Research Paper: Neurotoxicity and Behavioral Alterations Following Subchronic Administration of Aqueous Extract of Erythrophleum Ivorense Stem Bark in Mice

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ABSTRACT

Introduction: Erythrophleum Ivorense (EI) is a tree found across tropical Africa. The bark of EI is widely used as hunting poisons for animals and ordeal poison in humans. Eating this plant causes paralysis, respiratory distress, and amnesia. In folklore, these behavioral changes have been attributed to guilt in victims; nonetheless, no scientific evidence supports this claim. Thus, the mechanism of neurotoxicity and behavioral alteration of this plant should be investigated.

Methods: A total of 48 BALB/c male mice were randomly divided into four groups. The three experimental groups were administered an aqueous extract of EI in a single daily dose of 5, 10, and 15 mg/kg bodyweight for 28 days, while the control group received distilled water. Afterward, the motor coordination, learning, memory, and grip strength of the mice were accessed with wire grip, Morris water maze, and inverted wire mesh grid grip tests. Histological staining of brain sections was also carried out.

Results: At all tested doses, the aqueous extract of EI caused a significant reduction in hanging latency, significantly increased escape latency, and decreased duration of the target platform in the Morris water maze test compared to control. Reduced grip strength was also observed in the test groups compared to the control. Histology revealed dysmorphic and disoriented Purkinje cells and loss of this cell layer of the cerebellum.

Conclusion: Erythrophleum ivorense administration altered motor coordination, learning and memory, and grip strength in mice dose-dependently. It also caused disruption of granule cells layer, loss of Purkinje cells, and altered cerebellar anatomy leading to motor deficits in mice.

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Highlights

- The supposed alteration in behavior due to guilt following administration of stem bark of E. ivorense is due to memory impairment, loss of grip strength, and motor coordination.

- The Purkinje cell loss is part of a cascade of pathomechanistic events involved in its neurotoxicity.

- Active components in Erythrophleum ivorense can be developed and used as rodenticide rather than its current use as an ordeal poison in humans.

Plain Language Summary

Erythrophleum Ivorense (EI) is a common tree found in tropical regions. Its bark is usually used as hunting poisons for animals and poison in humans. Ingestion of this plant causes paralysis, respiratory distress, and amnesia. In folklore, these behavioral changes have been attributed to guilt in victims; nonetheless, there is a lack of scientific evidence to support this claim. We, therefore, investigated the mechanism of neurotoxicity and behavioral alteration of this plant. Forty-eight BALB/c male mice were randomly divided into four groups. The test groups were administered an aqueous extract of EI in a single daily dose of 5, 10, and 15 mg/kg body weight for 28 days while the control received distilled water. After that, motor coordination, learning, memory, and grip strength were accessed. Histological staining of brain sections was also carried out. At all tested doses, the aqueous extract of EI caused a significant reduction in hanging latency, significantly increased escape latency, and decreased duration in the target platform in the Morris water maze test compared to control. Reduced grip strength was also observed in the test groups compared to the control. Histology revealed dysmorphic and disoriented Purkinje cells and loss of the Purkinje cell layer of the cerebellum. Erythrophleum ivorense administration altered motor coordination, learning and memory, and grip strength in mice in a dose-dependent manner. It also caused disruption of granule cells layer, loss of Purkinje cells, and altered cerebellar anatomy leading to motor deficits in mice

1. Introduction

For several years, plants have been used in traditional medicine and primary healthcare systems (Malik, Bhat, Ballabha, Bussmann, & Bhatt, 2015). The use of plants has increased due to the search for novel compounds in pharmaceutical industries. They are believed to be cost-effective and lack the complexity of synthetic pharmaceutical preparations (Nasri & Shirzad, 2010).

Plants produce various phytochemicals, such as mucilage, polysaccharides, and tannin, that mediate cell functions; however, some of these constituents are neurotoxins. For example, acetogenins from the Annonaceae family has been documented to cause tremor, subcortical dementia, neuronal cell loss, and gliosis in the brain stem and basal ganglia (Champy et al. 2004; Höllerhage et al. 2009; Höllerhage et al., 2015). In the same vein, toxic exposure to Cycas circinalis and Lathyrus sativus cause several neurological diseases in humans and animals (Spencer et al., 1986).

Erythrophleum ivorense (A Chev.) is a large tree growing in many tropical regions in Africa. The stem bark is taken orally in Sierra Leone as an emetic (Betti, 2004). Its analgesic property has also been documented (Richter & Dallwitz, 2000). Bosch (2006) reported that extract from young branches of crushed E. ivorense is used to treat chickenpox. Despite these therapeutic values, the plant is toxic to humans and animals. Smoke from the burnt stem bark is reportedly used as a mosquito repellent in Cameroon (Youmsi et al., 2017). The bark is widely used as hunting poison for animals and ordeal poison in humans (Bakarr & Janos, 1996). In some cultures, suspects accused of witchcraft are given an aqueous extract of E. ivorense; ingestion of this plant causes paralysis, amnesia, and several morbidities (Dongmo et al., 2001). Despite the widespread toxicity of this plant, no evidence supports that behavioral alterations and mortalities following consumption of E. ivorense are equated with guilt or otherwise in suspects. Thus the present study aimed to bridge this gap. The study aimed at mimicking the folklore used to investigate the effects of the plant on the Central Nervous System (CNS) activities and its impact on behavior using an animal model.

2. Methods

Experimental animals

A total of 48 male BALB/c mice of about 6 weeks old weighing between 18 and 22 g were randomly divided into four groups (A–D) of 12 mice each. They were housed in a temperature and light-controlled room (27 °C-29 °C, 60%-70% relative humidity, a 12-h photoperiod starting at 08:00 h) and were fed a standard laboratory animal diet and allowed to drink water ad libitum. The experiments were conducted in a quiet laboratory between 10:00 and 13:00. Ethical clearance and approval for the study were given by the Animal Care and Use Research Ethics Committee of the University (UI-ACUREC/18/0026) following the Guide for Care and Use of Laboratory Animals, NIH, Department of Health Services Publication, USA, No. 83-23, revised in 1985. Efforts were made to minimize pain, suffering, and the number of animals used.

Plant Material

The stem bark of Erythrophleum ivorense (A Chev.) was collected from Omo Forest Reserve, Area J4, Ogun State, Nigeria, between May and June 2017 (rainy season). The plant was identified and authenticated at the Department of Botany, University of Ibadan, Nigeria; a voucher specimen (FHI. 109950) was preserved at the herbarium. The stem bark of E. ivorense was cleaned, air-dried for two weeks, and ground into powder using an electric blender (Blender/Miller III, model MS-223, Taiwan, China). Extraction was carried out by cold maceration of 1350 g of the coarse powder with 10 L of distilled water for 72 h, with constant shaking using the GFL shaker (No. 3017GBh, Germany). The resultant mixture was filtered using Whatman filter paper (No. 1), and the filtrate was concentrated to dryness in vacuo at 40°C using a rotary evaporator. The dried extract was dissolved in distilled water and administered by gastric gavage to the animals.

Experimental protocol

Based on the previous toxicity report of E. ivorense described by Adu-Amoah, Agyare, Kisseih, Ayande and Mensah (2014), we limited the maximum dose in the present study to 1/10 of the lowest observed adverse effect level. Group A received distilled water and served as control while mice in groups B, C, and D were administered 5, 10, and 15 mg/kg body weight aqueous extract of E. Ivorense Stem Bark (EISB), respectively in a single oral dose for 28 days.

Behavioral tests

Test for Memory Using the Morris Water Maze

The water maze was a circular pond with a diameter of 120 cm, filled with water at a temperature of 26°C±1°C and divided into four quadrants (north, east, south, and west). The water was made opaque with non-fat milk, and a hidden escape platform (submerged 1 cm below the surface) was placed at the same spot in one of the quadrants throughout the training/acquisition session. During the acquisition (short-term memory) trial, each mouse underwent four trials per day with an inter-trial interval of 30 min for 4 days (Vorhees & Williams, 2006; Barnhart, Yang, & Lein, 2015). At the beginning of each trial, the mouse was gently immersed into the water at one of the four quadrants. The same procedure was repeated for the other trials, starting the mouse at different quadrants for each of the four trials. Each mouse was allowed 120 s to find and climb onto the hidden platform. If the mouse failed to find it within this time, it would be guided onto the platform and allowed to remain there for 15 s. The mouse was returned to its home cage between trials. The learning curve for each animal was constructed by plotting the trial number on the x-axis and latency to find the platform in seconds on the y-axis (Li et al., 2008; Morris, Garrud, Rawlins, & O'Keefe, 1982).

The probe trial was performed 24 hours after the last acquisition trial, and this time, the platform was removed. The number of crossings over the position at which the platform had been located and the duration of swimming time within the quadrant that initially had the hidden platform were recorded (Golchin, Golchin, Vahidi, & Shabani, 2013). This is a measure of memory retention in mice.

Inverted Mesh Grid Grip Test

Mice were placed on the center of a 43-cm square wire mesh grid consisting of 12-mm squares of 1-mm diameter wire. The grid was surrounded by a 4-cm thick wooden frame which prevented mice from climbing to the opposite side. Once a mouse was positioned, the grid was turned upside down and elevated about 1 m over some soft bedding to force the mouse to grip the wire to avoid falling. The time taken for a mouse to fall and the number of mice that fell in each group were recorded. If an animal did not fall, the experiment was concluded after 60 s.

Wire Grip Test

The forepaws of the mice were placed on a horizontally suspended metal bar (measuring 2 mm in diameter and 1 m in length), placed 1 m above a soft bedding-filled landing area. The latency to fall (i.e., length of time each mouse could stay suspended before falling off the wire) was recorded with a stopwatch. A maximum time of 120 s was given to each mouse, after which it was removed. Each mouse was given two trials with a 6-h rest interval (VanWijk, Rijntjes, & Van De Heijning, 2008).

Grip Strength Test

Mice were placed over a base plate in front of a grasping bar of the grip strength meter (UGO Basile®). The bar was fitted to a force transducer connected to the peak amplifier. Mice were pulled by the tail, and the mice grasped the bar. Within 20 s, the maximal grip force was measured.

Histology

At the end of the experiment, the mice were anesthetized through intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). The mice were killed by intracardial perfusion of normal saline followed by 10% neutral buffered formalin. Their brains were dissected, the cerebella separated and post-fixed for 72 h in the same solution, and embedded in paraffin wax by conventional methods (Sidhu & Nehru, 2004). Mid-sagittal sections of the cerebella, measuring 5 µm in thickness, were prepared with a rotary microtome and then stained with hematoxylin-eosin. The sections were then observed under the light microscope for histopathological changes, and their photomicrographs were captured using a high-resolution digital camera (Sony® Cybershot DSC-W53 digital camera).

Statistical analysis

Morris Water Maze data were analyzed using a 2-way repeated-measures ANOVA with the group as the between-subject factor and training days as the within-subject factor. The independent variables were groups (control or extract-treated) and day of training; the dependent variable was the escape latency (time taken by the mice to find the escape platform). The wire grip and inverted mesh tests were analyzed with a 1-way ANOVA test. Values are presented as Means±Standard error of the mean. P values less than 0.05 were considered statistically significant. All statistics were performed on Graphpad Prism version 7.05.

3. Results

Morris Water Maze Test

In the Morris water test acquisition trial, a significant difference between groups ($P<0.0001$) and training days ($P<0.001$) was observed. The ability to learn the location of the hidden platform was unperturbed across the groups because mice gradually spent shorter times locating the platform with successive training trials. However, on day 4, the average escape latency in searching for the hidden platform in the 15 mg/kg EISB mice was significantly ($P=0.0038$) more prolonged than that in the control mice (Figure 1-A). On day 5, the probe trial (following the removal of the platform) revealed an overall difference between groups. The mice administered 5, 10, and 15 mg/kg of EISB spent less time ($P=0.002$, $P=0.0019$, and $P=0.0032$, respectively) in the target quadrant and fewer times passing through the original position of the hidden platform ($P=0.0016$, $P=0.0071$, $P=0.0055$, respectively) compared with the control mice (Figure 1-B). The EISB treated mice hardly recognized the quadrant when they were in it and so spent less time there quickly moving on in search of the platform (Figure 1-C).

Inverted Wire Mesh Grid Grip Test

This test revealed that hanging latency in the extract-treated groups was inversely proportional to the dosage of EISB extract administered (Figure 2-A). In addition, during the 60-s test, almost all mice in the extract-treated groups fell while all the control mice still remained on the inverted grid (Figure 2-B). Consequently, we further investigated if the increased tendency to fall in the extract-treated groups was related to grip strength, lack of coordination, or both using the wire grip test.

Wire Grip and Grip Strength Tests

The 10 mg/kg and 15 mg/kg groups also had significantly lower latency on the wire grip test, suggesting that the reduced hanging latency could be due to the inability of the mice to grip (Figure 3A). Grip strength was evaluated using a grip strength meter. EISB mice had lower forelimb grip strength (Figure 3B), suggesting impaired balance, motor, and coordination systems.

Brain histology

The Purkinje cells in control mice were observed at regularly spaced intervals between the molecular and granule cell layers. Cell bodies were round and clearly defined, and dendrites were visible (Figure 4-A). Vari-

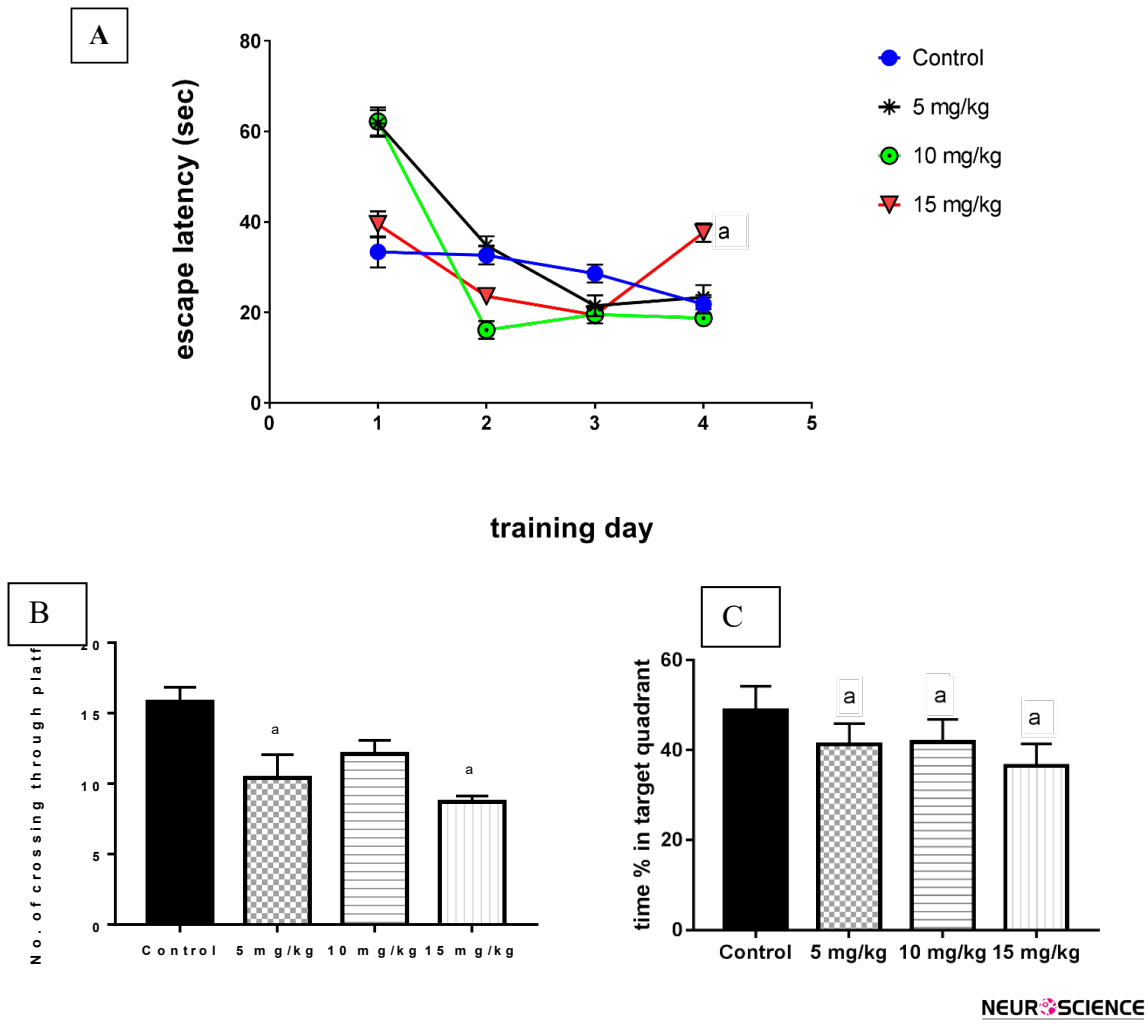

Figure 1. The morris water test showing

A: Learning Curve During the Acquisition Trials (Two-way ANOVA With Bonferroni Repeated Measures Post Hoc Tests; B: Effect of Erythrophleum ivorense Administration on the Number of Crossing Through Platform; and C: Mean percentage time spent swimming in the target quadrant in the probe trial of morris water maze

[a] significantly different from the control group at P<0.05, n=12; Mean±SD (Standard Deviation).

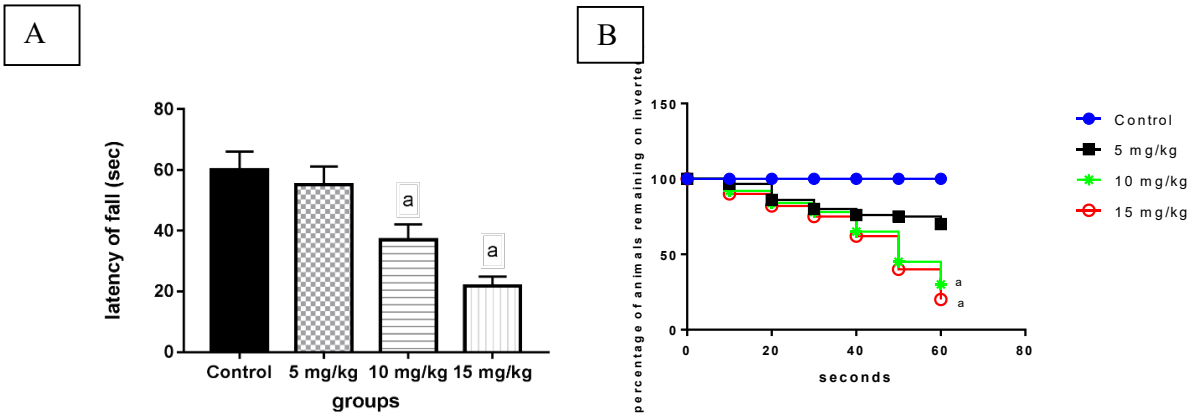

Figure 2. Latency of fall and percentage of animal remaining on the inverted wire mesh grid grip test

n=12; Mean±SD (Standard Deviation); [a] significantly different from the control group at P<0.05.

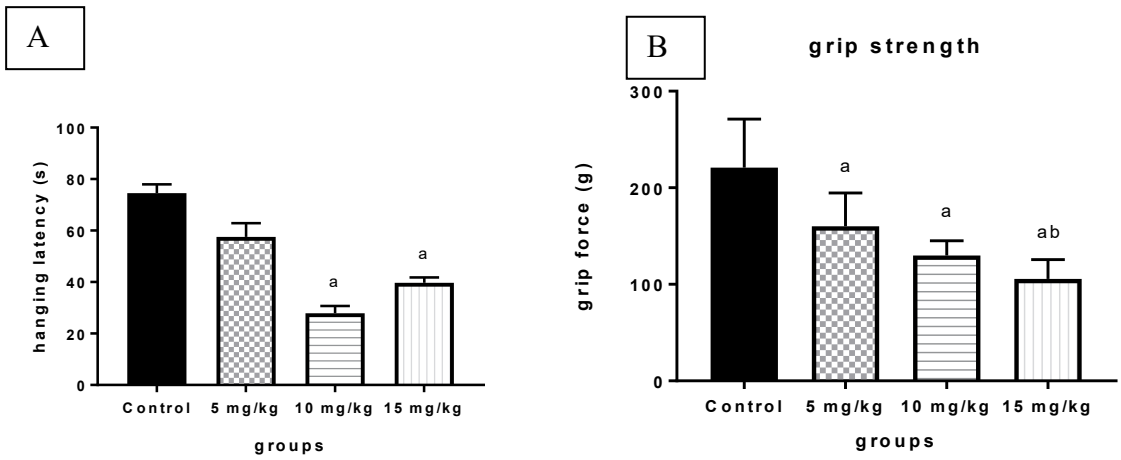

Figure 3. Hanging latency following administration of graded doses in experimental mice

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A: Latency on the Wire Grip Test; and B: Grip Force of Experimental Mice.

n=12; Mean±SD (Standard Deviation); [a] significantly different from the control group at P<0.05; [b] significantly different from the 5 mg/kg group at P<0.05.

ous cytoarchitectural differences in Purkinje cells were observed following the administration of EISB. In the 5 mg/kg mice, the disorientation of Purkinje cells was evident in the cerebellar cortex (Figure 4-B). In the 10 mg/kg group, there was no distinct Purkinje cell layer, and loss of axons of Purkinje cells was evident; how-

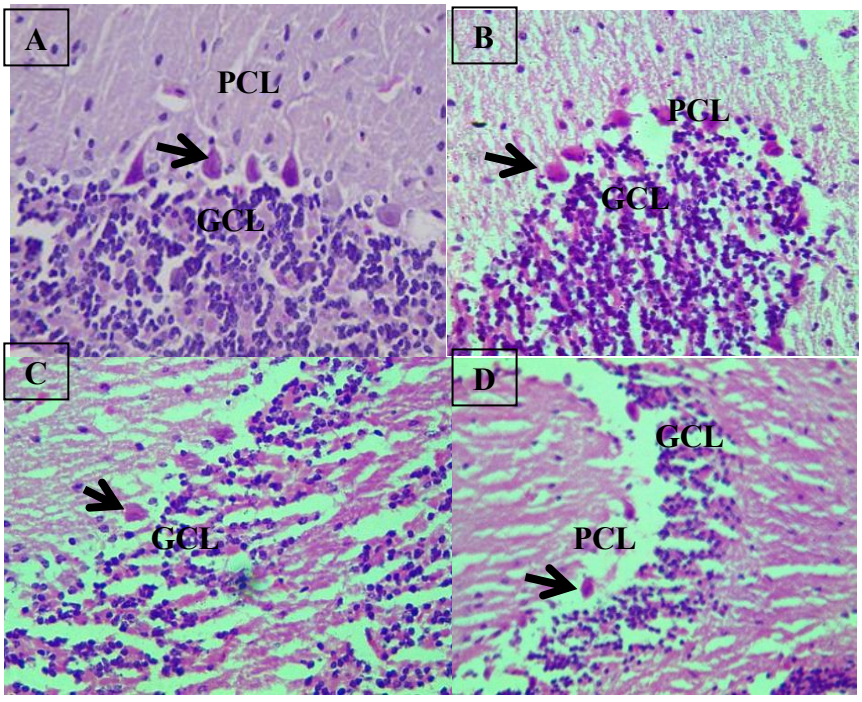

Figure 4. The brain sections of experimental animals showing the layers of the cerebellum

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The Cerebellar Section of Mouse in the (A) Control Group Showing Purkinje Cells (Arrows) of the Cerebellar Cortex Normally Arranged Between the Molecular and Granular Layer (B) 5 mg/kg Group Showing Disorientation of the Purkinje Cells (Arrows) With Loss of Axon (C): 10 mg/kg Group Showing No Distinct Purkinje Cell Layer (Arrows) and Loss of Axon (D) 15 mg/kg Group Showing Loss of Purkinje Cell Layer (Arrows) (H&E stain x400 magnification).

PCL: Purkinje Cell Layer; GCL: Granule Cell Layer.

ever, other layers of the cerebellar cortex remained intact (Figure 4-C). There was a loss of the Purkinje cell layer, loss of axon of the Purkinje cells with poorly defined soma which are now found in the granular layer in the 15 mg/kg group (Figure 4-D). Reduction in the number of granule cells was also noted in the cerebellum of the extract-treated mice.

4. Discussion

Many medicinal plants contain active constituents that interfere with the normal biological processes of the nervous system. They act by modulating intracellular processes of neurons, blocking synaptic communication, blocking or mimicking neurotransmitters, and initiating abnormal signals. The scientific data on the CNS activities of E. ivorense is scarce as the plant is regarded sacred (Quiroz & van Andel, 2015). Thus, we encountered several challenges during plant collection for the present study. To the best of our knowledge, this study represents the first examination of the behavioral changes and neurotoxicity associated with administering an aqueous extract of EISB. Ethno-medicine associates signs such as seizures, behavioral changes, shortness of breath, seizures, and cardiac arrest to guilt in victims; thus, the activities of this plant are worth exploring in an "innocent" animal model.

In the acquisition trial of the Morris Water Maze (MWM) task, animals across the control, 5 mg/kg, and 10 mg/kg groups show no difference in learning abilities as indicated by a progressive reduction in escape latency with subsequent trials. However, learning ability or short-term memory was significantly altered in the 15 mg/kg group as mice did not learn the task as effectively as the other animals. This impaired learning is dose-related as mice exposed to the highest dose of EISB had significantly increased escape latency with multiple trials. In the probe trial of the MWM, impairment in memory retention was observed in all test groups relative to control, as indicated by a significant decrease in the number of crossings of the platform area. Furthermore, EISB also reduced the duration in the target quadrant relative to the control group suggesting a decline in spatial memory as well. This result is similar to the report by Braida, Donzelli, Martucci, Capurro and Sala (2011) that salvinorin A from the extract of Salvia divinorum declined cognitive ability and memory. This might be due to the bioactive constituents of the stem bark; Youmsi et al. (2017) had previously stated that E. ivorense contains toxic alkaloids, and these phytoconstituents might be responsible for this memory deficit.

The motor and coordination systems of the mice were also assessed using the wire grip and inverted wire mesh tests (Osborne et al., 2012). The EISB mice performed poorly in coordination, muscle strength, and balance, as shown by a significant reduction in latency in the tests. Previous studies show that the cerebellum is heavily involved in regulating these systems (Becker et al., 2009). Therefore, EISB administration not only interferes with memory but also affects motor functions and leads to decreased motor activities and grip strength in mice.

Specific neuronal populations show selective susceptibility to toxic insults, which may ultimately result in losing those cells. The Purkinje cells are a type of cerebellar neurons, especially susceptible to stress (Sieber, Palmon, Traystman, & Matrin, 1995; Yoshida et al., 2002) and neurodegenerative-associated conditions (Dove, Nahm, Murchison, Abbott, & Griffith, 2000). They are large, GABAergic neurons, which serve as the sole output of the cerebellar cortex. Purkinje cells are typically located in a single row at the border of the granular and molecular layers. Their myelinated axons terminate on neurons of the cerebellar nuclei trees, are flattened and oriented perpendicular to the parallel fiber. The degeneration of the dendrites of these cells by EISB extract in the present study is in congruence with findings by Furukawa et al. (2012) that Purkinje cell dendrites are the first to degenerate as an early consequence of high dose-rate intoxication, then somal and axonal changes developed subsequently. Although the symptomatic loss of function following neurotoxicity is frequently attributed to the loss of neurons, this study demonstrates that there may be events before neuronal loss resulting in cerebellar dysfunctions such as impaired balance control, motor coordination, and motor learning (Zhang, Chung, & Chow, 2014). It is likely that a unique manifestation of these subtle Purkinje cell changes is more closely linked with the onset of tremor seen in humans following the consumption of this plant which has been interpreted as guilt in folklore use. The response of Purkinje cells to stress has similarly been documented in axotomies and diseases of cerebellar dysfunction. It represents a partly degenerative and partly compensatory response to various cellular injuries (Rossi, Gianola, & Corvetti, 2006). The relationship between Purkinje cell loss and motor deficits is not surprising given that the regions that control cognitive and motor functions via reciprocal connections to the prefrontal cortex, posterior parietal cortex, and cortical motor regions (Strick, Dum, & Fiez, 2009). They are all connected to the cerebellum. Thus, this damage to the Purkinje cell leads to impaired communication between the cerebellum and its efferent targets. The impaired motor coordination, according to the disconnection hypothesis, is, therefore, due to loss of connectivity between two or more brain areas (Catani &

Ffytche, 2008). This agrees with findings that the cerebellum regulates the motor coordination systems (Isaacs et al., 2003; Becker et al., 2009).

5. Conclusion

The administration of Erythrophleum ivorense elicits toxicity in the central nervous system and alters behavior in mice. It is advised that the use of this plant as ordeal poison be reconsidered and discouraged.

Ethical Considerations

Compliance with ethical guidelines

This study was carried out following the Guide of the National Institute of Health for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Approval was also given by the institutional Animal Care and Use Ethics Committee. Efforts were also made to reduce the pain and stress of the experimental animals.

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Authors' contributions

Conceptualization, supervision: Olamide Adebiyi; Methodology, resources: All authors; Investigation: Oluwasina Ajayi; Writing the manuscript, data analysis: Oluwasina Ajayi and Funmilayo Olopade.

Conflict of interest

The authors declared no conflict of interest.

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