) of the extract was detected. Also, there was a significant decrease in plasma GST activity at 100 and 150 mg/kg extract. The decrease in these enzymes is similar to Rambo which was significant when compared to the control group. There was no significant difference in the activity of alkaline phosphatase in the test groups as compared to the control group. A dose-dependent increase in protein concentration was observed.

Histopathological examination: Histopathological investigation of liver sections in control rats showed normal cellular integrity and normal lobular architecture with central veins and radiating cords of hepatocytes, separated by blood sinusoids of the liver. However, no marked lesion or congestion was observed in the groups treated with 50, 100, and 150 mg/kg of *B. Sapida* extract. A mild lesion and portal congestion were observed in the group that was administered 10% (w/v) Rambo pesticide (Plate 1).

In this study, no death was recorded during the acute and sub-acute toxicity tests with graded doses of ethanol extract of *B. Sapida.* No symptom of toxicity was noticed and no significant changes in appearances were observed when the treated groups were compared with the control group. The results of this study showed that *B. Sapida* decreased the activity of cholinergic and antioxidant enzymes in Wistar rats administered with high doses of the extract in a similar pattern as a synthetic pesticide (Rambo). According to Malhat *et al*. (2018), this effect is typical of botanicals that interrupt the proper functioning of the insect nervous system, causing uncontrollable excitation, tremor, and, death as a result of an accumulation of acetylcholine. Accumulation of acetylcholine at neuron synapses and neuromuscular junctions has been reported to cause effects such as paralysis and muscle weakness (Yılmaz *et al*., 2016). Glutathione S-transferases (GST) is the main enzyme that catalyzes the conversion of foreign chemicals to hydrophilic, less toxic, and easily excreted materials from the system (Lee *et al*., 2015; Dobritzsch *et al*., 2020). A low dose (50 mg/kg) of the extract exhibited no significant reduction in GST activities, however, higher doses of 100 mg/kg and 150 mg/kg caused a significant reduction in the activity of GST.

The findings of this study are similar to those of Oni *et al*. (2019) in which the administration of *Acalypha wilkesiana* extract which reduced GST killed *Callosobruchus maculatus.* Similarly, studies by Ebadollahi *et al*. (2013) and Tarigan *et al*. (2016) have also reported the pesticidal activity of different botanical extracts that reduced GST activity.

The observed effects of the *B. Sapida* extract on the activities of AChE and GST indicate that the metabolites in the extract may serve as an effective botanical pesticide.

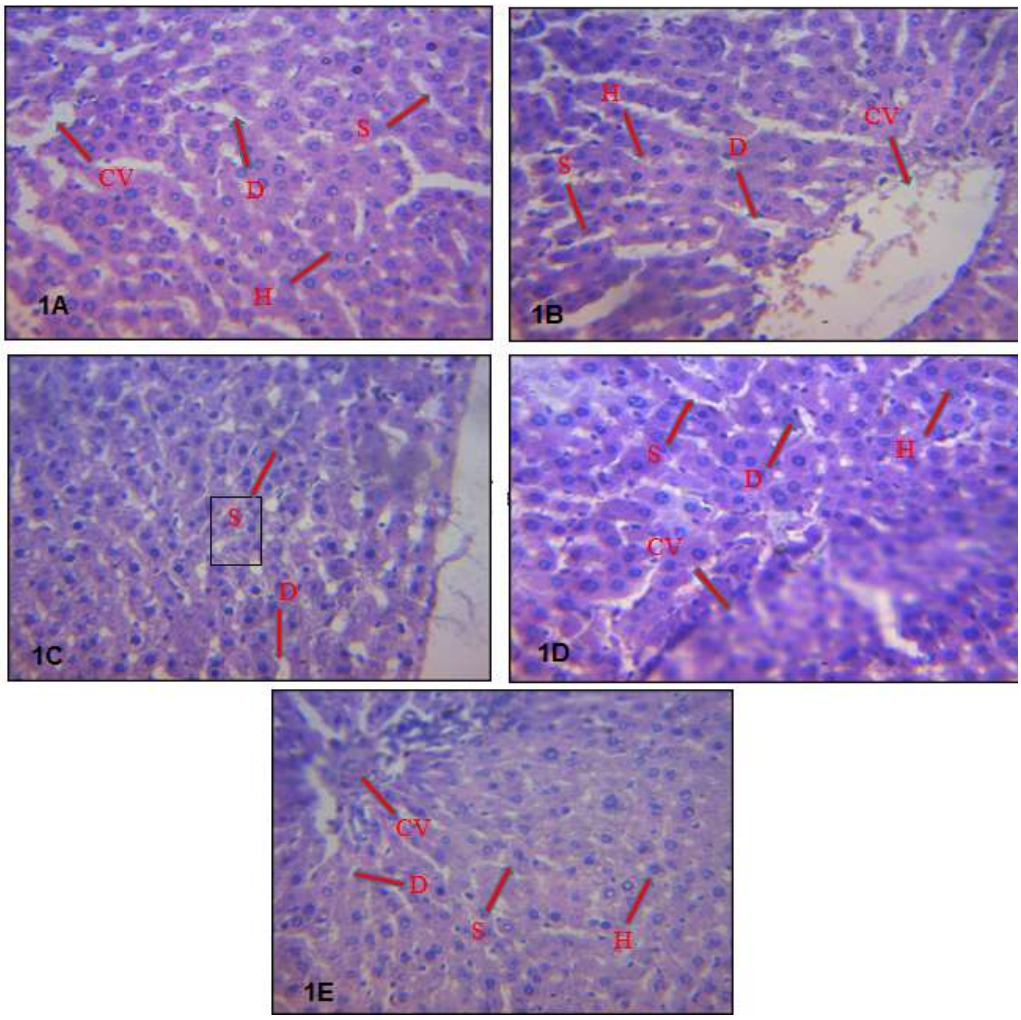

Plate 1: Photomicrographs of liver sections from control, 10% Rambo and *B. Sapida* treated rats.
NOTE: Central veins (CV), Hepatocytes (H), Sinusoids (S), Bile duct (D) and Magnification (x300

Plate 1A: Micrograph of the liver of rats in control group showing no lesion (x 300): Plate 1B: Micrograph of the liver of rats treated with 10% synthetic Rambo showing mild portal congestion (x300); Plate 1C: Micrograph of the liver of rats treated with 50mg/kg of BSE showing no lesion (x300): Plate 1D: Micrograph of the liver of rats treated with 100mg/kg of BSE showing no lesion (x 300): Plate 1E: Micrograph of the liver of rats treated with 150mg/kg of BSE showing no lesion (x300)

Table 1: Effect of Hydro-alcohol Extract of *Blighia sapida* on Biochemical Parameters in Rats

Groups	Control	Synthetic	Extract	Extract	Extract
Parameters	0 mg/kg	10 % (w/v)	50 mg/kg	100 mg/kg	150 mg/kg
AChE (U/L)	240.14 ± 6.18*	175.67 ± 30.42***	226.85 ± 2.46*	224.43 ± 4.47*	210.16 ± 2.03**
GST Liver (µmol/mg)	256.48 ± 12.73*	134.37 ± 7.42***	211.57 ± 7.06*	209.61 ± 20.09**	196.64 ± 8.44**
ALP (U/L)	0.022 ± 0.003	0.024 ± 0.008	0.017 ±0.001	0.019 ± 0.001	0.022 ± 0.003
AST (U/L)	290.87 ± 5.83	302.94 ± 10.49	300.02 ± 11.48	302.37 ± 5.87*	308.19 ± 13.54*
ALT (U/L)	8.79 ± 1.09	19.07 ± 0.97**	9.46 ± 1.50	17.47 ± 1.30*	19.13 ± 0.84**
TP Liver (g/L)	126.26 ± 0.75	135.15 ± 6.58	103.79 ± 0.62**	117.42 ± 7.17*	125.03 ± 6.97

Values are presented as Mean ± SEM of seven (6) replicates. Values with (*) are significantly different at $p < 0.05$ when compared to the control group (group 1); AChE: Acetylcholinesterase, ALP: Alkaline phosphatase; GST: Glutathione S-transferases, TP: Total protein, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase.

Alkaline phosphatase is a membrane-bound enzyme and it plays a critical role in the transportation of metabolites across the cell membrane, conditions such as liver damage and bone disease lead to its alteration which affects the transport of metabolites and membrane permeability (Simon *et al.*, 2019). Also, alkaline phosphatase has been referred to as a family of zinc metalloenzymes, and an increase in its activity is an indication of biliary process obstruction (Shirode *et al.*, 2019). The ALP activity of the rats treated with

different doses of ethanol extract of *B. Sapida* exhibited no significant difference at ($p > 0.05$) when compared to the control rats which indicates that the extract may not cause cellular leakage of the enzyme and obstruction of normal biliary process. The results of ALP in this study implied that phytoconstituents in the plant extract were neither toxic nor caused damage to the membrane at the doses considered, hence transportation of metabolite remains intact.

This result was also confirmed by histopathological examination of the liver Plate 1, in which the micrograph of the liver of rats treated with different doses of the plant extract revealed no lesion. Plasma alkaline phosphatase is one of the liver biomarker enzymes, whose evaluation provides details of pathological damage to the tissue (Mu'azu *et al*., 2020). A high level of plasma ALP activity has been linked with hepatobiliary dysfunction which is likely a result of hepatobiliary injury and cholestasis (Ore and Olayinka, 2015). The nonsignificant value of ALP activities observed in this study implied that the doses of the extract considered may not affect the hepatobiliary function of the liver.

The assessment of the activities of enzymes such as ALT and AST provides information on liver function. The ethanol extract of *B. sapida* stem-bark displayed a dose-dependent increase in the activities of ALT as well as AST in the rats. The results of this study indicated that administration of the plant extract as biopesticide may not alter the functions of the liver as displayed by liver marker enzymes. Higher levels of these liver enzymes indicating hepatotoxicity have been reported by different authors (Ogunleye *et al*., 2020). The liver is mainly involved in the synthesis of plasma protein, damage to which causes a reduction in the protein concentration (Adeoti *et al*., 2017). A dose-dependent increase in protein concentration was observed in the groups that were administered with different doses of ethanol extract of *B. Sapida* which implies that the extract is not toxic at the doses considered because a decrease in the level of total protein has been associated with damage to the liver function caused by toxicity (Simon *et al.,* 2019). The results of this study imply that bioactive compounds present in the study plant may cause a reduction in the activities of enzymes involved in the metabolism of foreign substances (pesticides) thereby leading to consequences such as tremor, reduction in the rate of survival, and death. The bioactive compounds in the plant may also serve as growth retardants, cause a reduction in the rate of reproduction, and loss of weight in the larva, pupa, and adult (Kaur *et al*., 2016).

The toxic effects of natural products on animals and humans can be evaluated by using physiological parameters such as but not limited to histological examination (Kasthuri and Ramesh, 2018). The histopathological examination of the liver in control rats showed normal cellular integrity and normal lobular architecture with central veins (CV) and radiating cords of hepatocytes (H), separated by blood sinusoids (S) of the liver. However, no marked lesion or congestion was observed in the groups treated with 50, 100, and 150 mg/kg of *B. Sapida* extract. A mild lesion and portal congestion were observed in the group that was administered with 10% (w/v) Rambo pesticide. This implies that the plant extract could be used on food materials to prevent insect infestation, without any toxic effect on the consumers of the treated food. However, Adekola *et al*. (2020) reported distinct hepatocytes, clear central veins, and mildly congested sinusoids in rats administered with 250 mg/kg ethanol extract of *B. Sapida*.

Conclusion: Natural products that can reduce cholinergic and metabolizing enzymes serve as safer alternatives for pest control. The hydro-ethanol extract of *B. sapida* significantly reduced the activities of AChE and GST which are involved in neurotransmission and detoxification, respectively. This is suggestive of *B. Sapida* as having neurodegenerative ability peculiar to pesticide agents. Hence, these properties of *B. sapida* could be harnessed in the development of pest agents as an alternative for the mitigation of pests, not only this but also prevent environmental contamination by synthetic pesticides.

Acknowledgments: The assistance rendered by Prof. O. O. Babalola and Dr. J. I. Olawuni, Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, Nigeria during this research is highly appreciated.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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