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The Amelioration of Cyanide Induced Liver Toxicity with Bentonite Using Wistar Rat as Experimental Model

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Authors' contributions

This work was carried out in collaboration between both authors. Author ONF designed the study, supervised the work, managed the analyses of the study and wrote the protocol and the first draft of the manuscript. Author NEC performed the experiment and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The objective of this study was to investigate the ameliorating effect of a natural bentonite found in Nigeria on liver enzymes of Wistar rat fed with cyanide from cassava waste water. The enzymes assayed were alkaline Phosphatase (ALP), alanine amino transferase (ALT) and aspartate amino transferase (AST).

Materials and Methods: A total of 45 Wistar rats were used. The rats were divided into four groups and a control group. Each group had nine rats including the control. Groups 1, 2, 3, and 4 were given a daily dose of 0.4ml of cassava waste water containing a sub-lethal amount of cyanide (2.16×10^{-3} mg) for three weeks. Groups 2, 3, and 4 were counter administered with 0.2 ml, 0.4 ml, and 0.8 ml of 7% bentonite solution respectively. Administration of bentonite was done simultaneously with the cyanide administration. Serum samples were assayed weekly for ALP, ALT and AST activities.

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Results: A significant increase in ALP, ALT and AST activities (p=0.05) for each week was recorded for group 2 (0.5 ml cyanide) when compared to the groups fed with bentonite. Significant decreases (p=0.05) were seen in the ALP, ALT and AST levels of groups fed with bentonite when compared to the control group and to the group fed only cyanide, with group 5 (0.8 ml bentonite) having the lowest ALP for each week. Body weight gain and reduction was also observed.
Conclusion: The results of this study suggest that bentonite has a detoxifying effect on the liver of cyanogenic Wistar rats.

Keywords: Alanine amino transferase; aspartate amino transferase; alkaline phosphatase; amelioration; bentonite; cyanide; detoxifying; enzymes; liver.

1. INTRODUCTION

Bentonite has been found to be effective in counteracting toxins [1,2]. However, the ability of bentonite to bind these toxins depends on pH, molecular arrangements, and its geographic region of origin [3,4,5,6].

Cyanide is considered a toxic, deadly substance, and has been used as a poison for thousands of years. The effects of a high dose of cyanide are quick, and death occurs within minutes. Antidotes are effective if administered in time [1,7].

Cassava (*Manihot esculentagrantz*) is a woody shrub that is widely cultivated in many tropical countries for its edible, starchy and tuberous root. It is consumed in many developing countries as a major source of carbohydrates and forms the basis of many traditional foods such as lafun, fufu, garri, and tapioca flour. In terms of annual production, cassava is the sixth most important food crop globally and is a staple food for approximately 800 million people [8]. Storage roots of cassava, also known as tapioca or manioc provides an important source of carbohydrate to some 500 million people in 30 tropical countries [9].

Cassava is a starchy staple whose roots are very rich in carbohydrates, a major source of energy. The cassava plant is the highest producer of carbohydrates among crop plants with perhaps the exception of sugarcane [10]. It is one of the most perishable tuber crops with a high postharvest loss [11]. Anatomically cassava root is not a tuberous root, but a true root, which cannot be used for vegetative propagation. The mature cassava storage root has three distinct tissues: bark (periderm), peel (cortex) and parenchyma. The parenchyma, which is the edible portion of the fresh root, comprises approximately 85% of the total weight, consisting of the xylem vessels radially distributed in a matrix of starch containing cells [12]. The cyanide concentration in cassava varies in different parts of the plant, according to variety, location, age, and environmental conditions. Consequently, cassava is of lower nutritional value than cereals, legumes, and even some other root and tuber crops such as yams [8]. Cassava root contains significant amount of iron, phosphorus, calcium and vitamin C, but is a poor source of proteins. The root contains carbohydrates, 64 to 72% of which is made up of starch, mainly in the form of amylose and amylopectin. About 17% sucrose is found in sweet varieties. The lipid content of cassava is only 0.5%, Proteins content is about (1 to 2%), and the amino acid profile of the cassava root is very low in some essential amino acids, particularly lysine, methionine, and tryptophan. Cassava is reasonably rich in calcium and vitamin C, but the thiamine, riboflavin, and niacin contents are not high [10,13,14].

Cassava is classified as either sweet or bitter. Like other roots and tubers, both bitter and sweet varieties of cassava contain antinutritional factors and toxins, with the bitter varieties containing much larger amounts. They must be properly prepared before consumption, as improper preparation of cassava can leave enough residual cyanide to cause acute cyanide intoxication, goiters, and even ataxia or partial paralysis. The more toxic varieties of cassava are a fall-back resource (a "food security crop") in times of famine in some places. Farmers often prefer the bitter varieties because they deter pests, animals, and thieves. Cassava grows well in a tropical climate and is an important root crop in Pacific Island countries, Latin America, Africa and regions of Asia. However, if eaten raw or processed inadequately, its consumption may be potentially harmful due to the presence of cyanogenic glycosides, which break down to produce hydrogen cyanide [15,16,17].

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase [18].

Aspartate amino transferase (AST) catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Alanine amino transferase (ALT) is found in plasma and in various body tissues, but is most common in the liver. Serum ALT and AST levels and AST/ALT ratio are commonly measured clinically as biomarkers for liver health. These enzymes have become increasingly important in clarifying the etiology, pathogenesis as well as diagnosis of a number of diseases associated with the different organs in which they are present or concentrated.

Research on the cyanogen of cassava has been focused largely on the biochemistry and physiology of linamarin synthesis and metabolism as it accounts for 95% of the total cyanogenic glycosides present in intact tissues [9]. Numerous researches have also shown that bentonite has electrical attractions that draw toxins into its space and bind them there. Studies also have revealed that bentonite can absorb pathogenic viruses, herbicides and pesticides [4].

This research is aimed at evaluating the *in vitro* effect of administration of cassava waste water (which is cyanogenic) and the effect of the counter treatment with antitoxic bentonite on the liver enzymes, such as ALP, ALT and AST of Wistar rats. Considering all these facts, the present study is undertaken to investigate the ability of natural bentonite to ameliorate the effect of cyanide toxicity in the liver of Wistar rats.

2. MATERIALS AND METHODS

Reagent kits were bought from Randox Laboratories Ltd. Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom BT29 4QY.

A total of forty five male and female Wistar rats (Ratus rattus) were obtained from the small animal holding unit of the Department of Biochemistry, University of Port-Harcourt, Choba Nigeria. The average weight of the rats was 100 g. They were housed in clean metabolic cages which were cleaned of wastes twice daily at 12 hours each of day and night at room temperature.

2.1 Sample Collection

Calcium bentonite clay was obtained from bentonite deposit at Anambra state in Nigeria.

Cassava (*Manihott esculenta)* root was bought from the Choba local market, Rivers State Nigeria, and was identified in the Plant Science and Biotechnology Laboratory of the University of Port Harcourt.

2.2 Sample Preparation

Fresh cassava tuber was collected and pilled. 15 g of the pilled cassava was soaked in 200 ml of water for 3 hours.

2.3 Determination of Cyanide Content in the Cassava Extract [19]

15 g of sample was measured into 800ml kjedahl flask containing 200 ml distilled water and allowed to stand for 3 hours at 25±5°C. Autolysis was carried out with the apparatus connected to a distiller. A 150 ml distillate was collected in 20 ml 25% of NaOH solution and further distilled to 250ml with distilled water. 100 ml of the distilled distillate was mixed with 8.0 ml of 6M NH_4OH and 2.0 ml of 5% KI indicator solution and titrated against 0.002M $AgNO_3$. The end point was indicated with a faint permanent turbidity appearance. The cyanide content (mg/100 g cassava wet weight) of the sample was evaluated from the expression:

- 1.0 ml 0.02M $AgNO_3$ ≡ 1.08 mg HCN

After the test, it was observed that 200 ml of cassava waste water from 15 g of cassava (with the back pilled off) contains 1.08 mg of hydrogen cyanide (HCN). Ronald [20] had earlier reported the LD50 of cyanide to Wistar rat and found that the sub-lethal dose of cyanide to Wistar rat is 0.1 to 10 mg CN/kg body weight of rat.

1.4 g of bentonite was mixed with 20 ml of distilled water to obtain 7% of bentonite solution.

Table 1. Administration of cyanide and bentonite to the wistar rats

	Administration		Animals sacrificed at the		
	Cassava waste water administered	Bentonite (in solution) administered	7^{th} day	14^{th} day	21^{st} day
Group 1	0.4 ml	Nil	3	3	3
Group 2	0.4 ml	0.2 ml	3	3	3
Group 3	0.4 ml	0.4 ml	3	3	3
Group 4	0.4 ml	0.8 ml	3	3	3
Control	Nil	Nil	3	3	3

Note: 0.4 ml of cassava waste water contains 2.16 x 10^{-3} mg of hydrogen cyanide (HCN)

2.4 Alkaline Phosphatase Determination

2.4.1 Principle

Alkaline phosphatase (ALP) catalyzes the hydrolysis of a wide variety of physiologic and nonphysiologic phosphoric acid esters in alkaline medium (pH optimum 10). The liver and biliary tracts are the source of alkaline phosphatase in normal sera. Normal alkaline phosphatase levels are age dependent being higher in children and adolescents in comparison to adults. ALP is one of the tests of choice for evaluating cholestasis and obstructive jaundice. Elevated levels are found in many diseases including hepatitis, cirrhosis, malignancy, and in bone diseases.

Colorimetric determination of alkaline Phosphatase activity according to the following reaction:

$$\text{Phenyl phosphate} \xrightarrow[pH10]{ALP} \text{Phenol} + \text{Phosphate}$$

2.4.2 Reagent composition

The reagents used are labeled as follows:

Composition of reagents for ALP assay

Reagent	Composition	Concentration
Reagent 1 (R1 Buffer) pH 10	Disodium phenylphosphate	5.0 mmol/L
	Carbonate-bicarbonate buffer	50 mmol/L
Reagent 2 (R2 Standard)	Phenol	Equal to 140 U/l
Reagent 3 (R3 Blocking reagent)	4-aminoantipyrine	60 mmol/L
	Sodium arsenate	240 mmol/L
	Buffer pH 10	
Reagent 4 (R4 Color reagent)	Potassium ferricyanide	150 mmol/L

2.4.3 Procedures

The following tubes were set up:

	Reagent blank	Standard	Serum blank	Serum sample
R1	1 ml	1 ml	1 ml	1 ml
The solution was incubated for 5 minutes at 37ºC.				
R2		25 µl		
Serum				25 µl
The solution was incubated for 15 minutes at 37ºC.				
R3	250 µl	250 µl	250 µl	250 µl
The solution was mixed properly.				
R4	250 µl	250 µl	250 µl	250 µl
Serum			25 µl	
Distilled water	25 µl			

The solution mix was properly mixed and allowed to stand for 10 minutes before being read with the spectrophotometer against reagent blank at wavelength of 510 nm and at 1 cm light path [21].

Calculations:

$$Absorbance\ of\ sample = \frac{absorbance\ of\ serum\ blank - absorbance\ of\ serum\ sample}{absorbance\ of\ standard}\ \text{X n}$$

n = 140 (IU/L)

2.5 Aspartate Amino Transferase Determination

Aspartate reagent kit contained 1: Phosphate buffer (100 mol/l, pH 7.4), L-aspartate (100 mmol/l), α-oxoglutarate (2 mmol/l). 2: 2, 4-dinitrophenylhydrazine (2 mmol/l).

Aspartate levels were determined by colorimetric test. The principle of this method is that Glutamic – Oxaloacetic transaminase is measured by monitoring the concentration of oxaloacetate hyrazone formed with 2, 4-dinitrophenylhydrazine [22]. Solution 1 (0.5 ml) was mixed with 0.1 ml of the sample. The blank tube contained 0.5 ml of solution 1 and 0.1 ml of distilled water. The mixtures were incubated for 30 minutes at 37°C. Solution 2 (0.5 ml) was then added to the tubes. They were then allowed to stand for 20 minutes at 25°C. 5 ml of sodium hydroxide was added to the tubes. The absorbance of the mixture was read against the reagent blank after 5 minutes using 1 cm light path cuvette at 546 nm with spectronic 20 spectrophotometer.

2.6 Alanine Amino Transferase Determination

Alanine reagent kit contained 1: Phosphate buffer (100 mol/l, pH 7.4), L-alanine (100 mmol/l), α-oxoglutarate (2 mmol/l). 2: 2, 4-dinitrophenylhydrazine (2 mmol/l).

Alanine levels were determined by colorimetric test. The principle of this method is that Glutamic – Pyruvic transaminase is measured by monitoring the concentration of pyruvate hyrazone formed with 2, 4-dinitrophenylhydrazine [22]. Solution 1 (0.5 ml) was mixed with 0.1 ml of the sample. The blank tube contained 0.5 ml of solution 1 and 0.1 ml of distilled water. The mixtures were incubated for 30 minutes at 37°C. Solution 2 (0.5 ml) was then added to the tubes. They were then allowed to stand for 20 minutes at 25°C. 5 ml of sodium hydroxide was added to the tubes. The absorbance of the mixture was read against the reagent blank after 5 minutes using 1 cm light path cuvette at 546 nm with spectronic 20 spectrophotometer.

2.7 Statistical Analysis

Data analysis was performed using the Statistical package for the Social Sciences software (SPSS, version 11.0). Data is displayed in mean ± SD. The statistical method of one way analysis of variance (ANOVA) was used to compare the mean values obtained among different groups. Differences were considered significant whenever the p-value is p=0.05.

3. RESULTS

The ameliorating effect of bentonite on cyanide-fed Wistar albino rats and its effect on their ALP levels are shown in Tables 3 to 5. The results are given as mean and standard deviations at p=0.05. Significant increases in ALP, ALT and AST levels were observed per week in group 2 (HCN) when compared with the bentonite fed groups (p=0.05). Significant decreases in ALP levels of bentonite fed rats were observed per week in each group (p=0.05). Group 5 (0.8 Bentonite) had the lowest ALP levels for all the weeks of treatment, followed by Group 4 (0.4 Bentonite) and then Group 3 (0.2 Bentonite).The ALP levels of the control group (without bentonite and HCN) remained consecutively constant throughout the period of the experiment.

The body weights of the animals before and after analysis are given in Table 2. Significant increases in body weight with time increment were seen among the control group (group 1). Significant decrease in body weight was observed in the group that was treated with only hydrogen cyanide (group 2), p=0.05. The groups which were fed with bentonite indicated increase in body weight. The weight gains were significant (p=0.05) in group 3 (16.67 g gain in weight) and group 4 (20 g gain in weight). Loss in body weight was seen in group 5 on the 21st day of the analysis.

4. DISCUSSION

In the first week of the experiment, the rats did not show any signs of toxicity in their appearance. Gradually, at week two (2), the rats fed with 0.5 ml of cassava waste water (group 2) developed lesions around their mouth, pale

colour in their skin and retarded growth rate. This was in agreement with the work done by Lewis et al. [23] and Sousa et al. [24]. Rats receiving bentonite had their normal appearance. The body weight comparison of animals fed with cyanide and those treated with bentonite were also noted. From the results, it was evident that there was a significant increase in weight (10.64%) of cyanide-fed animals treated with 0.2 ml of 7%w/v bentonite (group 3) from the third week. There was also a gain in weight (12.5%) for cyanide-fed animals treated with 0.4ml of bentonite (group 4) from the second week. The increase in weight gain (11.76%) was also seen in cyanide-fed animals treated with 0.8 ml of bentonite (Group 5) from the second week. This was followed by a weight reduction (17.25%) which was observed on the fourth week of the experiment. The increase in weight on treatment with bentonite agreed with the work of Santurio et al. [2] where the treatment of aflatoxin-intoxicated broiler chickens with sodium bentonite improved their body weights and improved their efficiency.

Loss in body weight of rats receiving only cyanide (group 2) was in agreement with Lewis et al. [23] and Sousa et al. [24].

Table 2. Body weights (in grams) of animals before and after analysis

Groups	Group 1 (control)	Group 2 (HCN)	Group 3 (0.2 Bentonite +HCN)	Group 4 (0.4 Bentonite + HCN)	Group 5 (0.8 Bentonite + HCN)
Day 0	140.00	120.00	140.00	140. 00	200.00
Day 7	140.00	120.00	140.00	140.00	200.00
Day 14	160.00	110.00	140.00	150.00	226.67
Day 21	160.00	100.00	156.67	160.00	193.33

Table 3. Alkaline phosphatase levels in blood samples (IU/L)

Groups	Group 1 (control)	Group 2 (HCN)	Group 3 (0.2 ml Bentonite +HCN)	Group 4 (0.4 ml Bentonite+ HCN)	Group 5 (0.8 ml Bentonite + HCN)
Day 7	110.30±27.64^{a}	40.00±4.23^{b}	20.33±0.55^{b}	19.67±2.82^{b}	19.50±0.69^{b}
Day 14	110.30±27.64^{a}	88.50±28.42^{b}	46.33±19.38^{b}	62.67±17. 35^{b}	40.33±5.65^{b}
Day 21	110.30±27.64^{a}	95.67±1.41^{a}	106.67±2.82^{a}	100.00±24.95^{a}	97.67±12.60^{a}

Results are means of triplicate determinations ± standard deviation.
$^{a, b,}$ Values without the same superscripts within a row differ significantly (p=0.05)

Table 4. Alanine amino transferase levels in blood samples (IU/L)

Groups	Group 1 (control)	Group 2 (HCN)	Group 3 (0.2 ml Bentonite +HCN)	Group 4 (0.4 ml Bentonite+ HCN)	Group 5 (0.8 ml Bentonite + HCN)
Day 7	8.00 ± 3.04^{a}	16.00 ± 3.21^{b}	15. 55± 0.35^{b}	14.27 ± 1.32^{b}	14. 3.0 ± 2.61^{b}
Day 14	8. 50 ± 1.04^{a}	17.50 ± 8.42^{b}	14.13 ± 4.38^{b}	12.67 ± 10. 15^{b}	11 .33 ± 4.25^{b}
Day 21	8.50 ± 1.54^{a}	19.57 ± 1.91^{b}	12.20 ± 2.02^{b}	11.00 ± 14.05^{b}	10.80 ± 1.50^{b}

Results are means of triplicate determinations ± standard deviation.
$^{a, b,}$ Values with the same superscripts within a row differ significantly (p=0.05)

Table 5. Aspartate amino transferase levels in blood samples (IU/L)

Groups	Group 1 (control)	Group 2 (HCN)	Group 3 (0.2 ml Bentonite + HCN)	Group 4 (0.4 ml Bentonite + HCN)	Group 5 (0.8 ml Bentonite + HCN)
Day 7	32.30 ± 5.64^{a}	19.00 ± 3.21^{b}	20.25± 0.15^{b}	23.67 ± 2.82^{b}	24. 4.0 ± 3.69^{b}
Day 14	32.40± 7.64^{a}	15.50 ± 8.42^{b}	22.13±14.38^{b}	25.67 ± 17. 35^{b}	25 .33 ± 4.25^{b}
Day 21	32.50 ± 7.64^{a}	11.67 ± 1.91^{b}	24.60 ± 3.82^{b}	27.00 ± 24.95^{b}	28.67 ± 2.60^{b}

Results are means of triplicate determinations ± standard deviation.
$^{a, b,}$ Values with the same superscripts within a row differ significantly (p=0.05)

The results also showed that there was a significant decrease in the ALP activity of Wistar rats fed with 7% bentonite (p=0.05) when compared to the ALP levels of the control group (group1) and group 2. The results also showed significant increase in ALT activity. The highest increase was observed on day 21 in group 2, the group that was given just the cyanide (19.57 ± 1.91 vs control 8.50 ± 1.54 IU/L). Nevertheless, group 5, the group that was given 0.8ml of bentonite solution showed the lowest decrease in ALT activity at 10.80 ± 1.50 vs control 8.50 ± 1.54 IU/L. Furthermore, the results showed significant decrease in the AST activity. The highest decrease was observed on day 21 in the group that was given just cyanide (11.67 ± 1.91 vs control32.50 ± 7.64 IU/L). However the highest increase in AST activity was observed on day 21, in the group that was given the highest concentration of bentonite. This means that bentonite had a clearly protective, antitoxic effect on the cyanide-fed rats, leading to lowered ALP levels. This was supported by Okotie –Eboh and Kubena [25] who reported that dietary addition of bentonite has been shown to reduce some toxic effects of some toxins and improve the performance of birds. Furthermore, some other studies showed also that bentonite has the ability to protect birds and other animals to some extent against the hepatoxicity of Aflatoxins [26,27,28,29]. Similarly, a recent study by Okoye and Uwhen, [1] stipulated that cyanide exposure caused a significant increase in the plasma urea level and that bentonite administration was effective in mitigating the resultant alteration. They suggested that bentonite might have some preventive and therapeutic effects on cyanide poisoning.

Progressive increase in ALP activity was recorded in rats fed with cyanide from cassava waste water. This increased ALP activity may be due to the de novo synthesis of the enzyme molecule. ALP is a marker enzyme for the plasma and endoplasmic reticulum of the tissues [30]. It is often employed to assess the integrity of the plasma membrane [31] since it is localized predominantly in the microvilli in the bile canaliculli, located in the plasma membrane. Since ALP hydrolyzes phosphate monoesters, the enzyme's hyper production could constitute a threat to the life of the cells that are dependent on a variety of phosphate esters for their vital processes [32] as it may lead to indiscriminate hydrolysis of phosphate ester metabolite of the liver. Consequently, this may adversely affect the facilitation of the transfer of metabolites across the cell membrane. If follows therefore that such hyper production of ALP may also have severe consequences on the architecture of the cells of affected organs. Cassava varieties are often categorized as either sweet or bitter, signifying the absence or presence of toxic levels of cyanogenic glucosides, respectively. The so-called sweet (actually not bitter) cultivars can produce as little as 20 milligrams of cyanide (CN) per kilogram of fresh roots, whereas bitter ones may produce more than 50 times as much (1 g/kg). Cassavas grown during drought are especially high in these toxins [33,34]. A dose of 25 mg of pure cassava cyanogenic glucoside, which contains 2.5 mg of cyanide, is sufficient to kill a rat. Excess cyanide residue from improper preparation is known to cause acute cyanide intoxication, and goiters, and has been linked to ataxia. It has also been linked to tropical calcific pancreatitis in humans, leading to chronic pancreatitis [35].

5. CONCLUSION

In line with the results of the study, administration of 0.5 ml/L cassava waste water containing 2.16 x 10^{-3} mg HCN induced toxicity in Wistar rats as was evident in their reduced body weights and high ALP, AST and ALT activities. The body weights of animals fed with bentonite as well as their ALP, AST and ALT levels showed that bentonite is efficient in ameliorating cyanide toxicity as well as improving body weights of cyanide intoxicated animals.

ETHICAL APPROVAL

This research work was carried out with the approval of the University of Port Harcourt research ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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