World Journal of Biology Pharmacy and Health Sciences

eISSN: 2582-5542

Cross Ref DOI: 10.30574/wjbphs

Journal homepage: https://wjbphs.com/

(Research Article)

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Evaluation of the effects of different cassava (*Manihot esculanta*) processing methods on locomotor, anxiety-like and nesting behaviors in Swiss mice

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World Journal of Biology Pharmacy and Health Sciences, 2026, 26(01), 185-194

Publication history: Received on 12 March 2026; revised on 19 April 2026; accepted on 22 April 2026

Article DOI: https://doi.org/10.30574/wjbphs.2026.26.1.0199

Abstract

Cassava (*Manihot esculenta*) is a major food source consumed by millions of people, especially in the tropics. Processing methods affect the concentration of cyanogenic glycosides in its products. Effects of its processing methods on locomotor, anxiety-like, and nesting behaviors in mice were investigated. Thirty-five mice were randomly divided into 5 groups: control, fermented + palm oil, fermented without oil, unfermented + oil and unfermented without oil, with 7 rats/group. Duration of daily feeding with the different cassava products was 8 weeks. Neurobehavioral assessments were conducted using the Open Field Maze, Elevated Plus Maze, and nesting setup. Significant level was P<0.05. Results showed a significant increase in grooming in the unfermented without oil group compared with the other groups and a decrease in rearing frequency in all cassava product-fed groups compared with the control. Stretch attend posture was significantly higher in the unfermented groups than in control and fermented groups, and also higher in the unfermented without oil than in unfermented+oil groups. Line crossing was significantly reduced in all products-fed groups compared with control. Open arm entry frequency was significantly lower in the fermented+oil than in control and higher in the fermented without oil and unfermented+oil groups than in the fermented+oil group. Duration of open arm entry was significantly reduced in both fermented groups and in unfermented without oil group compared with control. Close arm entry duration was significantly higher in the fermented groups and unfermented without oil than in the control groups. Nesting behaviour showed no significant difference. We conclude that both fermented and unfermented cassava products with/without palm oil, may be anxiogenic and affect locomotor activity. This effect is worse with unfermented products.

Keywords: Cassava;processing; methods; Anxiety-like; locomotor; Nesting; behaviors

1. Introduction

Cassava (*Manihot esculenta*) is a widely cultivated and consumed root crop that serves as an essential food source for millions of people in tropical and subtropical regions of the world [1]. Cassava tubers contain many substances of nutritional value. It contains cyanogenic glycosides which though, naturally-occurring, can be converted to a highly toxic compound, hydrogen cyanide on hydrolysis. The processes involved in the preparation of cassava products like peeling, cutting, grating or boiling cause enzymatic hydrolysis of primary cyanogenic compounds like lotaustralin and linamarin into hydrogen cyanide [2]. Cassava also contains carbohydrates which make up to 80-90% of its dry weight, low amount of proteins (<2%), low fat (<0.5%) as well as micronutrients like vitamins A and C, iron, zinc and fibre [3, 4].

Hydrogen cyanide is a potent mitochondrial toxin and is rapidly absorbed through the gastrointestinal tract, lungs and skin. The primary mechanism of cyanide toxicity involves inhibition of cytochrome C oxidase (complex IV) in the mitochondrial electron transport chain which disrupts the final stage of oxidative phosphorylation leading to tissue

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hypoxia, and ultimate cell death [5]. Chronic exposure to cyanide often from consumption of cassava has been said to have health implications including cardiac arrest, hypotension, loss of consciousness, konzo, an irreversible upper motor neuron disease; tropical ataxic neuropathy, a progressive neurological disorder associated with unsteady gait [6] and hypothyroidism [7]. Exposure to cyanide has also been said to induce oxidative stress and to promote lipid peroxidation. The accumulation of reactive oxygen species triggers inflammatory cascades and apoptosis [8].

Processing methods used in the preparation of cassava products are known to alter the cyanide content of such products. These processes include boiling, fermentation, drying, roasting/frying, and soaking. Boiling of peeled cassava at high temperature results in hydrolysis and leaching of the water-soluble cyanogenic compounds into the surrounding water, thereby reducing the cyanide content of the product [9]. Fermentation of cassava [2, 10, 11] and washing, frying, grating, addition of palm oil and pressing of cassava mash as done traditionally, are also known to reduce the cyanide content of cassava products [12, 13]. Cassava is processed into several cassava products including fufu, garri, African salad, chips , flakes etc [14, 15].

Palm oil is a widely consumed vegetable oil extracted from the mesocarp of the fruit of the tropical oil palm tree, *Elaeis guineensis* [16]. Palm oil is usually a mix of saturated and unsaturated fatty acids, with palmitic and Oleic acids being the major components. It also contains Co-enzyme Q10, ascorbic and stearic acids as well as potent antioxidants, carotenoids, tocopherols and tocotrienols which are precursors of vitamins A and E respectively [17], Omega-3 and Quercetin [18].

Locomotor behaviors refer to the coordinated physical movements an organism makes to navigate its environment, including walking or exploratory activities and are primarily controlled by the central nervous system particularly motor circuits in the brain and spinal cord [19]. Disruptions in central nervous system function from neurotoxicity, neurodegeneration or dietary factors can impair motor function and reduce spontaneous locomotion [20].

Anxiety is an emotional and physiological response to perceived threats or stress and characterized by heightened alertness, restlessness and sometimes avoidance behaviour and is regulated by the limbic system including the hypothalamus, amygdala, hippocampus and prefrontal cortex [21].

Since hydrogen cyanide induces neurotoxicity in part due to oxidative stress, its content in cassava products influenced by processing methods and the fact that palm oil contains compounds that have demonstrated strong antioxidant property [18, 22], we investigated possible effects of different cassava products on some neurobehavioral parameters. The parameters investigated were locomotor, anxiety-like and nesting behaviours in Swiss mice.

2. Materials and methods

2.1. Experimental animals

Thirty five male adult Wistar rats were housed in well ventilated plastic cages in the Animal House of the Department of Physiology, University of Calabar under a 12 hour light/dark cycle and allowed access to rodents feed and water ad libitum.

2.2. Experimental design

Animals were acclimatized for one week and then randomly divided into 5 groups of 7 mice each. Group 1 served as control and fed with rat chow, group 2 was fed with fermented cassava product (garri) mixed with palm oil (fermented+oil) while group 3 was fed with fermented plain cassava (fermented without palm oil) product. Group 4 was fed with unfermented cassava product mixed with palm oil (unfermented+oil) while group 5 was fed with unfermented plain cassava product (unfermented without oil). Animals in groups 2, 3, 4 and 5 were allowed free access to their respective modified rat feed composed of mixture of rat feed and individual cassava products for 8 weeks.

2.3. Preparation of processed cassava products (GARRI)

The traditional method of preparing this cassava product was used. In brief, fresh cassava tubers were harvested from a local farm, after which they were peeled and washed with clean water to remove dirt and contaminants. The tubers were then grated into fine mash which was divided into two portions. One portion of the cassava mash (unfermented) was placed in a porous bag and dewatered under a presser for 6 hours. It was then sieved and roasted in a metal pot to produce granules called 'garri'. While half of this unfermented portion was roasted without addition of oil, the other half was mixed with 30g oil/100g of the sieved dry mash and then roasted. The other portion of the mash (fermented) was kept/pressed for 72 hours and then treated in a similar way as described above for the first portion by adding oil to only one part of it, altogether producing 4 products: fermented+oil, fermented without oil, unfermented+oil and unfermented without oil.

2.4. Constitution of modified animal feeds

The various forms of the cassava product (garri) were each mixed with equal amount of rat feeds and given to the rats according to their groups.

2.5. Determination of locomotor and anxiety-like behaviours

These were conducted in the Neuro-behavior Laboratory of the Department of Physiology, University of Calabar, using the Open Field Maze (OFM) and Elevated Plus Maze (EPM) apparatus. Illumination was by a 60 Watt light bulb.

2.5.1. Open Field Maze test

The open field maze test provides simultaneous measurement of locomotion, exploration and anxiety in rodents [23]. The apparatus was constructed following the description by [23]. It was made of plywood and measured 72 x 72 × 36cm^3. The walls and floor were both painted white. Red lines were drawn on the floor to divide the floor into sixteen 18 x 18 cm squares. A central square of equal size was drawn in the middle of the open field (18 x 18cm) and the floor was covered with a 72 x 72 cm piece of clear Plexiglas.

Procedure

This was carried out as demonstrated by Brown et al., [24] and also used by Siebenhener and Wooten [23]. In brief, each mouse in turn was scooped up from its home cage and placed at the centre square of the open field apparatus, then allowed to explore the apparatus for 5 minutes and its behaviour in the maze observed and recorded. After 5 minutes, the mice were returned to their home cages and the open field maze cleaned with cotton wool soaked with 70% ethyl alcohol (Methylated spirit) and allowed to dry before introducing another animal for exploration. Behaviors observed and recorded were line crossing, rearing, stretch attend posture and grooming.

- Line crossing refers to the frequency with which each mouse crossed one of the grid lines with all four paws.
- Rearing is the frequency with which the mice stood on their legs in the maze without aid from the wall of the maze.
- Stretch attend posture refers to the frequency with which the mice demonstrated a forward elongation of the head and shoulder followed by retraction to its original position. Stretch attend posture is a “risk-assessment” behavior which indicates the animal's resistance to move from its present location to a new position [25].
- Grooming refers to the frequency and duration which the animal spent licking or scratching itself while stationary.

2.5.2. Elevated Plus Maze

This apparatus is used to test anxiety and exploratory activity in rodent [26, 27]. It is based on rodents natural aversion to elevated and open spaces and their preference for closed against open arms [28]. The apparatus consists of two “open” and two “closed” arms in the shape of a 'plus' (+). The open arms are aversive to mic. The close arms provide a sense of safety because they are enclosed. The maze was built according to the description lby Walf and Frye [26]. The maze has two open arms (45x5cm^2) with slight edges of 0.25cm to prevent the mice from slipping and falling off and two closed arms (45x5cm^2) with 15 cm high walls, all four arms radiating from a central square (5x5cm^2). This task exploits the conflict between the natural tendency of mice to explore the novel areas and fear of open spaces and height [29].

Procedure

One mouse at a time was taken out of its home cage and placed at the squared center, the junction of the open and closed arms facing the open arm opposite to where the experimenter stood and a stopwatch started immediately. The behavior of the mouse upon placement was monitored with a video camera for 5 minutes [26]. Prior to commencement of a procedure, the Maze was cleaned with ethanol and allowed to dry to eliminate olfactory cues and other contaminants. Behaviours monitored were: Open arm entry frequency and duration, close arm duration, grooming and stretch attend posture.

- Open arm entry frequency is the number of times a rodent entered the open arms of the maze with all four paws.

- Open arm duration is the total length of time that the mouse spent in both open arms.
- Close arm entry duration is the total length of time a mouse spent in the closed arms of the maze with all four paws.

2.6. Nesting behavior

The nesting behavior test is a simple and natural rodent assay used to evaluate animal social and cognitive decline by assessing the quality of a nest built from material (papers, cotton etc) provided to the animal to shred and formed into a nest over hours. The resulting nest is then scored on the Deacon 5-point scale (1-5), ranging from no nesting activity to a perfect nest with high walls covering the mouse [30, 31] as shown in Table 1.

2.6.1. Procedure

The mice were housed individually and tested in their home cages. One hour before giving the mice nesting materials, all enrichment objects in the home cages of the mice were removed. About 3.0g of the nesting material was supplied to each mouse in its home cage and allowed for 24 hours. Twenty four hours later, the nests were assessed using the rating scale supplied by Deacon [30]. The unshredded nesting material was weighed in order to calculate the percentage of the nesting materials used for building the nest.

Table 1 Nesting behavior rating scale

Rating	Requirements
1	Nestlet not noticeably touched (90% or more intact)
2	Nestlet partially torn (50-90% intact)
3	Nestlet mostly shredded, often no identifiable nest site, 50-90% shredded, also, less than 50% remains intact, but less than 90% is within a quarter of the cage floor (i.e., not gathered into a nest site but spread throughout cage)
4	An identifiable, but flat nest, more than 90% of the nestlet is torn, the nest is uneven, material is gathered into a nest within a quarter f the cage floor, but the nest is flat with walls higher than mouse body height for less than 50% of its circumference
5	A (near) perfect nest, more than 90% of the nestlet is torn, nest is fairly even, the nest is a crater, with walls higher than the mouse body for more than 50% of its circumference

2.7. Statistical analysis

Data collected from the study were expressed as mean ± SEM and analyzed using Analysis of variance (ANOVA) followed by a Post hoc Tukey test of least significant difference. A p-value of < 0.05 was considered statistically significant.

3. Results

Comparison of neurobehavioral parameters

3.1. Open Field Maze test

3.1.1. Frequency of line crossing

The frequencies of line crossing (/5min) for control, fermented + oil, fermented without oil, unfermented + oil and unfermented without oil groups were 97.86 ± 10.68, 31.43 ±4.98, 53.14 ± 10.96, 62.00 ± 14.26 and 42.71 ± 9.99 respectively. It was significantly ($p<0.05$) reduced in all cassava products-fed groups compared to the control as shown in Table 2.

3.1.2. Rearing frequency

The frequency of rearing (/5min) for control was 28.00 ± 2.60, fermented + oil was 10.00 ± 2.90; fermented without oil, 12.43 ± 2.08; unfermented + oil 15.14 ± 4.27 and unfermented without oil, 16.71 ± 4.13. It was significantly decreased in all cassava products-fed groups compared with the control as shown in Table 2.

3.1.3. Stretch attend posture

The frequencies of stretch attend posture (/5min) were 0.00 \ 0.00, 1.86 ± 0.40, 0.43 ± 0.43, 4.14 ± 1.79, and 6.43 ± 2.34 for control, fermented + oil, fermented without oil, unfermented + oil and unfermented without oil groups respectively. It was significantly increased in the unfermented products groups compared with the control and the fermented groups and also higher in the unfermented without oil group than in the unfermented+oil groups. This is shown in Table 2.

3.1.4. Grooming frequency

The frequencies of grooming (/5min) for control, fermented + oil, fermented without oil, unfermented + oil and unfermented without oil were 1.71 ± 0.57, 1.43 ± 0.30, 1.43 ± pm 0.30, 1.43 ± 0.20 and 2.86 ± 0.74 respectively. This was significantly elevated in the unfermented without oil group compared with the fermented groups and the unfermented+oil group as shown in Table 2.

3.1.5. Grooming duration

The mean durations of grooming (sec) were 5.42 ±**2.**75, 4.26 ± 0.86, 2.71 ± 0.70, 6.49 ± 1.72 and 10.63 ± 3.70 for the control, fermented + oil, fermented without oil, unfermented + oil and unfermented without oil groups respectively. This was significantly increased in the unfermented without oil compared with the fermented without oil groups as shown in Table 2.

3.2. Elevated Plus Maze test

3.2.1. Open arm entry (OAE) frequency

The OAE frequencies (/5 min) for the control, fermented+oil, fermented without oil, unfermented + oil and unfermented without oil groups were 0.80 ± 0.20, 0.50 ± 0.22, 1.63 ± 0.42, 0.67 ± 0.21 and 0.83 ± 0.40 respectively. It was significantly decreased ($P < 0.05$) in fermented + oil and unfermented without oil groups compared with the control though higher in both ferm without oil and unfermented + oil than in the fermented + oil groups. It was also significantly ($P < 0.05$) reduced in the unfermented without oil group compared with fermented without oil and unfermented + oil groups as shown in Table 3.

3.2.2. Open arm duration (OAD)

The open arm durations (sec) were 22.80 ± 10.39, 7.67 ± 4.89, 30.00 ± 8.35, 10.50 ± 3.77 17.50 ± 9.69 for the control, fermented + oil, fermented without oil, unfermented + oil and unfermented without oil respectively. It was significantly ($P < 0.05$) reduced in the fermented + oil, fermented without oil and unfermented without oil groups compared with control and also significantly higher ($P<0.05$) in the unfermented + oil group than in the fermented + oil and unfermented without oil groups.. It was also significantly lower in the unfermented without oil group compared with the unfermented + oil group as in Table 3.

3.2.3. Close arm entry duration (CAD)

The close arm entry durations (sec) for the control, fermented+oil, fermented without oil, unfermented+oil and unfermented without oil groups were 277.20 ± 10.39, 291.00 ± 4.76, 270.00 ± 8.35, 290.50/4.03 and 282.50 ± 9.69 respectively**. It** was significantly ($P < 0.05$) increased in fermented + oil, fermented without oil and unfermented without oil groups compared with control and significantly decreased in the unfermented + oil group compared with the fermented without oil and unfermented without oil groups. It was also significantly higher ($P < 0.05$) in the unfermented without oil than in the unfermented + oil group. This is shown in Table 3.

3.2.4. Grooming frequency (GF)

Grooming frequencies (/5 min) were 4.00 ± 1.30, 5.17 ± 0.31, 5.13 ± 2.78, 4.83 ± 1.38 and 12.67 ± 4.71 for the control, fermented+oil, fermented without oil, unfermented+oil and unfermented without oil groups respectively. The GF was increased ($P < 0.05$) in the fermented without oil group compared with control and reduced ($P < 0.05$) in the unfermented + oil than in the fermented without oil group. This is shown in Table 3.

3.2.5. Stretch attend posture (SAP)

The frequencies of SAP (/5 min) for the control, fermented+oil, fermented without oil, unfermented+oil and unfermented without oil groups were 35.00 ± 6.38, 46.33 ± 4.26, 31.63 ± 4.29, 26.00 ± 0.77 and 26.17 ± 4.21 respectively. It was significantly higher in the fermented+oil, unfermented+oil and unfermented without oil than in the control groups and elevated in the unfermented without oil compared with unfermented+oil groups as shown in Table 3.

3.3. Nesting score

The nesting scores for the control, fermented+oil, fermented without oil, unfermented+oil and unfermented without oil groups were 2.86 ± 0.26, 2.72 ± 0.18, 2.68 ± 0.40, 2.71 ± 0.20 and 2.29 ± 0.18 respectively. There were no significant differences among the groups as shown in Table 3.

Table 2 Comparison of anxiety-like and locomotor behaviors in the OFM test among different experimental groups.

Group	Line Crossing (/5min)	Rearing Frequency (/5min)	Stretch Attend Posture (/5min)	Grooming Frequency (/5min)	Grooming Duration (sec)
Control	97.86 ± 10.68	28.00 ± 2.60	0.00 ± 0.00	1.71 ± 0.57	5.42 ± 2.75
Fermented + oil	31.43 ± 4.98*	10.00 ± 2.90*	1.86 ± 0.40	1.43 ± 0.30	4.26 ± 0.86
Fermented without oil	53.14 ± 10.96*	12.43 ± 2.08*	0.43 ± 0.43	1.43 ± 0.30	2.71 ± 0.70
Unfermented + oil	62.00 ± 14.26*	15.14 ± 4.27*	4.14 ± 1.79*[a]	1.43 ± 0.20	6.49 ± 1.72
Unfermented without oil	42.71 ± 9.99*	16.71 ± 4.18*	6.43 ± 2.34*[ab]	2.86 ± 0.74[abc]	10.63 ± 3.70[b]

* = Significantly different from control at P < 0.05; a = Significantly different from fermented + oil at P < 0.05; b = Significantly different from fermented without oil at P < 0.05; c = Significantly different from unfermented + oil at P < 0.05

Table 3 Comparison of anxiety-like and locomotor behaviours in the EPM among experimental groups

	Frequency of OAE (/5min)	Duration of OAD (Sec)	Duration of CAD (Sec)	Frequency of GF (/5min)	Frequency of SAP (/5min)
Control	0.80± 0.20	22.80± 10.39	277.20± 10.39	4.00± 1.30	35.00± 6.38
Fermented+Oil	0.50± 0.22*	7.67± 4.89*	291.00± 14.76*	5.17± 0.31	46.33* ± 4.26
Frემented without Oil	1.63± 0.42a	30.33± 8.35*	27.00± 8.30*	5.13± 2.78*	31.63± 4.29a
Unfermented+Oil	0.67a± 0.21a	10.50*± 3.77ab	290.50± 4.03 ab	4.83± 1.38b	26.08± 0.77*b
Unfermented without Oil	0.83± 0.40*b,c	17.50± 9.69*c	282.50± 9.69*c	12.67± 4.71	26.17± 4.21*ab

* = Significance difference from control at p<0.05; = significant difference from fermented + oil at p<0.05;b = significant different from fermented without oil at P<0.05; c = significant different from fermented with oil at p<0.05

Table 4 Comparison of nesting behavior among experimental groups

Group	Nesting Score
Control	2.86± 0.26
Fermented + Oil	2.71 ± 0.18
Fermented without Oil	2.86 ± 0.40
Unfermented + Oil	2.71 ± 0.29
Unfermented without Oil	2.29 ± 0.18

No significant differences among groups

4. Discussion

Cassava (*Manihot esculenta*) is a widely consumed tropical and subtropical root tuber root tuber which contains cyanogenic compounds capable of negatively affecting central nervous system function [6, 32. Cassava processing methods have been known to affect the cyanide content of the final cassava products. We therefore sought to investigate the possible effects of these methods on anxiety-like, locomotor and social behaviors in mice model.

Our results indicate that the grooming frequency in the OFM test was increased in the group fed with plain, unfermented product (not mixed with oil) compared with other groups. A similar observation was made for the grooming duration. In neurobehavioral experiments in rodents, an increase in grooming frequency is seen as a sign of stress or anxiety [33]. This suggests that fermentation and addition of palm oil during cassava processing might reduce the anxiogenicity of cassava products, probably by reducing the concentrations of anxiogenic substances like hydrogen cyanide in the cassava products [34, 35].

The frequency of rearing in the OFM test was observed to be reduced in all test groups compared with control. A decrease in the frequency of rearing is an index of fear and low exploration [36]. This result suggests that the different cassava products might still have had varying degrees of anxiogenic effect.

The decrease in the frequency of line crossing in the test groups that was observed in this study compared with the control also supports the possible anxiogenic effect of these cassava products. A decrease in the frequency of line crossing in the OFM is used as an index of anxiety-like behavior [37]. The reduction in line crossing might have been due to exaggerated aversion, anxiety or fear for open, brightly lit space [23].

The significantly increased frequency of stretch attend posture in groups fed unfermented products compared with the control and the fermented groups suggests that fermented cassava products have lesser tendencies to cause anxiogenesis. Rats fed with unfermented cassava product without oil had a higher stretch attend posture frequency than those fed on unfermented + oil, indicating the possible effects of fermentation and palm oil on the anxiogenic potential of cassava products. These differences may be due to different concentrations of cyanogenic substances in the different products [2]. A similar result was observed in the EPM test, supporting the fact that processing methods affect the anxiogenicity of cassava products [9, 38].

Decreases in the open arm entry frequencies in the EPM that were noted in all test groups further buttresses the fact that these products are anxiogenic to varying degrees. The tendency for anxiogenicity appears to be more with the unfermented without oil group. The frequency of open arm entry is an established index to evaluate anxiety-like behaviors in rodents [40].

The reduction in the open arm duration observed in the cassava products-fed rats and which was differentially so among the products-fed groups in the EPM, also supports the fact that these products may have varying levels of anxiogenic effect. An increase in the duration of close arm entry is a pointer to anxiety [39]. This therefore indicates that the duration in the unfermented without oil group was lower than in the unfermented with oil group, suggesting that different processing methods for cassava have differential tendencies for anxiogenesis.

Close arm entry in the EPM is the total number of times or total amount of times that the mice spent in the close arm of the apparatus. Our findings indicate that this duration was increased in all test groups and more significantly so in the unfermented group without oil. These results also buttress the fact that processed cassava products may be anxiogenic to varying levels, depending on the nature of product. An increase in the close arm duration is well accepted as a pointer to anxiety-like behavior in rodents [39].

Our findings did not show any significant differences in the nesting scores among the experimental groups suggesting that the products might not have affected the cognitive and social functions of the animals. Nesting test is a behavioral assay used in rodents to assess cognitive function and social behavior in rodents [30, 40].

5. Conclusion

From the data analysis of the OPM, EPM and nesting tests obtained in this study, it can be concluded that consumption of the various forms of cassava products, may cause increase in anxiety, with lesser tendencies following consumption of fermented products, with/without addition of palm oil. Consumption of these cassava products do not appear to affect cognitive and social functions in mice.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that there are no competing interests.

Statement of ethical approval

Ethical approval for this study was obtained from the Animal Research Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Calabar (Approval No: 621PHY11025)

Funding

This study did not receive funding, directly or indirectly from any individual or organization.

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