

NEUROBEHAVIORAL AND AMELIORATIVE EFFECT OF COMPLAN MILK AND BAMBARA NUT ON RATS FED WITH BITTER CASSAVA – A NUTRITIONAL APPROACH

ABSTRACT

Cassava products have been introduced to the market in different regions of the world as part of diet, despite containing more than one toxic substance. Roots and leaves of cassava of all varieties contain cyanogenic glucoside, mainly as linamarin, but also as lotaustralin, in different concentrations in their cellular vacuoles. Twenty (20) albino Wistar rats were used for this research and were randomly selected into 4 groups of five 5 rats each. The result obtained from the study showed that there was a significant reduction in weight of the Konzo induced Wistar rats group from week 1 to week 4. A weight gain of 21.7g to 34.7g was observed in the cassava-induced Konzo and complan milk fed group Wistar rats and cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats when compared with the cassava-induced Konzo group Wistar rats. There was a decrease in Na^+ , K^+ , Urea, Creatinine and HCO_3^- in cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats when compared with cassava-induced Konzo Wistar rat group and control group. In evaluating the motor coordination/impairment using rotarod test, cassava-induced Konzo Wistar rat group, cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara nut (Okpa) fed Wistar rats group spent a significantly less mean time 20.65 ± 0.33 , 24.93 ± 0.67 and 25.71 ± 0.72 respectively when compared to the control group mean time of 39.45 ± 0.42 . Also, a significant improvement in motor coordination was observed in cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara nut (Okpa) fed Wistar rats group when compared with cassava-induced Konzo Wistar rat group. From the histological evaluation of the brain of the Wistar rats showed neurons that are vacuolated as a result of bitter cassava and alpha motor neurons. It is hence concluded that the toxic effect of bitter cassava can be ameliorated using proteins or balanced diets such as milk and bambara nuts (Okpa).

Key Words: Neurobehavioral, Ameliorative, Bitter cassava, Konzo disease, Complan, Bambara Nut

INTRODUCTION

Cassava (*Manihot esculenta*) is a perennial 1 to 3-meter-high tropical shrub. The leaves have a high content of protein and vitamins, and normally they are consumed after processing, which removes cyanogens. The major harvested organ is the root. The roots have a high content of

carbohydrate and also small amounts of some vitamins and minerals. Their protein content is low and deficient in SAAs such as cystine and methionine (Moller and Seigler 1999; Diasolua *et al.*, 2002, Nassar and Sousa 2007). The bromatological analysis of cassava tuber has reported the presence of water (62.2%), protein (1 %), fat (0.4%), total carbohydrates (32.8%), fiber (1%), ash (0.6%), calcium (40 mg), phosphorus (34 mg), iron (1.4 mg), thiamine (0.05 mg), riboflavin (0.04 mg), niacin (0.6 mg), ascorbic acid (19 mg), and an inedible portion (32%) in 100g of fresh samples (Food and Agriculture Organization (FAO), 2008).

Cassava products have been introduced to the market in different regions of the world as part of diet, despite containing more than one toxic substance (Suarez-Guerra *et al.*, 2011). Roots and leaves of cassava of all varieties contain cyanogenic glucoside, mainly as linamarin, but also as lotaustralin, in different concentrations in their cellular vacuoles (Mkpong *et al.*, 1990; White *et al.*, 1994). Both these chemical compounds have been involved in the etiology of diverse motor alteration in humans and experimental animals. In different geographic regions the excessive consumption of cassava has been consistently associated with some neuropathies. cyanogenic glucoside in cassava can be reduced by appropriate processing of the plant material prior to consumption. In research done by Mlingi (1995), Cardoso *et al.* (2005), processing also improves palatability and increases shelf life, as the root suffers rapid post-harvest deterioration if preserved in the fresh state for more than a few days. The final product may be flour (tapioca) or granules (garri). The consumption of improperly processed cassava derivatives, combined with an unbalanced diet that is deficient in sulfur amino acids, can lead to chronic cyanide poisoning (Jorgensen *et al.*, 2010). Sulfur is essential in the cyanide detoxification process by converting cyanide to thiocyanate, which is eliminated in urine (Duarte *et al.*, 2002). The consumption of fresh cassava or its derivatives has been associated with the development of neurological

disorders such as Konzo and tropical ataxic neuropathy (Enefa *et al.*, 2020; Nzwalo and Cliff, 2011).

Konzo is an upper motor neuron disease that causes irreversible paralysis of the legs and occurs mainly in children and young women of child bearing age. The paralysis occurs quite suddenly, does not progress over time and is irreversible. It is associated with the consumption of monotonous diet of high cyanide(bitter) cassava, by poor rural people in Africa, many of whom suffer from malnutrition. Specifically, konzo is associated with a high cyanide diet of bitter cassava consumed over a period of several weeks combined with a low intake of protein, particularly a shortfall of essential S-containing amino acids that are needed to detoxify cyanide to thiocyanate in the body. Although Konzo affects both men and women, adult males are less frequently affected, and no studies have reported Konzo in children younger than 2 years of age. The disease primarily affects children above the age of three and women in the fertile age group for reasons that are yet to be elucidated (OkitunduLuwa *et al.*, 2014; Cliff *et al.*, 2011; Mlingiet *al.*, 2011; Nzwalo and Cliff, 2011). The total number of persons affected by Konzo in Africa has been estimated to hundreds of thousands with majority of cases occurring in the Democratic Republic of Congo (DRC). Accurate prevalence estimates (as high as 5% in certain rural areas) have been difficult to obtain because of unreliable demographic data and poor surveillance systems (Tshala-Katumbay *et al.*, 2013). Konzo-affected regions face the challenges of the agricultural, educational, and public health capacity and infrastructure needed to implement the necessary dietary changes. For the same reasons, these regions fail to diversify their food staples. Presently, the key to eradicating Konzo is prevention. As there is no cure for the neurological damage that Konzo causes, the battle against the disease must focus on prevention.

METHODOLOGY

Experimental Design: A laboratory based experimental design was used in this study. Twenty (20) male Wistar rats weighing between 200g to 250g were used for this research work and they were acquired from the Animal House of the Department of Anatomy. All animals were housed in their individual standard metal cages. Animals were allowed to acclimatize for one week in their cages, with pellet animal feed and water. The experimental animals were used for this research and were randomly selected into four (4) groups of five (5) rats each. The rats were fed with animal feed (finisher) for the period of two weeks during acclimatization period and were fed with the bitter cassava for 4 weeks. Mode of feeding was by oral ingestion. Animals were weighed weekly with an electric weighing scale and the weights recorded. Animals were closely observed for physical manifestations and clinical signs. The experiment lasted for duration of five weeks.

Plant Collection and Identification: The bitter cassava roots were collected from the Ministry of Agriculture, Agricultural Development Programme and were identified in the Faculty of Agricultural Science, University of Port Harcourt, Rivers State

Inducing the rats with Konzo Disease: After two weeks of acclimatization, 15 Wistar rats were allowed to feed freely on inappropriately processed bitter cassava for the period of 4 weeks. Research into the neurobiology of responsiveness to placebo has addressed placebo analgesia; accordingly, the neurobiology of placebo effects is commonly considered in terms of opioid and non-opioid mechanisms (Kirsch, 1985; Price *et al.*, 1999).

Rehabilitation Group: After period of Konzo disease induction, the rehabilitation group (group 3 and group 4) were completely stopped from consuming the bitter cassava and replaced by feed

+ Complan for group 3 and Bambara nut (Okpa) for group 4. Mode of feeding was by oral ingestion.

Processing of the Bitter Cassava

- Roots were cleaned into water to remove any soil clinging to them.
- Peel of the cassava roots were removed using a clean knife.
- The roots were cut into smaller pieces of chips (2-5cm) to hasten the drying process and also to reduce the cyanide content of the cassava roots. Chopping of the cassava were done in an open area with good ventilation so as to avoid sickness caused by the release of chemicals from the cassava.
- Drying cassava chips. The pieces of cassava were placed on a tray and dried in the sun and this took about 4-5 days. The drying was dependent on weather and as such, it took a longer period because of the unavailability of enough sunlight.
- Grinding of cassava chips was done using a grinding machine; the chips were grounded smoothly until the powdered form was attained. This was to ease digestion of the cassava by the rats.
- Cassava induction: dried powdered cassava was weighed to measure 86g per 1kg rats following the food restrictions strategy by the IACUC and PA, USA. The normal rat chow in the food dishes of the rat cages was then replaced with the powdered cassava. The cassava was consumed adequately by the rats.

Cyanide Analysis

Preparation of test samples for analysis of free cyanide (As HCN equivalent)

- 10g of each sample was mixed with 50ml of water in a corked conical flask

- Allowed to stand for 24h to extract the residual cyanoglucosides in the samples.
- The mixture was subsequently filtered to obtain the soluble extract containing cyanoglucosides.
- The same procedure with standard KCN solutions was followed to determine the free cyanide concentration (as HCN equivalent) in the sample filtrate.
- The absorbance of the sample solution was equally measured at 510nm wavelength against a blank devoid of KCN solution.
- Cyanide levels of the test samples were evaluated from the standard calibration curve by extrapolation.

Behavioral Variables

Rotarod Test

Before testing:

1. Acclimation: subjects in home cage were placed in testing room for at least 20 minutes before testing to minimize effects of stress on behavior during testing.
2. Subject training: Subjects should be able to walk forward on rotating rod.
 - After 60s on rod, animals were returned to home cage and apparatus is cleaned withbefore next trials.

Testing procedures

- Animals are placed on the rod individually.
- The apparatus will be manually rotated. It will be rotated at 30rpm.

- Trial will begin when acceleration starts and ends when animal falls off rod. Timer will be stopped for each animal



Figure 1:Manual Rotarod

- Procedure will be repeated for total of three trials separated by 10min inter-trial intervalsbut no more than four trials should be run per animal.
- Theparameter that will be collected for analysis is latency to fall

Open Field Activity Test

Before testing

1. Acclimation: subjects in home cage were placed in testing room for at least 1hr before testing to minimize effects of stress on behavior during testing.
2. Subject training: none required.

Testing procedures

- Animal was placed in corner of arena and allowed to move freely for 10min while being monitored by automated tracking system. Trial will begin once animal is placed at the

center of the arena and is able to touch the four corners of the arena and will end when defined duration has elapsed. The defined duration is 5 minutes

- Animal was returned to home cage and number of fecal pellets was recorded.
- Arena was cleaned between each trial.



Figure 2: Open field apparatus

- The following parameters were collected for analysis; corners (area) reached and time spent in the arena

Histological Procedures: For the histological analysis, the rats were first anesthetized with chloroform for about 5 minutes then trans-cardiac perfusion was performed. This involved the cutting open of the auricle and sending 100ml of 10% formalin to the brain through the left ventricle. The head and neck were then cut off down to the extent of the thoracic vertebrae. The bones of the skull were then separated and the brain and spinal cord were then removed and stored in a universal bottle containing formalin.

Tissue Processing: The fixed tissues were further dissected by coronal sectioning into smaller bits and placed in different bottles to enable proper identification. The specimens were then dehydrated with alcohol, cleared with xylene, infiltrated with paraffin wax for a period of 30 minutes, embedded and then sectioned into very fine sections. The sections were floated in a warm water bath and then picked up and placed on microscopic slides. The slides were then stored in the laboratory until the time for staining.

Staining Technique

Hematoxylin and eosin (H&E) staining technique

The slides were deparaffinized in xylene for 1 minute and then immersed in absolute alcohol for 30 seconds to 1 minute. They were then immersed 90% and 70% alcohol for 30 seconds each and rinsed in water afterwards. The slides were put in hematoxylin stain for 30 minutes. Then washed under a running tap for 5 – 10 minutes till the colour of the section became blue – this process is called bluing. They were then differentiated using 1% acid alcohol for very few seconds. The slides were then put in 1% aqueous eosin solution for 5 minutes. Immersion of slides into 70%, 90% and absolute alcohol for 30 seconds was done respectively. Clearing was next done in xylene solution and the slides cleaned, blotted and mounted.

Data Scoring and Statistical Analysis: Data was analyzed using Statistical Package for the Social Sciences (SPSS IBM version 23.0) and Microsoft excel 2019 edition. Values were expressed as mean $\pm$ SD in descriptive statistics. One-way analysis of variance (ANOVA) was used to analyze the difference between the groups followed by least significant difference (LSD)

post-hoc test. Confidence interval was set at 95%, and therefore $p < 0.05$ was considered significant.

RESULT

The results are presented in tables and bar charts as shown below;

Table 1: Effect of Konzo disease on Weight of Wistar rats

Group	Week 1	Week 2	Week 3	Week 4	Mean Weight
Group 1 (Control) (g)	263	265	260	273	265.25±2.78
Group 2 (Induced) (g)	175.7	181.1	171.8	172.4	175.25±2.13*
Group 3 (Induced + Complan) (g)	194.1	192.5	223.1	230.1	209.95±9.72*
Group 4 (Induced + Bambara Nut) (g)	188.3	190	200.6	208.9	196.95±4.82*

*Significant at $p < 0.05$

Each value represented in mean±SD, values marked (*) differ significantly from control. From this study, it was observed that there were significant changes in the body weight of the experimental animals during the 4 weeks duration of the experiment. The result shows weekly body weight differences in experimental animals. There was significant weight reduction in the body weight of the Konzo induced group experimental Wistar rats from week 1 to week 4 with a mean weight of 175.25±2.13g compared to the significant weight increase observed in the Konzo induced and complan milk fed group Wistar rats with a mean weight of 209.95±9.72g. There was also a significant weight increase observed in the Konzo induced and Bambara nut (Okpa) fed group Wistar rats with a mean weight of 196.95±4.82g when compared with the konzo induced group. There was a mean weight loss of 90±0.65g observed from the cassava-induced

Konzo Wistar rats compared to the mean weight loss of $55.3 \pm 6.94\text{g}$ and $68.3 \pm 2.04\text{g}$ in cassava-induced Konzo and complan milk fed group Wistar rats and cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats. A weight gain of 21.7g to 34.7g was observed in the cassava-induced Konzo and complan milk fed group Wistar rats and cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats when compared with the cassava-induced Konzo group Wistar rats which indicated the ameliorative effect of complan milk and bambara nut.

Table 2: Effect on Renal function of Wistar rats

Group	Na ⁺ (mEq/l)	K ⁺ (mEq/l)	Urea (mg/dl)	Creatinine (mg/dl)	HCO ₃ (mEq/l)
Group 1 (Control)	220.84	1.39	115.69	1.89	25.6
Group 2 (Induced)	174.01	2.99	73.64	1.54	14.32
Group 3 (Induced + Complan)	136.81	2.43	58.82	2.02	15.18
Group 4 (Induced + Bambara Nut)	148.69	2.7	64.92	1.53	12.95
<i>P-Value</i>	0.04	0.03	0.01	0.03	0.04

P<0.05* means statistically significant when compared with the control

A decrease in Na⁺, K⁺, Urea, Creatinine and HCO₃ was observed in cassava-induced Konzo Wistar rat group when compared with control group while there was a decrease in Na⁺, K⁺ and Urea and an increase in Creatinine and HCO₃ in cassava-induced Konzo and complan milk fed group Wistar rats when compared with cassava-induced Konzo Wistar rat group and control group. Also, there was a decrease in Na⁺, K⁺, Urea, Creatinine and HCO₃ in cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats when compared with cassava-induced Konzo Wistar rat group and control group.

Table 3: Result from Rotarod Test on effect of Konzo disease on motor coordination/impairment

Group	Week 1	Week 2	Week 3	Mean (Sec)
Group 1 (Control)	39.87±2.00	38.60±1.73	39.87±1.74	39.45±0.42
Group 2 (Induced)	21.20±1.19	20.67±0.82	20.07±0.98	20.65±0.33*
Group 3 (Induced + Complan)	23.67±0.60	25.20±0.90	25.93±0.64	24.93±0.67*
Group 4 (Induced + Bambara Nut)	24.40±0.94	25.87±1.03	26.87±0.62	25.71±0.72*

*Significant at $p < 0.05$

The results are expressed as mean $\pm$ SEM per group and the respective control group. Level of significance values are $p < 0.05$. In evaluating the motor coordination/impairment using rotarod test, cassava-induced Konzo Wistar rat group, cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara nut (Okpa) fed Wistar rats group spent a significantly less mean time 20.65±0.33, 24.93±0.67 and 25.71±0.72 respectively when compared to the control group mean time of 39.45±0.42. Also, a significant improvement in motor coordination was observed in cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara nut (Okpa) fed Wistar rats group when compared with cassava-induced Konzo Wistar rat group indicating the ameliorative effect of complain milk and Bambara nut (Okpa).

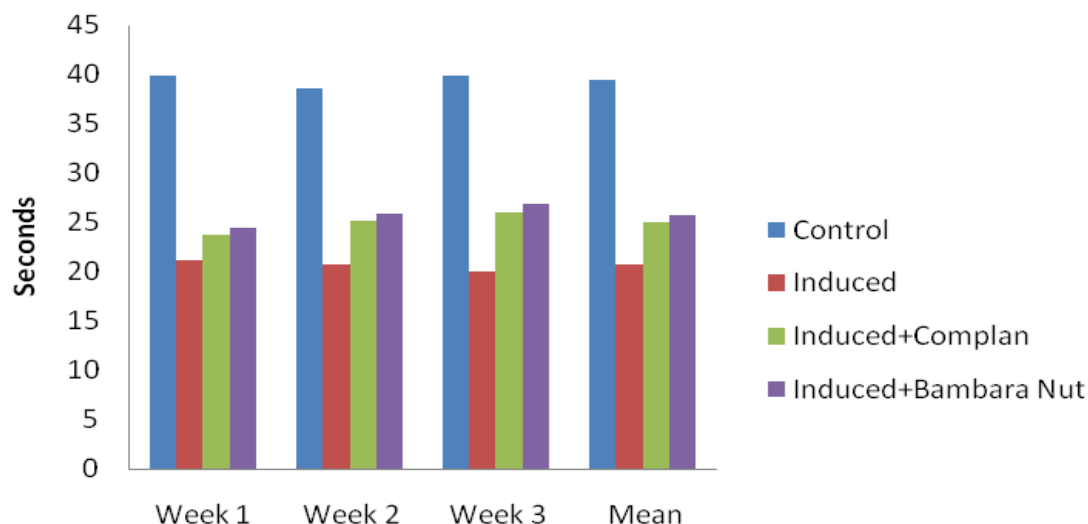


Figure 3: Comparison charts from Rotarod Test on the effect of induced Konzo disease on motor coordination/impairment

Table 4: Result of Locomotion Activity level/impairment, anxiety and willingness to explore in Wistar rats using Open Field Test

Group	Week 1	Week 2	Week 3	Mean (Sec)
Group 1 (Control)	208.33±20.46	177.00±17.95	165.33±18.93	183.55±12.84
Group 2 (Induced)	173.20±27.36	247.80±16.92	239.07±22.18	220.02±23.55*
Group 3 (Induced + Complan)	270.33±12.17	277.87±12.72	248.93±16.58	265.71±8.67*
Group 4 (Induced + Bambara Nut)	234.67±20.22	269.73±12.74	278.20±9.48	260.87±13.33*

*Significant at $p < 0.05$

The results are expressed as mean $\pm$ SEM per group and the respective control group. Level of significance values are $*P < 0.05$. In evaluating the locomotion impairment and anxiety level of wistar rats using Open Field Test, cassava-induced Konzo Wistar rat group, cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara

nut (Okpa) fed Wistar rats group spent significantly more time with a mean time of 220.02 ± 23.55 , 265.71 ± 8.67 and 260.87 ± 13.33 respectively when compared to the control group mean time of 183.55 ± 12.84 . Also, a significant improvement in locomotive activity and anxiety level was observed in cassava-induced Konzo and complan milk fed group Wistar rats group and cassava-induced Konzo and Bambara nut (Okpa) fed Wistar rats group when compared with cassava-induced Konzo Wistar rat group indicating the ameliorative effect of complan milk and Bambara nut (Okpa).

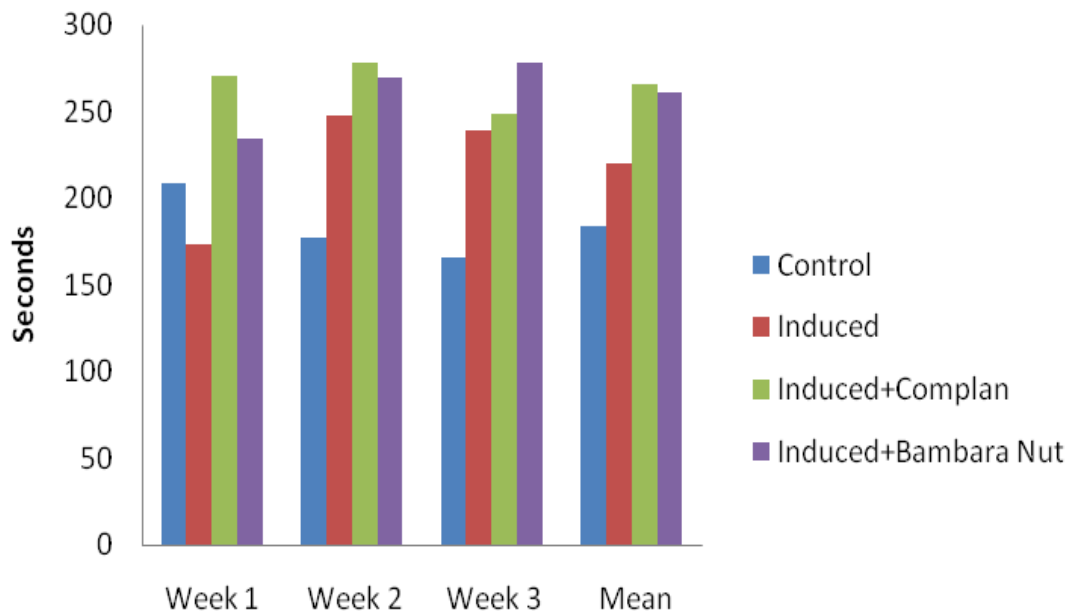


Figure 4: Comparison charts from Open Field Test on the effect of induced Konzo disease on Locomotion Activity level/impairment

4.2 HISTOLOGY

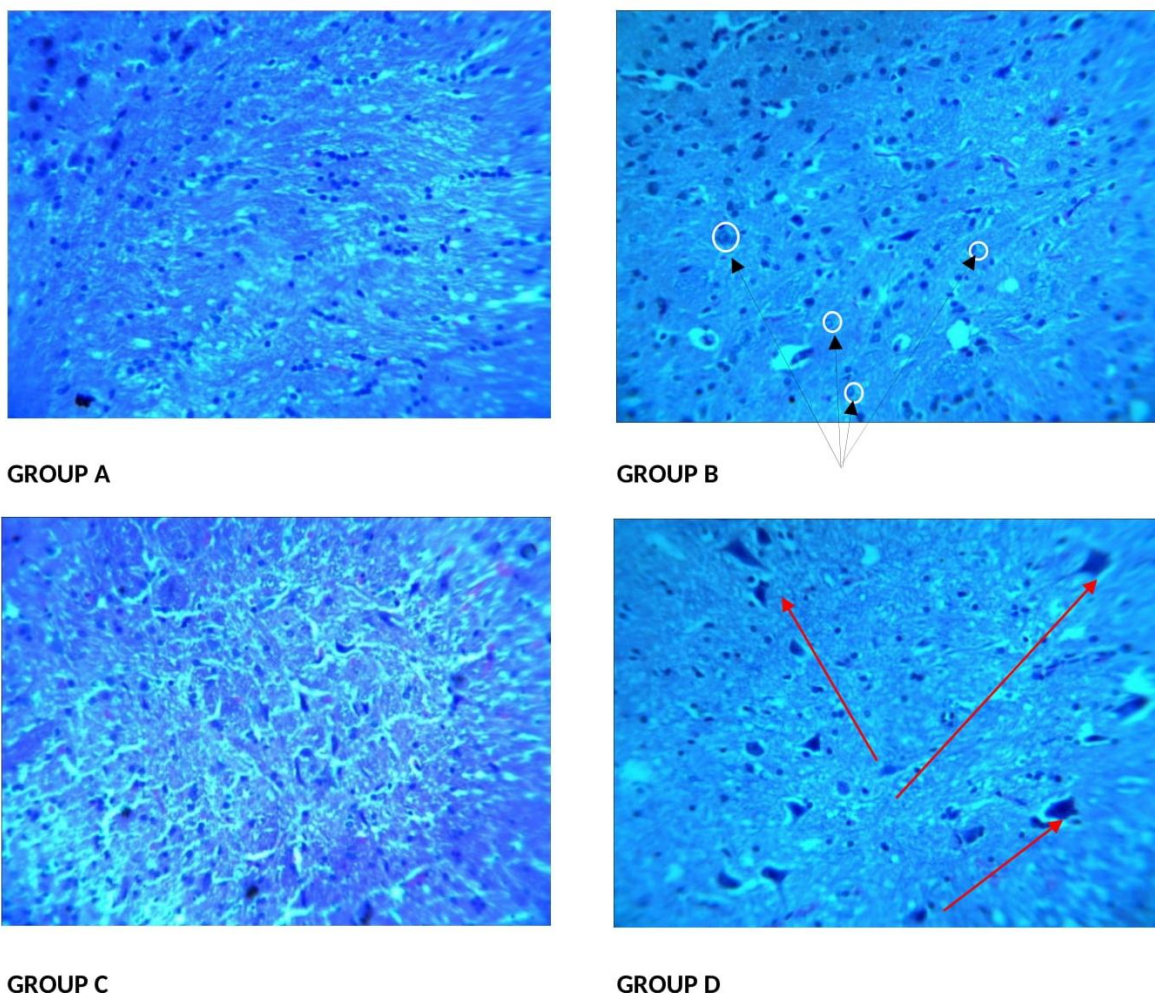


Figure 5: Photomicrograph of the spinal cord of adult Wistar rats (H and E Mag X40). Group A – Control (Fed with normal feeds – finishers), Group B – Fed with bitter cassava, Group C - fed with bitter cassava then treated with Complan, Group D - Fed with bitter cassava then treated with Bambara Nut. Black arrow indicating neurons that are vacuolated as a result of the effect of bitter cassava toxicity. Red arrow in group C indicates α -MNs (alpha motor neurons) in spinal cord.

DISCUSSION

Neurotoxicity studies have utilized experimental animal models to evaluate the effects of diverse substances on motor function and their relationship with brain (especially the central nervous system) disorders (Gerhart *et al.*, 1982; Kulig *et al.*, 1985; Fowler *et al.*, 1990) and the effect of bitter cassava has been studied extensively and has been reviewed in details. This was first

monitored from the weight of the rats. The weight of the animals was taken accurately at significant value of $P < 0.05$, there was a significant difference from the weight of the induced group with mean $\pm$ SEM of 175.25 ± 2.13 compared to the mean $\pm$ SEM value of the control group 265.25 ± 2.78 . This is in line with the observation made by Enefa *et al.* (2020). From the present study, a nutritional approach to ameliorate the effect of bitter cassava induced konzo disease was studied/experimented. A weight gain of 21.7g to 34.7g was observed in the cassava-induced Konzo and complan milk fed group Wistar rats and cassava-induced Konzo and Bambara nut (Okpa) fed group Wistar rats when compared with the cassava-induced Konzo group Wistar rats which indicated the ameliorative effect of complan milk and bambara nut (nut). But when compared with the control group, a decrease in weight was observed which conform to the findings of Enefa *et al.* (2020) which opined that a weight loss is expected in wistar rats injected with cyanogenic glycoside and in laboratory Wistar rats feed with cassava root chips and cassava flour.

In evaluating the motor coordination/impairment using rotarod test, the rats treated with the inappropriately processed bitter cassava quickly fell from the rotarod, thus indicating a motor coordination impairment, which is something that has been suggested to be associated with the neurotoxic compound content in cassava pellet by Rivadeneyra-Domínguez *et al.* (2016) and Rivadeneyra-Domínguez *et al.* (2017). At significant value of $P < 0.05$, the mean value of the induced group was 20.65 ± 0.33 compared to the mean value of the control group 39.45 ± 0.42 which shows that there is a decrease in the time the induced group spent on the apparatus which signifies that there is an impairment in the motor coordination of the rats. Previous studies have suggested that impaired motor coordination is likely associated with the toxic action of cyanide that is derived from linamarin content in cassava juice during their biodegradation (Díaz-Sobac

al., 2019). This concurs to the findings of Eduardo *et al.* (2020) who explained that the effect of cassava juice also affects motor impairment. The mean value of the cassava-induced and complan fed group and cassava-induced and Bambara nut (Okpa) fed group showed that they spent longer time on the apparatus compared to cassava-induced group. It means that the treatments of Complan and Bambara nut given to the induced group were effective and could help minimize the cyanide level in the animals. Prolonged intake of insufficiently processed bitter cassava roots is associated with impaired motor coordination and these neurotoxicity effects of cassava can be ameliorated with consumption of cassava along with sulphur amino acid proteins and vitamins according to Enefa *et al.*, (2020).

The present study was also carried to investigate the level of anxiety and willingness to explore using the open field test. The induced group took a longer time to explore and reach the corners of the apparatus compared to the time the control group took to perform the same task. It was observed that for the group that was treated with complan milk, from the first apparatus used which is the rotarod, it corrected the impaired motor coordination but could not perform the task in the open field as a result of its excessive weight gain. The weight gain contributed to its dull movement in the apparatus. It could walk around the corners but was quite unable to touch the corners of the apparatus on time compared to the control group.

Notably, cyanide that is derived from cassava causes the demyelination of spinal cord neurons, which is related to a lack of limb coordination (Ernesto *et al.*, 2002). The daily consumption of cyanide products, such as those from cassava, has been linked to such neurological disorders as tropical ataxic neuropathy and Konzo (Rivadeneyra-Dominguez *et al.*, 2013). The findings agree to the report that the cyanogen found in bitter cassava affects the Neurons of the spinal cord which also leads to lack of limb coordination. This is seen as pointed in group B (cassava –

induced group) as shown in figure 5. Voluntary muscles controls were lost at the forelimb of some animals and at the hind limb of other animals. This was due to loss of the alpha motor neurons that relay voluntary signals from the upper motor neurons to skeletal muscle fibers, while involuntary control loss was as a result from interruption of the reflex circuit which could lead to reduced muscle tone and flaccid paresis. As seen in the histology slides (figure 5, group D), there was ameliorating effect which brought about the visible alpha neurons.

CONCLUSION

This study has shown that inappropriately processed bitter cassava is toxic and has neurotoxic effects on the brain (especially central nervous system and upper motor neurons). The toxic effect of bitter cassava can be ameliorated using proteins or balanced diets such as milk and bambara nuts (Okpa) as established from this study. Adherence to the findings from this study can reduce toxicity from bitter cassava consumption and nutritional insufficiency may likely increase the potential toxicity of various processed bitter cassava roots consumption.

RECOMMENDATION

It is recommended that the findings from this study be used to enlighten rural dwellers who consume different variety of bitter cassava processed roots on the need to also balance their diet while consuming varieties of processed cassava roots. Also, further studies to corroborate the findings of this study are recommended.

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