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Evaluation of neurotoxicity and hepatotoxicity effects of acute and sub-acute oral administration of unripe ackee (*Blighia sapida*) fruit extract

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ABSTRACT

Ackee (*Blighia sapida*) is a commonly eaten fruit that is indigenous to West Africa and Jamaica. Ackee poisoning in young children have been reported in parts of Nigeria due to consumption of the unripe fruits. This study was designed to identify potential mechanisms of acute and sub-acute toxicity of unripe *B. sapida* fruit extract (BSE).

Acute toxic effect was investigated in mice of either sex administered BSE 2000 mg/kg. The sub-acute toxicity effects were investigated in mice of either sex that received 28 days repeated administration of BSE (100 and 500 mg/kg, p.o.). Locomotor activity and memory performance were measured as well as seizure vulnerability in PTZ-induced model. Liver enzymes were assessed in the serum. Acetylcholinesterase, oxidative stress parameters and histopathological changes were assessed in the brain and liver tissues.

Signs and symptoms of toxicity such as urination, tremor, depressed locomotion and death were observed in acute toxicity test. Sub-acute dosing caused significant impairment in locomotor activity and memory performance in mice. Seizure threshold was shortened in BSE-treated compared to control mice. Brain acetylcholinesterase activity was significantly increased. Alkaline phosphatase (ALP) was significantly elevated in mice that received BSE (500 mg/kg). Furthermore, BSE caused significant increase in oxidative stress expressed in nitrite, malondialdehyde, reduced glutathione and catalase in the brain and liver tissues. Histological staining revealed neuronal damage of brain hippocampus and hepatocellular swelling and necrosis.

Blighia sapida unripe fruit extract increased susceptibility to seizure and impaired locomotor and memory function. The biochemical and histopathological findings revealed potential toxicity mechanisms related to neurotoxicity and hepatotoxicity.

1. Introduction

Blighia sapida is a tree bearing the ackee fruit, which is indigenous to West Africa. The fruit is known as ackee apple fruit in English, a national fruit in Jamaica [1,2]. The tree belongs to the family sapindaceae, the fruits consist of the pod, the pulp and the aril covering the seeds. The fruit arils only become edible when the matured fruit opens up spontaneously to reveal the seeds and the fleshy aril [3].

Blighia sapida is considered as nature's gifts because of the potential in using different parts of the tree as woods for furniture, fruits for food and medicines, seeds for oil and in soap making [4]. The fruit has been utilized purposely for its food purpose since the arils can be eaten as vegetable either raw or roasted. The edible arils are rich source of protein, fat and vitamins A, B1, B2 and C [1]. The matured fruit is considered a staple food prepared by cooking or roasting among the poor population in West Africa or canned food in Jamaica [5,6].

Clinical and chemical studies carried out on unripe ackee reveals that it contains a toxic substance known as hypoglycin A (HGA) [6,7]. HGA concentration in the unripe fruit compared to the ripe ackee fruit is fold higher [8]. It is believed that the toxic substance is dispelled by light as the jacket of the ripped fruit opens [9]. HGA is thought to be translocated to the seeds and converted to the dipeptide hypoglycin B during the fruit maturation [7]. Hypoglycin B being found mainly in the seed is known to possess less potent hypoglycaemic activity as compared to hypoglycin A [10]. The mechanism of ackee poisoning has been closely associated with hypoglycaemia [5]. This toxic component in the immature fruit is shown to exhibit toxic mechanism by inhibition of the enzyme in fatty acid β-oxidation, flavoprotein acyl-CoA

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dehydrogenase as well as other acyl-CoA dehydrogenases (the short-chain acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase and isovaleryl-CoA dehydrogenase [11].

Carboxycyclopropyglycine (CPG) is another central nervous system (CNS) active agent isolated from the unripe ackee fruit arils [12]. CPG are selective agonists at the group II metabotrophic glutamate receptor (mGluR2) considered to be critical in several of the unexplained neurological disorders that characterized ackee poisoning. Activation of group II (mGluR2 and 3) reduces the concentration of cyclic adenosine monophosphate (cAMP) by inhibiting the activity of adenylyl cyclase (AC) [13]. Studies have also shown that stimulation of mGluR2 and -3 activates microglial cells, and release of tumor necrosis factor-alpha (TNFα), and Fas ligand, resulting in depolarization of the mitochondrial and neuronal apoptosis [14,15].

Ackee constitute an affordable dietary element among poor populations suffering from undernutrition. Natural disasters or internal displacement due to war have consequences on local food productions, hence internally displaced depends on wild fruits for survival. Lethal epidemic encephalopathy associated with ingestion of unripe ackee fruit has been severally reported in West Africa and the Caribbean [16,17]. The symptoms of ackee poisoning in young children of lower socio-economic groups have been reported to include severe vomiting, followed by seizure and coma [18]. The toxicity is dose dependent occurring within the first 6–48 h post ingestion and a gradual recovery within 1 week [3]. Reported cases of poisoning broadly referred to as "ackee poisoning" numbering about 500 since 1976 have been documented from the West Africa and the Caribbean countries [16,17,19,20].

Recently in Nigeria, there was a clinical case report on acute ackee poisoning among eight siblings in Kwara-State, North central Nigeria. The Boko haram war in the North eastern part of Nigeria and around Cameroun borders have caused internal displacement and food scarcity. Displaced children feeds on any available wild fruits and there could be more cases of ackee poisonings [21]. Foungbe et al. [22] stated that the toxicity of hypoglycin is enhanced when starving children are exposed to daily ingestion of infratoxic doses, producing a cumulative and delayed hypoglycemic effect that can be lethal. Even the ripe arillus, that contain less hypoglycin A, could also produce cumulative and delayed effect on starving children. Given the accessibility and affordability factor among the less privileged populations, there is need to provide further evidence on the toxicity profile of ackee fruits as a step in raising awareness of the potential toxicity of ackee fruits. There are a number of studies that have investigated the mechanism of toxicity of hypoglycin, several key areas of uncertainty remain with the extract of ackee [6]. Therefore, this study investigated the neurotoxicity and hepatotoxicity effects of oral administration of unripe *Blighia sapida* fruit extract in mice.

2. Materials and methods

2.1. Chemicals

Ellman's Reagent [5^1, 5^1 -Dithios-nitrobenzoic acid (DTNB)], acetylthiocholine, thiobarbituric acid (TBA) were obtained from Sigma Chemicals Co., (USA). All other reagents such as petroleum ether and methanol were analytical grade.

2.2. Experimental animals

Swiss mice of either sex weighing 18–20 g were obtained from the central animal house were housed in polypropylene plastic cages in the Department of Pharmacology And Therapeutics. The experimental animal facility was well ventilated space with temperature of 28 ± 2 °C, and subjected to natural photoperiod of 12:12 light: dark cycle. The animals were acclimatized for one week before the commencement of the experiment. All animals were allowed free access to water and fed with standard commercial rat chow pellets (Vital feeds Ltd, Ibadan, Nigeria). The experimental protocol followed were in compliance with the "Principle of Laboratory Animal Care" (NIH Publication No. 85-23) [23].

2.3. Collection of fruits and preparation of extract

The unripe fruit of *Blighia sapida* were collected from the University of Ibadan premises in the month of May. The fruit was identified and authenticated at Forestry Research Institute of Nigeria (FRIN), Jericho Ibadan. Herbarium voucher number FHI-111210 was issued. The pods of unripe fruit were cracked opened and the aril separated from the seed. The arils were then oven dried at 55 °C to a constant weight for seven days. The method of extraction was a slight modification to the methods earlier described by Foungbe et al. [22] and Bareness et al. [9]. Briefly, the dried aril were pulverized and 200 g of the pulverized aril was deffated by sohxlet apparatus using petroleum ether. The fat free macerate was extracted by maceration at room temperature for 48 h in methanol (80%). The macerate was filtered first with a white cotton muslin cloth and doubly filtered with a Whattman filter paper. The filtrate was evaporated in a rotary evaporator at 40 °C under reduced pressure. The concentrated extract was dried completely in a vacuum oven at 50 °C. The resultant yield was a semi-solid brownish color extract with a percentage yield was calculated as 12.32%. The extract which was denoted as *Blighia sapida* extract (BSE) was kept in a dark amber bottle wrapped with dark sheet to prevent it from light and kept in the refrigerator at 4 °C till needed for acute and sub-acute animal studies.

2.4. Acute toxicity test

Male and female Swiss mice weighing 18–20 g were used to test for the acute toxicity effect of BSE according to the method described by the Organization of Economic Cooperation and Development 423 guidelines (OECD) [24]. The animals were divided into two groups (n = 3) for the male and female mice. Three males and three females mice were administered 2000 mg/kg of extract while three male and three female mice were administered with vehicle (normal saline). The animals were individually monitored for signs of acute toxicity within the 0–4 h and then every day for 14 days for delayed toxicity signs. Thereafter, the animals were sacrificed on the 14th day and the organs were observed macroscopically for any changes.

2.5. Experimental design and administration of extract for sub-acute toxicity test

Sixty (60) Swiss mice (30 male mice and 30 female mice) were randomly divided into six groups of ten animals per group and received treatment with *Blighia sapida* extract (BSE). Groups 1–3 which were male mice received vehicle (10 mL/kg), BSE (100 mg/kg), BSE (500 mg/kg), while the female mice in groups 4–6 received vehicle (10 mL/kg), BSE (100 mg/kg) and BSE (500 mg/kg). The extract and vehicle (normal saline) were administered by oral gavage daily for 28 days. The effect of treatment on body weights were monitored every 3 days, and volume administered adjusted accordingly. The effect on locomotor activity, memory performance and seizure vulnerability were assessed 24 h after the last treatment by trained observers blinded to the treatment.

2.5.1. Assessment of BSE sub-acute dosing on mice locomotor activity

Twenty-four hours after the last administration of BSE, spontaneous motor activity was monitored in an Ugo Basile activity cage. Mice (n = 5) per groups were randomly selected from their home cage and individually placed for 5 min in a novel environment in the activity cage. The movement the animals made in the activity cage was recorded as horizontal beam breaks counts and vertical beam breaks

count for spontaneous locomotor activity and rearing movements, respectively.

2.5.2. Assessment of BSE sub-acute dosing on mice memory performance

The Y-maze test was used to assess memory performance in mice repeatedly treated with BSE for 28 days. The Y-maze consist of three equally spaced arms (120°, 41 cm long ×15 cm high), spontaneous alternation in this arms can be used as a measure of spatial working memory. The floors of each arm (A, B and C) consist of wood (5 cm wide). A mouse movement into all three arms (i.e., ABC, BCA, CAB but not ABA) defines a correct alternation. The mouse is placed initially in arm A and allowed free movements for a 5 min duration. The percentage alternation is expressed as the ratio of actual alternations to possible alternations (defined as the total number of arm entries minus two) multiplied by 100 [25].

2.5.3. Assessment of seizure vulnerability following repeated administration of BSE

Following uninterrupted daily oral administrations of unripe *Blighia sapida* extract for 28 days, mice (n = 5/group) were intraperitoneally injected with a bolus dose of pentylenetetrazole (PTZ) intraperitoneally (40 mg/kg). In a study by Alele and Rujumba [26], 40 mg/kg dose of PTZ was shown to reliably induce seizures without causing death in most of the animals. The onset time to first sign of seizure (seizure latency), were recorded for each animal.

2.5.4. Preparation of serum, brain and liver tissues for biochemical studies

Following the completion of the behavioral function test, animals were deeply anaesthetized by ether and blood was collected through the retroorbital vein into plain tubes. The blood was centrifuged in a benchtop centrifuge at 3000 rpm for 15 min to obtain the serum. The organs (brain and liver) were removed, wiped clean and kept in cold container. A piece of the liver and the whole brain were homogenized in sodium phosphate buffer (0.1 M, pH 7.4), centrifuged for 10 min at 10,000 rpm at 4 °C, and the supernatants were separated into aliquots. The brain and liver supernatants were used the determination of brain acetylcholinesterase (AChE), and oxidative stress biomarkers namely, reduced glutathione (GSH), malondialdehyde (MDA) and nitrite.

2.6. Determination of serum liver enzymes

The liver enzymes, serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were determined with Randox diagnostic test kits (County Antrim, BT29 4QY, UK) following manufacturer's instruction.

2.7. Determination of brain acetylcholinesterase enzyme activity

Acetylcholinesterase enzyme as a brain biomarker of cholinergic function and memory was assessed by the Ellman's method [27]. Briefly, 100 µLs of brain supernatant was diluted 10 times in phosphate buffer saline (0.1 M, pH 7.4) followed by addition 50 microliters of DTNB (0.01 M). The reaction mixture was incubated for 5 min and baseline absorbance measured at 412 nm in a cuvette. Then, 25 µL of acetylthiocholine iodide (0.028 M) was added for the reaction to proceed for 3 min, and final absorbance was measured again. The change in absorbance per minute was determined and using the molar extinction coefficient (1.36×10^4), the rate of AChE activity was calculated and expressed as µmol/min/ mg protein.

2.8. Determination of brain and liver oxidative parameters

2.8.1. Lipid peroxidation level

Tissue malondialdehyde was measured as a marker of lipid peroxidation using the assay method of measuring thiobarbituric reacting substances (TBARs) by Nagababu et al., [28]. A twenty-fold dilution of supernatant (100 µL) in 0.15 M Tris-KCl buffer was mixed with 30% trichloroacetic acid (500 µL) and 0.75% TBA (500 µL). The mixtures were heated at 80 °C for 1 h and extracted with 1 mL butanol. The organic phase was separated by centrifugation for 5 min at 3000 *g* and measured at 532 nm. The amount of MDA formed was calculated using the molar extinction coefficient 1.56×10^5/M/cm. The concentration of TBARS in the brain and liver tissues were expressed as nmol MDA/ mg protein.

2.8.2. Reduced glutathione level

Reduced glutathione as non-enzymic antioxidant were measured in brain and liver supernatants as described by Jollow et al., [29]. Briefly, ten-fold dilution of supernatant (100 µL) was deproteninzed with 1000 µL Trichloroacetic acid (20%) and then centrifuged at 10,000 rpm at 4 °C for 10 min. 250 µL deproteinized supernatants were mixed with 750 µL sodium phosphate buffer (0.1 M, pH 7.4) and 1000 µL of 5^1, 5^1 -Dithios-nitrobenzoic acid (DTNB, 0.0006 M). The absorbance was read after 5 min at 412 nm in a UV/Vis Spectrophotometer (725 N INESA, China). The glutathione concentration was extrapolated from standard curve of glutathione (0–200 µM) and expressed as a µM GSH/mg protein.

2.8.3. Determination of nitrite

The accumulation of nitrite in the brain and liver supernatant was measured as an indicator of the production of nitric oxide using the Griess reagent colorimetric assay method [30]. Griess reagent was freshly prepared by mixing equal volumes of 0.1% N-(1-napththyl) ethylene diamine dihydrochloride and 1% sulphanilamide (in 5% phosphoric acid). Equal volumes of the diluted tissue supernatant and Greiss reagent were mixed and incubated for 10 min at room temperature in the dark. The absorbance was read using a UV/Vis Spectrophotometer at 540 nm. The concentration of nitrite in the brain and liver supernatants were determined from sodium nitrite standard curve and expressed as µM nitrite/mg protein.

2.8.4. Catalase enzyme assay

Catalase activity in brain and liver tissue was determined using the colorimetric assay based on the yellow complex with molybdate and H_2O_2, which was described by Goth [31]. Briefly, 100 µL of supernatant was diluted 10 times in a reaction mixture containing 65 mmol/mL of H_2O_2 in sodium-potassium phosphate buffer (60 mM, pH 7.4). The enzymatic reaction was incubated for 3 min and stopped with 2 mL ammonium molybdate (64.8 mM). The absorbance at 405 nm was measured in a in a UV/Vis Spectrophotometer. The catalase enzyme activity unit was expressed kU/ mg protein.

2.9. Histopathological staining and light microscopy of brain and liver tissue

A brain and liver tissues were separately fixed in 10% buffered formalin, the fixed tissues were dissected longitudinally, placed in embedding cassettes, embedded in paraffin, and then cut into 4 µm section. Sectioned tissues placed on slides were stained using hematoxylin and eosin (H & E) and observed under light microscope. Histological photomicrographs were captured using Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a Leica ICC50 E Digital Camera (Germany) and a computer interface (MagnaFire)

2.10. Data presentation and analysis

All data were recorded and presented as mean ± standard error of mean (SEM). Statistical significance was set at $p < 0.05$. Data from the acute toxicity were analysed using the student *t*-test to compare means. Behavioral and biochemical data were analyzed using one-way analysis of variance (ANOVA), significant differences were further analyzed by Newman-Keuls *post hoc* test (multiple comparison) with GraphPad

Table 1
Effect of single acute oral administration of *Blighia sapida fruit extract* (2000mg/kg) on mice.

Treatment	Acute toxicity			
	Behavioral alterations and symptoms of toxicity	Dead mice/total mice	% Mortality	Locomotor activity count (5 min)
Male				
Control	None	None	0	595.0 ± 38.1
2000 mg/kg	Urination, diarrhoea, hypomotility, convulsion, death	1	33.3	274.0 ± 39.0*
Female				
Control	None	None	0	456.0 ± 3.6
2000 mg/kg	Urination, diarrhoea, hypermotility	None	0	551.0 ± 50.3

* $p < 0.05$ vs control male using student's *t*-test.

Prism® software version 5.01 (GraphPad Software, Inc. La Jolla, CA 92037 USA).

3. Results

3.1. Acute toxicity test

The extract of unripe *Blighia sapida* fruit showed symptoms of acute toxicity in both the males and females as shown in Table 1. There were behavioural alterations and a significant reduction in spontaneous locomotor activity count in the male mice. The extract at a single oral dose of 2000 mg/kg showed a percent mortality of 33.3% in male mice but no mortality was recorded in the female mice.

3.2. Sub-acute toxicity test of unripe Blighia sapida fruit extract (BSE)

3.2.1. Blighia sapida fruit extract (BSE) affect body weights

The repeated administration of unripe fruit extract of *Blighia sapida* at 100 and 500 mg/kg for 28 days caused a significant decrease in body weights of male and female mice (Fig. 1A). The area under the curve for the percentage change in body weight (Fig. 1B) showed a significant ($p < 0.05$) difference in BSE 100 and 500 mg/kg (20.21% and 31.22%) compared to control (58.07%) in male mice. Similarly, in the female mice, there was a significant difference in percentage change in body weight in BSE 100 and 500 mg/kg (7.01% and 30.43%) compared to control (67.33%).

3.2.2. Blighia sapida fruit extract (BSE) induced hypolocomotion in mice

The locomotory activity 24 h after the last treatment was analysed and presented in Fig. 2. Repeated oral administration of BSE caused significant reduction in spontaneous locomotor activity in male ($F(2, 14) = 4.279$, $p = 0.0396$) and female ($F(2, 14) = 12.09$, $p = 0.0013$) mice. Similarly, BSE (100 and 500 mg/kg) caused significant reduction in rearing (vertical beam break) as shown in Fig. 2B in treated male mice ($F(2, 14) = 30.80$, $p < 0.0001$) and treated female mice ($F(2, 14) = 54.33$, $p < 0.0001$).

3.2.3. Blighia sapida fruit extract (BSE) caused memory impairment in mice

Cognitive memory effect of repeated administration of extract of unripe ackee (*Blighia sapida* extract) fruit in male and female mice were assessed in the Y-maze. As shown in Fig. 3, percentage correct alternation were significantly decreased in male ($F(2, 17) = 6.193$, $p = 0.0109$) and female ($F(2, 17) = 6.853$, $p = 0.0077$) mice administered BSE (100 and 500 mg/kg) relative to their controls. *Post hoc* tests showed statistical significant reduction in correct alternations in male mice administered 500 mg/kg BSE and female mice administered 100 and 500 mg/kg BSE.

3.2.4. Blighia sapida fruit extract (BSE) lowers seizure latency in PTZ-induced seizures

Seizure vulnerability was assessed in male and female mice repeatedly administered with BSE (100 and 500 mg/kg) using the PTZ-induced model. BSE (100 and 500 mg/kg) significantly lowered the time to onset of convulsion in male ($F(2, 11) = 23.81$, $p = 0.0003$) and female mice ($F(2, 11) = 174.8$, $p < 0.0001$). *Post hoc* tests showed statistical significant reduction in the seizure latency in both male and female mice administered 100 and 500 mg/kg BSE compared to the control groups, respectively (Fig. 4).

3.3. Effect of Blighia sapida fruit extract (BSE) liver weight and liver serum liver enzymes

As shown in Table 2 below, there was a significant increase in the relative liver weight of female mice sub-acutely treated with BSE (500 mg/kg). Results of the serum liver enzymes assays showed no significant increase in the levels of AST and ALT in both male and female treated mice (Table 2). However, there was a significant ($p < 0.05$) elevation in the ALP levels in both male and female mice sub-acutely administered 500 mg/kg BSE when compared to control groups.

3.4. Effect of Blighia sapida fruit extract (BSE) brain acetylcholinesterase enzyme activity

The effect of sub-acute oral administration of BSE (100 and 500 mg/kg) in mice revealed significant increase ($p < 0.05$) in brain acetylcholinesterase enzyme activity in both male and female mice when compared to the control mice (Fig. 5). In the male mice, acetylcholinesterase enzyme activity increase dose dependently by 2 fold and 3 fold in mice administered 100 and 500 mg/kg BSE, respectively.

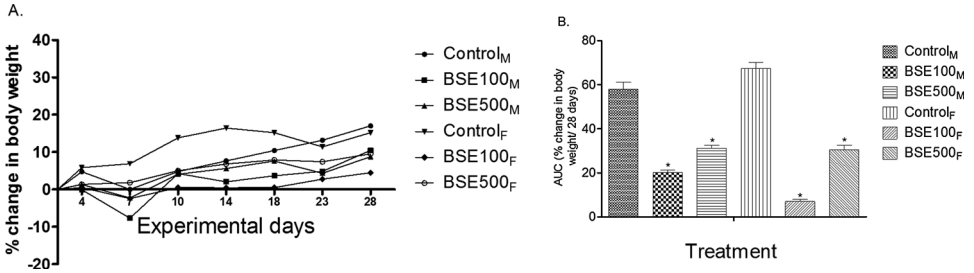

Fig. 1. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) on body weight (A) Percent change in body weight, (B) Area under the curve (% change in body weight/ 28 days). Values represents Mean ± SEM (n = 10). *$p < 0.05$, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

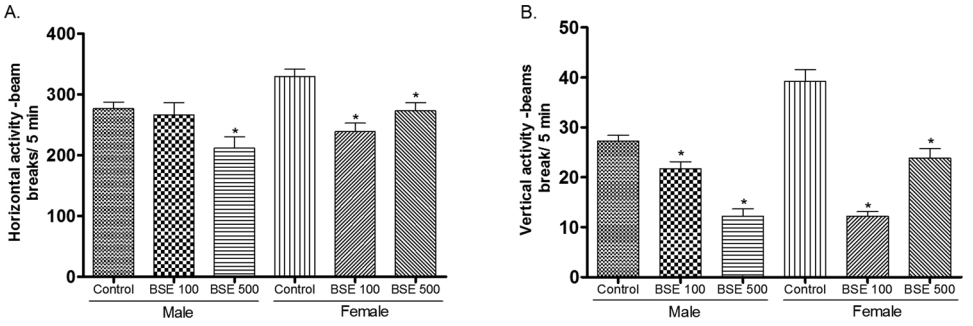

Fig. 2. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) locomotor activity (A) Horizontal activity count, (B) Vertical activity count. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

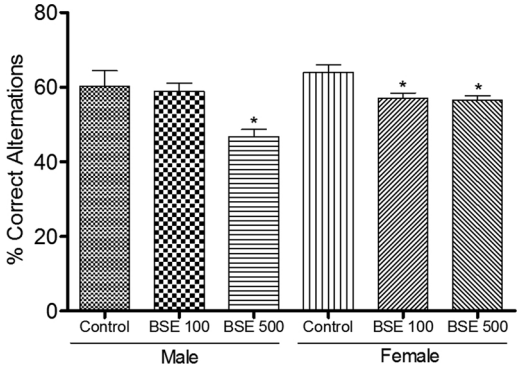

Fig. 3. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) memory performance on the Y-maze test. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

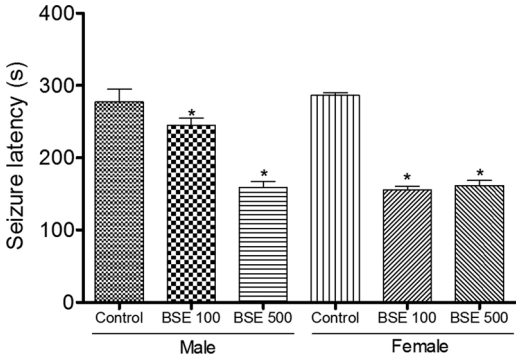

Fig. 4. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) on seizure latency in PTZ-induced model. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

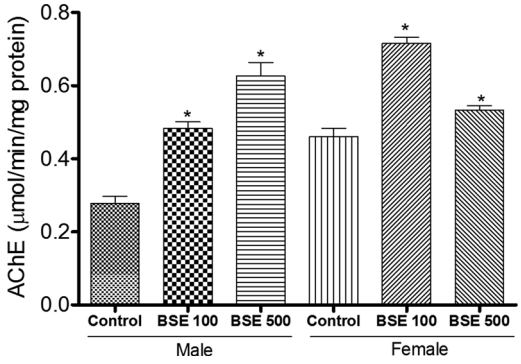

Fig. 5. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) on brain acetylcholinesterase enzyme activities. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

3.5. Effect of Blighia sapida fruit extract (BSE) on brain nitroso-oxidative stress parameters

Sub-acute administration of extract of unripe fruit of *B. sapida* showed a significant ($p < 0.05$) elevation of nitroso-oxidative stress parameters in the male and female mice as shown in Fig. 6. The brain nitrite level was significantly elevated by 31.4% and 37.7% in males and 34.4% and 7% in female mice administered with 100 and 500 mg/kg, respectively (Fig. 6A). Lipid peroxidation in brain tissue was measured by the amount of TBARs formed. Brain MDA level (Fig. 6B) was significantly increased by 22.5% and 32.6% in male and 35.9% and 39.4% in female mice repeatedly treated with 100 and 500 mg/kg BSE, respectively. As shown in Fig. 6C, repeated administration of BSE (100 and 500 mg/kg) caused significant depletion of GSH in both male and females' brain by 22.6%, 22.6% and 16.1% and 19.4%, respectively. The brain catalase activity was reduced significantly in male mice administered 500 mg/kg BSE (13.6%, $p < 0.05$), and by 10.6% and 18.8%, respectively in female mice treated with 100 and 500 mg/kg

Table 2
Effect of sub-chronic administration of unripe Blighia sapida fruit extract on liver weight and liver enzymes.

Treatment	Liver Enzymes [a]			Relative liver weight (%)
	AST	ALT	ALP	
Males				
Control	42.7 ± 2.85	31.7 3.33	96.7 ± 0.96	4.08 ± 0.20
BSE (100 mg/kg)	42.0 ± 2.89	30.7 ± 1.76	100.0 ± 2.56	4.39 ± 0.05
BSE (500 mg/kg)	43.3 ± 1.67	32.0 ± 1.00	104.0 ± 1.16*	4.36 ± 0.01
Females				
Control	40.7 ± 1.38	31.7 ± 2.03	102.3 ± 3.71	4.07 ± 0.13
BSE (100 mg/kg)	42.0 ± 2.00	29.7 ± 1.86	104.0 ± 1.16	4.10 ± 0.11
BSE (500 mg/kg)	44.3 ± 0.33	33.3 ± 0.33	± 2.19*	4.66 ± 0.10*

[a] Data represent mean ± SEM (n = 4). * $p < 0.05$ when compared to sex-matched controls using one-analysis of variance followed by Newman-Keuls *post hoc* test.

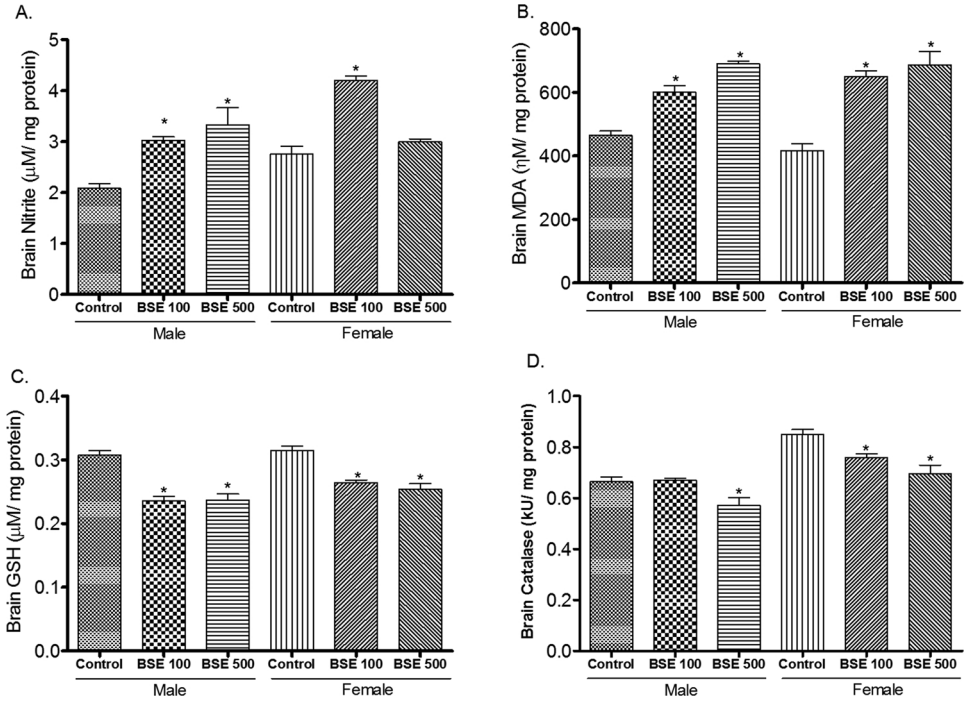

Fig. 6. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) on brain nitroso-oxidative stress parameters. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

BSE (Fig. 6D).

3.6. Effect of Blighia sapida fruit extract (BSE) on liver nitroso-oxidative stress parameters

Nitroso-oxidative stress parameters in the livers of male and female mice sub-acutely treated with BSE (100 and 500 mg/kg) were significantly affected (Fig. 7). The levels of nitrite assayed in the liver supernatants revealed significant ($p < 0.05$) increase by 19.0%, 21.6%, and 19.6, 21.7% in male and female mice, respectively (Fig. 7A). The MDA levels were significantly increased by 39.8% and 40.2% in males and 54.0 and 54.3% in female mice, respectively (Fig. 7B. There was significant depletion of liver glutathione content in males (29.8% and 61.7%) and females (14.3% and 32.1%) administered with 100 and 500 mg/kg BSE (Fig. 7C). As shown in Fig. 7D, catalase enzyme activity in the liver was also significantly reduced in males (27.4% and 38.2%) and female (16.5% and 15.1%) mice that received 100 and 500 mg/kg BSE for 28 days.

3.7. Histolopathological findings

Using H&E staining methods, male and females sub-acutely

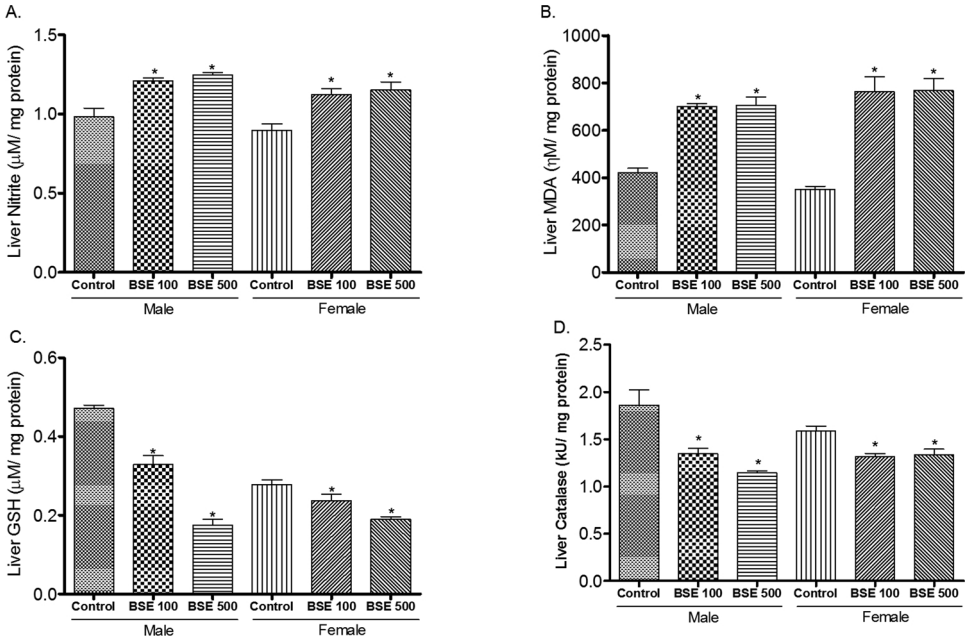

Fig. 7. Effect of sub-acute oral administration of unripe Blighia sapida fruit extract (BSE) on liver nitroso-oxidative stress parameters. Values represents Mean ± SEM (n = 5). *p < 0.05, vs control in either sex, using 1-way ANOVA (Newmann Keuls test). Subscript M denotes male mice; F denotes female mice.

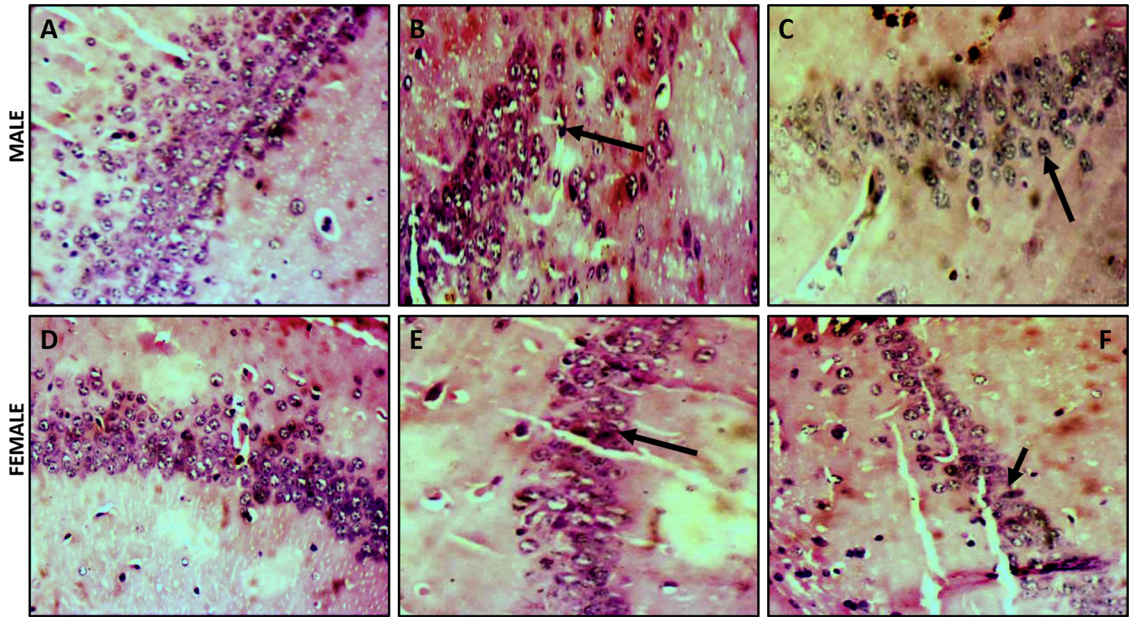

Fig. 8. Photomicrographs of histopathological changes in the brain hippocampal region (Heamatoxylin and Eosin Staining 400x). The cytoarchitecture of H&E of hippocampal (CA2) region revealed pyramidal neurons are normal and in a lamina arrangement in control male and female (A &D). The pyramidal neurons are normal except the disruption of the lamina arrangement in male (B) but moderate atrophy of pyramidal cells and increased eosinophilia of the neurons (necrosis, arrow) in females (E) mice treated with BSE 100 mg/kg. There is moderate atrophy and esinophilia of pyramidal neurons in males (C), and diffuse atrophy of pyramidal neurons in females (F) mice treated with BSE 500 mg/kg. A- Control male, B- BSE (100 mg/kg)-treated male, C- BSE (500 mg/kg)-treated male, D- Control female, E- BSE (100 mg/kg)-treated female, and F- BSE (500 mg/kg)-treated female.

administered with 100 and 500 mg/kg BSE revealed increased deposition of pyknotic cells with darkly stained nuclei (arrow) in the hippocampal region of mice brain. Hippocampal section of CA2 region revealed the pyramidal neurons are normal and in a lamina arrangement in the control animals (Fig. 8A&D). In the female treated BSE (100 mg/kg; Fig. 8E), it revealed moderate atrophy of pyramidal cells and increased eosinophilia of the neurons. As shown in Fig. 8C &F, there is diffuse atrophy of pyramidal neurons (arrows) in the hippocampal region (CA2) of both male and female mice treated with BSE (500 mg/kg).

Liver section stained in H&E revealed that the hepatocytes are normal, arranged in cords and with distinct nuclei with no observable lesion (Fig. 9A&D). In male treated with BSE (100 mg/kg), Fig. 9B revealed there is diffuse hepatocellular swelling, degeneration (arrows) and coagulation necrosis, while BSE (500 mg/kg) showed there is vascular congestion, severe diffuse vacuolar degeneration and necrosis of zone 1 hepatocytes (Fig. 9C). In female mice, there is moderate vascular congestion, diffuse coagulation necrosis of hepatocytes and foci of inflammatory cells (arrows) within the parenchyma (Fig. 9E) and moderate congestion of central vein and vacuolar degeneration of centrilobular to mid-zonal hepatocytes (Fig. 9F).

4. Discussion

Ingestion of unripe ackee (*Blighia sapida*) fruits has continued unabated in West Africa sub-region as a result of food shortages among internally displaced people in troubled regions, and is the cause of several unexplained episodes of deaths among malnourished children. In this study, we report findings on the neurotoxic and hepatotoxic consequences of acute and sub-acute oral administration of the extract of unripe *Blighia sapida* fruit.

The study demonstrated toxicity in both male and female mice given a single dose of 2000 mg/kg BSE. The observed signs of acute toxicity including tremors, urination, and hypomotility were similar to observations reported elsewhere [6]. The acute toxicity findings revealed that the male mice were more susceptible to the extract of unripe ackee. Convulsion and death was observed in 33% of the male mice population that received this extract. In acute toxicity studies, when animals died shortly after an episode of convulsion, it is postulated that the extract might be working by some action on the nervous system [32]. Although, the primary objective of the acute toxicity study of the extract was not to obtain the median lethal dose (LD_{50}), as it has been previously reported as 2.1 g/kg by Barennes et al., [9]. However, the results help in dose selection and design for the sub-acute toxicity study. The acute toxicity helps to predict likely target organ systems and possible outcome when human population are exposed [33].

A significant reduction in body weight both in male and female mice was observed in the sub-acute toxicity study. This finding was consistent with Blake et al., [6] in case of unripe ackee fruit diet fed to rats. Changes in body weights is one of the first critical sign of toxicity [34]. Measures of animal growth using the body weight index is a routine evaluation in toxicological studies as it helps in the interpretation of compound-related effects [35,36]. The unripe fruit of *Blighia sapida* have shown to contain large amounts of Hypoglycin A. HGA is not known to stimulate insulin secretion, but its mechanism involve inhibition of secondary gluconeogenesis, limiting the co-factors (CoA and carnitine) essential to the oxidation of long chain fatty acids thereby causing depletion of glycogen stores [19].

This study reported that locomotor activity and rearing of mice were affected by sub-acute oral administration of BSE. This behavioural deficit was consistent with abnormal motor movements in rats fed with unripe ackee fruit diet rich in hypoglycin A [6]. The open field test which measures motor activity endpoint is one the observational assessments that can be used to characterize impairment of neurological functions. It's a sensitive measure of neuronal function [38]. The hypolocomotion effect in this study showed disruption of the sensory, motor and integrative neural function in the animals. Locomotion is considered an excitatory behavior mediated mainly through dopaminergic pathways and a decrease in locomotor activity in rodent is suggestive of a possible CNS-depressant activity [39]. Unripe ackee has been reported to cause CNS depression in human toxicity case studies [40,17].

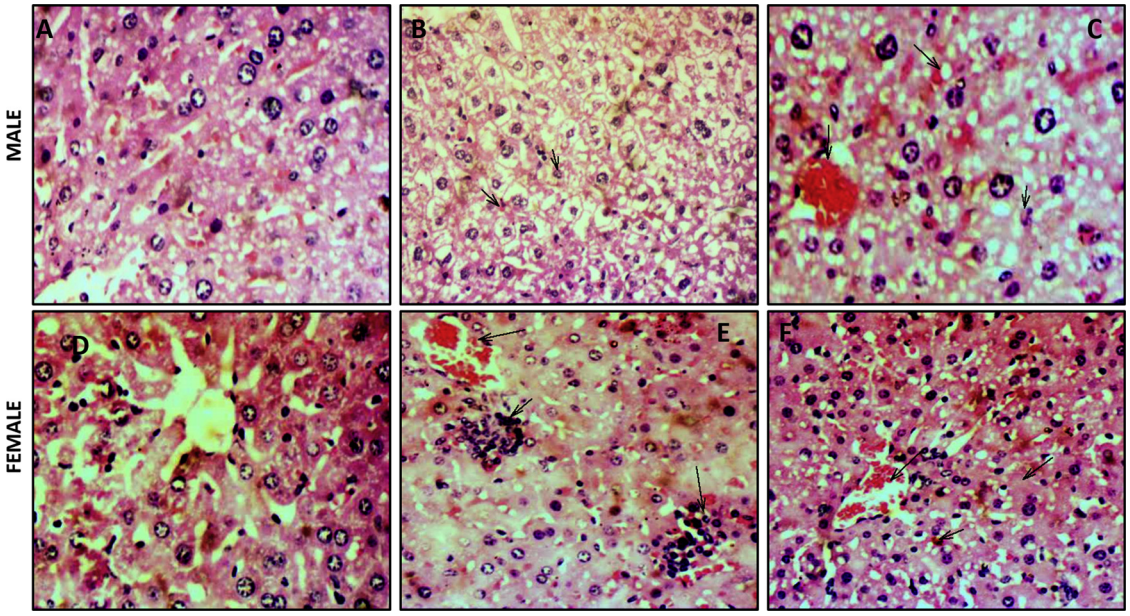

Fig. 9. Photomicrographs of histopathological changes in the liver section (Heamatoxylin and Eosin Staining 400x). (A) Normal hepatocytes (B) There are diffuse hepatocellular swelling, degeneration (arrows) and coagulation necrosis, (C) There is vascular congestion, severe diffuse vacuolar degeneration (arrow) and necrosis of zone 1 hepatocytes. (D) The hepatocytes are normal, arranged in cords and with distinct nuclei (no observable lesion). (E) There is moderate vascular congestion, diffuse coagulation necrosis of hepatocytes and foci of inflammatory cells (arrows) within the parenchyma. (F) There is moderate congestion of central vein (blue arrow) and vacuolar degeneration of centrilobular to mid-zonal hepatocytes (arrows). A- Control male, B- BSE (100 mg/kg)-treated male, C- BSE (500 mg/kg)-treated male, D- Control female, E- BSE (100 mg/kg)-treated female, and F- BSE (500 mg/kg)-treated female.

Sub-acute administration of BSE showed impairment of spatial working memory in male mice as revealed by the Y-maze test. The measure of spatial working memory in the Y-maze test is the number of correct alternations demonstrated by a rodent's ability to recall it former location. The Y-maze model is used to measure short term memory and stereotypic behavior [41]. No mechanistic studies have been designed to find out the molecular targets implicated in the cognitive impairments but modulation of the cholinergic system could be associated with the impaired memory in mice.

Human consumption of unripe ackee fruit is associated with hypoglycin A toxicity characterised by severe vomiting followed by seizures, loss of consciousness, and possibly death [3,16]. The extract shortened seizure latency in both male and female mice after an acute dose of PTZ in this study. Our findings demonstrated that sub-acute BSE modified the seizure reactivity of mice to PTZ. Chemical stimuli that induce seizures may decrease inhibitory or enhance excitatory neuronal activity in the brain [42]. PTZ's convulsive activity is related to its selective antagonism of the receptor of $GABA_A$ chloride ionophore complex. It affects GABAergic and Glutamergic systems in many brain regions including hippocampus [43]. This effect demonstrated by unripe ackee fruit extract suggest it might be acting by a facilitatory action on glutamatergic neurotransmission, leading to enhanced brain excitability and seizures [44]. Earlier reports have indicated the involvement of carboxycyclopropylglycine compounds (glutamate analogs) in the CNS effects during Jamaican vomiting sickness [45].

The liver may be the target of organ toxicity, particularly as it serves as the site of elimination reactions of the hypoglycin A. Hypoglycin is generally considered as liver hepatotoxin [19]. In our study, the aspartate aminotransferase (AST) and alanine aministransferase (ALT) were increased insignificantly, indicating the hepatocyte integrity is not significantly compromised. However, there was significant elevation of alkaline phosphatase enzyme in both male and female mice. Elevated ALP is often a prognostic indicator of liver damage due cholestasis [46]. Alkaline phosphatase (ALP) serves as an enzyme marker for the plasma membrane as well as the endoplasmic reticulum and is present in the cell lining of the biliary duct of the liver [47]. The increase in serum ALP levels in our study may indicate alterations in permeability of the plasma membrane, which may progress to cholestatic diseases such as gall stone [48]. Toxic liver injuries such as Cholestatic jaundice [49], and fulminant hepatic failure [50] have been described after chronic ingestion of ackee fruit.

Our results also showed an increased level of acetylcholinesterase activity in mice brain. Increased level of acetylcholinesterase activity in the cortex and hippocampus has been shown to be directly related to occurrence of seizures in mice [51]. Up-regulation of AChE in rodents contributes to epileptogenesis [52]. In cholinergic regions of the brain such as in the cortex and hippocampus, AChE acts primarily to terminate cholinergic neurotransmission by hydrolysing acetylcholine neurotransmitter implicated in learning and memory [53]. Decreased level of brain acetylcholine has also been linked to memory disturbance [54]. Taken together, the toxicity effect of unripe ackee fruits might be related to modulation of the cholinergic neurotransmission system via up-regulation of the acetylcholinesterase enzyme.

Xenobiotic or drug-induced oxidative stress is implicated as a mechanism of toxicities affecting several tissues and organs, including the liver, kidney, heart and brain. Hypoglycin A, is a water soluble liver toxin that induces hypoglycaemia by inhibiting gluconeogenesis, resulting in hypoglycaemia. Hypoglycaemia stimulates increased production of reactive oxygen species (ROS), resulting in oxidative stress that plays an important role in tissue damage [55]. Our study showed significant changes in the nitro-oxidative stress markers in the brain and liver. Significant increases in tissue nitrite and malondialdehyde and depletion of reduced glutathione and catalase activities were observed in male and female mice. ROS degrade membrane polyunsaturated fatty acids through a multi-step lipid peroxidation processes. Malondialdehyde and 4-hydroxylnonenal (4-HNE) are the major products of lipid peroxidation [56]. Significant elevation of MDA levels in the brain and liver serves as a marker to measure tissue oxidative stress levels. It is well known that reduced glutathione (GSH) is involved in protection of normal cell structure and functions by maintaining the redox homeostasis, quenching free radicals and by participating in detoxification reactions [57]. GSH is a major endogenous

antioxidant which counter balances free radical mediated damage. Reduction of non-enzymatic antioxidant GSH in neuronal and hepatic cells following sub-acute oral administration of unripe fruit extract of *Blighia sapida* could be a consequence of increased utilization for trapping free radicals. Depletion of GSH in the liver correspondingly affects the glutathione system in interrupting lipid peroxidation chain reaction [58,59]. The result from this study thereby establishes that unripe *Blighia sapida* fruit induces oxidative stress. Moreover, the liver rather than the brain appeared to be more prone to oxidative stress unlike the reports of Ritesh et al., [60] wherein the brain was more prone to oxidative damage than the liver. There are evidences suggesting that the production of ROS and oxidative stress are involved in hypoglycemic-induced neuronal damage and cognitive impairment [61,62].

In this study, histological examinations of the liver revealed clear hepatocellular swelling, coagulation, microvesicular steatosis and necrosis in the liver. This is an indication of toxicity induced by sub-acute oral administration of unripe *Blighia sapida* fruit extract. In reported cases of ackee toxicity, in addition to glycogen depletion in the liver, histologic appearance of toxic liver injury similar to that seen in acetaminophen overdose has been documented [63]. Also the microvesicular steatosis seen in hepatocytes of ackee fruit toxicity was linked to hypoglycin A metabolite (methylenecyclopropylacetate) found in the unripe ackee fruit [64]. The liver degeneration and microvesicular steatosis is as result of mitochondrial injury and often occurs as adverse effect of drug/toxins or in Reye syndrome [65,66].

The histologic examination of the brain showed diffuse atrophy of pyramidal neurons in the hippocampal region (CA2). Neuronal cell examinations of the hippocampal region of the brain revealed significant neuronal cell loss. The hippocampal function is considered a major influence in spatial performance as well as alternation learning [67], damage to hippocampus typically produces temporally graded retrograde amnesia [68]. Hippocampal-dependent cognitive functions rely on production of new neurons, however, hypoglycaemia is known to slow the hippocampal neurogenesis [69,70].

Furthermore, a recent report showed nearly above estimated daily intake levels of aluminium, arsenic, cadmium and lead in ackee fruits [71], also mercury have been found in many fruits and vegetables [72,73]. There are reports that accumulation of low dose metals have potential neurobehavioral and neurotoxicants effects in rodents [74], which can influence the development of neurodegenerative diseases [75]. Moreover, evidence has been accumulating that neurotoxicant contained in food cause neurodevelopmental disabilities [76,77], hence children exposed to chronic ingestion of unripe ackee fruit as a result of food scarcity may develop learning disabilities.

5. Conclusion

The behavioural, biochemical and histopathological findings revealed potential toxicity mechanisms via induction of acetylcholinesterase and oxidative stress mediators, which showed that the unripe fruit extract of *Blighia sapida* possesses hepatotoxic and neurotoxic effects.

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