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Investigating the Role of Cassava-Derived Products as Etiological Agents in Immunodeficiency: Pathological Mechanisms and Public Health Implications

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Abstract: Cassava roots provide important sources of energy-rich food for millions of people. It is rich in two toxic cyanogenic glycosides. The study aimed to assess the impact of cyanide-containing food (Cassava) on hematological and histological parameters in experimental rats. Freshly prepared cassava products (fried garri and cassava paste) were obtained. Thirty-five (35) inbred male Swiss Rattus norvegicus were used for the study. There were two major animal groups, each divided into four other subgroups that contained five rats each. Subgroups were designated as A (only cassava form administration), B (cassava form and immunosuppressor), H (1mg/kg KCN), and I (only feed and water). All subgroups B received cyclophosphamide as a single dose on the 9th day. Animals were sacrificed on the 14th day. The data were analyzed using SPSS. Cyanide concentration was 133.16 ± 16.23 mg/kg in cassava paste and 59.40±8.07 mg/kg in fresh fried garri. Results of white blood cell, red blood cell, monocyte, granulocyte, and neutrophil counts revealed lower levels in groups A, B, and H in rats exposed to freshly fried garri compared to group I. In rats exposed to fresh cassava paste, hematological parameters were similarly lower in groups A, B, and H compared to the normal control, where they were higher. When compared to the control group, there were various histological deformations observed in the liver, thymus, spleen, and kidney of the tested rat species like thickened centriole, adipose tissue, prominent dilated central artery, mild atrophic, and inflamed cells.

Keywords: Neutrophils, Hematology, Cassava, Deformities, Cyanide.

1. Introduction

Many people in their millions depend on the energy-rich and nutritional food obtained from Manihot esculenta Crantz (Cassava). Although highly nutritional, cassava has a shortcoming due to the presence of toxins in the form of cyanogenic glycosides. Notable among the toxic glycosides include linamarin and lotaustralin (Agbor-Egbe et al. 2001; Peprah et al. 2020).

Millions of people about 500 million in both Latin America and Africa regard cassava as their staple food (Alves 2002). In African populations, there are more than 200 million low-income people who get more than 40% of their calories from cassava/cassava products (Bradbury et al. 2008; Thomas and Turk 2023). Apart from linamarin and lotaustalin, there is another cyanogenic glycoside in cassava (Lunsinet al. 2012; Emmanuel et al. 2021). When cyanogenic glycosides are ingested, there are a series of steps leading to intoxication and they include acid hydrolysis and microbe-mediated enzymatic hydrolysis during digestion.

Consumption of improperly prepared cyanogenic glycoside foods, such as cassava, may lead to cyanide intoxication. The toxin is rapidly transported by the blood to all parts of the body and could potentially inhibit iron, copper, or molybdenum-containing enzymes (Seidl et al. 2003; Briffa et al 2020). Cyanide can inhibit the electron transport system by binding to iron in cytochrome C oxidase and hemoglobin. Once this happens, there is a shift from aerobic to anaerobic process, ultimately leading to energy depletion and death of cells (Seidl et al. 2003; Nnoliet al 2013; Miller et al. 2017).

Hematological parameters are often used to indicate changes in physiology and general health status in animals (Burgos-Aceves et al. 2019). Toxins have been reported to negatively affect hematological parameters thereby inhibiting their functions. The level of damage by immune-toxicant can be accessed by determining how it affected the amount of some parameters such as hemoglobin, red blood cell, white blood cells, etc. immune-toxicant can alter the balance among these parameters or cause a decrease in their concentration, with concomitant negative health implications (Manoharan et al. 2021).

Hydrogen cyanide present in cassava has been reported to possess toxicity to different organs in experimental animals. Organ toxicity has been reported in the liver, kidney, thyroid gland, etc, as a result of exposure to hydrogen cyanide (Dhaset al. 2011; Schrenk et al 2019). Hydrogen cyanide present in improperly processed cassava has been reported to cause intracellular depletion of oxygen, creating an anaerobic environment that ultimately leads to cell death and tissue/organ injury, with resultant impairment of tissue/organ functions and/or structures (Nnoliet al. 2013; Zuhra and Szabo 2021). Therefore, this study aimed to evaluate the potential of cassava products to cause alterations in hematological and histological properties using Wistar rat as a biological model.

2. Materials and Methods

2.1 Samples Collection

Freshly harvested cassava tubers were purchased from farmers in Ovbiogie community, Ovia North East Local Government Area, Edo State. Cassava was processed into cassava paste and fried garri. Cyanide concentration in cassava paste (after grinding) and fried garri was determined by the alkaline titration

method as described by AOAC (1980). In this method, 10 g of sample was allowed to soak for 4 hr in 200 ml of water in a round bottom flask. Steam distillation was then carried out until the distillate collected was up to 150 ml. Then, 8.0 ml of 6 N NH_4OH and 2 ml of 5% potassium iodide were added to the distillate. Titration with 0.02 N $AgNO_3$ until there was a faint but permanent turbidity, was carried out. Cyanide was calculated by using the formula; 1ml of 0.02 N $AgNO_3$ = 1.08 mg HCN.

Forty (40) inbred Swiss Rattus norvegicus(albino rats) weighing between 120-180 g were purchased from the Anatomy Department, University of Benin, and used for the in vivo study. Approval for the experimental use of the rats was obtained from the ethical committee at the University of Benin, Benin City, Edo State. The animals were acclimatized for a period of 14 days. The albino rats were divided into two major groups, designated Fried Garri (FG) and Cassava Paste (CP). Each of the major groups was divided into four (4) subgroups of five animals each, designated as A (only cassava form administration), B (cassava form and immunosuppressor), H (1mg/kg KCN), and I (only feed and water). All groups were given food and water appropriately. At the end of 14 days, all rats were sacrificed under mild anesthesia with chloroform, and all blood was withdrawn into EDTA bottles.

2.2 Hematological Analysis

Total white blood cell and differential cell counts were analyzed according to Brown and Wittwer (2000), using a Beckman Coulter hematology analyzer to apply total and differential peripheral blood cell counts. Parameters analyzed include hemoglobin, red blood cells, white blood cells, monocytes, lymphocytes, and platelets. A blood film assay was used to determine neutrophil counts.

2.3 Quantitative Determination of CD_4 and CD_8 cells

CD_4 and CD_8 cell counts were estimated by use of a flow cytometer according to Brown and Wittwer (2000). The CD4 + lymphocytes were estimated using the Cyflow Automated Cell Counter (Partec, Germany).

2.4 Histological Analysis

The kidney, thymus, spleen, and liver specimens harvested from each rat were immediately stored in 10%v/v formalin in normal saline after gross histological examination and dehydrated using increasing concentrations of isopropyl alcohol (80–100%). Paraffin sections at 5 μm thickness were made from the paraffin-embedded organs using a Leica rotary microtome (Bright B5143 Huntington, England). This was followed by routine staining with hematoxylin and eosin which involved the process of deparaffinization, hydration, staining, rinsing, and clearing in xylene. Slides were viewed under a light microscope with photomicrographs taken with a Leica DM750 Camera Microscope (X 400).

2.5 Data Analysis

All data analyses were carried out using SPSS version 23. Descriptive statistics and Analysis of Variance (ANOVA) were used to analyze data. Data are presented as mean ± standard error (mean±SEM). Descriptive statistics were used to analyze measured data, and ANOVA was used to establish statistical significance among variables.

3. Results

3.1 Results of Cyanide Concentration in Cassava Forms

The result of cyanide concentrations in the different forms of cassava revealed that cyanide concentration was highest in the raw cassava paste compared to fried garri. Cyanide concentration in raw cassava paste was 133.16 ± 16.23 mg/kg compared to freshly fried garri with a cyanide concentration of 59.40±8.07 mg/kg (Table 1).

3.2 Results of the Effect of Cyanide in Different Cassava forms on some Hematological Parameters in Rat

The effect of fried garri on some hematological parameters in the rat is presented in Table 2. Cells of the innate immune system including white blood cells, monocytes, granulocytes, and neutrophils revealed a drastic reduction in cell population in groups A, B, and H while in groups I. In group A, WBC concentration was 4.98±0.49 $x10^8$µL compared to 7.88±1.01 $x10^8$µL in group I (normal control). While for the Neutrophil count, a value of 46.80±0.86 % was found compared to 52.80±0.96 % in group I. There were significant differences in cell counts among the different groups. Monocytes and granulocytes were also found to be higher in group I compared to group A.The lymphocytes cell population in the normal control Group 'I' (received feed and water alone) was high. When compared to the group of animals that received fried garri alone, 'A', fried garri and immunocompromised 'B' and H (received potassium cyanide as positive control), cell populations were significantly reduced. Similar occurrences were observed in the cell populations of CD_4 and CD_8. Groups A, B and H had reduced cell populations compared Group I which had the highest cell population.

Table 3 shows the effects of cassava raw paste on some hematological parameters in rat. Raw cassava paste had negative effects on innate cells of the immune system in experimental animals. Cells including white blood cells, monocytes, granulocytes and neutrophils were found to higher in group I that received feed and water only. While in group A, that received only cassava raw paste, there was reduced cells population. In group A, white blood cell, monocytes, granulocytes and neutrophil counts were 5.68±0.26$x10^8$µL, 0.50±0.03 $x10^8$µL, 0.42±0.02 $x10^8$µL, 35.20±1.93 % respectively. However, in the normal control group, white blood cells, monocytes, granulocytes and neutrophils counts were 7.88±1.01 $x10^8$µL,

0.56±0.17 x10^8µL, 0.76±0.26 x10^8µL and 52.80±0.96%. Lymphocyte counts were low in groups A and H compared to group I, where it was considerably high. Similar trend was observed in the CD_4 and CD_8 cell populations. There were observed significant difference in cells population among the different groups.

3.3 Results of Phagocytotic Activities in Rats Administered with Different Forms of Cassava Effects of fried garri on the phagocytic ability of the reticule-endothelial cells, as presented in Figure 1, revealed lower carbon clearance from the blood system of experimental animals while higher carbon clearance activity was observed in groups D, E, and I with the highest value in group E. There was a significant difference in phagocytic activities among the different groups.

Figure 2 shows the effect of raw cassava paste on carbon clearance activity in experimental animals. Carbon clearance activity in experimental animals administered with raw cassava paste was found to increase in groups D, E, and F, treated with mushroom, algae, and Vit B_{12} compared to groups A, B, C, G, and H with lower values. While in group I, carbon clearance activity was highest. There was a significant difference

Table 1: Cyanide concentrations (mg/kg) in different cassava forms

Cassava forms	Cyanide content (mg/kg)
Cassava paste	133.16±16.23^{b}
Fried garri (day 1)	59.40±8.07^{a}
p-value	0.000

p-value < 0.05 means there is a significant difference. Different superscript alphabets indicate significant differences among values

Table 2: Effects of cyanide in fried garri on some hematological parameters in rat

Groups	WBC (x10^8µL)	MO(x10^8µL)	GR(x10^8µL)	Neutrophil (%)	Lym(x10^8µL)	CD_4 (cell/µL)	CD_8 (cell/µL)
A	4.98±0.49^{abc}	0.40±0.08^{ab}	0.52±0.15	46.80±0.86^{bc}	2.56±0.39^{a}	6.52±1.26	1.96±0.38
B	3.16±0.38^{ab}	0.26±0.07^{a}	0.28±0.02	45.80±3.5^{bc}	1.92±0.29^{a}	6.20±1.01	2.10±0.27
H	2.92±0.07^{a}	0.30±0.10^{a}	0.32±0.21	37.60±2.46^{a}	1.16±0.39^{a}	5.94±1.24	1.78±0.37
I	7.88±1.01^{d}	0.56±0.17^{ab}	0.76±0.26	52.80±0.96^{d}	5.56±1.03^{c}	9.20±0.39	2.76±0.12
p-value	0.004	0.040	0.167	0.000	0.000	0.099	0.103

p-value < 0.05 indicates a significant difference in parameters among different groups. Different superscript alphabets show that values are significantly

different from each other while similar alphabets show values are not significantly different. Legend: A= only fried garri (FG); B= FG plus immunosuppressor;H= only potassium cyanide (KCN) positive control; I= normal control (just feed and water administration). WBC = white blood cell, MO= monocyte, GR = granulocyte.

Table 3: Effects of cyanide in raw cassava paste on some hematological parameters in rat

Groups			Parameter					
	WBC ($x10^8$µL)	MON($x10^8$µL)	GR($x10^8$µL)	NEUT		Lym($x10^8$ µL)	CD_4(cell/ µL)	CD_8(cell/ µL)
A	5.68 ± 0.26^{ab}	0.50 ± 0.03	0.42 ± 0.02	35.20 ± 1.93^{ab}		2.48 ± 0.11^{a}	6.78 ± 1.25	2.03 ± 0.37
B	3.80 ± 0.62^{a}	0.28 ± 0.04	0.42 ± 0.06	31.00 ± 0.44^{a}		2.48 ± 0.71^{a}	7.16 ± 1.14	2.15 ± 0.34
H	2.92 ± 0.07^{a}	0.30 ± 0.10^{a}	0.32 ± 0.21	37.60 ± 2.46^{a}		1.16 ± 0.39^{a}	5.94 ± 1.24	1.78 ± 0.37
I	7.88 ± 1.01^{d}	0.56 ± 0.17^{ab}	0.76 ± 0.26	52.80 ± 0.96^{d}		5.56 ± 1.03^{c}	9.20 ± 0.39	2.76 ± 0.12
p-value	0.000	0.106	0.413	0.000		0.000	0.075	0.076

p-value < 0.05 indicates a significant difference in parameters among different groups. Different superscript alphabets show that values are significantly different from each other while similar alphabets show values are not significantly different. Legend: A= only cassava paste (CP); B= CP plus immunosuppressor. H= only potassium cyanide (KCN) positive control; I= normal control (just feed and water administration). WBC= white blood cell, MON= monocyte, GR= granulocytes, NEUT: neutrophil, RBC= red blood cell, PLT= platelets.

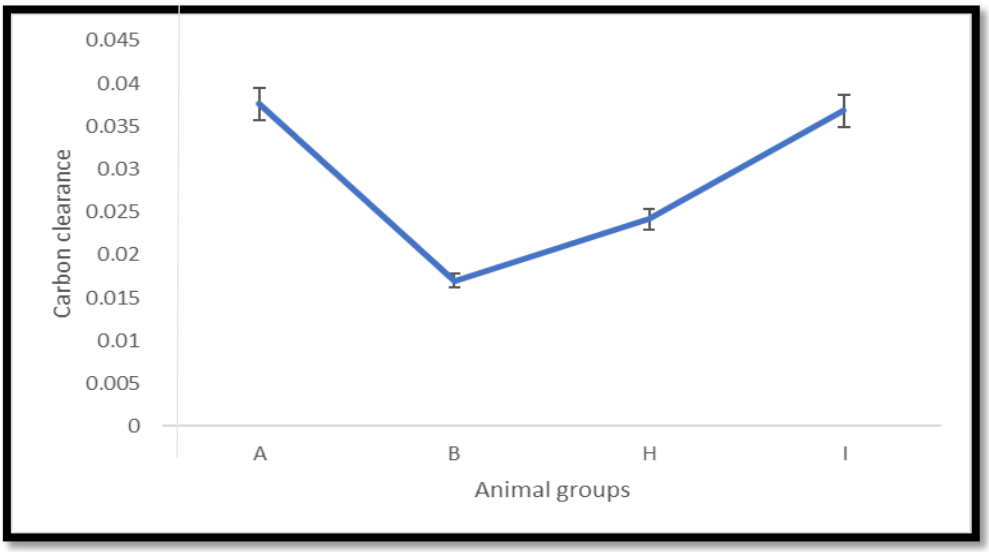

Figure 1 Phagocytotic activity in rats administered with fried garri

p-value < 0.05 Legend: A= only fried garri (FG); B= FG plus immunosuppressor; H= only potassium cyanide (KCN) positive control; I= normal control (just feed and water administration)

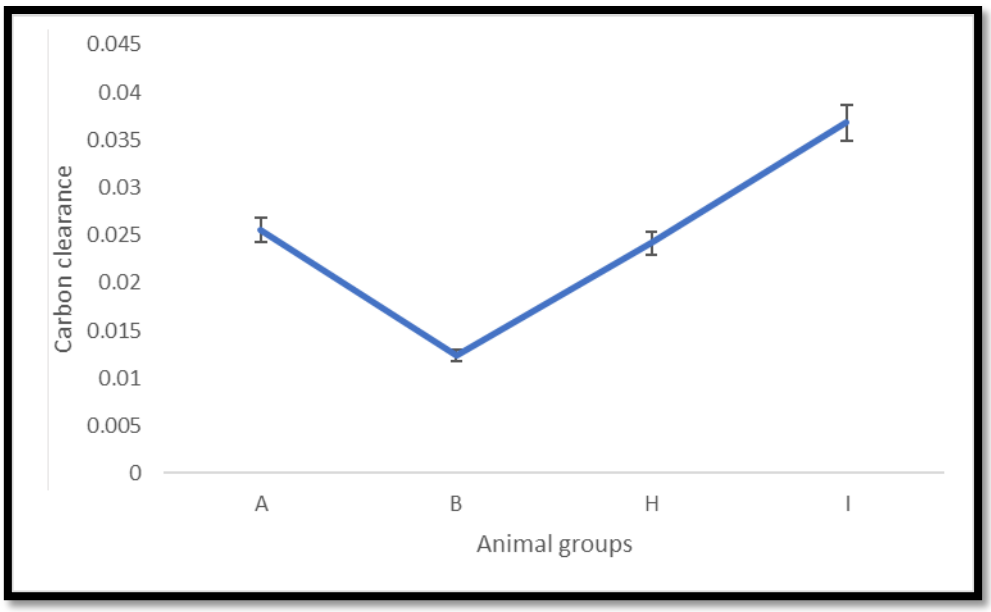

Fig. 2: Phagocytotic activity of rats administered with cassava paste p-value >0.05 Legend: A= only cassava paste (CP); B= CP plus immunosuppressor; H= only potassium cyanide (KCN) (negative) control; I= normal control (just feed and water administration.

3.4. Results of Organs (Liver, Kidney, Spleen, and Thymus) Toxicity in Animals Exposed to Different Fried garri

Plate 1 shows histology slides of a rat's liver exposed to fried garri (A). The liver reveals thickened centriole with congestion (long arrow). Hepatocytes show a vacuolated nucleus (short arrow) while in B (normal control), liver histology reveals a visible centriole with mild inflammatory cells surrounding it (arrow) and hepatocytes with the pyknotic nucleus.

Plate 2 reveals histology slides of the rat's thymus exposed to fried garri (A). Thymus reveals cortex surrounding (long arrow) adipose tissue (short arrow) and medulla. While in (B, normal control), the thymus reveals cortex surrounding (long arrow) adipose tissue (short arrow) and medulla.

Plate 3 shows histology slides of the rat spleen exposed to fried garri (A). In A, Spleen's histology reveals white and red pulp with a prominent dilated central artery (arrow) and prominent lymphocytes. In B (normal control), Spleen histology reveals white and red pulp with a prominent central artery with mild dilation (arrow) and prominent lymphocytes.

Plate 4 reveals histology slides of a rat's kidney exposed to fried garri (A). Kidney reveals mild atrophic (short arrow) renal corpuscle with mild tubular necrosis

(long arrow). In B, (normal control) kidney reveals renal corpuscles with mild inflammatory cells in the interstitial.

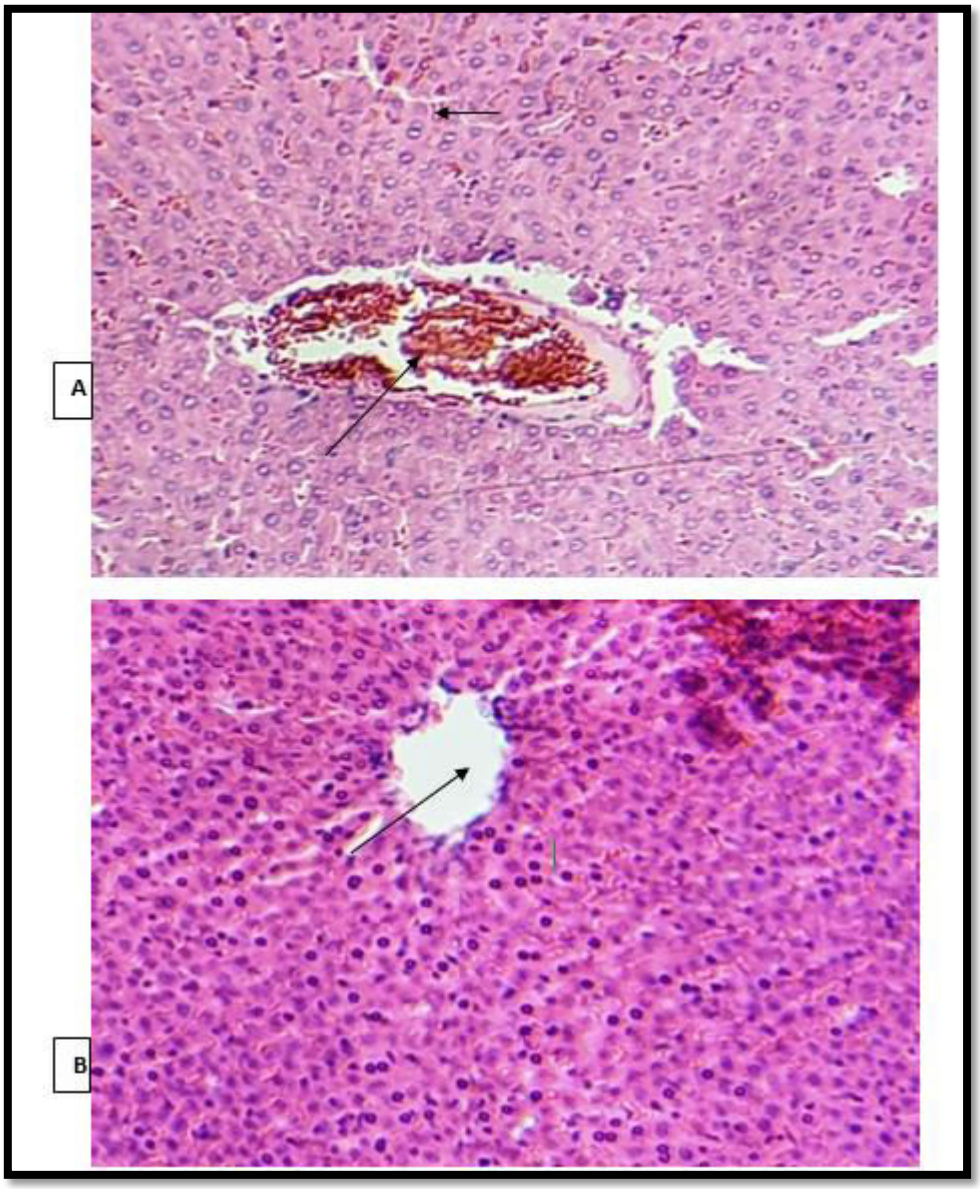

Plate 1: Histology slides of rats liver (400x) exposed to fried garri (A) and normal control group (B) using hematoxylin and eosin stains In A, long arrow = thickened centriole with congestion while short arrow = Hepatocytes vacuolated nucleus of hepatocytes. In B, the arrow represents a normally visible centriole.

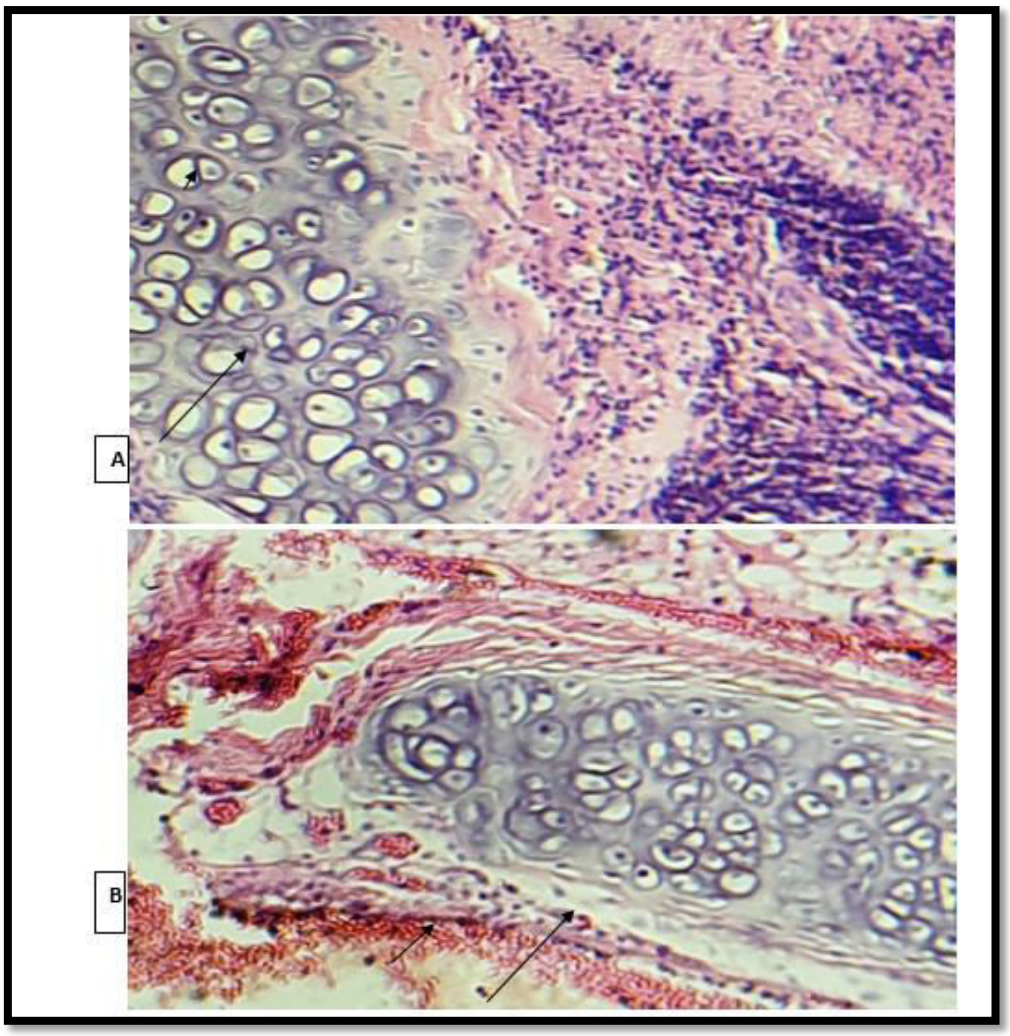

Plate 2: Histology slides of rats thymus (x400) exposed to fried garri (A) and normal control group (B) using hematoxylin and eosin stains. In A, long arrow = Thymus cortex surrounding abnormal adipose tissue and medulla (short arrow). In B, the long arrow represents normal adipose tissue while the short arrow points to the normal medulla.

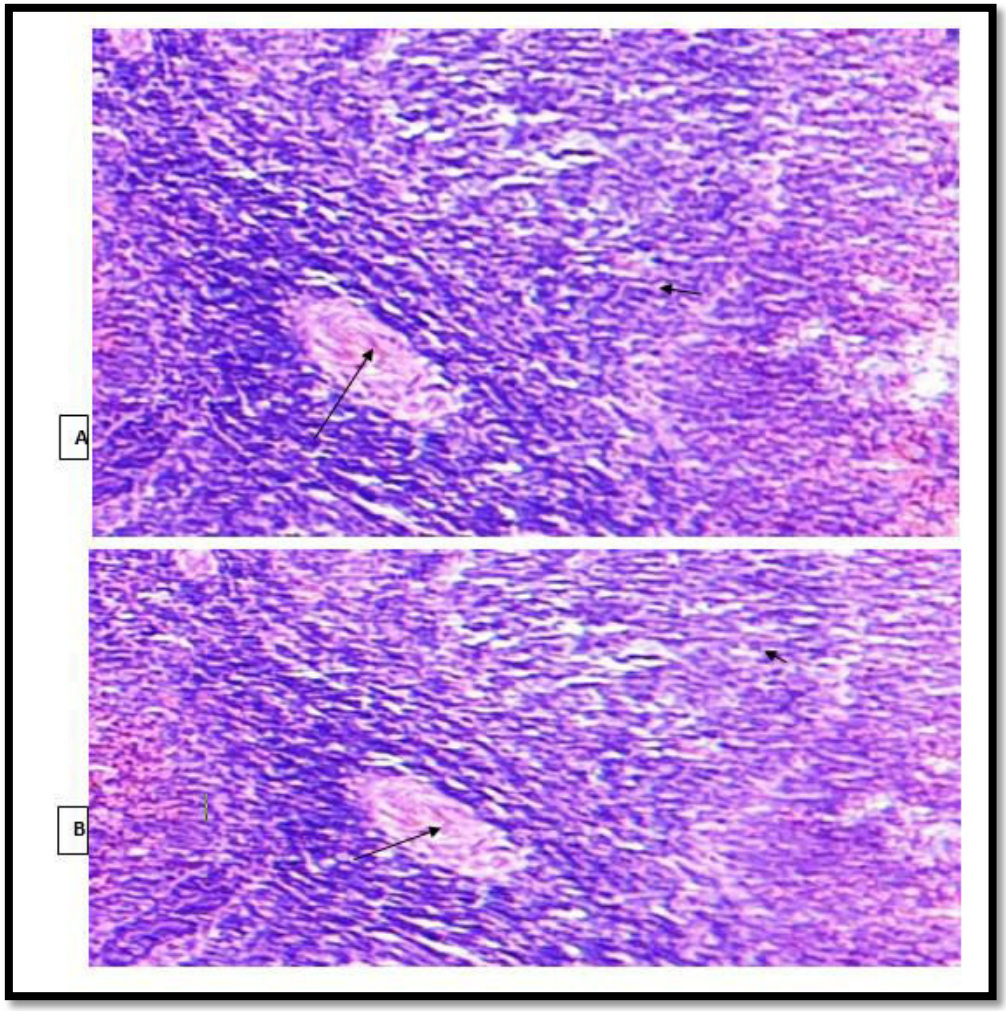

Plate 3: Histology slides of rat's spleen (x400) exposed to fried garri (A) and normal control group (B) using hematoxylin and eosin stains. In A, the long arrow = white and red pulp with a prominent dilated central artery while the short arrow reveals prominent lymphocytes. In B, the long arrow represents white and red pulp with a prominent central artery with mild dilation while the short arrow shows prominent lymphocytes.

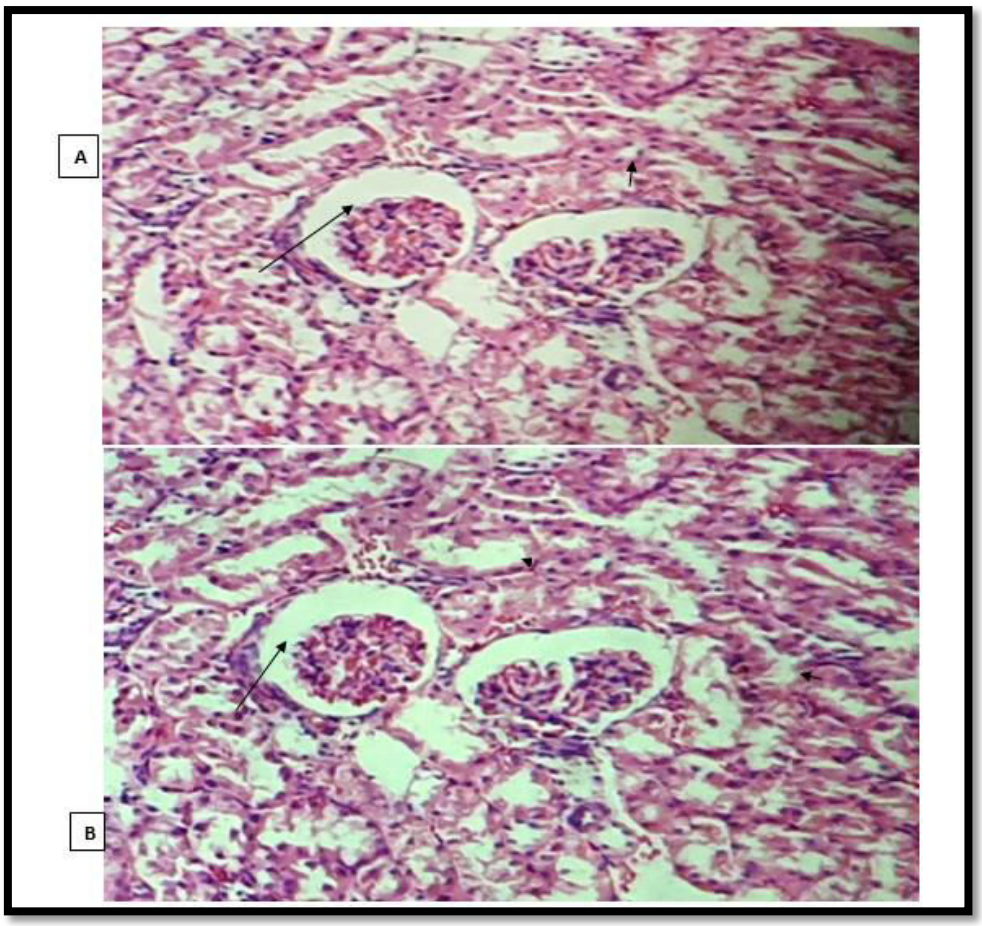

Plate 4: Histology slides of rat's kidneys (x400) exposed to fried garri (A) and normal control group (B). In A, the presence of mild atrophic (short arrow), renal corpuscle with mild tubular necrosis (long arrow) while in B, renal corpuscles with mild inflammatory cells in the interstitial are present.

4. Discussion

Cyanide has been recognized as a potential toxicant in animals, especially ruminants (Belani et al. 2012). Plants such as Manihot esculenta are major sources of cyanide in which case cyanide is present as a cyanogenic glycoside (Anseeuwet al. 2013).

It was observed in this study that cassava paste had the highest cyanide content compared to fried garri. The difference may be a result of the different processing methods such as peeling, washing, grating, fermentation, drying/dewatering, milling/pulverizing, and frying which may significantly reduce the cyanogens contents (Ndam et al. 2019). At drying temperatures above 55 °C, linamarase activity is inhibited and, therefore, linamarin starts to accumulate in dried cassava. Hamel(2011) showed a decrease in the cyanide content of cassava products at 75 °C during garri processing. The result of the present study confirms the report of Mowanget al. (2011) that there was a significant difference in cyanide content between fried garri (yellow and white

garri) and cassava paste. The result of the study by Ezeigboet al. (2015) showed that an increase in cooking time decreased the starch and cyanide contents of the cassava samples. Improperly processed cassava products contain some amount of residual linamarin and hydrogen cyanide. The significant difference between total cyanide contents in cassava products obtained in this study is consistent with the report of Howardet al. (2011) and Adeniji (2013) who found a significant difference between HCN concentrations in cassava varieties. This result is in agreement with the study of Thompson and Marrs (2012) who reported that the levels of cyanide in cassava paste were generally higher than that in processed cassava.

When there is physiological stress, it usually results in the alteration of hematological parameter balance in the host (Abuh et al. 2011). Findings from this study indicate showed reduction in hematological indices of rats exposed to cassava raw paste and fried garri compared to control rats which had normal counts. In the treatment groups, there were marked changes in hematological parameters such as white blood cells, monocytes, granulocytes, and neutrophils with a decrease in concentrations compared to the control group. This finding is in agreement with the studies of Abuh et al. (2011) who reported that cyanide from extracts of Manihot utilissima decreased the concentrations of hematological parameters in exposed rats compared to the normal control group.

The decline in WBC levels in groups treated with fried garri compared to the normal control group may have been linked to the cytotoxic effects of cyanide in the product. Potassium cyanide showed deleterious effects against WBC, causing the positive control group to demonstrate a significant decline in WBC which indicates the probability of systemic damage to the immune system due to the persistence of high doses of KCN. This report is in agreement with the findings of Lohner et al. (2001) and Shahi and Singh (2014) who reported a decline and increase in WBCs respectively under different potassium cyanide concentrations. Janeway et al.(2005), explained that decreased number of WBC may be due to reduced production or rapid removal from circulation and subsequent destruction. The reduction of WBC is indicative of the immunosuppressive effects of potassium cyanide which has been previously reported. Moreover, the results obtained by Goel et al.(2006) explained that during cyanide poisoning, the decrease in WBC could be a result of their slow production, or inhibited release into the circulation. It is also possible for immune-toxicant such as cyanide, to directly cause cytotoxicity of WBC, leading to its decrease in circulation.

It was observed in this study the fried garri and raw cassava paste administered to experimental rats reduced the monocyte and neutrophil counts when compared to the normal control group. This finding agrees with a previous report by Abuh et al. (2016). In their study, a group of experimental rats administered with different concentrations of sweet cassava had reduced monocytes and neutrophil counts compared to a normal control group not administered.

Lymphocytes, especially T cells are critically important in the cell-mediated arm of immunity and any alteration in its balance will ultimately affect its functionality (Oloruntola 2020). In this study, the lymphocyte population including CD4 and CD8 cells was reduced following exposure to cassava products. The reduction is ascribed to high levels of hydrocyanic acid. This finding is supported by findings from the work of Dennis (2005) and Oloruntola (2020) who reported lower concentrations of lymphocytes in rabbits fed with cassava peel meal and identified hydrocyanic acid as the cause of the reduction.

The result of carbon clearance activities in rats exposed to cassava products revealed a reduced phagocytic activity of phagocytes. These phagocytes play an important role in removing debris such as dead cells, immune complexes, and other particles by phagocytosing them, from the circulation which is critical to healthy living. In this work, cyanide in freshly fried cassava (garri) and raw cassava paste has been proven to have negative effects on the particle clearance potential of the reticule-endothelial system. This study has shown that cyanide in cassava hurts immune cells.

The effect of cyanide on the histology of the liver of Wistar rats reveals visible centrioles with mild inflammatory cells surrounding them and hepatocytes with pyknotic nuclei. This result differs from the report of Fasogbonet al. (2015) who reported that guinea pigs injected intramuscularly with 1ml of 30 mg dose of potassium cyanide did not have any physical or histological effect on the liver of the guinea pigs. FAO (2006) reported that at exposure to low doses of cyanide compounds, most of the cyanide and its product leaves the body within the first 24 hours after exposure. The way cyanide enters and leaves the body is similar in people and animals (FAO, 2006).
The liver tissues showed gross abnormalities with Haematoxylin and Eosin stains. Thickened centriole with congestion, vacuolated nucleus, renal corpuscle with glomerulus that appears atrophied, and interstitial space with inflammatory cells and tubules were the microscopical observation when compared with the finding from their controls. This result is different from the report of Okolie and Iroanya (2003) who stated that no histopathological changes were observed in the kidneys of rats and monkeys exposed to 25 ppm cyanogen (12.5 ppm cyanide) 6 hours/day, 5 days/week for 6 months. Okolie and Iroanya (2003) also reported a decrease in renal activities of male rabbits ingested with 15 mg CN^- /kg/day of sodium cyanide in feed for 4 weeks. Changes in absolute and relative kidney weights were observed in rats and mice exposed to 0.2–12.5 mg CN^- /kg/day and mice exposed to 0.3– 28.8 mg CN^- /kg/days sodium cyanide in the drinking water for 13 weeks (Barbara et al. 2006). Histopathological lesions of the kidney have been reported in animals exposed to cyanide (Manzano et al. 2007). Thickened congested centrioles with radiating hepatocytes with pyknotic nuclei

were observed in rats exposed to potassium cyanide. Histopathological lesions were marked in the fish injected with the higher dose (10 ml), the fish revealed severe necrosis, hypertrophy, and vacuolation of hepatocytes (Adeyemo 2005). histopathological examination of the kidney, gill, and liver of the treated fish indicated damages, ranging from edema and telangiectasis of the gill lamella and gill hyperplasia to vacuolation of the liver cells and necrosis. Adewoyeet al. (2016) reported Degenerative changes were congestion, vacuolization of hepatocytes, cellular infiltration, and necrosis. Furthermore, the liver revealed slight vacuolated cells which is an indication of fatty degeneration of hepatocytes and histological degradation in most organs of Claras gariepenus

5. Conclusion

This study has shown that cassava products including fried garri and raw cassava paste not properly processed are rich sources of cyanide. Due to the presence of cyanide, these products significantly reduced hematological parameters and cells that mediate the functionality of the immune system. It is therefore important that processors must ensure that level of cyanide is drastically reduced by following the protocols that significantly reduce the level of cyanide in cassava products.

6. Acknowledgments

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7. Statements and Declarations

Funding Declaration : This research did not attract funding from any Agency.

Conflict of interest: We declare no conflict of interest amongst us.

Author Contribution

All authors contributed to the research design, data collection, data analysis, writing, editing, and review of this manuscript. While Lucky Evbuomwan conceived the research

Declarations

"All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors and are aware that with minor exceptions, no changes can be made to authorship once the paper is submitted. All authors have agreed to publish the research in International Journal of Chemical and Biochemical Sciences

Ethics approval

"This study was performed in line with the principles of the Declaration of Helsinki". Approval was granted by the Ethics Committee of the University of Benin, Benin City Nigeria 2019/2020."

Consent to participate: No consent was needed in this study because of the used of animal subjects.

Consent to publish: The authors give the full consent for the publication of the full manuscript.

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