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Histochemical and Histomorphological Evaluation of Cerebellar Cortical Cells of Adult Wistar Rats Following the Administration of *Acanthus montanus* Ethanol Leaf Extract

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ABSTRACT

Acanthus montanus is a shrub used in traditional medical practice as a herbal remedy for pains. This study investigated the effect of consuming leaf extract of this plant on neuronal histology and Nissl substance in the cerebellum of adult Wistar rats. Eighteen adult Wistar rats weighing an average of 185 g were randomly divided into three groups of six rats each: A control received appropriate volumes of distilled water, and two test groups administered 200 mg/kg and 500 mg/kg of *Acanthus montanus* extract. The administration was done with an orogastric tube for 14 consecutive days. After 24 hours of the last administration, animals were anaesthetised with 20 mg/kg ketamine hydrochloride, intraperitoneally, and humanely sacrificed. Their cerebella were dissected out, processed, and stained with haematoxylin and eosin and Cresyl fast violet, as well as neuron-specific enolase immunoreactivity to investigate neuronal integrity. The cells were counted with ImageJ software, and the data was analysed with SPSS version 25.0. In the 200 mg/kg group, cerebellar cortex hyperplasia was observed in the granular layer. In the 500 mg/kg group, hyperplasia was observed in the granular, molecular, and Purkinje layers. Hypertrophy of Purkinje cells was observed in the 200 mg/kg group compared with other groups. Nissl substance increased in the 200 mg/kg group, and positive expression of neuron-specific enolase reactivity was observed in the test groups. In conclusion, these changes in histology and histochemistry suggest that *Acanthus montanus* extract has a negative effect on cerebellar cytology. As a result, long-term use may not be beneficial.

Keywords

Acanthus montanus, Cerebellar cytology, Cerebellar histochemistry

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INTRODUCTION

There is an increasing awareness and interest in the use of plants as medicine and food rather than synthetic ones by the majority of the population in developing countries like ours, especially with the increasing inflation in the country and economic recession globally. Despite their popularity, many herbal medicines and nutraceuticals lack scientific validation and need proper usage monitoring to avoid serious health risks (Santini *et al.*, 2018). They sometimes pose grave health challenges.

Apart from providing between 30 to 40% of today's conventional drugs, the medicinal and curative properties of various plants are also employed in herbal supplements, botanicals, and teas (Adesina *et al.*, 2017). Different plant species with useful nutritional and medicinal benefits are abundant in Africa. About 80% of the people in developing countries rely on folk medicine to treat health conditions because of familiarity, availability, and cost-effectiveness (Okaiyeto and Oguntibeju, 2021).

Acanthus montanus is a shrub in the Acanthaceae family that grows in Africa, Romania, Greece, and Mediterranean countries (Ale *et al.*, 2021). It is commonly known as false thistle, leopard's tongue, bear's breech, and white ginger. In Nigeria, its local names include Ahun ekun dudu (Yoruba), Gautar fadama (Hausa), Nyin-yiogwu or

Agamsoso (Igbo) (Oshadu *et al.*, 2022), and Mbara ekpe (by the Efik and Ibibio). It is one of the most important species of the Acanthus family (Matos *et al.*, 2022).

Bioactive substances like alcohol, hydroxybenzoic acids, flavonoids, triterpenoids, alkaloids, steroids, fatty acids, phenols, saponins, and phytate oxalate have been found in the leaf of *Acanthus montanus* (Okiemen *et al.*, 2018; Nwachukwu *et al.*, 2020; Matos *et al.*, 2022; Ndukwe*et al.*, 2023). It has a wide range of medicinal uses and biological activities, which include antimicrobial (Igwe *et al.*, 2014; Ndukwe *et al.*,, 2023), antiulcer (Okorie *et al.*, 2023), hypolipidermic (Onuoha *et al.*, 2018), hypoglycemic (Ukwe *et al.*, 2011), anti-inflammatory and analgesic (Odoh *et al.*, 2010), antinociceptive, and hepatoprotective properties (Matos *et al.*, 2022). The methanol extract is rich in flavonoids and has good antioxidant activity (Igwe *et al.*, 2014). Olasunkanmi and Adebayo (2021) also reported its use in treating inflammatory conditions, gonorrhoea, syphilis, and boils.

Acanthus montanus may affect the cerebellum, the largest part of the hindbrain. The cerebellum is a tightly folded organ with grey matter located on the surface and the white matter underneath it. The white matter embeds several deep nuclei and is responsible for motor coordination (D'Angelo, 2018). It coordinates gait and maintains posture, controls muscle tone, and voluntary muscle activity (Jimsheleishvili and Dididze, 2023). It is also involved in motor learning and plays a role in cognitive function like language processing and memory. Damage to the cells of the cerebellum can lead to cerebellar disorders resulting in impaired motor control, posture, and language, causing balance problems, gait disorders, and difficulty in coordination (Knierim, 2020).

Nissl substance, or granule, is a part of neurons that makes up the endoplasmic reticulum. It is unique to cells in the cerebellum and other parts of the brain. Nissl substance is important for protein synthesis, growth, and repairs of neurons and is necessary for nerve cell regeneration (Bhati *et al.*, 2023). Identifying neurons immunohistochemiclly include the neuron-specific enolase (NSE), an isoenzyme of the glycolytic enzyme enolase. that is useful in neural crest cell maturation. The gamma form of it is widely distributed in neurons (Wang *et al.*, 2018) and is a specific marker for neurons and neuroendocrine cells as well as tumours arising from them (Jones *et al.*, 2017). NSE activity can be measured to assess brain injury and some neurological insults (Janardhan *et al.*, 2018). The gamma enolase is widely recognised and a reliable marker for neuronal function (Horvat *et al.*, 2024).

This study was to investigate the possible side effects of *Acanthus montanus* use on the cerebellar cortex, as there is not much information on this area. And so it will provide scientific data on the potential effect the plant may have on the users while managing other health conditions, including joint pains, ulcers, and inflammation. The information will raise awareness among users, especially the elderly, who may be vulnerable to other cerebellar-related disorders.

MATERIALS AND METHODS

Plant Extract and Administration

Acanthus montanus leaves were obtained from a farm in Calabar municipality and authenticated in the Department of Botany, University of Calabar with a voucher number, Bot/Herb/UCC/356. The leaves were washed, air dried, blended to fine substances to obtain 700 g of the powdered material of *Acanthus montanus* which was immersed in absolute ethanol, well shaken and left for 48 hours to dissolve properly which was then extracted (Syahputra *et al*., 2021). The filtrate was evaporated to dryness in a water bath at 40 ℃. The obtained paste was weighed yielding 10.2 g which was kept in the refrigerator.

Duration and Route of Administration

The extract solution was administered at 200 mg/kg and 500 mg/kg once daily using orogastric tubes for fourteen days.

Experimental Animals

Animal Grouping

Eighteen adult Wistar rats, weighing between 150 and 220 g, were obtained from the animal house of the College of Medical Sciences, University of Calabar. They were randomly grouped into three groups of six rats each and kept under standard conditions. The control group received feeds and water ad libitum with equivalent volumes of distilled water as the extract for the test groups. The test groups were administered 200 mg/kg and 500 mg/kg of *Acanthus montanus* extract. The administrations were done using the orogastric tube once daily for fourteen days.

Animal Sacrifice and Sample Collection

At the end of the administration, the animals were anaesthetised with a 20 mg/kg ketamine hydrochloride (from Pfizer, USA) injection intraperitoneally. They were perfused with 10% buffered formalin by passing the fluid through the left ventricle. Each of the skulls was later opened, and the cerebellum was removed, preserved in the same fixative for 7 days, and subsequently processed for further analysis using the methods stated below.

Tissue Processing

Cerebellar tissues were processed using the routine paraffin-embedded histological procedure, sectioned at 5 µm with a rotary microtome, and stained with haematoxylin and eosin for the cerebellar cytoarchitecture. Histochemical staining was done using the cresyl fast violet staining method for Nissl substance (Suvarna *et al.*, 2019).

For the immunohistochemical staining, tissues were sectioned at a thickness of 4 µm using a rotary microtome. The sections were deparaffinized in xylene, rehydrated in descending concentrations of alcohol, washed in water, and then in citrate buffer solution. Heat antigen retrieval in citrate buffer was carried out, and 3% hydrogen peroxide was used as the blocking solution, Tris buffered saline as the wash buffer. The primary antibody used was a mouse monoclonal antibody, while the secondary antibody was

horse radish peroxidase from Vitro Master Diagnostica, Turkey, and diaminobenzidine was the chromogen used. Sections were counter-stained with haematoxylin, stabilised with distrene plasticiser xylene as a mounting medium, and viewed under the microscope (Abcam 2013). The presence of brown stains on sections denotes a positive expression of NSE.

Stereological Method

The number of cells in the layers of the cerebellum was counted using ImageJ software, an open-source image processing software developed by the National Institutes of Health (Schneider *et al.*, 2012; Rasband, 2018). The volume of Purkinje cells was determined using Cavalieri's method. After five random counts, the cells were picked for counting.

Statistical Analyses

Data were presented as mean + standard error of mean. One-way analysis of variance was used to determine the significant difference between groups. The statistical package Statistical package for Social Sciences (SPSS, version 25.0) was used for the statistical analysis, and Fisher's least significant difference was used as the post hoc test.

RESULTS

Histological Observation

The sections from the control group showed normal cerebellar cortex histology with the three distinct well-delineated layers. Cells in the molecular layer of the 200 mg/kg *Acanthus montanus* group were not affected: However, there was hypertrophy of the Purkinje cells and an increase in the number of cells in the granular cell layer of this group compared with the control. There was an increase in the number of cells in all the layers of the cerebellar cortex in the 500 mg/kg *Acanthus montanus* group compared with the control group (Fig. 1).

Histochemical Observation

The sections of cerebellar cortex from the control group showed the presence of Nissl substance indicated by the blue stain, which serves as the marker for Nissl substance distribution, while sections from the 200 mg/kg *Acanthus montanus* group had increased intensity of the Nissl substance. In the 500 mg/kg *Acanthus montanus* group, there was less Nissl substance (less stain intensity) on the sections compared to the control group (Fig. 2).

Immunuhistochemical Observation

The sections from the control group did not have any brown staining from NSE, which was seen in the sections of cerebellar cortex from the test groups. The brown stain indicated a positive expression of the enzyme. The 200 mg/kg *Acanthus montanus* extract group had a positive expression of NSE. Sections from the 500 mg/kg *Acanthus montanus* extract group had a weak expression of the enzyme indicated by the reduced brown colouration (reduced stain intensity) compared with the 200 mg/kg *Acanthus montanus* group (Fig. 3).

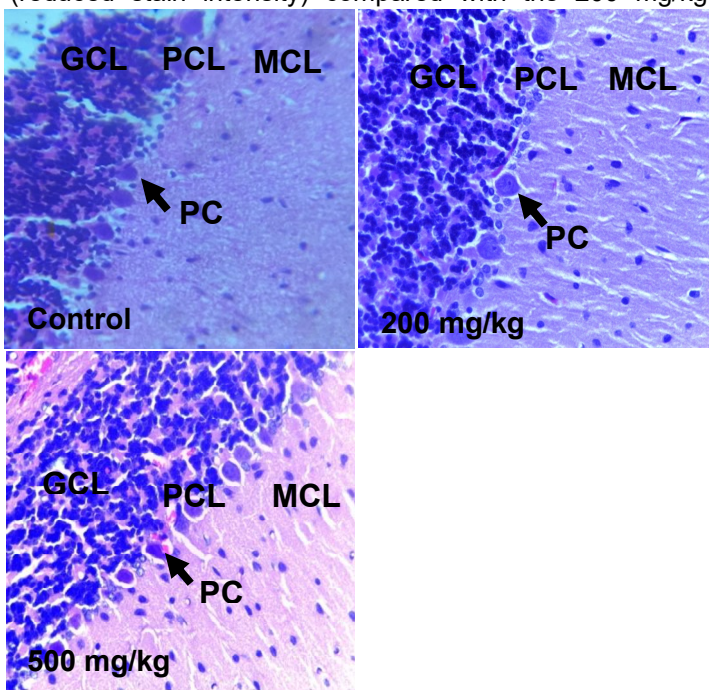

Fig. 1: Photomicrographs of the cerebellar cortex from experimental animals. The control group exhibits normal histology, displaying three distinct layers: GCL, the granular cell layer; PCL, the Purkinje cell layer; and MCL, the molecular cell layer. Sections from the 200 mg/kg *Acanthus montanus* group show hypertrophy of Purkinje cells (PC) and hyperplasia of cells in the GCL. The 500 mg/kg *Acanthus montanus* group shows hyperplasia of cells in the GCL. H&E Stain, ×400.

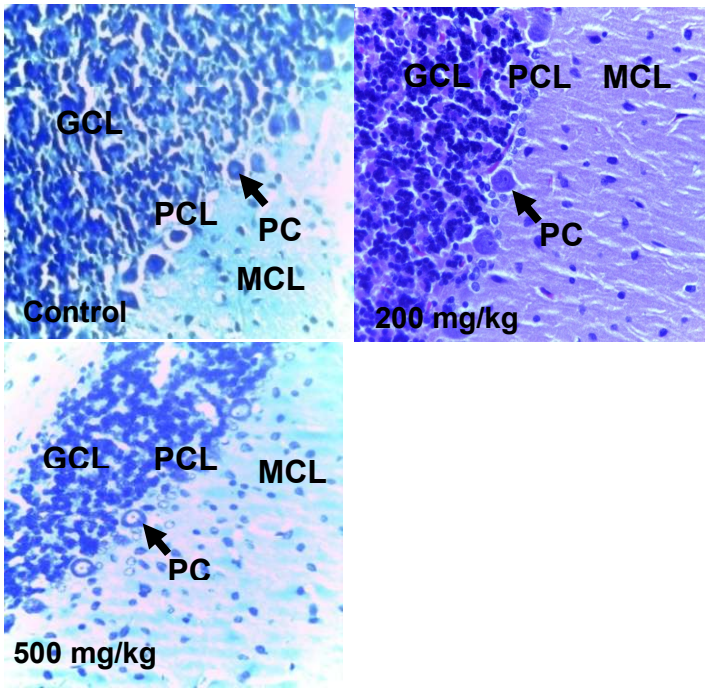

Fig. 2: Photomicrographs of the cerebellar cortex from experimental animals. Control group section shows the presence of Nissl substance (blue stain). The 200 mg/kg *Acanthus montanus* group has increased blue stain intensity (a marker for the Nissl substance) on the section. The 500 mg/kg *Acanthus montanus* group shows a reduced cerebellar stain intensity

compared with the control group. VPC = vacuolated Purkinje cell. GCL=Granular cell layer, ML=Molecular layer, PC=Purkinje cell; cresyl fast violet stain, ×400

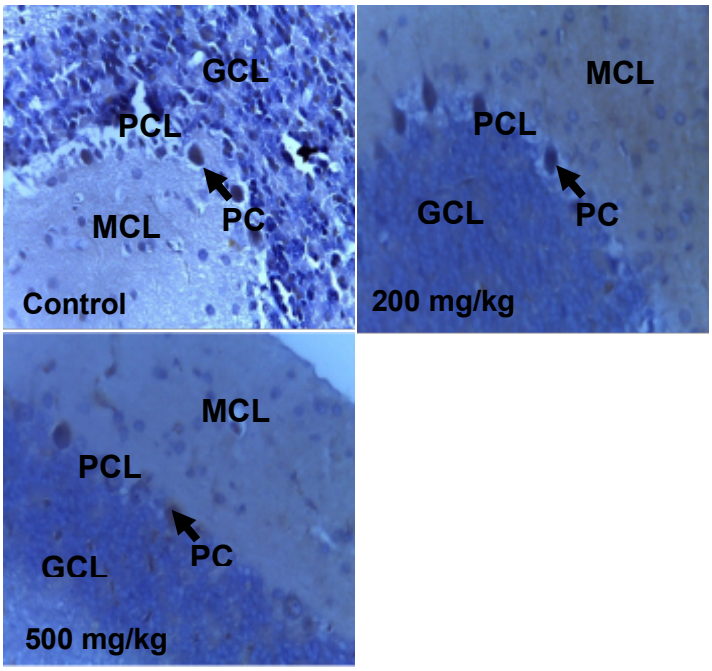

Fig. 3: Photomicrographs of the cerebellar cortex showing NSE immuno-expression. The section from the control group has a negative expression of NSE (no brown stain seen). The 200 mg/kg *Acanthus montanus* group shows a positive expression of NSE indicated by the brown stain (BS). Sections from the 500 mg/kg *Acanthus montanus* group shows a brown colour having less stain intensity compared with the 200 mg/kg group. PC = Purkinje cell; NSE (neuron-specific enolase); ×400.

Stereological Result

It was observed that the number of cells in the molecular layer of the cerebellar cortex from the 200 mg/kg *Acanthus montanus* group was not affected compared with the control. The number of Purkinje cells in the 200 mg/kg *Acanthus montanus* group was significantly reduced. In the 500 mg/kg *Acanthus montanus* group, the number of cells in the three layers of the cerebellar cortex was significantly increased ($p < 0.05$) compared with the control (Table 1).

The volume of Purkinje cells was significantly increased in the 200 mg/kg *Acanthus montanus* group but significantly reduced in the 500 mg/kg group compared with the control group ($p < 0.05$) (Table 2).

Table 1: Cell counts in the different layers of the cerebellar cortex

Layers	Control	*Acanthus montanus* (200 mg/kg)	*Acanthus montanus* (500 mg/kg)	p-value
Molecular	47.20± 0.20	47.60± 0.40	56.80± 0.37[a,b]	<0.001
Purkinje	6.40± 0.24	5.40± 0.24[a]	8.60± 0.24[a,b]	<0.001
Granular	367.80± 0.37	397.20± 0.37[a]	480.80± 0.58[a,b]	<0.001

Values are expressed as mean ± SEM, n = 5.
a = significantly different from Control group at p<0.05.
b = significantly different from 200 mg/kg of *Acanthus montanus* group at p<0.05.

Table 2: The volume of Purkinje cells in the cerebellar cortex
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Groups	Volume of Purkinje cells (μm^3)
Control	22390.74 ± 1549.01
Acanthus montanus (200 mg/kg)	25402.65 ± 1203.16
Acanthus montanus (500 mg/kg)	20270.95 ± 800.26[b]
p-value	0.045*

Values are expressed as mean ± SEM, n = 5.
* = significant at $p < 0.05$; a = significantly different from control group at $p < 0.05$; b = significantly different from 200 mg/kg of *Acanthus montanus* group at p<0.05

DISCUSSION

This study investigated the effect of *Acanthus montanus* leaf extract administration on the cerebellar cortex microstructure in rats. The 200 mg/kg *Acanthus montanus* group had mild hyperplasia of cells in the granular cell layer of the cerebellar cortex. The 500 mg/kg *Acanthus montanus* group had hyperplasia of cells in all layers. These suggest that the extract may have phytochemical constituents that are capable of inducing cell proliferation. *Acanthus montanus* contains hydrogen cyanide, tannins, saponins (Nwachukwu *et al.*, 2020), flavonoids, alkaloids, polyphenols, and steroids (Ndukwe *et al.*, 2023). Flavonoids have been reported to induce the differentiation of stem cells into neurons and 'stimulate stem cell proliferation, migration, and survival' (Lofti and Kalalinia, 2023). This reaction may be an attempt by cells to overcome the extract's toxicity. *Acanthus montanus* leaf extract has anti-inflammatory potential proposed to be due to its inhibitory effect on nitric oxide (Foyet *et al.*, 2008). As such, it could adversely affect the cerebellum, given the role of nitric oxide signals in the molecular layer of the cerebellar cortex (Larson *et al.*, 2020). Although nerve cell regeneration is minimal, different types of stem cells from different tissues have been used together with natural stimuli to manage neurological disorders in neuronal tissue engineering (Lofti and Kalalinia, 2023). The phytochemicals of *Acanthus montanus* may have induced endogenous stem cells to proliferate. Research on the implication of phytochemicals and stem cell differentiation for the management of neurological disorders has been documented (Choudhary *et al.*, 2021; Safitri *et al.*, 2024). However, the observed results lack a clear explanation, prompting the need for further research.

An increase in the number of cells in the granular layer may have caused the hypertrophy of Purkinje cells in the 200 mg/kg *Acanthus montanus* group. Granular neurons are potent regulators of Purkinje cell development and can influence Purkinje cell survival (Baptista *et al.,* 1994; Morrison and Mason, 1998). Purified Purkinje cells survived and differentiated poorly, but when co-cultured with granular neurons, they improved survival and triggered dendritic differentiation (Baptista *et al.*, 1994).

These authors attributed their findings to a balance between neurotrophins and activity-dependent signalling. A previous study on Purkinje cell pathology demonstrated that these cells exhibit significant structural plasticity, including hypertrophy, in response to injury. This suggests that they can adapt morphologically to compensate for the loss of other neurons, thereby maintaining cerebellar functions despite cell death (Kemp *et al.*, 2016). Purkinje cells represent the sole output neuron of the cerebellar cortex, and thus changes in their structure may affect function, thus having a significant impact on the cerebellum as a whole (Redondo *et al.*, 2014). The cerebellum modulates motor neurons and thereby controls proprioception. A dysfunction may lead to a gait disorder with difficulty in coordination and impaired motor function (Knierim, 2020).

The weak cresyl fast violet stain intensity in the 500 mg/kg *Acanthus montanus* group suggests a reduction or loss of Nissl substance in the neurons, probably due to insults from the extract. Nissl substances are responsible for protein synthesis. Nissl substance are necessary for neuronal growth and synaptic plasticity and, as such, are important for the regeneration of injured axons and brain healing. Loss of Nissl substance may consequently affect protein synthesis, leading to impaired nerve function, loss of synaptic plasticity, neuronal degeneration, and death (Bhati *et al.*, 2023). The strong cresyl fast violet stain intensity in the 200 mg/kg *Acanthus montanus* group may be attributed to cell proliferation, probably induced by proliferating endogenous stem cells (Lofti and Kalalinia, 2023): Consequently, more cells picked up the stain. Although there is limited information on adult neurogenesis in the cerebellum (Ahlfeld *et al.*, 2017), the present result may suggest this.

NSE is an intracellular enzyme that catalyses the conversion of glucose into pyruvate, which releases adenosine triphosphate (Vizin and Kos, 2015). It is one of the enzymes of the glycolytic pathway, which the gamma form is specifically located in neurons (Isgro *et al.*, 2015; Jones *et al.*, 2017; Babkina *et al.*, 2024). NSE's activity can serve as a marker for neuronal damage in brain injury. The cerebellar cortex of rats from the control group did not express this enzyme, suggesting a normal neuronal condition. Neuronal cells in the 200 mg/kg *Acanthus montanus* group expressed NSE in their cytoplasm, suggesting a neuronal injury, impaired neuronal activity, or a neurodegeneration. A similar result was reported on rats' cerebellum after administration of tramadol (a pain reliever) and coffee (Umanah *et al.*, 2024).

The weak expression of NSE in the 500 mg/kg *Acanthus montanus* group suggests an adaptive survival process leading to neuroprotection. Although NSE activity is a key biomarker for neurodegenerative diseases and neurological conditions, it is also necessary in nerve cell formation and neuroprotection, exhibiting neurotrophic-like properties (Horvat *et al.*, 2024). NSE has similar characteristics as neurotrophins, which are essential for the development, survival, and functions of neurons. Therefore, it is critical for the growth and protection of neurons and has been suggested as a possible treatment for neurodegenerative diseases (Horvat *et al.,* 2024). Neuronal injury can affect intracellular enzymes, which in turn will affect their ability to form high-energy compounds of adenosine triphosphate and other aspects of neuronal functions since NSE is important in glycolysis and cell metabolism, especially during critical ill health (Babkina *et al.*, 2024).

The mechanism of action of *Acanthus montanus* is therefore proposed to be through the interaction of its phytochemical constituents, like the polyphenols, flavonoids, steroids, and alkaloids, with some signalling pathways, stimulation of endogenous stem cells, and inhibition of nitric oxide.

Conclusion

Acanthus montanus leaf extract induced histomorphological alterations in the cerebellar cortex 200 mg/kg group and in all its layers in the 500 mg/kg group. It increased the Nissl substance and positive expression of NSE in the 200 mg/kg group, suggesting neuronal pathology. The decreased Nissl substance concentration and NSE expression in the 500 mg/kg group suggest a cell adaptative process and recovery.

DECLARATION

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None was received

Conflict of Interest

None declared.

Ethical Approval

Ethical approval for the study was obtained from the ethical committee of the Faculty of Basic Medical Sciences, University of Calabar with approval number 150-1/2023.

Consent to Participate and Publish Data

Not Applicable.

Authors' Contribution

AOA: Conceptualisation, methodology, supervision, draft and final preparation; GOD: Methodology, collection of data, draft and final preparation; TEI: Methodology, and editing; MRA-E: Software and analyses.

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