PRINT ISSN 1119-8362
Electronic ISSN 2659-1499

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

J. Appl. Sci. Environ. Manage.
Vol. 29 (2) 393-400 Feb. 2025

Preliminary Evidence Of Possible Neurotoxic Activity Of Aqueous *Annona muricata* (Soursop) Leaf Extract In The Cerebellum Of Adult Wistar Rats

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ABSTRACT: Global trends show a rising adoption of plant-based diets due to their therapeutic benefits, however, studies show that some medicinal plants may induce neurotoxicity. Accordingly, this study investigated the preliminary evidence of possible neurotoxic activity of aqueous *Annona muricata* (Soursop) leaf extract (AMLE) on the cerebellum of adult Wistar rats using appropriate standard procedures after the cerebella were assayed for antioxidant enzymes activity, lipid peroxidation and histological changes. Findings revealed no significant difference (p>0.05) in the body, brain, cerebellar, relative cerebellar weights, and cerebellum-brain weight ratio of all AMLE-treated rats compared to control. There was no significant difference (p>0.05) in CAT activity in AMLE-treated rats compared to control, however; there was a significant decrease (p<0.05) in SOD activity and a significant increase (p<0.05) in MDA concentration in rats treated with 5000 mg/kg BW of AMLE compared to control. Also, there was a significant decrease (p<0.05) in ambulation, line crossing, and movement initiation score, and a significant increase (p<0.05) in immobility in rats treated with 5000 mg/kg BW of AMLE compared to control. Histological findings reveal relatively normal and intact histology of the cerebellum in all AMLE-treated rats following comparison to control. Conclusively, treatment of the experimental rats with AMLE did not adversely affect the cerebellum except for a few parameters at 5000 mg/kg BW, thus indicating that AMLE may be toxic at higher doses.

DOI: https://dx.doi.org/10.4314/jasem.v29i2.7

Cite this Article: EKWEAGA, E. C; TOBALU, F. O; ORHERUATA, A. R; MOMODU, O. I; IDEMUDIA, O. U; ENOGIERU, A. B (2025). Preliminary Evidence Of Possible Neurotoxic Activity Of Aqueous *Annona Muricata* (Soursop) Leaf Extract In The Cerebellum Of Adult Wistar Rats. *J. Appl. Sci. Environ. Manage.* 29 (2) 393-400

Dates: Received: 23 December 2024; Revised: 27 January 2025; Accepted: 09 February 2025; Published: 28 February 2025

Keywords: Neurotoxicity; Cerebellum; *Annona muricata*; Soursop; Neurobehaviour

The brain is an intricately complex organ, comprising billions of neurons, and it governs a wide range of vital functions, including cognition, memory, emotions, sensory perception, motor skills, and autonomic processes such as respiration, hunger and temperature regulation (Stiles and Jernigan, 2010). It consists of three major parts; cerebrum, brainstem and cerebellum, which together with the spinal cord forms the central nervous system (Orheruata and Enogieru, 2024). The cerebellum also known as the 'little brain' is the largest structure of the hindbrain, located below the temporal and occipital lobes of the cerebrum (Orheruata and Enogieru, 2024). The cerebellum's main functions include; balance and equilibrium, muscle tone regulation, movement coordination, and supporting various cognitive abilities (Enogieru and Momodu, 2021a). Globally, neurological disorders pose a significant threat to

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public health, affecting over one billion people worldwide and highlighting the urgent need for effective solutions and support (Zahiruddin *et al.*, 2020). Current medical treatments for neurological disorders typically only address symptoms and are often pricey, consequently, natural remedies, supplements, and nutrition-based therapeutics to support brain health and performance continue to rise (Orheruata and Enogieru, 2024; Zahiruddin *et al.*, 2020).

According to the World Health Organization (WHO), a staggering 80% of the global population now relies heavily on traditional medicine, primarily derived from plants, as their primary means of healthcare (Jamshidi-Kia *et al.*, 2017). Studies have shown that medicinal plants serve as effective therapeutic agents, offering a natural treatment approach for the treatment of neurological (Enogieru and Idemudia, 2024; Enogieru and Momodu, 2021b), and metabolic disorders (Enogieru *et al*., 2015a; Enogieru *et al*., 2015b). *Annona muricata*, a member of the Annonaceae family, has garnered significant scientific interest in recent years due to its notable medicinal properties (Zubaidi *et al.*, 2023a). Studies have demonstrated the medicinal properties of *Annona muricata*, showing its potential in treating various health conditions through its anti-cancer, anti-microbial, antioxidant, anti-ulcer, anti-diabetic, anti-hypertensive, anti-inflammatory and wound healing activities (Orak *et al.*, 2019; Zubaidi *et al.*, 2023a). Several reports have identified approximately 212 bioactive compounds in *Annona muricata*, with a significant presence of acetogenins, as well as notable amounts of alkaloids, phenolics, and several other bioactive substances (Coria-Téllez *et al.*, 2018). These phytochemicals possess pharmacological activity against neurological and neurodegenerative disorders (Enogieru and Ezennia, 2024; Omoruyi *et al.*, 2021). Despite the broad therapeutic potential of *Annona muricata*, the effects on the cerebellum remain largely unexplored. Accordingly, the objective of this paper is to investigate the preliminary evidence of possible neurotoxic activity of aqueous Annona muricata (soursop) leaf extract (AMLE) on the cerebellum of adult Wistar rats.

MATERIALS AND METHODS

Plant material: *Annona muricata* leaves were harvested in the Staff Quarters of the University of Benin, Benin City, Edo state, Nigeria. It was identified and authenticated in the Herbarium unit of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, and assigned a herbarium number UBH-A356. Aqueous extraction was carried out as previously described (Okhah and Enogieru, 2023). Briefly, leaves weighing 3000 g were washed with tap water, chopped into pieces and then blended into smooth powdered specimens using an electric grinder. The powdered specimen was mixed in 10 litres of water and thoroughly mixed at intervals for 48 hours. The mixture was filtered using Whatman paper into a clean jar. The resulting solution was freeze-dried to obtain a dark greenish paste weighing 54.7g. The resulting extract was reconstituted with distilled water to give the desired concentrations used in this study.

Experimental Design: Thirty (30) adult Wistar rats were randomly assigned into five (5) groups (A-E) of six (6) rats each. Group A (control) received 1 ml distilled water, group B received 100 mg/kg body weight (BW) of AMLE, group C received 1000 mg/kg BW of AMLE, group D received 2500 mg/kg BW of AMLE and Group E received 5000 mg/kg BW of AMLE. All administration was done via an orogastric tube and lasted for 28 days.

Evaluation of Neurobehavioral Activity: Twenty-four (24) hours after the last administration, neurobehavioral activities were evaluated using open field, string and movement initiation tests.

Open Field Test (OFT): This is a test widely used to assess locomotor activity and exploratory behaviour in rodents and was performed as previously described by our laboratory (Enogieru and Inneh, 2022; Enogieru and Omoruyi, 2022). This was performed in a wooden open box apparatus (72 cm × 72 cm × 20 cm) with lines on its floor sharing it into 18 cm by 18 cm (Enogieru and Inneh, 2022). The OFT parameters assessed were ambulation (time spent making movements), line crossing (regularity of rats crossing lines in the box) and central square entry (regularity of rats moving into the center square in the box) and immobility (time spent motionless) (Enogieru and Inneh, 2022; Enogieru and Omoruyi, 2022).

String Test: This test, often utilized to evaluate grip strength, was performed with the rats using the forepaws to hold a steel wire (2 mm – diameter x 60 cm – length x 50 cm – height), as previously described (Enogieru and Idemudia, 2024; Ijomone *et al.*, 2014). The duration of time (maximum 120 s) spent by the rats in holding the wire till it fell was recorded.

Movement Initiation Test (MIT): This test, often utilized to evaluate motor functions, was performed as previously described (Ijomone *et al.*, 2014). Here, the trunk of the rat is held, with its hindlimbs and forelimb raised above the table to allow for weight

support by only one forelimb. The time taken to initiate one step is recorded for each forelimb and averaged to arrive at the movement initiation score.

Evaluation of Weights: After euthanizing the rats under mild anesthesia, their brains and cerebellum were harvested, and weighed, and the relative weights (%) were calculated as previously described (Enogieru and Egbon, 2022; Kim *et al.*, 2008). Thereafter, the cerebellum was used for biochemical and histological assessments, respectively.

Assessment of Biochemical Parameters: The harvested cerebella were homogenized in ice-cold 20 mM Tris–HCl buffer (pH 7.4), and the homogenates were then centrifuged at 10,000 g for 10 min at 4 °C (Montilla *et al.*, 2005). The supernatants were collected and evaluated for superoxide dismutase (Misra and Fridovich, 1972), catalase (Cohen, 1983) and malondialdehyde (Buege and Aust, 1978).

Histological Evaluation: The cerebella were fixed in 10% buffered formal saline for 72 hours, followed by the paraffin wax embedding as well as hematoxylin and eosin staining as previously reported (Drury and Wallington, 1980).

Statistical Analysis: The data were analyzed using the GraphPad Prism, version 9. Values were presented as mean ± standard error of mean (SEM), and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test was used to determine statistical significance (p<0.05).

RESULTS AND DISCUSSION

Effect of Treatment on Weights: Table 1 shows the weight findings of rats across experimental groups. There was no significant difference (p>0.05) in the weight parameters following treatment with 100 mg/kg, 1000 mg/kg, 2500 mg/kg, and 5000 mg/kg AMLE when compared to the control group.

Table 1: Weights across experimental groups

	Concentration of aqueous *Annona muricata* (soursop) leaf extract (AMLE) received by experimental groups				
Parameters	Group A (control): 1 ml distilled water	Group B: 100 mg/kg body weight (bw) of AMLE	Group C: 1000 mg/kg bw of AMLE	Group D: 2500 mg/kg bw of AMLE	Group E : 5000 mg/kg BW of AMLE
Initial Body Weight (g)	194.8 ± 18.25	191.0 ± 8.48	190.7 ± 4.17	199.0 ± 13.69	201.8 ± 11.54
Final Body Weight (g)	213.8 ± 16.67	212.3 ± 7.36	216.0 ± 2.48	214.8 ± 14.08	216.3 ± 10.73
Weight Change (g)	19.0 ± 2.48	21.3 ± 2.06	25.3 ± 3.36	15.8 ± 0.85	14.5 ± 1.50
Whole Brain Weight (g)	1.65 ± 0.03	1.53 ± 0.03	1.65 ± 0.03	1.58 ± 0.63	1.58 ± 0.05
Relative Brain Weight (%)	0.79 ± 0.06	0.72 ± 0.02	0.76 ± 0.01	0.74 ± 0.02	0.73 ± 0.03
Cerebellar Weight (g)	0.38 ± 0.03	0.35 ± 0.03	0.38 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
Cerebellum-Brain Ratio (g)	0.23 ± 0.01	0.23 ± 0.02	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Relative Cerebellar Weight (%)	0.18 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01

Table 2: Neurobehavioural Assessment of experimental rats.

	Concentration of aqueous *Annona muricata* (soursop) leaf extract (AMLE) received by experimental groups				
Parameters	Group A (control): 1 ml distilled water	Group B: 100 mg/kg body weight (bw) of AMLE	Group C: 1000 mg/kg bw of AMLE	Group D: 2500 mg/kg bw of AMLE	Group: E - 5000 mg/kg BW of AMLE
OFT					
Ambulation (s)	257.5 ± 4.52	233.8 ± 6.25	255.3 ± 11.92	228.0 ± 11.11	218.0 ± 5.76 #
LC (f)	73.8 ± 3.86	69.0 ± 2.12	79.0 ± 2.80	63.8 ± 2.96	53.0 ± 2.55 #
CSE (f)	4.3 ± 0.48	4.0 ± 0.41	4.3 ± 0.48	4.0 ± 0.41	3.8 ± 0.48
Immobility (s)	42.5 ± 4.52	66.3 ± 6.25	44.8 ± 11.92	72.0 ± 11.11	82.0 ± 5.76 #
STRING TEST					
Latency to grip loss (s)	148.3 ± 18.4	132.5 ± 17.1	149.8 ± 17.5	119.8 ± 8.4	106.5 ± 4.7
MIT					
Movement Initiation score (s)	1.3 ± 0.14	1.3 ± 0.14	1.2 ± 0.16	1.5 ± 0.08	1.9 ± 0.12 #

# $p < 0.05$ following comparison to control.

Effect of Treatment on Neurobehavioural Activity: The findings from the OFT, string, and MIT evaluation are presented in Table 2. For OFT, a significant decrease (p<0.05) was observed in ambulation and line crossing in rats treated with 5000 mg/kg AMLE when compared to the control. Similarly, a significant increase (p<0.05) in immobility was observed in rats treated with 5000 mg/kg AMLE when compared to control. For the MIT, there was a significant increase (p<0.05) in the movement initiation score in rats treated with 5000 mg/kg AMLE when compared to the control

Effect of Treatment on Antioxidant Enzymes and Lipid Peroxidation: The findings from the activity of antioxidants and MDA in the cerebella across experimental groups are presented in Table 3. A significant decrease (p<0.05) in cerebellar SOD was observed in rats treated with 5000 mg/kg AMLE when compared to control. Similarly, a significant increase (p<0.05) in MDA concentration was observed in rats treated with 5000 mg/kg AMLE when compared to the control.

Table 3: Antioxidant Enzymes and Lipid Peroxidation in the cerebellum of experimental rats.

	Concentration of aqueous *Annona muricata* (soursop) leaf extract (AMLE) received by experimental groups				
Parameters	Group A (control) - 1 ml distilled water	Group B - 100 mg/kg body weight (bw) of AMLE	Group C - 1000 mg/kg bw of AMLE	Group D - 2500 mg/kg bw of AMLE	Group E - 5000 mg/kg BW of AMLE
SOD (KU/L)	11.28 ± 0.41	9.67 ± 1.10	11.77 ± 1.29	9.22 ± 0.48	7.23 ± 0.63 #
CAT (KU/L)	2.30 ± 0.21	2.02 ± 0.08	2.36 ± 0.19	1.99 ± 0.12	1.86 ± 0.12
MDA (μM/mg Protein)	0.17 ± 0.03	0.20 ± 0.04	0.18 ± 0.04	0.26 ± 0.04	0.33 ± 0.02 #

# $p < 0.05$ following comparison to control.

Effect of Treatment on the Histology of the Cerebellum: Plate 1A-D shows the representative cerebellum of rats in the control and AMLE-treated groups, displaying normal layers of the cerebellar cortex. The cortex is organized in layers, starting with the outer molecular layer, which houses baskets and stellate cells. Further layers include the Purkinje cell layer, containing Purkinje cell bodies, and the granular layer, which is densely packed with granular cells. However, in plate 1E, very few degenerating Purkinje cells with nuclei appearing irregular were observed in the Purkinje cell layer.

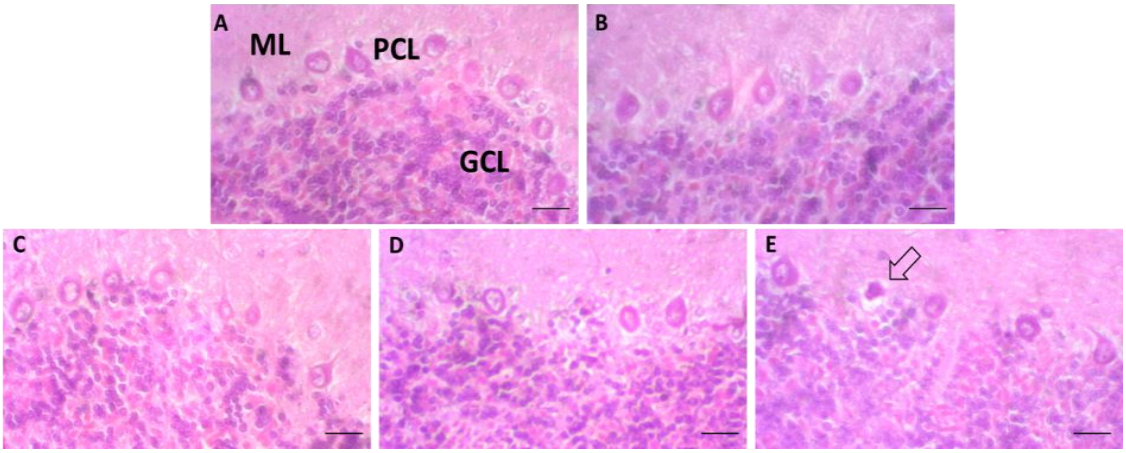

Plate 1: Representative histology of the cerebellum across experimental groups. (A-D) Normal histological structure of cerebellum layers – Molecular layer (ML); Purkinje Cell layer (PCL); Granular Cell layer (GCL); (E) Normal histological structure of cerebellum layers observed. However, a degenerating Purkinje cell with nuclei appearing irregular [arrow] is also noticed. (H&E 400x; Scale bar: 25μm)

Annona muricata has garnered significant attention due to its rich nutritional profile and potential health benefits (Santos *et al.*, 2023). The bark, leaves, and roots are considered to have sedatives, antispasmodic, hypoglycemic, hypotensive, diuretic, and neuralgia properties (Adewole and Caxton-Martins, 2006). Studies show that *Annona muricata* is one of the plants that contain important phytochemicals that are beneficial for body and brain health (Gajalakshmi *et al.*, 2012; Kim *et al.*, 2020). Beyond its conventional applications, studies have revealed that the acetogenins present in *Annona muricata* leaf possess potent anti-cancer properties, selectively targeting and destroying cancerous cells (Pieme *et al.*, 2014; Ragasa *et al.*, 2012).

Studies have shown that changes in body weight can be a critical indicator of overall health status in rodents and other experimental animals, offering valuable insight into their general well-being and health condition (Al-Shabanah *et al.*, 2002). Organ weights are widely recognized as reliable indicators of toxicity, including toxicity caused by plant-based substances (Akhigbe, 2014). The ratio of body weight to organ weight provides valuable insights, and research suggests that exposure to toxic substances

can lead to decreased body and organ weights, serving as a potential marker of toxicity (Akhigbe, 2014). Findings from this study showed no significant change in body weights of AMLE-treated rats when compared to control. This is consistent with findings from previous studies which demonstrated no significant change in body weights of rats treated with *Annona muricata* extract when compared with control (Opara *et al.,* 2021; Zubaidi *et al.,* 2023b). Also, there was no significant difference in cerebellar weights of AMLE-treated rats when compared to control.

Accumulating evidence emphasizes the importance of incorporating behavioural evaluations into the assessment of neurotoxins (Roberts and Stout, 2023). Neurobehavioral assessments are crucial diagnostic tools for evaluating brain functions and examining the behavioural and cognitive effects of neurological disorders or exposure to environmental toxins (Kim and Kim, 2012). These assessments play a vital role in identifying the effects of neurotoxic exposure, which can lead to changes in cognitive abilities, motor skills, emotional control, mood, and social behaviour (Kaufer, 2015). The open field test is a fundamental and widely used neurobehavioral assessment that evaluates locomotor activity, exploratory behavior, and motor function in rodents, providing valuable insights into their neurological health (Ijomone *et al.,* 2014). Significant decrease in ambulation and line crossings, as well as a significant increase in immobility, are indicative of impaired motor function and reduced exploratory behaviour in rodents, suggesting potential neurological deficits (Enogieru and Idemudia, 2024; Enogieru and Iyoha, 2023). In this present study, 5000 mg/kg AMLE-treated rats showed a significant reduction in ambulation, line crossing, and a significant increase in immobility, suggesting the toxicity of AMLE at higher doses. The movement initiation test is a valuable diagnostic tool for assessing motor functions in rodents. Better motor performance is typically indicated by a decreased movement initiation time, conversely, increased movement initiation time is an indicator of impaired central control of motor functions (Fleming *et al.*, 2004). Findings from the present study revealed a significant increase in movement initiation score in the 5000 mg/kg AMLE-treated rats when compared to the control, thus indicating the possibility of toxicity at higher doses.

Antioxidants are natural compounds present in the body or various food supplements, which play a crucial role in mitigating the debilitating effects of oxidative stress and promoting overall physiological well-being (Enogieru *et al.,* 2018; Mamta *et al.,* 2014). Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are essential antioxidant enzymes that work together to provide a robust defence system against oxidative damage caused by reactive oxygen species and free radicals (Enogieru and Idemudia, 2024; Enogieru and Momodu, 2022). SOD catalyzes the breakdown of superoxide radicals into hydrogen peroxide and oxygen molecules, thereby mitigating cellular damage (Bhattacharya, 2015). CAT protects cells by breaking down hydrogen peroxide into water and oxygen molecules, thereby preventing its accumulation and potential toxicity (Andrés *et al.,* 2022). Consequently, the enzyme activities of SOD and CAT can serve as common biomarkers for assessing oxidative stress levels within cells (Enogieru and Idemudia, 2024). Reduced activities of SOD and CAT enzymes have been implicated in the lipid peroxidation process, which is a key indicator of oxidative damage (Karoui-Kharrat *et al.,* 2017). Findings from the present study showed a significant decrease in SOD activity, and a significant increase in MDA concentration in the 5000 mg/kg AMLE-treated rats when compared to the control, suggesting the induction of oxidative stress in the experimental rats treated with 5000 mg/kg AMLE.

The examination of tissue structure at the microscopic level is pivotal for the advancement of biomedical and clinical research by providing critical insights into tissue morphology and organization at the microscopic level, which informs research and clinical applications (Gurcan *et al.,* 2009). Histological examination is a gold standard for the diagnosis of many pathological diseases, for which staining is an essential component (Gurina and Simms, 2020). Histological findings from this study revealed that rats administered 100 mg/kg, 1000 mg/kg, and 2500 mg/kg BW of AMLE exhibited a normal cerebellar microstructure, with clear preservation of the Molecular, Purkinje, and Granular cell layers, comparable to the control group. However, very few degenerating Purkinje cells were observed in the Purkinje layer of the cerebellum of rats treated with 5000 mg/kg BW of AMLE, indicating that higher doses of the extract could alter the microstructure of the cerebellum.

Conclusion: This study demonstrates that *Annona muricata* is not toxic to the experimental rats at lower doses following comparison to control, however, doses of 5000 mg/kg and higher could be neurotoxic to the cerebellum, consequently causing locomotor and exploratory impairments. Further studies are needed to investigate the possible neuroprotective activity of *Annona muricata* against experimental

neurodegenerative models, particularly at lower doses.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability Statement: Data are available upon reasonable request from the corresponding author.

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