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Effects of lima beans (*Phaseolus lunatus* L.) cyanide on the glutathione (GSH) and some selected enzymes of liver damage in Wistar rats

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ABSTRACT: Lima beans (*Phaseolus lunatus* L) are recognized, vining leguminous plant with cyanide constituent. The effects of Lima beans cyanide on the glutathione (GSH) level and liver damage enzymes like Aspartate aminotransferase (AST), Alanine aminotransferase (ALP) and Alkaline phosphate (ALP) were investigated. Twenty-four (24) self-breed male albino Wistar rats were adopted for this study. The control (group 1) had 12 rats and the test animals categorized as groups 2-4 had 4 rats each. Test rats were fed for 21 days with a Lima beans diet containing 47.5 mg Cyanidekg^{-1} diet meal and a 10% protein supplement. Animal sacrifice was conducted thrice (on the 7day, 14day and 21day). After an overnight fast, animals were sacrificed by cervical dislocation. Biochemical analysis was conducted with urine and serum. The urine and serum cyanide and thiocyanate levels revealed a significant difference ($p<0.05$) between the test and the control groups. The mean serum and urinary cyanide (CN) were 2.51±3.70 and 22.49±1.18 µg mL^{-1} after 7 days; 7.77±3.56 and 29.32±3.10 µg mL^{-1} after 14 days; and 9.88±7.22 and 31.50±3.88 µg mL^{-1} after 21 days, respectively. Mean serum and urinary thiocyanate (SCN) were 14.86±0.61 and 36.59±1.66 µg mL^{-1} after 7 days; 15.08±0.94 and 39.13±1.77 µg mL^{-1} after 14 days; 19.64±0.87 and 43.07±2.31 µg mL^{-1} after 21 days, respectively. A progressive depletion in blood glutathione level by 41%, 54% and 72% (after 7, 14 and 21 days, respectively) compared to control, was observed. Increases in serum activity of ALT (172%), AST (112%) and ALP (93%) were quantified after 21 days of *Phaseolus lunatus* dieting. Total bilirubin level was elevated in the test animals, while total protein and albumin levels remained unchanged from normal. Significantly, this study elucidates that cyanide toxicity from diet intake or other sources might be conciliated via antioxidant depletion such as glutathione and increased liver damage biomarkers.

Keywords: Cyanide, glutathione, *Phaseolus lunatus* L, thiocyanate.

INTRODUCTION

The quest to alleviate food insecurity and enhance availability in low income nations has culminated in cascades of plant investigations which can suffice either as nutrient supplements or for medicinal purposes (Galhena *et al.*, 2013). Nations and localities that are unable to measure up to their protein needs augment via plant base products. Fortunately, one plant-based food source constituting a significant portion of traditional diets is grain legumes. Aside been nutritious, the seeds of legumes contribute significantly to biodiversity, environmental alleviation, food security, and agricultural sustainability (Boukid *et al*., 2019). These highly dependable food materials by impoverished localities are relatively cheaper protein sources than animal protein which makes it easily obtainable by indigent farmers. Periodic investigations have demonstrated preventive and curative propensity of legumes as it relates to health conditions such as marasmus, diabetes, obesity etc, thereby upgrading health and wellness (Bassett *et al*., 2010).

Lima beans (*Phaseolus lunatus*) is a tropical leguminous plant (Sandoval-Peraza, 2020) popularly recognized among rural dwellers of the South-Western and South-Eastern regions of Nigeria, hence ascribed locally as “Kapala” (Yoruba) and “Ukpa” (Igbo) (Ezeagu and Ibegbu, 2010; Seidu *et al*., 2014; Seidu *et al*., 2018). Nutritionally, *Phaseolus lunatus* are rated and scrutinized as an excellent source of nutrients (Jayalaxmi *et al*., 2016). Such nutritional efficacy as evaluated by previous researchers reveals that lima beans contain sufficient amounts of proteins ranging from 14.24 - 24.92% (Ibeabuchi *et al*., 2019) with an exceptionally abundant amount in globulin and albumin which are primarily storage proteins (Agarwal, 2017; Alghamdi *et al*., 2019). Compositionally, it is been evaluated to consist of essential amino acids such as lysine (FAO, 2016; Palupi *et al.*, 2022) and macro-minerals such as zinc, copper, iron, etc. (Campos-Vega *et al*., 2010). To a great degree, Lima beans are less consumed due to reduced productivity, biodiversity and hard seed texture (Diniyah *et al.,* 2020).

Beyond the nutritional predominance, *Phaseolus lunatus* possesses myriads of anti-nutritional components, which consist of bio-compounds like phytic acid, saponins, trypsin inhibitors, phenolic compounds, and toxic effluents of cyanogenic glycosides (Bolade *et al*., 2017; Sandoval-Peraza et al., 2020). Linamarin and lotaustralin are cyanogenic glycosides domicile in *Phaseolus lunatus* (Frehner *et al*., 1990; Speijers 1993).

Like *Manihot esculenta*, *Phaseolus lunatus* has been demonstrated to possess elevated content of cyanogenic glycoside (Palupi *et al*., 2022). Under prevailing environmental conditions such as cell maceration or tissue damage, myriads of cyanogenic plants (Lima beans inclusive) via cyanogenesis, releases toxic prussic acid or HCN (Coursey, 1973; Bolarinwa *et al*; 2016).

Evidentially, the virulence and lethality of cyanogenic plants alongside their by-products are conspicuously connected to the free HCN released from *Phaseolus lunatus* as a residuum of cyanogenic glycosides hydrolysis (Coursey, 1973). Cyanide (HCN) toxicity is conventionally and scientifically ascribed to cytochrome C oxidase (COX) inhibition, a complex IV enzyme domiciled inside the electron transport chain (ETC). COX once compromised alters mitochondria oxidative phosphorylation culminating in cytotoxic hypoxia or oxygen starvation. Other biochemical alterations or disturbances of cyanogenesis include the amplification of the N-methyl-D-aspartate (NMDA) receptor (Arden *et al*., 1998; Sun *et al*., 1999) and elevated concentration of extracellular glutamate (Patel *et al*., 1991).

Cyanogen toxicity has been hypothesized to propagate free radical injuries and cell damage (Mcmillian and Geervarghese, 1979). This further depletes endogenous antioxidants like glutathione, a biological agent that provides defenses against carcinogens, drugs, and metabolic reactive intermediates (Meister and Anderson, 1983).

Although literature correlating cynogenic substances and ROS damage is scanty, equivocacy still exists as it recounts its mechanism of action. Hence, the cruxes of this research piece rely on evaluating *Phaseolus lunatus* cyanogenic glycosides on some bio-identifiers and their corresponding antioxidant status in Wistar rats.

MATERIALS AND METHODS

This research was executed between May to September 2022 in the Department of Medical Biochemistry, Delta State University Abraka, Delta State Nigeria. Analytical grade chemicals and materials were utilized.

Experimental animals

Twenty-four (24) male Wistar rats weighing an average of 125 g and bred at the Novena University Animal House, Ogume were used. Animals were subjected to environmental temperature of 25-30°C with access to portable water and diet. Acclimatization to environmental conditions and diet inducement was permitted for 2 weeks prior to experimentation. Male rats were adopted for this study to avoid hormonal interference during the menstrual cycle and bioaccumulation of toxicant (cyanide).

Experimental design

The rats were sub-grouped randomly into four groups of which groups 2, 3, and 4 had four animals each while group 1 representing the control comprised of 12 rats. Groups 2, 3, and 4 were fed lima beans diet containing 47.5 mg CN kg^{-1}DM for 7, 14, and 21 days, respectively while the control rats had commercially manufactured rat pellets bought at Abraka main market, Nigeria. The method of Siedu *et al*. (2018) was adopted for the preparation of lima beans diet. Debris free lima beans were cracked and soaked in H_2O for about three (3) hours. They were further dehulled, washed and dried for 24 hours at 60±2^{o}C before milling. The milled products were sieved to obtain 40 mm mesh size flour. The lima bean flour was preserved in plastic containers at 4^{o}C until needed. After seven days of Lima diet intake, Group 2 animals together with four (4) rats from the control group were sacrificed under anesthesia using diethylether. Blood was collected via retro-orbital venous plexus into plain tubes using a 5 mL syringe. The same procedure was adopted for Group 3 and 4 rats at 14 and 21 days respectively.

Glutathione (GSH) determination

Glutathione estimation was established spectrophotometrically using Ellman's reagent as described by Sedlak and Lindsay (1968). GSH was proportional to the absorbance at 412 nm and was further quantified (GSH) by adopting a standard curve designed with different GSH concentrations.

Cyanide (CN) and Thiocyanate (SCN) determination

Serum and urinary thiocyanate were determined using the previously described method of Haque and Bradbury (1999). The cyanide content of *Phaseolus lunatus* fed samples was estimated by the method of Essers *et al*. (1993).

Enzyme assay procedures

Diagnostic or marker enzymes as an indicator of organ damage and apoptosis were carried out by analyzing the following biochemical assays: Aspartate aminotransferase (AST), (Bergmeyer *et al*., 1985), Alanine aminotransferase (ALT) (Klauke *et al*., 1993), Albumin and Alkaline phosphatase (ALP) (Tietz and Shuey, 1986), Total protein (Koller, 1987) and Total bilirubin (Schlebusch *et al*., 1995).

Statistic evaluation

Results were reported as Mean ±SD of four repeated determinations and evaluated using the student's t-test. The weekly variations between treatment groups were conducted by Duncan's multiple-range test using Sigma-stat 4.0 software. The p-value was set at <0.05.

RESULTS

Results obtained from the weekly administration of *Phaseolus lunatus* L cyanide are shown in Table 1. Table 1 revealed mean *Phaseolus lunatus* cyanide concentrations of 2.51±3.70, 7.77±3.56 and 9.88±7.22 μg mL^{-1} in serum after 7, 14 and 21 days respecttively, while urine concentration was expressed as 22.49±1.18, 29.32±3.10 and 31.50±3.88 μg mL^{-1} after 7, 14 and 21 days respectively.

Within the period of investigation represented in Table 1, there was statistically, a significant increase ($p<0.05$) in *Phaseolus lunatus* cyanide levels of serum and urine in the experimental groups compared to the normal control. A similar significant ($p<0.05$) increase in the thiocyanate level was obtained when the experimental groups were compared with the control group. Glutathione concentration dropped from 11.80±0.46 μg mL^{-1} (for the control) to 6.99±0.67, 5.33±0.34 and 3.29±0.69 μg mL^{-1} (for the test groups) after 7, 14 and 21 days respectively. With respect to the control, the percentage (%) changes of glutathione for the test groups obtained were 41, 54 and 72% after 7, 14 and 21 days respectively (Table 1).

The serum ALT, AST, ALP and Total bilirubin levels when juxtaposed with the control group, were significantly ($p<0.05$) elevated in experimental subjects after 21 days. No significant differences in serum total protein and albumin levels were observed (Table 2).

DISCUSSION

Erroneously, it is been assumed that plant-based products are non-toxic and rather devoid of side-effects. This disposition is rather too hasty and inconclusive, except scientifically proven otherwise. *Phaseolus lunatus* because of its numerous health-beneficial ingredients might be perceived likewise. Lima beans contain multiform and diverse anti-nutrient factors plus cyanogenic glycoside (Bonita-chanu *et al*., 2020). Scientific documentation has postulated that the only *Phaseolus* species containing cyanogenic glycosides are *Phaseolus lunatus.* Cyanogenic glycosides principally domiciled in *Phaseolus lunatus* are linamarine and lotaustralin (Frehner *et al*., 1990; Speijers, 1993). These glycosides are situated in the vacuoles of cells and are released upon maceration or wound to cell tissues (Montero-Rojas *et al*., 2013). Results from this study unequivocally demonstrated that exposure to *Phaseolus lunatus* cyanide consequentially affected the glutathione status of animals fed with Lima beans or possibly their dietary byproducts. As evidenced from this study, GSH reduction in the blood of rats was obviously recorded in rats fed *Phaseolus lunatus* containing 47.5 mg cyanide kg^{-1}DM.

The depletion of GSH level (endogenous non-enzyme antioxidant) upon *Phaseolus lunatus* consumption is a significant finding in this present research. The reduction in glutathione status among other mechanisms could be the pathway by which cyanide exerts its numerous and enormous toxicities (Gänzle, 2020). Contextually, the depletion of GSH is indicative of increased bioactivity of *Phaseolus lunatus* cyanide. GSH has been established to be an indispensable biomolecule protecting against chemically initiated cytotoxicity. This they carry out through free radical quenching, conjugation with ROS intermediate or H_2O_2 reduction (Usoh *et al*., 2005).

GSH occupies a vital position in the detoxification

Table 1. Cyanides, thiocyanate and glutathione concentration in blood and urine of rats fed *Phaseolus lunatus* L cyanide.

Groups	Blood (µgmL^{-})			Urine (µgmL^{-})	
	CN	SCN	GSH	CN	SCN
Group1 (Control)	ND	2.33±0.07	11.80±0.46	0.98±0.22	8.60±0.04
Group 2 (After 7 days)	2.51±3.70	14.86±0.61	6.99±0.67 (41%)*	22.49±1.18	36.59±1.66
Group 3 (After 14 days)	7.77±3.56	15.08±0.94	5.33±0.34 (54%)*	29.32±3.10	39.13±1.77
Group 4 (After 21 days)	9.88±7.22	19.64±0.87	3.29±0.69 (72%)*	31.50±3.88	43.07±2.31

Values expressed as Mean±SD. Each group possesses an average of 3 determinations. ND-Non-detectable. *Changes with respect to control.

Table 2. Level of liver damage biomarkers in rats fed *Phaseolus lunatus* L cyanide.

Groups	ALT	AST	ALP	TP	T-BIL	Albumin
Group 1 (Control)	32.4±4.0	23.3±2.72	4.81±0.46	7.7±0.22	0.45±1.1	4.20±0.48
Group 2 (After 7 days)	61.2±3.1^{y} (89%)*	35.6±3.66^{y} (53%)*	6.99±0.67^{y} (45%)*	8.2±0.18 (6%)*	0.69±3.6^{y} (53%)*	4.45±0.41 (5%)*
Group 3 (After 14 days)	77.5±4.6^{y} (139%)*	45.8±2.94^{y} (97%)*	8.73±0.34^{y} (81%)*	8.4±0.20 (9%)*	0.75±1.8^{y} (67%)*	4.49±0.55 (7%)*
Group 4 (After 21 days)	88.1±2.2^{y} (172%)*	49.4±3.87^{y} (112%)*	9.29±0.69* (93%)*	8.6±0.33 (11%)*	0.70±2.5^{y} (76%)*	4.57±0.31 (9%)*

Values expressed as Mean±SD. ^{y}($p < 0.05$) *Phaseolus lunatus* cyanide compared with control. *Changes with respect to control.

of toxic metabolites, with liver necrosis initiated when GSH repositories are exhausted remarkably (Davis *et al*., 1974; Mitchell *et al*., 1973). In this result (Table 1), the apparition of cyanide in the blood of the animal after 7, 14 and 21 days of diet consumption indicates exposure to *Phaseolus lunatus* cyanide which is consistent with reactive oxygen species (ROS) bio-availability. Absorption of cyanide occurs in the gastrointestinal tract (GIT) where it undergoes gastrointestinal circulation (Seigler, 1992). The experimental subject had blood cyanide concentrations of 6.99±0.67, 5.33±0.34 and 3.29±0.69 µg mL^{-1} after 7, 14 and 21 days respectively, expatriates the fact that animals exposed to *Phaseolus lunatus* cyanide culminate into toxic manifestation represented in blood glutathione depletion.

Cyanide exhibits neurotoxicity stimulating the intracellular generation of ROS (Isom and Way, 1984). The urine cyanide of test rats was significantly elevated ($p<0.05$) than the control which further endorses the exposure of the rats to *Phaseolus lunatus* cyanide. A significant increase ($p<0.05$) in blood and urine thiocyanate (Table 1) was also observed. Thiocyanate, on the contrary, is the most reliable biomarker of cyanide exposure. This is because thiocyanate is a stable metabolite of free radicals (Haque and Bradbury, 1999; Rosling, 1994). The report on the plasma half-life of thiocyanate is 3 days (Rosling, 1994) and this level is reflective of the mean daily load observed during the last 21 days.

As shown from this research, the elevated levels of SCN determined are indications of the test animals attempting to detoxify the ingested cyanide. Studies have established that linamarase a cell wall-associated β-glycosidase enzyme utilizes sulphur-containing amino acids like cysteine for cyanide detoxification (Nagahara *et al*., 1999). Another biochemical implication of cyanide detoxification is decreased concentration of glutathione as the exposure days extend. Cysteine is a limiting amino acid in glutathione synthesis and hence its utilization in cyanide detoxification could invariably contribute to reduced synthesis of this important biological compound. Furthermore, the reduced level of GSH as replicated in this study could be traced to the scavenging potential of GSH in mopping up reactive intermediates generated via the metabolism of glucosidic and non-glucosidic cyanide.

The susceptibility of the body to oxidative stress is a function of glutathione depletion with the concomitant release of ROS. Another implication of *Phaseolus lunatus* cyanide exposure with concomitant glutathione depletion is observed in the statistically significant ($p<0.05$) increases of serum AST, ALT, ALP and bilirubin (Table 2). An increase in serum concentration of these enzymes indicates damage to the isostructural integrity of cell membranes such as hepatocytes (Ribeiro *et al.,* 2019). Consequently, a myriad of biochemical activities of cyanide either from dietary sources or otherwise could be facilitated via antioxidant status reduction (especially glutathione) of the animal cell.

Conclusion

The assumptions that plant-based therapeutics are nontoxic or free from side effects are hypothetical and need further scientific justification. However, this current research (in consonant with previous studies) has shown that *Phaseolus lunatus* (lima beans) ingestion for intervals of 7, 14 and 21 days dropped the level of vital endogenous defensive mechanism (antioxidants) in the administered subject with an elevation of some tissue bio-makers. Since cyanogenic glucosides in *Phaseolus lunatus* are enzymatically converted to HCN when tissue disruption occurs, it is expedient therefore that processing methods such as soaking, boiling, roasting, cooking, germination, and fermentation are introduced at some point (to reduce cyanide content) before raw lima bean seeds are fed to animal subjects.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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