



Tissue correlation of nitrite in plant parts of cassava (*manihot esculenta crantz*) and nitrosamine toxicity in wistar rat

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ABSTRACT

Aim: This study was designed to determine the correlation in nitrite content of cassava (*Manihot esculenta Crantz*) as present in the various parts (roots, stems and leaves) and the possible hepatotoxicity when Wistar rats are exposed to N-nitrosamine precursors. **Methods:** Cassava cultivar used for this experiment was collected from International Institute of Tropical Agriculture, Ibadan, Nigeria (IITA). Various parts (Roots, Stems and Leaves) was weighed and homogenized separately. The homogenate was filtered to get clear solution and nitrite content therein was analyzed. Thirty Wistar rats divided into three groups, classified into; Group 1, Group 2 and Group 3 were used for the in-vivo experiment. The urine nitrite content and serum biomarkers of toxicity namely; Serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) was estimated and the histopathological changes in the liver was examined in order to evaluate the extent of toxicity. **Results:** The nitrite levels in the roots, stems and leaves of these cassava cultivars were estimated as follows: roots; $110.8 \pm 23.7 \mu\text{g}/50 \text{ g}$, stems; $14.4 \pm 17.7 \mu\text{g}/30 \text{ g}$ and leaves; $112.0 \pm 30.4 \mu\text{g}/5 \text{ g}$. The nitrite correlations between the roots and leaves is $r = 0.97$, correlations between the roots and stems is $r = 0.65$ while the correlations between the stems and leaves is $r = 0.63$. Urinalysis test carried out shows urine is the major sources of excretion of N-nitrosamines from the system. Both Group II and Group III animals had a significant increase in ALT, AST, ALP and GGT levels in the serum. Histopathology study of liver is in agreement with these results. **Conclusion:** This study shows that there was a correlation in nitrite levels between the roots and leaves of cassava and the level of toxicity found in the liver of rat administer with N-nitrosamine precursors (DMA.HCL and NaNO_2).

KEY WORDS: Cassava leaves; Cassava roots; Cassava stems; Dimethylamine hydrochloride; Nitrite.

INTRODUCTION

Globally, cassava (*Manihot esculenta Crantz*) is ranked the sixth most important source of calorie in human diet. In tropical countries, including sub-Saharan Africa, where cassava is primarily grown as a staple food, it serves an essential dietary carbohydrate source for approximately 800 million people [1-3]. In recent years, increase in cassava production in Africa has been recorded because of its potential to feed the rapidly increasing population and its agricultural advantages such as ability to withstand drought stress, tolerance to soil infertility and the fact that it can be stored underground for several months after maturation without water and still retains its nutritional value [4, 5].

Cassava roots are excellent source of carbohydrates and the leaves are believed to be rich in protein, vitamins and minerals. It has been reported that some nutrients are not optimally distributed within the plant and that the roots and leaves are deficient in sulfur-containing amino acids (methionine and cysteine) [5]. Depending on the amount consumed, cassava also contains antinutrients such as phytate, fiber, nitrate, polyphenols, oxalate, and saponins that can have either positive or adverse effects on health. Although some of these compounds act as antioxidants and anticarcinogens (for example tannins one of the polyphenol

content), they can interfere with nutrient absorption and utilization and may have toxic side effects [5, 6].

One of the toxicology implications of nitrate and nitrite ingestion in cassava-eating populations is that thiocyanate, which is present in high amounts in the stomach of such individuals, may act as catalyst for the nitrosation of amines in the stomach to form carcinogenic nitrosamines, arising from microbial conversion of nitrate to nitrite which acts as the nitrosating agent enzymatically or spontaneously [7]. Nitrite is naturally present in soil; however under unfavourable conditions, nitrite may enter the food chain through microbial reduction of nitrate thereby endangering human health [8]. Nitrite levels in food are very low (generally well below 10mg/kg and rarely exceed 100mg/kg). Exceptions to this are vegetables that have been damaged, poorly stored, or stored for extended periods as well as pickled or fermented vegetables [9].

It has been reported in literature that nitrite is a known precursor of toxic and carcinogenic N-nitrosamines [10]. Nitrite and N-nitroso compounds which form when nitrite binds to other substances like amines, amides, etc., before or after ingestion are toxic and can lead to severe pathologies in humans [11].

Nitrosamines are toxic chemical compounds found low in quantity, but widely spread in the environment. The nitrosating agent nitrous anhydride is usually used in food materials that are formed from nitrite in acidic or aqueous solution. Nitrosamines are very reactive once activated. Some foods contain volatile nitrosamines, examples include cured meats, primarily cooked bacon, beer, some cheeses, nonfat dry milk, and sometimes fishes. The volatile nitrosamine n-nitrosodimethylamine (NDEA) occurs to a major extent as nitrosopyrrolidine (Npyr) in food stuffs. Several researchers have estimated that the average daily intake of volatile nitrosamines from foods is approximately 1 microgram/person [12, 13]. In view of this consideration, the present study aims to investigate the correlation of nitrite levels in parts of cassava (roots, stems and leaves) and to evaluate the toxicity of the precursor of N-nitrosamines: sodium nitrite and dimethylamine hydrochloride in the liver of Wistar rat.

MATERIALS AND METHODS:

Preparation of the cassava extracts

Root tubers, stems and leaves of cassava cultivars were obtained from the international institute of tropical agriculture (IITA) Ibadan, Nigeria. This was washed and cut into small pieces. The cassava peels were removed from the root tubers prior to homogenization. Cassava roots (50 g), stems (30 g) and leaves (5 g) were weighed and were homogenized separately in 250ml - 350ml of 0.1 % orthophosphoric acid (0.2µl) pH 7.4 at 4°C using electric blend. The homogenate was filtered through four layers of muslin. The filtrate was centrifuge at 2960 x g for 5 minutes at 4°C. The supernatant was gently decanted and centrifuged at 19,400g for 15 minutes and the amount of nitrite and nitrate present were analyzed.

Experimental animals, grouping and treatment

Thirty Wistar male rats weighing between 180 - 200 g were obtained from the Department of Veterinary Physiology, University of Ibadan. The animals were acclimatized for two before the commencement of the experiment and were fed with rat chow and water *ad libitum*. The animals were randomly distributed into three groups of ten rats each. Animal grouping was as follows:

Group I was given water and serves as control, Group II was given 8.4 mg NaNO₂ /kg and 50 mg DMA.HCL/kg while Group III was given 8.4 mg NaNO₂/kg. Both compounds were dissolved in distilled water and a single dose was administered by oral gavage. The animal experimental protocol was according to the committee for the Care and Use of Laboratory Animals.

Collection of urine, blood and tissue samples

The rats were placed in metabolic cages with separate facilities for collection of urine. Urine sample was collected for a period of 24 hours after administration of NaNO₂ and

DMA.HCL. The rats were scarified by cervical dislocation 24 hrs after the administration of the dose sodium nitrite and dimethylamine hydrochloride to the animals. Blood samples were collected and allowed to coagulate at room temperature. The clear, non-haemolysed supernatant sera were quickly removed and stored at -20 °C for subsequent analysis. Liver samples were rapidly expunged, weighed and washed in ice-cold 1.15% KCl solution; they were homogenized in 56 mM Tris-HCl buffer (pH 7.4) comprising 1.15% KCl, and then centrifuged at 10,000 x g for 15 minutes. The supernatant were carefully separated and kept till required for the analysis. Histological assessment of the liver was carried out under a light microscope. Small liver samples were fixed in 10 % normal saline and then dehydrated and paraffin-embedded for histological assessment.

Biochemical parameters

The activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) were estimated using the method of Reitman and Frankel [14] while the activities of alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were determined in the serum using the method described by Klein et al., 1960 and Szasz, 1969 respectively [15, 16].

Statistical analysis

Results were expressed as mean ± standard deviation and the difference between the groups were tested by one-way analysis of variance (ANOVA) followed by the student t-test using SPSS software package (17.0). Results were considered as significant at $p < 0.05$.

RESULTS

Present on Table 1 are the mean values of nitrite obtained from the roots, stems and leaves (Table 1). The leaves have the highest nitrite value (112.0±30.4) followed by the roots (110.8±23.7) while the stems had the lowest nitrite value (14.4±17.7). Table 2 revealed that there was a strong positive significant correlation relationship in nitrite distribution when the roots were compared with the leaves ($r=0.97$). In like manner, there was a positive correlation relationship in nitrite distribution when the roots and stems ($r= 0.65$) were compared with stems and leaves ($r= 0.63$). Table 3 shows that there was a significant increase in the level of N-nitrosamine excreted in the urine of rats after 24 hrs; following oral administration of NaNO₂ and DMA. HCl when compared to the control.

Table 1. Nitrite Concentration in the roots, stems and leaves of cassava

| Samples | Nitrite Concentration (µg) |
|---------|----------------------------|
| Roots | 110.8± 23.7 |
| Stems | 14.4±17.7 |
| Leaves | 112.0±30.4 |

Table 2. Distribution of Nitrite in the roots, stems and leaves of cassava

| Samples | Regression equation | Correlation coefficient (r) |
|-----------|---------------------|-----------------------------|
| Root-Stem | Y=9.47-0.161x | 0.65 |
| Stem-leaf | Y=108.86-0.219x | 0.63 |
| Root-leaf | Y=108.86-0.029x | 0.97 |

Table 3. Excretion of N-nitrosamine in the urine of rat after 24 hrs following oral administration of NaNO₂ and DMA.HCl

| Rat Groups Treatment | Mean N-values of urine in (µg/ml) |
|----------------------|-----------------------------------|
| Group I | 20±7.5 |
| Group II | 1573.9±99.9* |
| Group III | 772.7±79.2* |

A significant increase in serum ALT, AST, ALP and GGT activities was observed in the animals treated with NaNO₂ and DMA.HCl (Group II and III animals) when compared with the control (Fig 1). Histological examination of the liver section reveals that there was no visible lesion in the control animals (Group I). However, Group II and III animals showed various form of alterations in the liver architecture. Specifically, Group II (DMA.HCL+ NaNO₂) showed liver tissue with very severe portal and central venous congestion, while Group III (NaNO₂) showed liver tissue with marked infiltration of mononuclear cells and periportal cellular infiltration (Fig 2).

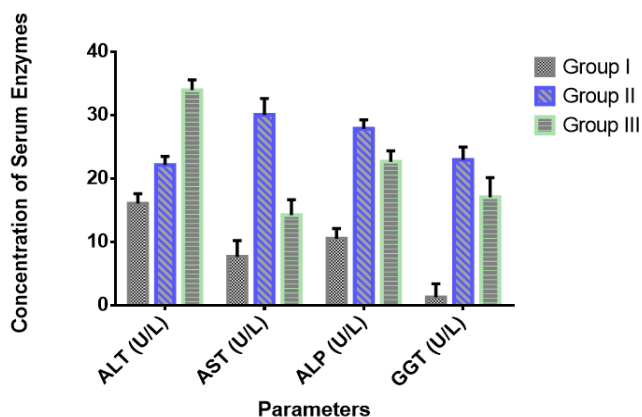


Fig 1. Serum enzyme activities in rats following oral administration with dimethylamine hydrochloride and sodium nitrite.

DISCUSSION

There has been an exponential increase in cassava production in last 10 years. Cassava (*Manihot esculenta Crantz.*) is an essential root crop cultivated for its root starch, which is consumed primarily as food (48%) and livestock feed (34%) in many of the developing countries in subSaharan Africa, Asia, and Latin America, but also used as feedstock (18%) for biofuels and biochemicals [17, 18]. It is prepared and consumed in various forms as

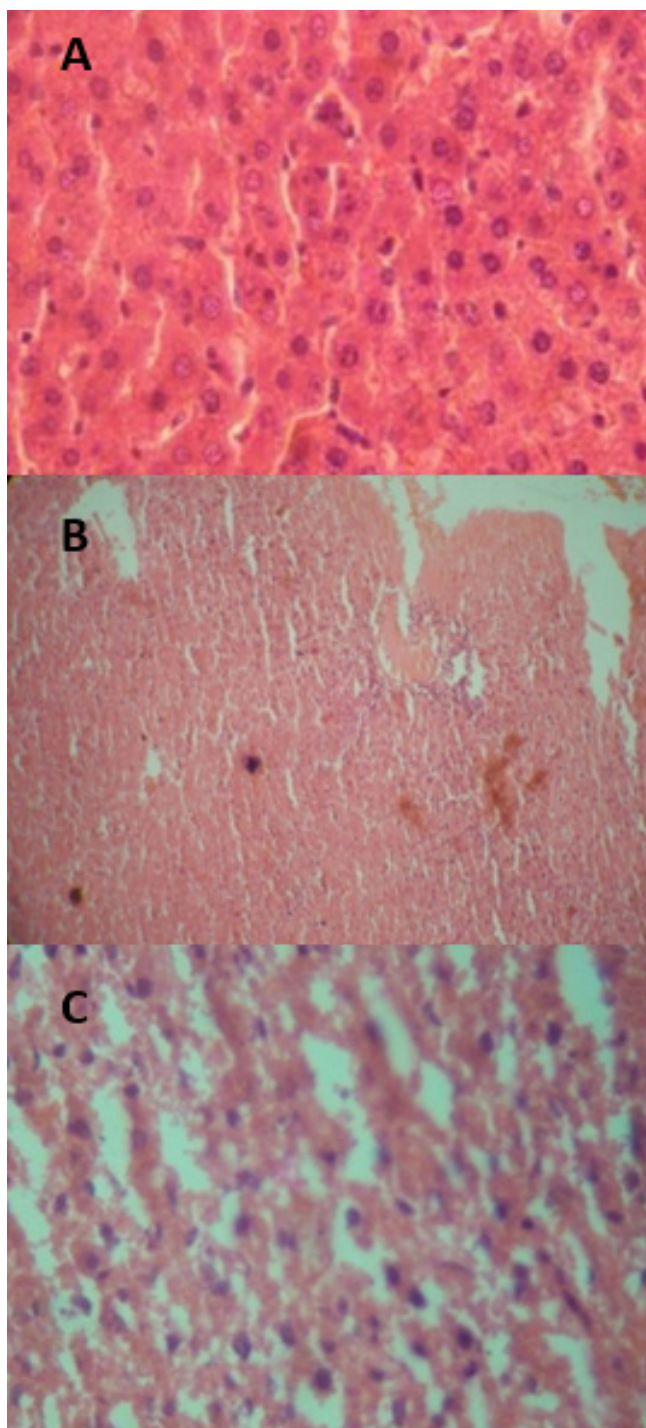


Fig 2. Photomicrograph of liver section of the rats stained with hematoxylin and eosin (H&E). A). Group I showing no visible lesion. B). Group II showing liver tissue with very severe portal and central venous congestion. C). Group III showing liver tissue with marked infiltration of mononuclear cells and periportal cellular infiltration.

a food; boiled, baked or often fermented into other foods and beverages [19]. The nutritional value of the cassava root is not enough to meet all dietary needs; it has little protein content and low ascorbic acid levels [20, 21], but it

has been reported that the leaves contained a high level of crude protein, vitamins and minerals [22].

Recent studies has revealed that both roots and cassava leaves are rich in antioxidant and have the potential to inhibit lipid peroxidation as well as to moderately scavenge free radicals especially reactive oxygen species (ROS) [20, 23]. However, toxicological implications of nitrite content in cassava are of great concern in human and animal health. Nitrite is well-known to interact with haemoglobin, forming methemoglobin by oxidation of ferrous iron to ferric state, preventing or reducing the ability of blood to transport oxygen, a condition known as methemoglobinemia. Nitrite is also implicated in the formation of nitrosamines; compounds known to be carcinogenic, mutagenic, embryopathic and teratogenic in experimental animals [24, 25].

In the present study, there was a strong positive significant correlation relationship in nitrite distribution when the roots were compared with the leaves, and also there was a positive correlation relationship in nitrite distribution when the roots and stems were compared with stems and leaves. The leaves have the highest nitrite value followed by the roots while the stems had the lowest nitrite value. These values are above the 0.06 mg/kg body wt. World Health Organization's Acceptable Daily Intake (ADI) for nitrite [26]. Nitrites are formed in nature by the action of nitrifying bacteria as an intermediate stage in the formation of nitrates, but concentrations in plants and water are usually very low [27].

However, microbiological conversion of nitrate to nitrite may occur during the storage of fresh vegetables, particularly at room temperature, when nitrite concentrations may rise to exceptionally high levels (about 3600 mg/kg dry weight). The high nitrite content in the leaves, roots and stems of cassava in this experiment may be as a result the increased use of synthetic nitrogen fertilizers and livestock manure in intensive agriculture [28, 29].

It was observed that the mean urinary N-nitrosamine excretion increased in the animals treated with NaNO₂ and DMA.HCl after 24 hr of administration. Urine serves as the extractable medium to get rid of nitrosamine from the system [30]. Rats treated with NaNO₂ and DMA.HCl showed significantly increased activities of ALT, AST, ALP and GGT in the serum when compared with the control. ALT, AST, ALP and GGT levels are widely used in animal's studies to diagnose and observed the development of hepatocarcinogenesis [31]. Increase in serum concentrations of these enzymes is an indication of hepatocellular damage. AST and ALT play a significant role in the metabolism of amino acids, an increase in these enzymes in this study probably resulted from hepatic dysfunction caused by exposure to NaNO₂ and DMA.HCl, especially as it was accompanied by a corresponding increase in GGT and ALP [32]. Histopathological changes in the liver of the treated rats are in agreement with these biochemical findings.

CONCLUSION

This study suggests that there is a strong correlation in the nitrite content of cassava root and leaves. Our findings also show that dimethylamine hydrochloride and sodium nitrite (precursors of N-nitrosamine) results in acute synergistic damage in the liver of experimental animals as demonstrated by the marked elevation witnessed in the levels of serum biomarkers of xenobiotic-induced hepatotoxicity in this study. Taken together, individuals that consumes both the roots and leaves of cassava should consume it with precaution and ensure proper processing and storage so as to reduce the nitrite contents in the roots and leaves of cassava before consumption, in order to avoid and prevent elevated risks of colon cancer and other related diseases that have been linked to nitrite (a potential precursor of endogenously formed carcinogenic N-nitroso compounds and or other derived nitrosating species, N₂O₃ and N₂O₄, and dietary amines) toxicity during nitrite metabolism.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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