Original Research Article

EFFECT OF BITTER CASSAVA ON SULPHUR-CONTAINING AMINO ACIDS IN KONZO INDUCED WISTAR RATS

ABSTRACT

Introduction: The present study is aimed at assessing the effect of bitter cassava on blood biochemical parameters of konzo disease induced Wistar rats. Nutritional modification with suphur rich amino acids was used as a measure to correct the impact in rats induced konzo disease. **Method:** 25 adult male Wistar rats were assigned to 5 experimental groups (i) Control n=5, (ii) cassava only n=5, (iii) cassava + animal feed n=5, (iv) cassava + Eggshell + Brown beans n=5, (v) Eggshell + Brown Beans only n=5. The bitter cassava foods were taken by oral ingestion for a period of 4 weeks. The weights of the rats were checked through the experiment and blood samples were taken from each group into EDTA-lined containers and analyses for blood cyanide, methionine and cysteine levels. **Results**: There was significant difference in weight and there was a progressive increase in their success rate as against the cassava only group which decreased in their success rate, hence, differs statistically and significantly ($p<0.05$) from the control group. It was observed that the cassava fed group had higher blood level of cyanide far above the normal blood reference range (2.60 – 2.90µg/ml) for cyanides, hence, was seen to be statistically significant as compared to that of the control group. The eggshell and brown beans only group showed high blood levels of methionine that statistically differ significantly ($p<0.05$) from both the control group and the cassava only group. Blood level of cysteine in the cassava plus eggshell and brown beans group differed significant statistically from the control group. The other groups also showed differences in their individual groups but did not significantly differ statistically. **Conclusion**: Sulphur amino acids such as methionine and cysteine are essential for detoxification of the residual cyanogens remaining in insufficiently processed cassava roots. Foods such as cereals and legumes as source of sulphur amino acids should be promoted to prevent paralytic neurotoxico-nutritional disease such as konzo among the poor population.

Key words: Bitter Cassava, Sulphur-containing Amino acids, Methionine, Cysteine, Konzo disease

INTRODUCTION

Cassava (*Manihot esculenta*) forms part of the staple diet for more than 600 million people across the world, particularly those that live in poverty and remote areas where food security is poor (Tshala-Katumbay *et al.,* 2013). The plant grows in poor soil and is relatively drought

resistant; the tubers are rich in carbohydrates and the leaves contain some protein. Cassava contains cyanogenic glucosides (linamarin and lotaustralin) that are released as hydrogen cyanide, which are thought to protect the plants from insects and other animals. For human consumption, the plants need to be detoxified, usually by soaking, drying in the sun, boiling, fermentation, or grating with roasting (Nzwalo and Cliff, 2011). These processes allow the cyanogenic glucosides to be released, but depend upon traditional practices, time taken, and the availability of water. Neurotoxicity is associated with incompletely detoxified cassava, although the exact mechanisms by which these compounds cause neurological damage is unclear. The toxicity of cyanide is reduced by its transformation to thiocyanate or cyanate, which requires sulphur donors, often limited in malnutrition. Two neurological conditions are mainly associated with bitter cassava: a myeloneuropathy and konzo. The myeloneuropathy manifests as a slowly evolving bilateral sensory polyneuropathy, optic atrophy and sensorineural deafness, and sensory ataxia, is seen in adults (particularly elderly) who have a solely cassava diet (Adamolekun, 2011). Konzo is a condition with selective upper motor neuron damage, manifesting as an acute or subacute onset of an irreversible, non-progressive, and symmetrical spastic paraparesis or quadriparesis (Howlett *et al.*, 1990). It is found in remote poor regions, often occurring as epidemics in times of drought, famine, and war, when the usual detoxifying preparations of cassava are not followed. Tshala-Katumbay and colleagues have been conducting seminal studies of konzo in the Congo, often in challenging circumstances of the remote areas in which this condition is found. They have clearly documented the neurophysiological impairment of the cortico-spinal tracts, (Tshala-Katumbay *et al.*, 2002), the hallmarks of konzo, and impaired sensation (Tshala-Katumbay *et al.*, 2002). This group were the first to demonstrate cognitive impairment with konzo (Tshala-Katumbay *et al.*, 2013; Boivin *et al.*, 2013), after earlier

electroencephalographic studies suggested cortical involvement (Howlett *et al.,* 1990; Tshala-Katumbay *et al.,* 2000). Not much study has been carried out on konzo disease using animal models, but in recent times, a neuroscience researcher in Nigeria, David Lekpa Kingdom, has been carrying out in-vivo study into the neurological effect of bitter cassava induced konzo disease using rat models to better understand the mechanism in which konzo affects the neural system (Enefa *et al.,* 2020; David *et al.,* 2021, 2021, 2021, 2022, 2022). The present study is aimed at assessing the effect of bitter cassava on blood biochemical parameters of konzo disease induced Wistar rats. Nutritional modification with suphur rich amino acids was used as a measure to correct the impact in rats induced konzo disease.

METHODOLOGY

Experimental Design

Twenty-five male Wistar rats weighing between 200g to 250g were used for this research work. They were acquired from the animal house of the Department of Pharmacology. All animals were housed in their individual standard cages. Animals were allowed to acclimatize for two weeks in their cages, with pellet animal feed and water. The experimental animals were then divided into five groups; Group 1 (negative control group) n=5, were fed with pelleted animal feed and water, Group 2 (cassava only group) n=5, were fed with bitter cassava flour and water, Group 3 (cassava + animal feed) n=5, were fed with animal feed, bitter cassava flour and water, Group 4 (cassava + Eggshell + Brown beans group) n=5, were fed with bitter cassava flour, Eggshell, brown beans and water, Group 5 (protein treated group) n=5, were fed with Eggshell, brown beans and water only. Animal 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 four weeks.

Ethical Clearance

The experimental animals were obtained from the Animal House of the Department of Pharmacology in the Faculty of Basic Clinical Sciences. All procedures carried out during this research were done in accordance with the guiding principles of research involving animals as recommended by the Research Ethics Committee of the University of Port Harcourt. Animals were kept in standard metal cages and at normal room temperature.

Sample Size

The sample size of the study was thirty (25) female Wistar rats. The sample size for this experimental animal study was determined using Power Method.

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, Nigeria.

Processing of Bitter

Cassava Root Fresh cassava roots were uprooted from the farm. Immediately after harvesting, the brownish peel (skin or cortex) was removed with knife to expose the white inner layer. Then the roots was sliced into smaller sizes like chips and allowed to sundry for 3 consecutive days. The dry cassava pieces were manually grinded into powdery form using a grinding machine and served to the experimental animals as cassava chow.

Processing of Protein Food supplement

A combination of egg shells and brown beans served as the protein food supplement for this research work. Egg shells were washed, broken into small pieces and allowed to sundry for 24 hours. Brown beans and the egg shells were grinded together using a grinding machine, into powdery form and served as sulphur amino acid protein food supplement and it was given exclusively to the "positive control group" (group 3) and to the "protein treated group" (group 5) rats.

Process of Inducing Konzo disease in Rats

After two week of acclimatizing in their cages, fifteen Wistar rats were allowed to freely feed on the inappropriately processed bitter cassava flour exclusively and constantly for a duration of four weeks to induce Konzo disease and its manifestations. The oral-consumption method used in this study better mimics a real-world consumption scenario wherein the food enters the mouth, then passes through the esophagus an into the stomach following the entire alimentary canal, contacting the relevant visceral organs along with their associated fluids, such as saliva in the mouth and gastric juices, including any contribution provided via sublingual or buccal absorption during digestion.

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 eggshell and brown beans for group 3 and animal feed for group 4. Mode of feeding was by oral ingestion.

Assessment of Parameters

Sampling

Cassava sample was collected and analyzed for cyanide content in cassava. Blood samples were collected into appropriate containers for analysis of cyanide, Cysteine and methionine content in blood. Description of the blood sampling and amino acid assays were according to Olsen *et al.* (2018). Blood was collected into EDTA-lined vacuum tubes for determination of methionine and cysteine.

Determination of Cyanide in Cassava

The alkaline titrimetric method for Cyanide was used where 10 to 20g of sample was placed on a ground to pass No. 20 Sieve in an 800mL Kjeldahl flask. 200mL of H_2O added and allowed to stand for 2-4hrs. Distil was steamed, 150-160mL distillate was collected in NaOH solution (0.5g in 20mL H2O), and distilled to a definite volume. 8ml 6*N* NH_2OH and 2mL 5% KI solution was added to 100mL distillate (it is preferable to dilute to 250mL and titrate 100mL aliquot) and titrated with 0.02*N* AgNO3 using micro-burette. A faint endpoint point with permanent turbidity was recognized, especially against black background.

Determination of Cyanide in blood

Chemicals and Reagent

Potassium cyanide (KCN), Acetonitrile (IS), Chloramine T, H_2SO_4, NaOH, and HCl were purchased from Chemical Laboratories within the University of Port Harcourt environment. Other chemicals used in this study were: Pyridine, Glacial acetic acid, and Barbituric acid.. Pyridine-barbituric acid reagent was prepared by adding 15mL of pyridine, 3mL of concentrated HCl and 7mL of H_2O to 3.0g barbituric acid. Water was obtained from a Milli-Q ultra-purifying system, 18.2 MW⁄cm (Millipore SA, Molscheim, France). All solvents and reagents were of analytical grade.

Spectrophotometric Method (VIS)

Glass Conway micro-diffusion cells were used (18 x 70mm o.d.; 8–10 x 41mm o.d., inner chamber). Adsorbing solution (2mL, 0.1M NaOH) was added to the inner compartment of each Conway cell, and the liberating solution (2mL, 50% H_2SO_4) was added to the outer compartment. Blood samples (1mL) were added to the opposite part of the outer chamber, as mixing had to be avoided. The cell was then quickly closed by a Teflon-lined screw cap and gently rotated to mix blood and liberating solution. After 30 minutes contact at 38^{o}C, 1mL of the inner chamber contents from each cell was taken up and transferred into a 10-mL volumetric flask. To each flask 3mL of 1M NaH_2PO_4 and 1mL of Chloramine-T (2.5g⁄L) were added, mixed, and allowed to stand for 2–3 minutes. Pyridine-barbituric acid reagent (3mL) was then added and the solution diluted to 10mL with H_2O. Absorbance was determined at 586 nm against a blank.

Spectrophotometric Analysis

VIS was Performed on a Varian CARY50 (Torino, Italy) Spectrophotometer. Standard cyanide solution was prepared by placing 25.0mg of KCN in a 100-mL volumetric flask, to yield a solution with a concentration of 100 lg⁄mL of CN; 0.1 N NaOH was used as diluent. In another volumetric flask, 10mL of this solution was transferred and added with 90mL of 0.1 N NaOH, to yield a solution with a concentration oaf 10 lg⁄mL. The standard cyanide solution was further diluted to yield six working solutions at concentrations in the range of 0.5– 10.0 lg⁄mL

Amino Acid Assays

Methionine and cysteine were measured by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) as described by Olsen *et al.* (2018). Diagnosis of methionine and cysteine was based on fasted plasma amino acids.

Method of Statistical Evaluation

Data was exported to excel sheet where tables and graphs was done. Analysis was done using the Statistical Package for Social Sciences (SPSS IBM version 23.0) and Microsoft excel 2019 edition. Values were expressed as mean ± SD in descriptive statistics. One-way analysis of variance (ANOVA) was used to compare the differences between the groups followed by Tukey's post-hoc test.

RESULTS

Table 1: Effects of Cassava on the body weight of Wistar rats

Groups	Week 1	Week 2	Week 3	Week 4
Control	238.72±21.45	245.82±21.05	245.82±21.05	245.82±21.05
Cassava only	245.82±21.05	250.60±50.74	247.60±51.13	238.60±40.04
Cassava+Animal feed	212.80±86.09	212.80±86.09	212.80±86.09	233.60±90.23
Cassava+Eggshell and Brown beans	250.00±66.33	252.40±59.24	257.00±60.06	259.30±59.38
Eggshell and Brown beans only	256.00±43.48	259.80±42.43	263.40±42.95	268.00±43.61

Each value represents mean±SD, Values marked with asterisk (*) differ significantly from control value ($p < 0.05$) while those marked with (#) differ significantly from cassava only group (#$p < 0.05$)*

From Table 1, it was observed that the weight of the control group progressively increased from week 1 to week 4 (238.72±21.45 to 280.60±48.30). It was also observed that the weight of the cassava only group decreased progressively from 260.40±52.37 to 238.60±40.04 (about 3 to 10g weight loss) over the four weeks of cassava only administration. The cassava plus animal feed group as well as the cassava plus eggshell and brown beans group showed progressive increase in weight as against the cassava only group that showed decrease in weight. The group fed with eggshell and brown beans only showed a progressive weight gain from 256.00±43.48 to 268.00±43.61 (4g to 12g weight gain). However, the difference in the weight across the groups was not statistically significant as compared to the control group.

Table 2: Blood levels of cyanide compared across the groups at weekly intervals

Groups	Week 1	Week 2	Week 3	Week 4
Control	0.02±0.00	0.05±0.00	0.03±0.00	0.03±0.00
Cassava only	3.42±0.02*	3.02±0.01*	4.29±0.20*	4.00±0.00*
Cassava + Animal feed	3.75±0.19*	3.01±0.00*	3.16±0.03*#	4.29±0.17*
Cassava + Eggshell and Brown beans	0.04±0.02#	0.04±0.00#	0.32±0.00#	0.44±0.00*#
Eggshell and Brown beans only	0.02±0.00#	0.04±0.00#	0.01±0.00#	0.02±0.00#

*Each value represents mean±SD, Values marked with asterisk (*) differ significantly from control value (*p<0.05) while those marked with (#) differ significantly from cassava only group (#p<0.05).*

It was observed from Table 2 that the cassava fed group had higher blood level of cyanide far above the normal blood reference range (2.60 – 2.90µg/ml) for cyanides, hence, was seen to be statistically significant as compared to that of the control group. The eggshell and brown beans only fed group had the least blood cyanide level as compared to that of the cassava plus animal feed group and cassava plus eggshell and brown beans fed group. These three groups have blood levels of cyanide that differ significantly from the blood of the cassava only group.

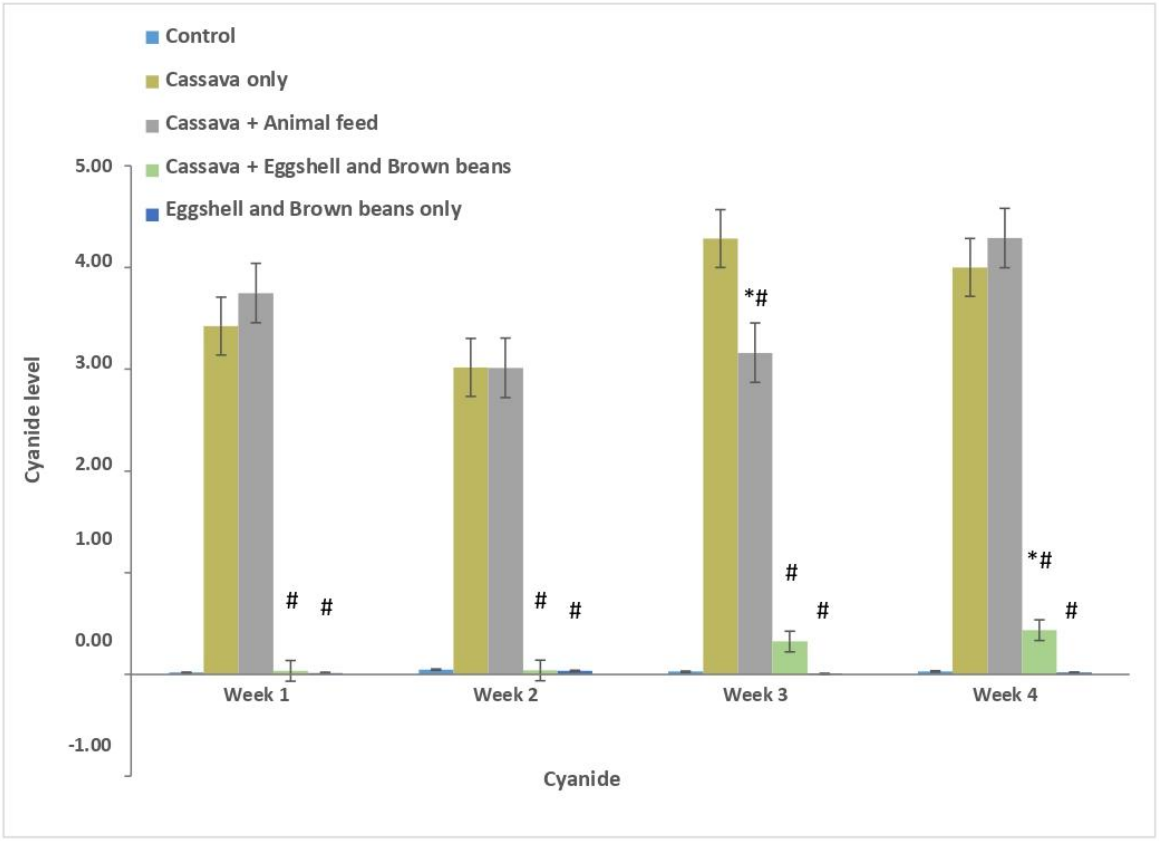

Figure 1: Bar chart showing the blood levels of cyanide compared across the groups at weekly intervals

Table 3: Blood levels of methionine compared across the groups at weekly intervals

Groups	Week 1	Week 2	Week 3	Week 4
Control	0.08±0.02	0.06±0.00	0.04±0.00	0.04±0.02
Cassava only	0.03±0.02*	0.02±0.00*	0.01±0.00*	0.03±0.02
Cassava + Animal feed	0.02±0.00*	0.01±0.00*	0.01±0.00*	0.02±0.00
Cassava + Eggshell and Brown beans	0.04±0.00	0.03±0.00*#	0.03±0.00*#	0.04±0.00
Eggshell and Brown beans only	0.07±0.00	0.04±0.00*#	0.03±0.00*#	0.06±0.00

Each value represents mean±SD, Values marked with asterisk (*) differ significantly from control value ($p < 0.05$) while those marked with (#) differ significantly from cassava only group (#$p < 0.05$).*

From Table 3, the eggshell and brown beans only group showed high blood levels of methionine that statistically differ significantly ($p<0.05$) from both the control group and the cassava only group. The cassava plus eggshell and brown beans group differ significantly from both the cassava only group and control group. The cassava plus animal feed only group and the cassava only group statistically differ significantly from the control group.

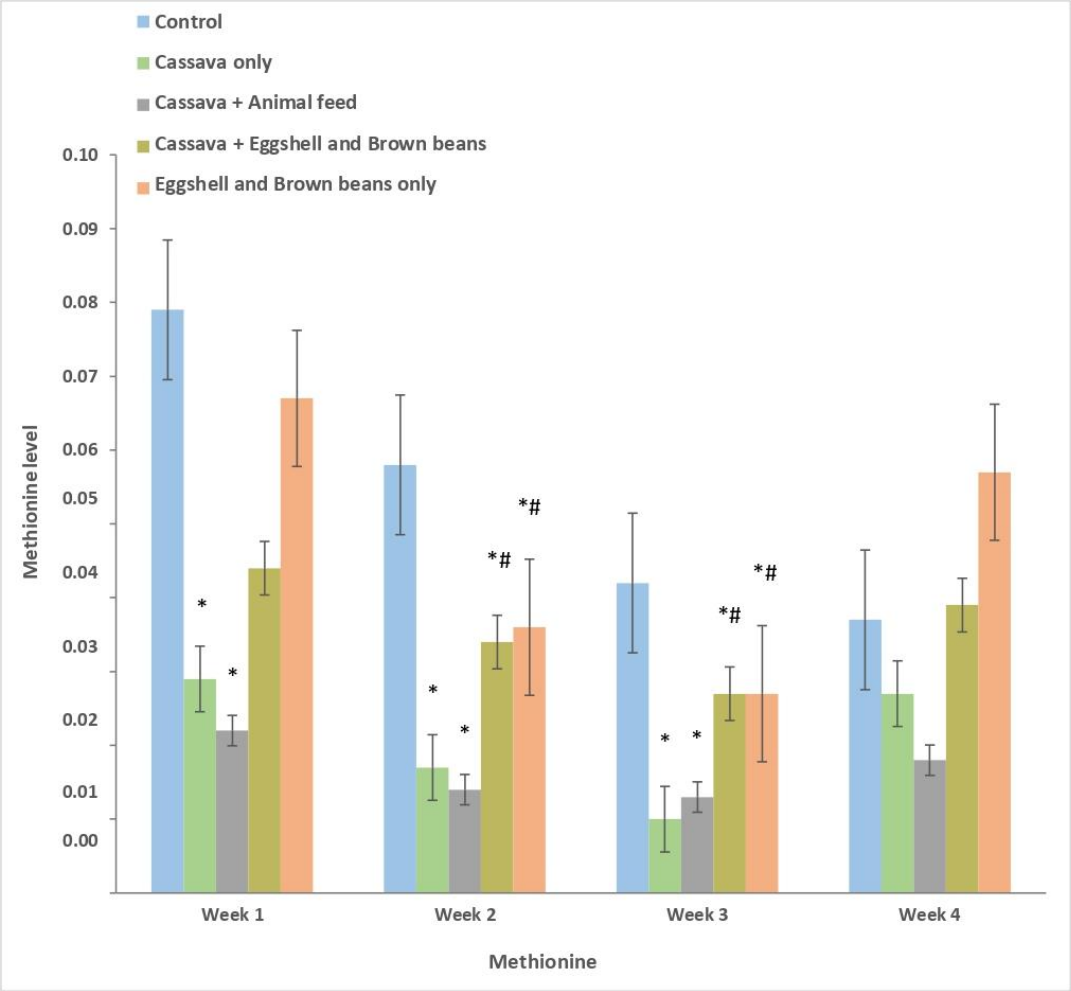

Figure 2: Bar chart showing the blood levels of methionine compared across the groups at weekly intervals.

Table 4: Blood levels of Cysteine (mmol/l) compared across the groups at weekly intervals

Groups	Week 1	Week 2	Week 3	Week 4
Control	0.06±0.03	0.04±0.03	0.04±0.00	0.03±0.02
Cassava only	0.02±0.02	0.02±0.01	0.01±0.00	0.02±0.01
Cassava + Animal feed	0.03±0.02	0.02±0.00	0.03±0.02	0.04±0.00
Cassava + Eggshell and Brown beans	0.04±0.00	0.03±0.01	0.04±0.00#	0.06±0.00
Eggshell and Brown beans only	0.03±0.00	0.03±0.00	0.01±0.00	0.03±0.00

Each value represents mean±SD, Values marked with asterisk (*) differ significantly from control value ($p < 0.05$) while those marked with (#) differ significantly from cassava only group (#$p < 0.05$).*

Table 4 showed that the blood level of Cysteine in the cassava plus eggshell and brown beans group differed significant statistically from the control group. The other groups also showed differences in their individual groups but did not significantly differ statistically.

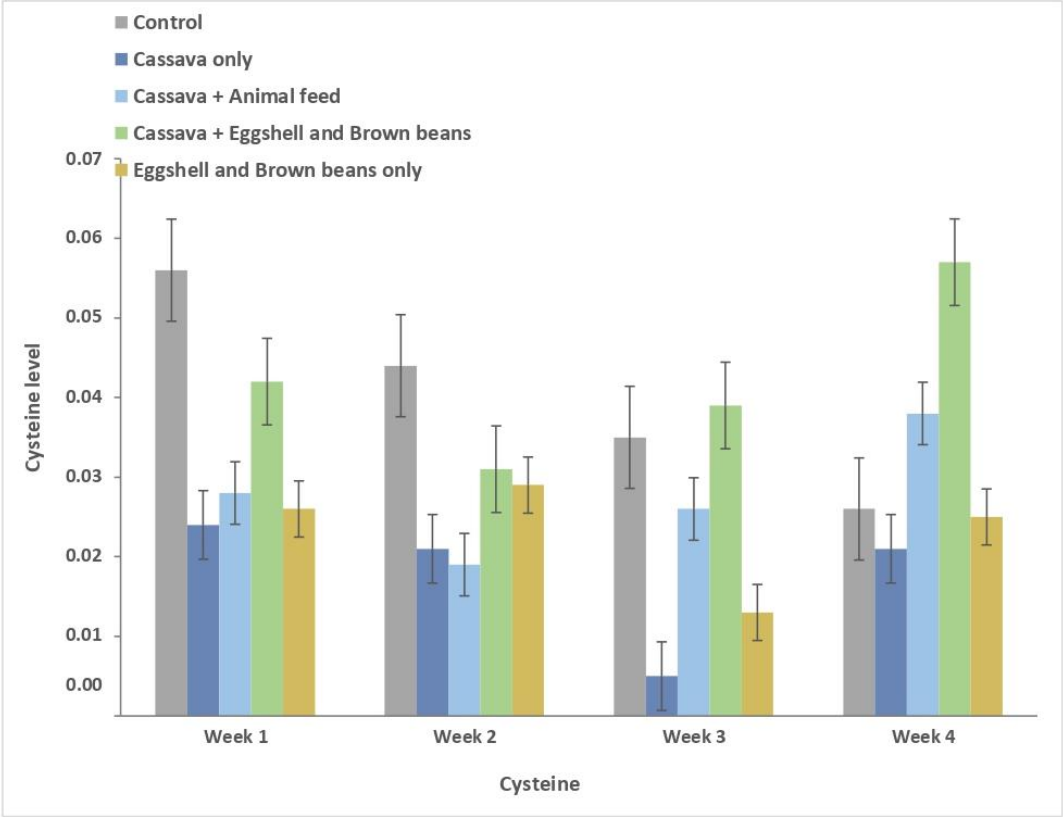

Figure 3: Bar chart showing the blood levels of Cysteine compared across the groups at weekly interval

DISCUSSION OF FINDINGS

Our study as seen in table Table 1 showed that the weight of the control group progressively increased from week 1 to week 4 (238.72±21.45 to 280.60±48.30). It was also observed that the weight of the cassava only group decreased progressively from 260.40±52.37 to 238.60±40.04 (about 3 to 10g weight loss) over the four weeks of cassava only administration. The cassava plus ordinary rat feed group as well as the cassava plus eggshell and brown beans group recorded progressive increase in weight as against the cassava only group that showed decrease in weight. The group fed with eggshell and brown beans only showed a progressive weight gain from 256.00±43.48 to 268.00±43.61 (4g to 12g weight gain). However, the differences in the weight across the groups was not statistically significant ($p<0.05$) as compared to the control group. The decrease in body weight as seen in the cassava only group could be attributed to the presence of cyanogenic glycoside present in the poorly processed cassava that was given to the animal. Ebeye (2018) in his work reported that there was a difference in the weight of animal fed with "garri and tapioca" a local diet made from cassava rich in cyanogenic glycoside as compared with the control he used, such that there was a reduction in weight of the animals fed with cassava meal. In a study also done by Enefa *et al.* (2020) there was significant reduction in weight in animal fed with cassava due to the presence of cyanide in cassava which was in keeping with our study. Shama et al. (2011) and David *et al.* (2022) posited also in their studies that there was a reduction in weight of the Wistar rats fed with unprocessed cassava rich cyanogenic glycosides also indicating the effect of cassava on weight.

It was observed from Table 2 that the cassava only fed group had increasing higher blood level of cyanide far above the normal blood reference range (2.60-2.90µg/ml) for cyanides from week to week 4, hence, was seen to be statistically significant ($p<0.05$) as compared to that of the

control group. Also the cassava plus animal feed group show high blood cyanide levels as compared to the control group which was also statistically significant ($p<0.05$) but this was lesser than that of the cassava only fed group. The eggshell and brown beans only fed group had the least blood cyanide level as compared to that of the cassava plus animal feed group and cassava plus eggshelland brown beans fed group, these three groups have blood levels of cyanide that differ significantly ($p<0.05$) from the blood level of cyanide of the cassava only group. Osuntokun (1970) in a similar work like ours found out in his research that animal fed with purupuru (a diet made from cassava) diet which is rich in cyanogenetic glycoside showed high thiocyanide levels in plasma which was seen to be significant. Tor-Agbidiye *et al*. (1999), also stated in his study that animal fed with sulphur amino acid free diet and potassium cyanide in double distilled water showed high plasma potassium cyanate than those fed with balanced diet (which was rich in methionine and cysteine amino acids) and potassium cyanide in double distilled water which showed a decreased plasma cyanate levels. This is in keeping with this study which also showed a similar trend.

Table 3 showed that the eggshell and brown beans only group showed high blood levels of methionine that statistically differ significantly ($p<0.05$) from both the control group and the cassava only group. The cassava plus eggshell and brown beans group differ significantly from both the cassava only group and control group. The cassava plus animal fed only group and the cassava only group differ significantly ($p<0.05$) from the control group. Also, Table 4 showedthat the blood level of cysteine in the cassava plus eggshell and brown beans group differed statistically ($p<0.05$) from the control group. The other groups also showed differences in their individual groups but did not differ statistically. In a similar research by Osuntokun *et al.* (1968), in their study, it was observed that there were reduced plasma levels of methionine and other

sulphur amino acids in patients with cyanogenetic glycoside based neuropathy. Williams and Osuntokun (1969) also stated in their research that there were reduced indices of plasma protein in the blood of patients fed with cyanogenetic glycoside which could be the basis for the neuropathy manifested in these patients. However, in our research, also it was observed that the rats fed with eggshell and brown beans showed high levels of methionine and cysteine in their blood (Table 3 and Table 4). These indicates the influence that sulphur amino acids could have on konzo disease patients as seen in the high blood levels of methionine and cysteine found in these rats when they were rehabilitated with eggshell and brown beans only therapy.

Both methionine and cysteine are incorporated into structural protein and are required for normal growth. The sulphur side chains help stabilize secondary and tertiary structures. Methionine is part of the co-enzyme S-adenosyl methionine, which influences and regulates the activity of number of enzymatic and cellular replication processes. According to a study by Milner (1979) who carried out a study on the assessment of indispensable and dispensable amino acids for immature dogs discovered that Puppies fed a methionine deficient diet experienced decreased food intake, weight loss and evidence of dermatitis. In puppies, methionine deficiency in combination with excess cysteine resulted in hyperkaratotic, necrotic foot pad lesion that resolved with reintroduction of methionine (Burns and Milner, 1982). In a study by Teeter *et al.* (1978) and Rogers and Morris (1979) discovered that feeding of a methionine deficient diet to kittens resulted in weight loss, lethargy and abnormal ocular secretions. Deficient methionine intake with adequate cysteine supplementation intake in kittens also resulted in severe perioral and foot pad lesions (National Research Council, 2006). With respect to the present study, the effect of methionine and cysteine deficiency was well expressed in the groups induced with konzo disease and rehabilitated with sulphur amino acid containing diets when compared with

the control group. The deficiency in methionine and cysteine levels in this group is possibly related to the high level of cyanide present in the in the blood resulting from the bitter cassava used in inducing konzo disease in Wistar rats.

Conclusion

The use of sulphur amino acid containing diets was able to compensate for the methionine and cysteine deficiency in konzo induced rats. This shows that the dietary requirement for methionine and cysteine needs to be adjusted for the loss caused by cyanide detoxification. This dietary methionine and cysteine requirement may be supplemented if rats were fed with sulphur amino acid containing diets for a longer period of time. Hence, consumption of foods rich in amino acids are advised to prevent cyanide toxicity from bitter cassava and hence, konzo disease.

Recommendation

Sulphur amino acids such as methionine and cysteine are essential for detoxification of the residual cyanogens remaining in insufficiently processed cassava roots. Foods such as cereals and legumes as source of sulphur amino acids should be promoted to prevent paralytic neurotoxico-nutritional disease such as konzo among the poor population.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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