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COMPARATIVE STUDIES ON THE EFFECT OF CONSUMPTION OF FRESH FRUIT JUICE AND THIAMIN ON THE METABOLISM OF LINAMARIN IN RATS.

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ABSTRACT: The effect of consumption of fresh fruit juices and thiamin on the metabolism of linamarin were investigated in rats. The experiment consisted of 45 rats of wistar strain divided into 9 groups of 5 rats per group. Five of the groups randomly received fresh fruit juices which were mango juice, grape juice, tomato juice, orange juice and pawpaw juice at the rate of 5mls of juice dissolved in 5mls of water. 3 groups randomly received graded levels of thiamin pills while the remaining one group served as control. The results showed that the groups on fresh fruit juices and thiamin had significantly lower($p>0.05$) total cyanide, unmetabolised linamarin, free cyanide and thiocyanate concentration in the urine after the first and second 24 hours compared to the control. There was accumulation of unmetabolised linamarin and free cyanide in the control group that neither consumed thamin nor fresh fruit juices. The significant rise in the free cyanide of the urine samples of the control may possible account for the toxicity effect of the ingested linamarin. Fresh fruit juices and thamin appear to be effective techniques for detoxification of linamarin at least in rats.
Key words: cassava, toxicity, fruits, thamin,cyanide.

INTRODUCTION

Cassava is a staple food for millions of Nigerians (Calsson *et al*. ,1999). It is also an important food ingredient for poultry and livestock. But it contains potentially toxic compounds called cyanogenic glycosides, primarily linamarin and a small amount of lotaustrain (ethyl linamarin)(Numbisan, 2010) Linamarin is also known as 2-hydroxy- isobutyronitrile- β- D-glucoside and is chemically similar to glucose except that it has cyanide (CN) ion attached.

Cyanogenic glycosides are toxic because they release hydrogen cyanide (HCN) by enzymatic hydrolysis as a result of maceration of the plant tissue (ATSDR, 2006). If cassava is consumed raw, HCN is also released for absorption by the action of B-glucosidases in the gastrointestinal tract (Calsson *et al*., 1999, ATSDR, 2006). Absorbed cyanide is converted in the liver to less toxic metabolites (Umoh *et al*., 1986, ATSDR, 2006, JECFA, 1993.) The major pathway involves conversion to thiocyanate by rhodanase or 3-mercaptopyruvate sulfur transferase(ATSDR,2006, JECFA, 1993). This major route of detoxification requires sulfur donors, which by different metabolic pathways are provided from dietary sulfur containing amino acids. Three minor pathways are also known, converting less than 20% of the total cyanide (ATSDR, 2006, JECFA, 1993). The first one involves conversion to 2-aminothiazolin-4-carboxylic acid. The second one involves incorporation into 1-carbon metabolic pool and the third one involves combining with hydroxycobalamin to form cyanocobalamin (ATSDR, 2006, JECFA, 1993).

Long term cyanide intoxication may result in neurological diseases such as Tropical Ataxia Neuropathy, endemic Goiter and Konzo(Oluwole *et al*., 2002). These diseases affect mostly children and women of child bearing age (Oluwole *et al*., 2002).

There are many methods available for reducing the level of cyanogenic glycosides in cassava. Many research efforts to reduce or eliminate cyanoglucoside have focused on the development of a acyanogenic cassava varieties by breeding; controlling its metabolism; and processing to remove cyanogens (Numbisan, 2010). These methods have been extensively reviewed (Numbisan, 2010).Many of these methods are very effective. For example, fermentation followed by moist heating effectively reduced cyanogenic glycosides level by ninety percent (Maduagwu, 1979). But, to achieve a safety level of 250µg of cyanide equiv./g of cassava, new methods of processing still remains a challenging problem (Numbisan, 2010).

Preparatory methods for some local delicacies make it difficult to apply effective methods to reduce the cyanide level. For example, local delicacies such as "Ekpang iwa , "Asa iwa"," iwoekpang", "abacha," "eberebe" etc common in the southeast and southsouth parts of Nigeria are prepared using freshly uprooted cassava without fermentation and dewatering which would have reduced the cyanide content. Thus, people consuming these delicacies may be consuming high level of cyanogenic glycosides. The safety of these delicacies has not been previously reported. Relevant research efforts in this case, may be directed towards invivo detoxicification that is detoxification of the cyanide in the subject consuming these delicacies. The roles of thiamin were investigated, but information is scanty (Umoh, 1985). Recent body of information suggest the possible role of fresh fruit juices as they are seen to influence the metabolism of drugs/toxins (Ozdemir *et al*., 1998; Bakalar, 2006; Hidaka *et al*, 2008; Niemin *et al*., 2010) and has made it imperative in the present study to beam searchlight on the effect of fresh fruits juices. Cyanide intoxication is common in areas of high cassava consumption (Umoh *et al*. 1985). Tropical Ataxic Neuropathy and endemic goiter were reported in eastern areas of Nigeria, now south east and south-south states (Ekpechi, 1967; Delange *et al* 1977; Barrett *et al*. 1977, Smith *et al*. 1963). The present study was designed to determine the effect of consumption of fresh fruit juice and compare to those of thamin on the metabolism of linamarin in rats. This study is significant because it will enlighten the public on the possible role of fresh fruits on the detoxification of toxic substances including linamarin in cassava.

MATERIALS AND METHOD

Animal and Treatment: Forty-five rats of wistar strain were purchased from the animal house of Department of Physiology, University of Calabar and recruited for the study. The animals were randomly divided into nine (9) groups of five rats in each group. Each group was fed for 40 days. Group 1 (control group) received basal di*et al*one formulated according to the methods of Cuthbertson (1957), Bassir and lobel (1968) (Table 1). Group 2,3,4,5 and 6 received basal diet and fresh juices (fresh fruit juices were mango juice, grape juice, tomato juice, orange juice and pawpaw juice respectively at 5ml of the juice dissolved in 5ml of water, that is, in the ratio of 1:1). Group 7, 8, and 9 received basal diet and thiamin pills. Group 7 received basal diet and half normal thiamin requirement of rats. Group 8 received basal diet and normal thiamin requirement of rats. While, group 9 received basal diet and twice normal thiamin requirement of rats. Feed and water were given ad libitum. The animals were kept within the same condition of temperature (28 ± 2^0C), relative humidity (46%) and lighting (12 hours light and dark cycle). Care of animals conformed to the principles of good laboratory practice and animal handlings (NIH) guidance for the care and use of laboratory animals, publication no: 85-23, 1985. At the end of the 40 days, the rats were transferred to metabolic cages. Urine and faeces were collected during the first 24 hours in the cages and labeled blank. That is, zero hours sample. Pure synthetic linamarin (2-hydroxy-isobutyronitrile-β-D-glycoside); melting point 145-147^{o}c $[\propto]_D$= 29.5 was purchased from Calibiochem, USA. The synthetic linamarin was dissolved in

water, and a single dose of 30mg/100g body weight was administered to the rats. Urine samples were collected for analysis after the first, second and third 24 hours of the administration of the synthetic linamarin. All samples were kept in the refrigerator at 0-4^{o}c in well labeled amber coloured bottles until required for analysis.

Table1. Percentage composition of the experimental diets (per dry weight basis)

INGREDIENTS	PERCENTAGE COMPOSITION (%)
Soya bean powder	46.47
Garri	47.47
Salt mix	4.74
L-lysine	0.20
L-methionine	0.10
Vitamin mix[2]	1.00
Corn oil	5.00

Measurement of Linamarin

Urine samples were analyzed for total, free and unmetabolised linamarin (bound cyanide). In addition, thiocyanate content was determined in the urine samples. Free and unmetabolised linamarin in the urine samples were determined by the enzymatic assay method of Cooke, 1968. The protocol involved neutralizing and incubating 0.2ml for bound cyanide, or 0.4ml for the free cyanide for 15min at 37^{o}C with appropriate crude linamarase preparation for bound cyanide prior to spectrophotometric analysis, but for the determination of the free cyanide, the colour was developed directly without any enzyme hydrolysis. The thiocyanate content of urine samples were determined by the methods of Bowler, 1944.

Preparation of Fruit Juice

Fresh fruit juices were extracted using manual Juice extractor. About Ikg of the fruit, in each case were purchased from Watt market Calabar, Cross River State. They were thoroughly washed, peeled and sliced. Juiced were extracted from the slices, filtered and stored in well-labeled containers in the refrigerator at 0-4^{o}C.

STATISTICAL ANALYSIS

Statistical analysis were conducted using students' 't' test and the difference in mean were considered significant at $P<0.05$.

RESULTS

The concentrations of total cyanide, unmetabolized linamarin, free cyanide and thiocyanate of urine samples collected during the first and second 24 hours is shown in table II and III. We did not detect free cyanide nor unmetabolized linamarin in the zero and 72-hour urine samples of any of these groups, by the analytical methods we used. No free or unmetabolized cyanide were detected in any of the faecal samples.

The animals in group I which received neither fruit juice nor thiamin excreted in the urine, the highest concentration of total cyanide (10:35$\pm$ 1.20mg/100ml of urine) within the first 24 hours (Table 2) of administration of linamarin. Group I animals had significantly higher ($P < 0.05$) concentration of urine total cyanide when compared to those of group 2, 3, 4, 5 and 6 that received fruits juice and group 7 and 8 that received thiamin pills. Mean total urine cyanide concentration of the groups on fruits juice were statistically not different ($P < 0.05$) when compared to those receiving thiamin pills except for those of the animals in group 9 that received twice normal thiamin requirement of rats. In these rats total urine cyanide was significantly higher ($p < 0.05$).

The concentration of the unmetabolized linamarin in the 24 hour urine samples followed the same pattern as the total cyanide with animals in group I excreting the highest amount of unmetabolised linamarin in urine (8.07± 1.02mg/100ml) and was significantly higher ($P < 0.05$) when compared with those of groups receiving fruits juice and thiamin pills.

Table 2: Concentrations of total cyanide, unmetabolised linamarin, free cyanide and thiocyanate of urine samples collected during the first 24-hours (mg/100ml)

Experimental group (treatment)	**Total cyanide**	**Unmetabolised linamarin**	**Free cyanide**	**Thiocyanate**
1 (control)	10.35± 1.20^{C}	8.07± 1.02^{b}	2.28± 0.18^{b}	3.50± 1.28^{b}
2 (5ml mango juice)	6.82± 1.32^{a}	5.20± 0.50^{a}	1.62± o.24^{b}	2.79± 0.40^{b}
3 (5ml grape fruit)	6.56± 1.20^{a}	5.20± 0.50^{a}	1.36± 0.70^{a}	2.70± 0.60^{a}
4 (5ml tomato juice)	6.60± 0.90^{a}	5.60± 0.85^{a}	1.00± 0.05a	2.81± 0.40^{a}
5 (5ml orange juice)	6.56± 0.80^{a}	5.30± 0.60^{a}	1.28± 0.20a	2.68± 1.20^{a}
6 (5ml pawpaw)	6.90± 1.62^{a}	5.70± 1.20^{a}	1.20± 0.42a	2.80± 0.50^{a}
7($^{1}/_{2}$ normal thiamin)	6.62± 1.86^{a}	4.01± 1.60^{a}	2.62± 0.26^{b}	3.20± 0.80^{b}
8 (normal thiamin)	6.58± 0.85^{a}	5.40± 0.8^{a}	1.68± 0.05^{a}	2.68± 0.50^{a}
9(twice normal thiamin)	8.70± 2.30^{b}	6.70± 1.50^{b}	2.oo± 0.80^{b}	1.04± 0.20^{c}

Source: Mean with different superscript are significantly different (P<O.05)
Mean 1 SE for 5 rats per group.

The concentration of free cyanide and thiocyanate did not follow the pattern of total cyanide and unmetabolised linamarin. The concentration of free cyanide in the 24 hour urine sample (Table 2) showed that animals in group I (that received neither thiamin nor fruit juice), group 2 (that received mango juice), group 7 (that received ½ normal thiamin) and group 9 (that received twice normal thiamin) had significantly ($P < 0.05$) higher urine free cyanide concentration when compared to those of group 3 (that received grape fruits Juice), group 4 (that received tomato juice), group 5 (that received orange juice), group 6 (that received pawpaw juice) and group 8 (that received normal thiamin requirement). Animals in group 3, 4, 5, 6 and 8 had mean urine free cyanide concentrations that were numerically but not statistically different.

The thiocyanate concentration followed similar pattern as the free cyanide except for group 9, which in this case, rather had a significantly lower ($P < 0.05$) mean urine thiocyanate concentration.

Table 3 shows, that after the second 24-hours, animals in group 1 that received neither fruit juices nor thiamin still excreted the higher total cyanide concentration (1.56 ± 0.93/100ml of urine). This was followed by animals in group 7 receiving half normal thiamin that had mean urine total cyanide concentration of 1.38± 0.03mg/100ml. The mean total cyanide of group 7 and 1 when compared showed significant mean difference ($P < 0.05$). Total cyanide concentration of the urine samples of group 2, 3, 4, 5, 6, 8 and 9 were not significantly different ($P < 0.05$) when compared to one another except when compared to those of group 1 and 7.

The concentrations of the unmetabolised linamarin in the urine samples of all the groups after the second 24 hours were similar. The free cyanide concentration followed the same pattern of the total cyanide. The thiocyanate also followed the same pattern of the total cyanide except for the group 5 and 9 that had lowest level thiocyante in the urine

Table 3: Concentrations of total cyanide, unmetabolised, linamarin, free cyanide and thioanate of urine samples collected during the second 24 hours (mg/100ml).

Experimental group (treatment)	**Total cyanide**	**Unmetabolised linamarin**	**Free cyanide**	**Thiocyanate**
1 (control)	$1.56\pm 0.03_b$	0.16 ± 0.20^a	1.40 ± 0.17^b	2.10 ± 0.40^b
2 (5ml mango juice)	0.98 ± 0.52^a	0.17 ± 0.01^a	0.81 ± 0.51^a	1.40 ± 0.52^a
3 (5ml grape fruit)	0.86 ± 0.32^a	0.17 ± 0.02^a	0.69 ± 0.30^a	1.00 ± 0.25^a
4 (5ml tomato juice)	1.00 ± 0.62^a	0.17 ± 0.62^a	0.93 ± 0.07^a	1.40 ± 2.15^a
5 (5ml orange juice)	0.87 ± 0.05^a	0.17 ± 0.01^a	0.70 ± 0.04^a	0.50 ± 0.16^c
6 (5ml pawpaw)	0.98 ± 0.20^a	0.19 ± 0.05^a	0.81 ± 0.15^a	1.32 ± 1.20^a
7($^1/_2$ normal thiamin)	1.38 ± 0.03^c	0.16 ± 0.15^a	1.12 ± 0.12^c	1.06 ± 0.50^a
8 (normal thiamin)	0.80 ± 0.25^a	0.15 ± 0.21^a	0.85 ± 0.04^a	1.25 ± 0.15^a
9(twice normal thiamin)	0.75 ± 0.05^a	0.12 ± 0.25^a	0.63 ± 0.20^a	0.40 ± 0.25^c

DISCUSION

The toxicity of the cyanogenic glycoside in cassava has been of interest to many scientists. The effect of consumption of fresh fruits juice on the metabolism of linamarin has not been previously reported. Limited information was available on the effect of thiamin. From our results, it seems that both thiamin and fresh fruit juices aid the metabolism of linamarin. The concentration of the total cyanide, unmetabolised linamarin, free cyanide and thiocyanate of urine samples collected during the first 24-hours showed that groups on fruit juice and thiamin had significantly lower total cyanide, unmetabolised linamarin, free cyanide and thiocyanate compared to the control group 1. The same was the case after the second 24-hour. All the fruits juice were effective, but it appears that the grape fruit Juice and orange juice impacted more on the metabolism of linamarin as their level of free cyanide in the urine were the lowest in the first and second 24-hours. From our results, it appears that deficiency of thiamin in addition to none consumption of fruit juice (group 1) may cause accumulation of unmetabolised linamarin and free cyanide. The free cyanide tends to be converted immediately to thiocyanate which resulted in significant rise in the concentration of thiocyanate in the urine of the controls. This result agrees with the work of Umoh *et al* (1985) and supports Clarks (1935) findings that patients suffering from chronic cyanide intoxication have higher amount of thiocyanate in saliva and urine compared to the healthy subjects. This work further supports findings that thiamin especially when administered in adequate amount, is required (at least to some extent) for the detoxification of cyanide (Umoh *et al* 1985). The results further shows that consumption of fresh fruits juice, under this study, may help in the detoxification of cyanide. The mechanism by which the fruits juices aid in the detoxification of cyanide is not known. But it might be due to the rich chemical components of these juices or its influence on cytochrome p450 activity and is recommended for further studies. Fresh fruit juices are known to be rich in ascorbic acid, free amino nitrogen, reducing sugars, carotenoids, organic acids, minerals, and pectic substances etc. (Ragals and EL-Nemr, 2006). The free amino acids, peptides, proteins, and other ammonium cations and organic nitrogenous compounds usually serves as nutrients for microbes and influences biomass formation, rate of fermentation, and production of various by-products. The juices may have increased the proliferation of gut microbial flora that is known to enhance localized detoxification reactions (ATSDR, 2006; EFSA, 2004; JECFA, 1993). Trinchieri *et al*. (2002) demonstrated that acute load of grapefruit juice increases urinary excretion of citrate and reduce the risk for the formation of renal stone. Multiple consumption of grapefruit juice also was reported to increase the metabolism of paracetamol and plasma concentrations of orally administered drugs (Samojlik *et al*, 1999).

None of the animals except in group 1 showed signs of cyanide intoxication. In this group, the animals ate less food. They latter developed stiffness of the hind legs and the hair on their body stood erect.

We did not estimate the total cyanide, unmetabolised linamarin, free cyanide and thiocyanate in the blood and tissue because previous analysis has shown that they do not accumulate in the blood and tissue following chronic exposure (Maduagwu, 1989, ATSDR, 2006; EFSA, 2004; JECFA, 1993). Comparatively, animals on the fresh fruit juices appeared to metabolize linamarin better than animals on thamin pills. We concluded that consumption of fresh fruit juices is effective (at least to some extent) in the metabolism of linamarin in rats. This technique may be employed in the management of cyanogenic glycoside toxicity particularly in the rural areas of the South-eastern and South-southern states of Nigeria where available techniques for detoxification of cyanide in cassava cannot be applied to some of their local delicacies. Fruits are available and cheap in the rural areas, so their consumption may be easily encouraged.

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