Original Research Article

In-vitro Antioxidant Capacity, Phytochemical Characterisation, Toxic and Functional Properties of African Yam Bean (*Sphenostylis stenocarpa*) *Seed*-Enriched Cassava (*Manihot esculenta*) Product (*Pupuru*)

ABSTRACT

Aims: This study aimed at determining the In-vitro antioxidant capacity, characterise phytochemical constituents, assess toxic and functional properties of African yam bean (*Sphenostylis stenocarpa*) seed-enriched Cassava product *Pupuru* flour blends using standard methods.

Methodology: *Pupuru* flour blends were produced from spontaneously-fermented cassava tubers substituted with African yam bean (*Sphenostylis stenocarpa) seed* (5% (EP5), 10% (EP10) and 15% (EP15), prior to toasting, cooling, milling, sieving and packaging. A commercial sample (CP) with 100% cassava and another produced in this study, were used as controls.

Results: In-vitro 2,2-Azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging abilities, total flavonoid content (TFC), ferric reducing antioxidant power (FRAP) and total phenol content (TPC) increased significantly (P = .05) as AYBS enrichment levels increased. The commercial and laboratory control samples showed no significant difference (P = .05) in all the antioxidants analysed except DPPH: ABTS (7.61 - 12.27%); DPPH (26.34 - 48.26%); TFC (0.10 - 0.25 mg CAE/g); FRAP (0.81 - 2.36 (mg/g) and TPC (15.74 - 24.15 mg GAE/g). All the phytochemicals except tannins increased significantly (P = .05) as levels of enrichment with AYBS increased. Tannins, phytates, saponins, alkaloids and oxalates were 1.46 - 2.87 (mg/g); 0.85 - 1.40 mg/100 g; 4.18 - 13.27 mg/g; 24.89 - 29.05 mg/g and 1.71 - 3.23 mg/g, respectively. The toxic constituent revealed that all the samples contained significantly different (P = .05) cyanide ranging from 0.87 - 2.51 mg/kg which reduced as AYBS level of inclusion increased. The functional properties of the samples were significantly (P = .05) enhanced with AYBS enrichment.

Conclusion: Utilisation of African yam bean (*Sphenostylis stenocarpa*) seed (AYBS) to enrich *Pupuru* increased its In-vitro antioxidant capacity and phytochemical constituents, reduced the toxic cyanide content enhanced the functional properties, hence, its suitability as a nutraceutical to delay aging process and prevent cardiovascular diseases.

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Keywords: African Yam Bean (*Sphenostylis stenocarpa*) seeds, Cassava, antioxidant, cyanide, phytochemicals, functional properties, nutraceuticals,

1. INTRODUCTION

A predictable upsurge in global request for food in the next few decades is looming owing to continuous population and consumption growth [1]. The last five decades have experienced a remarkable growth in food production, resulting in a marked decline in the percentage of the hungry people, globally, despite a double increase in the total population [2, 3]. The natural antioxidant content of food products has become the focus of most studies in recent years owing to its involvement in defence against diseases [4]. Also, there has been a recent growing indication to propose that countless age-associated human diseases such as cancer, arthritis, immune system degeneration, brain dysfunction, heart diseases and cataracts are the consequences of cellular impairment by free radicals, and that antioxidants in foods could play an imperative role in the prevention of such diseases [5, 6, 7]. The public attention in natural antioxidants has been much driven and steered towards an all-encompassing search for effective, yet natural antioxidants in foods [8, 9, 7, 10, 11]. The capacity of some inherent bioactive compounds in food products to work as antioxidants has been documented, resulting to the impending benefits of consuming the foods that are rich in such compounds [12, 7, 13].
Pupuru is a stiff dough meal traditionally prepared from fermented, smoke-dried cassava (*Manihot esculenta* Crantz) roots and consumed by the riverine dwellers of the South-Western States in Nigeria. Cassava root is primarily starchy [14], energy-dense, with about 80 to 90% carbohydrate on a dry weight basis, with toxic and anti-nutritional components like cyanide and phytate [15;]. There seemed to be scarce studies on the antioxidant activity, toxic, anti-nutritional and functional properties of AYBS-enriched *Pupuru* flour blends. In view of the anticipated shortage of foods, globally, this study aimed at determining the effect of AYBS enrichment of cassava on the in-vitro antioxidant activities, phytochemical constituents, toxic and functional properties of the developed *Pupuru* flour blends.

2. MATERIALS AND METHODS

2.1 Materials

Fresh cassava (*Manihot esculenta,* Crantz) (TME 0581) tubers were sourced from the research farm of the Federal University of Technology, Akure, Ondo State. African Yam Bean (AYB) (*Sphenostylis stenocarpa)* seeds (TSs 091) were from a farm in Efon Alaaye, Ekiti State and characterised by the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State. Reagents and chemicals of analytical grade were purchased from a local chemical store in Akure, Ondo State.

2.2 Methodology

2.2.1 Pre-processing of African yam bean (AYB) (*Sphenostylis stenocarpa*) seeds

African yam bean seeds (AYBS) were pre-processed into flour using modified method of [16] Oluwamukomi and Akinlabi, ~~2011~~. The seeds were manually cleaned to remove dirts, stones, defective seeds, dead insects, and other unwanted materials. The cleaned seeds were washed with and soaked in clean tap water for 24 h, manually dehulled, rinsed, cooked for about 45 min, drained of water, rinsed, dried in an oven at 60^{o}C for 5 h, cooled to about 30 ± 2^{o}C, milled, sieved and the resultant flour was packaged in an airtight container for further uses.

2.2.2 Production of *Pupuru* flour blends

One kilogram (1 kg) of peeled, chipped and washed cassava tubers was soaked in one litre of water in a sterile plastic container and left to ferment for 96 h. Fibrous materials were manually removed from the dewatered, fermented cassava which was then pressed with a hydraulic jack, for further removal of water before being pulverized. Various percentages (5, 10 and 15) of the African yam bean (*Sphenostylis stenocarpa)* seed (AYBS) flour were homogenously mixed with the fermented cassava paste before toasting in an open and wide pan until it was dry (Oluwamukomi and Akinlabi, 2011). The resultant cassava and AYBS blends (Table 1) were cooled to about 30 ± 2 ^{o}C, milled, sieved and packaged in an airtight container for further analyses. A commercial sample of *Pupuru*

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AYAŞAN, T., 2010. Use of cassava and products in animal nutrition (in Turkish). J Agric Fac Gaziosmanpasa University,, 27 (1): 73-83.

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flour (CP) with 100% cassava and another prepared during this study (P100) were both used as commercial and laboratory controls, respectively.

Table 1. Formulation of *Pupuru* flour blends

Sample code	Formulation ratio AYBS flour: Cassava (%)
P100	0 : 100
EP5	5 : 95
EP10	10 : 90
EP15	15 : 85

AYBS = African yam bean (*Sphenostylis stenocarpa*) seed.

2.3 In-vitro antioxidant capacity determination

2.3.1 Determination of 2, 2 - azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS) scavenging capacity

The 2, 2 - Azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS) scavenging ability of each sample was determined according to the method described by [17]. The ABTS was generated by reacting an (7 mM) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM/l, final concentration) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.700 with ethanol. Appropriate dilution of the sample (0.2 ml) was then added to 2.0 ml of the ABTS solution and the absorbance read at 732 nm after 15 m. The Trolox equivalent antioxidant capacity was subsequently calculated as follows:

$$\text{Radical scavenging}(\%) = 100 - [100 \times (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \quad \text{Eqn 1.}$$

Where:

A_{sample} = the absorbance of the ABTS mixed with sample,

A_{control} = the absorbance of the ABTS mixed with water, and

A_{blank} = the absorbance of sample mixed with water.

2.3.2 Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability

The free radical scavenging ability of each of the *Pupuru* flour blend extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated as described by [18]. Briefly, appropriate dilution of the extracts (1 mL) was mixed with 1 mL, 0.4 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability was subsequently calculated.

$$DPPH\ (\%) = \frac{Abs._{ref} - Abs._{standard}}{Abs._{ref}} x\ 100$$ Eqn. 2

Where,
$Abs._{ref}$ = Absorbance of the reference (reacting mixture without the test sample) and,
$Abs._{sample}$ = Absorbance of reacting mixture with the test sample.

2.3.3 Determination of total flavonoid Content (TFC)

Total Flavonoid Content was determined by aluminum chloride colorimetric assay [19] with slight modification. About 500 µl of methanol was added to 10 ml flask containing 500 µl of aqueous extract. To this 50 µl 10% $AlCl_3$ and 50 µl of 1 M CH_3CO_2K was added respectively. The total volume was made up to 2500 µl with distilled water. The solution was then incubated at room temperature for 30 min. Absorbance was read against blank at 540 nm with spectrometer. (JENWAY 6305, United Kingdom). The flavonoid was calculated using quercetin as standard.

$$Total\ flavonoid\ content\ \left(^{mg\ QE}/_{g}\right) = \frac{Abs._{sample}\ x\ Conc._{standard}\ (mg/ml)}{Abs._{standard}\ x\ Conc._{sample}(mg/g)}$$ Eqn. 3.

$Abs_{standard}$ is the absorbance of the solution containing 500 µl quercetin, About 50 µl 10% $AlCl_3$ and 1 M CH_3CO_2K. Blank is the mixture of 500 µl of distilled water, 500 µl of methanol, 50 µl distilled water and 1M $CH3CO_2K$.

2.3.4 Determination of ferric reducing antioxidant power (FRAP)

The reducing potential of each of the *Pupuru* flour blend extracts was determined by assessing the ability of the extract to reduce a $FeCl_3$ solution as described by [20]. A 2.5 mL aliquot was mixed with 2.5 mL, 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL, 1% potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min, and then 2.5 mL, 10% trichloroacetic was added and centrifuged at 650 g for 10 min. A 5 mL of the supernatant was mixed with an equal volume of water and 1 mL, 0.1% ferric chloride. The same treatment was performed to a standard ascorbic acid solution and the absorbance taken at 700 nm. The reducing power was then calculated and expressed as ascorbic acid equivalent.

$$FRAP\ (mg/g) = \frac{Abs._{sample}\ x\ Conc._{standard}}{Abs._{standard} x\ Conc._{sample}}$$ Eqn. 4

Where,
$Abs._{standard}$ = Absorbance of standard (Vitamin C),
$Abs._{sample}$ = Absorbance of reacting mixture with the test sample,
$Conc._{standard}$ = Stock concentration of standard in mg/ml, and
$Conc._{standard}$ = Stock concentration of sample in g/ml.

2.3.5 Determination of total phenol content (TPC)

The total phenol content (TPC) was determined by Folin–Ciocalteu assay [21] using gallic acid as standard. Fifty microliters (50 µl) of aqueous extract solution containing 0.5 mg of aqueous extract was dispensed into a test tube, 50 µl of distilled water and 500 µl of Folin–Ciocalteu reagent was added respectively into the test tube and shaken thoroughly. After 3 min, 400 µl of 7.5% sodium carbonate solution was added and the mixture was incubated at 45 ^{o}C in a water bath for 40 min. Absorbance was measured at 765 nm against blank. The same procedure was repeated to all standard gallic acid solution (0.1 mg/ml). The blank is a mixture of 100 µl of distilled water, 500 µl of Folin-Ciocalteu reagent and 400 µl of 7.5% sodium carbonate. The total phenolic content was expressed as gallic acid equivalent per gram of sample (mg of GAE/g sample) through the calibration curve of gallic acid and calculated as follows;

$$Total\ phenolic\ content\ \left(^{mg\ GAE}/_{g}\right) = \frac{Abs._{sample}\ x\ Conc._{standard}\ (mg/ml)}{Abs._{standard}\ x\ Conc._{sample}(mg/g)}$$ Eqn. 5.

2.4 Toxic and Phytochemical Content Determination

2.4.1 Determination of toxic property

2.4.1.1 Determination of cyanide content

Some 50 mg of the sample was weighed out into a small flat-bottomed plastic vial [22]. Phosphate buffer (0.5 ml of 0.1 M at pH 4–10) was added, followed by exogenous enzyme. A picrate paper

attached to a plastic backing strip (Bradbury *et al.*, 1999) was added and the vial immediately closed with a screw stopper. After about 16 h at 30 °C, the picrate paper was removed and immersed in 5.0 ml water for not less than 30 min. The absorbance was measured at 510 nm and the total cyanide content (ppm) determined by the equation:

$$\text{Total Cyanide content (ppm)} = \frac{396 \times \text{absorbance} \times 100}{z} \quad \text{Eqn 6}$$

where z = weight (mg) of sample.

2.4.2 Phytochemical characterisation

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2.4.2.1 Determination of tannin content

Tannin content of the flour blends were determined using the method of Medoua et al. [23]. Two grams (2 g) of each sample was weighed into a 250 ml flask followed by addition of 200 ml of 0.004 M $K_3Fe(CN)_6$ and 10 ml of 0.008 M $FeCl_3$ in 0.008 M HCl. The flask was allowed to stand for 20 minutes and stirred occasionally at 10 min interval and 1 ml aliquot was removed. This aliquot was added 2 ml of 0.008 M $FeCl_3$ in 0.008M HCl and 10 ml of 0.0015 M $K_3Fe(CN)_6$. After adding the final reagent, the absorbance was then read at 720 nm after 30 seconds against a blank.

$$Tannin\ (mg/g) = \frac{Absolute\ of\ the\ sample\ x\ concentration\ of\ the\ standard\ x\ Dilution\ factor}{Absorbance\ of\ the\ standard\ x\ sample\ size} \quad \text{Eqn. 7.}$$

2.4.2.2 Determination of phytate content

The determination of phytate in sample was done using the method described by Abulude [24]. Eight grams (8 g) of each *Pupuru* flour blend was dispersed in 200 ml of 2% HCl and extracted. Following *extraction*, the dispersion was filtered and 50 ml of the filtrate was mixed with 10 ml of 0.3% ammonium cyanide (NH_4SCN) and diluted with 107 ml of distilled water. The extract was titrated against 0.00195 g/ml of Ferric chloride solution until a brownish yellow colour persisted. Phytate content was estimated with the expression:

$$Phytate\ Phosphorous = (Iron\ equivalent\ x\ 1.95\ g\ of\ titre)\ x\ 3.65\ g \quad \text{Eqn. 8}$$

2.4.2.3 Determination of saponin content

The method described by Obadoni and Ochuko [25] was used to determine the saponin content of the *Pupuru* flour blends. Twenty grams (20 g) of each sample was put into a conical flask and 100 cm of 20% aqueous ethanol was added. This was heated over a hot water bath for 4 hours with continuous stirring at about 55 °C. The mixture was filtered and the residue re-extracted with another 200 ml 20% aqueous ethanol. The combined filtrate was concentrated to 40 ml with the water bath at about 90 °C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. This purification step was repeated. 60 ml of n-butanol was added; the combined butanol extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath; after evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated in mg/g.

2.4.2.4 Alkaloid content determination

Alkaloids in the samples were determined by the method described by Harborne [26]. Five grams (5 g) of the sample was soaked in 200 mL of 20% acetic acid in ethanol for 4 hours. The mixture was filtered and the filtrate was concentrated on a water bath to about three-quarter of the original volume. Concentrated ammonia solution was added dropwise to the extract to precipitate the alkaloids. The solution was allowed to settle, the precipitate filtered and weighed while the alkaloid content was calculated as below:

$$\text{Alkaloid content} = \frac{Weight\ of\ dry\ residue}{Weight\ of\ sample} \times 100$$

....Eqn. 9.

2.4.2.5 Determination of oxalate content

The method described by Ukpabi and Ejidoh [27] was used. Two grams (2 g) of each *Pupuru* sample was digested with 10 ml 6 M HCl for one hour and made up to 250 ml in a volumetric flask. The pH of the filtrate was adjusted with concentrated NH_4OH solution until the colour of the solution changed from salmon pink colour to a faint yellow colour. Thereafter, the filtrate was treated with 10 ml of 5% $CaCl_2$ solution to precipitate the insoluble oxalate. The suspension was centrifuged at 2500 rpm, after which the supernatant was decanted. The precipitate was dissolved in 10 ml of 20% (v/v) H_2SO_4 and the solution was made up to 300 ml. An aliquot (125 ml) was heated until near boiling point and then titrated against 0.05 M standardized $KMnO_4$ solution to a faint pink colour which persisted for about 30 seconds after which the burette reading was taken and used to estimate the oxalate content.

$$Oxalate\ (mg/g) = \frac{(titre\ value\ x\ volume\ of\ KMnO_4\ x\ dilusion\ factor)/5}{Sample\ size} \quad \text{Eqn. 10.}$$

2.6 Determination of Functional Properties

2.6.1 Foaming and emulsification capacities (%)

Foaming, emulsification, Water and oil absorbing capacities (%) were determined using the methods of AOAC [28].

2.6.2 Swelling capacity

The swelling capacity of each sample was determined by the method described by Takashi and Sieb [29]. One gram of flour was weighed into a 50 ml centrifuge tube. 50 ml of distilled water was added and mixed gently. The slurry was heated in a water bath at 90 ºC for 15 minutes. During heating, the slurry was stirred gently to prevent clumping of the flour. On completion, the tube containing the paste was centrifuged at 3,000 rpm for 10 minutes using a centrifuge machine. The supernatant was decanted immediately after centrifuging. The weight of the sediment was taken and recorded. The moisture content of sediment gel was thereafter determined to get the dry matter content of the gel.

Swelling capacity = Weight of mass of sediment
Weight of dry matter in the gel (4)

2.6.3 Bulk density

Bulk density was determined using the method described by Wang and Kinsella [30]. 10 g of sample was weighed into a 50 ml graduated measuring cylinder. The sample was packed by gently tapping the cylinder on the bench top. The volume of the sample was recorded.

Bulk density = Weight of the sample
Volume of the sample after tapping (5)

Packed Bulk Density (g/ml)
Loose bulk density (g/ml).

2.6.4 Dispersibility (%)

Dispersibility of the samples was determined by the method described by Kulkarni et al. [31]. 10 g of flour was suspended in a 100 ml measuring cylinder and distilled water was added to reach a volume of 100 ml. The mixture was stirred vigorously and allowed to settle for 3 hours. The volume of settled particles was recorded and subtracted from 100. The difference was reported as percentage dispersibility of the sample:

% dispersibility = 100 – volume of settled particle of sample Eqn. (6).

2.6.5 Least gelation concentration

Least Gelation Concentration (LGC) of the each sample was determined by the method described by Coffman and Grarcia [32]. Ten suspensions (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20% w/v) in 5 ml distilled water were prepared in test tubes. The test tubes containing the suspensions were heated in a boiling water bath (Thelco, model 83, USA) for 1 hour. The tubes and contents were cooled rapidly under running cold water and then cooled further for 2 hr at 4°C. The tubes were inverted to observe if the contents would fall or slip off. The least gelation concentration was that concentration when the sample from the inverted test tube did not fall or slip off.

2.6.6 Statistical Analysis

All the data obtained in this study were subjected to Analysis of Variance (ANOVA) using IBM SPSS version 21. Duncan Multiple Range Tests (DMRT) were carried out for the separation of means and determination of significant differences between means. Results are presented as mean ± standard deviation accepted at $P = .05$ confidence limit [33].

3 Results and Discussion

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3.1 In-vitro antioxidant activities

The in-vitro antioxidant activities of commercial and spontaneously-fermented African yam bean (AYB) (*Sphenostylis stenocarpa*) seed-enriched *Pupuru* flour blends are presented in Table 2. The observed trend was an increase in all the antioxidant activities as the levels of AYBS enrichment increased. The commercial and laboratory control samples showed no significant difference ($P = .05$) in all the antioxidant capacities except DPPH. The ABTS activities of all the flour blends were significantly different ($P = .05$) ranging from 7.61 (CP) to 12.27% (EP15). In the DPPH assay, radicals in the reaction medium were scavenged, resulting in a colour change from purple to yellow (indicating hydrogen donation ability of the sample), which led to a decrease in absorbance. The degree of solution discoloration, therefore, indicated the scavenging efficiency of the sample beverage [34]. The results of DPPH for all the samples were significantly different ($P = .05$) between a range of 26.34% (CP) and 48.26% in EP15. The flavonoid in-vitro antioxidant capacities of all the flour blends ranged from 0.10 (CP) to 0.25 (mg CAE/g) (EP15). Flavonoids are a group of phenolic compounds extensively found in plants. As discovered with other biologically active anti-nutrient compounds, flavonoids may stimulate beneficial and adverse physiological properties in humans [35].Millet has been reported to contain flavonoids that possess a strong inhibitory consequence on thyroperoxidase activity in goiter cases which were caused by consumption of peanuts [35]. The healthy properties of flavonoids could be due to their antioxidative characteristics as free-radical neutralisers. Though, some particular functions such as cancer prevention, anti-inflammatory and antiviral activities, positive effect on capillary fragility and vascular protection have been documented [35]. Ferric reducing antioxidant power (FRAP) assay is used to determine the electron-donating abilities of natural antioxidants. FRAP of the entire samples ranged between 0.81 (P100) to 2.36 (mg/g) (EP15). Polyphenols are plant secondary metabolites that are abundant in plant-based foods. Total phenolic in-vitro antioxidant capacities of all the *Pupuru* flour blends varied from 15.74 (CP) to 24.15 mg GAE/g (EP15). Plant phenolic compounds are of utmost interest to researchers because of their antioxidant potential to prevent degenerative diseases [36]. The consumption of plants rich in phytochemicals has been reported to have a positive regulatory effect in humans and that phenolics possess the ability to scavenge free radicals, which normally accumulate in the body due to an imbalance between the antioxidant system of the body and the formation of reactive oxygen species [37]. The addition of AYBS to cassava in producing *Pupuru* flour blends in this study increased the phenolic contents of the samples to significant ($P = .05$) levels, hence, their consumption could delay aging process, prevent degenerative diseases and enhance health.

Table 2. In-vitro antioxidant activities of commercial and spontaneously-fermented African yam bean (AYB) (*Sphenostylis stenocarpa*) seed-enriched *Pupuru* flour blends

Sample code	ABTS (%)	DPPH (%)	Flavonoid (mg CAE/g)	FRAP (mg/g)	Total Phenols (mg GAE/g)
CP	7.61±1.04[d]	26.34±1.60[e]	0.10±0.02[d]	0.78±0.04[d]	15.74±0.09[d]
P100	7.97±1.32[d]	29.41±1.37[d]	0.10±0.03[d]	0.81±0.02[d]	16.30±0.13[d]
EP5	9.19±0.84[c]	37.95±2.43[c]	0.15±0.02[c]	1.84±0.03[c]	17.14±0.80[c]
EP10	10.94±1.02[b]	43.21±1.69[b]	0.19±0.01[b]	2.27±0.06[b]	21.47±1.01[b]
EP15	12.27±0.96[a]	48.26±1.48[a]	0.25±0.02[a]	2.36±0.01[a]	24.15±0.62[a]

Values are means ± SD with different superscripts in the same column are significantly different (P = .05), n = 3. CP = Commercial 100% Cassava *Pupuru* Sample; P100 = Laboratory 100% Cassava *Pupuru*; EP5 = 5% AYB-enriched *Pupuru;* EP10 = 10% AYB-enriched *Pupuru*; EP15 = 15% AYB-enriched *Pupuru*.

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3.2 Toxic Property and Phytochemical ~~c~~ Characterisation

The toxic (cyanide) and phytochemical characterisation of commercial and spontaneously-fermented African *y*am bean (AYB) (*Sphenostylis stenocarpa*) seed-enriched *Pupuru* flour blends are as presented in Table 3. All the *Pupuru* flour blends contained significantly different (P = .05) cyanide ranging from 0.87 - 2.51 mg/kg in EP15 and CP, respectively. The results indicated reduction in cyanide content as level of inclusion of AYBS increased. Cyanide intoxication can either be by inhalation or ingestion [38] and its level in cassava has been reported by Hahn [39] to depend on cultivar, growth and environmental conditions, such as temperature, soil type, humidity and age of the plant [40, 41]. Cyanide has been reported as the major toxin that limits the consumption and utilisation of cassava roots which contains a range of 10 to 500 mg cyanide equivalents/kg DM [15]. Some cassava varieties, especially, the bitter one, contain more than 10mg cyanide equivalents/kg DM but the recommended limit to prevent acute toxicity in humans is < 10 mg cyanide equivalents/kg DM [42].The samples in this study are therefore, safe for consumption in terms their cyanide contents which are much lower than 10 mg/kg. Cyanide causes acute toxicity in humans, its residues (glucoside, cyanohydrin or free cyanide) in processed cassava, are as toxic as the unprocessed derivatives present in raw cassava roots but various effective detoxification methods which are usually in sequence and time-dependent have been documented to include peeling, grating, soaking, boiling/cooking, drying and fermentation [15, 43, 44, 45].

The phytochemicals such as tannins, oxalates, phytates, are natural or synthetic compounds found in all plant foods (in varied quantities and types), which reduce absorption and utilisation of essential nutrients like vitamins and minerals. The results indicated that all the phytochemicals except tannins increased significantly (P = .05) as levels of enrichment with AYBS increased. Tannin contents were significantly different (P = .05) ranging from 1.46 (in EP15) to 2.87 (mg/g) (in CP) in all the *Pupuru* flour blends. Tannins affect the nutritive values of food products, form a complex with protein (both

Table 3. Toxic and phytochemical characterisation of commercial and spontaneously-fermented African yam bean (AYB) (*Sphenostylis stenocarpa*) seed-enriched *Pupuru* flour blends.

Sample code	Cyanide (mg/kg)	Tannin (mg/g)	Phytate (mg/ 100g)	Saponin (mg/g)	Alkaloids (mg/g)	Oxalate (mg/g)
CP	2.51±0.02[a]	2.87±0.02[a]	1.12±0.01[b]	5.97±0.02[d]	27.78±0.02[c]	1.72±0.02[d]
P100	2.17±0.06[b]	2.09±0.02[b]	0.85±0.03[c]	4.18±0.30[e]	24.89±0.04[e]	1.71±0.10[d]
EP5	1.71±0.03[c]	1.71±0.05[c]	1.12±0.18[b]	10.00±0.28[c]	25.37±0.02[d]	1.84±0.08[c]
EP10	1.10±0.01[d]	1.69±0.02[d]	1.15±0.50[b]	12.36±0.30[b]	28.93±0.05[b]	2.76±0.04[b]
EP15	0.87±0.01[e]	1.46±0.04[e]	1.40±0.04[a]	13.27±0.24[a]	29.05±0.03[a]	3.23±0.05[a]

Values are means ± SD with different superscripts in the same column are significantly different (P = .05), n = 3. CP = Commercial 100% Cassava *Pupuru* Sample; P100 = Laboratory 100% Cassava *Pupuru*; EP5 = 5% AYB-enriched *Pupuru;* EP10 = 10% AYB-enriched *Pupuru*; EP15 = 15% AYB-enriched *Pupuru*.

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substrate and enzyme) and inhibit digestion and absorption of proteins. Tannins also bind Iron, inhibit nonheme-Fe absorption and make it unavailable for utilization by the body [46]. A report [47] documented that condensed tannins may cleave to DNAs in the presence of copper ions, thereby, increasing malnutrition rate. Presence of tannins in large quantities in foods could lead to intestinal tract damage, and carcinogenesis [48]. Phytate in all the samples significantly (P = .05) ranged from 0.85 (P100) to 1.40 mg/100 g (EP15), with the CP containing a lower value (1.12 mg/100 g) than in the laboratory control sample. The difference in these results might be due to different varieties used for both samples. Phytate possesses anti-nutritional activities in human diets due to its strong ability to chelate zinc, calcium and iron to form insoluble complexes which are not absorbed, hence, contributing to zinc and iron deficiencies [49]. Conversely, phytate has a beneficial antioxidant property which subdues Fe-mediated ·OH formation by Fe, that is complexed by phytate [50], and acts as an anticancer agent [51]. A report [35] revealed that a focus has been on the beneficial properties of phytates owing to their antioxidative characteristics, which are favourable to offset free-radical activities. Saponins in all the samples were significantly (P = .05) different, ranging between 4.18 (P100) and 13.27 mg/g (EP15), the commercial control (CP) sample had (5.97 mg/g) which was higher than what was obtained in the laboratory control (P100) sample. Saponins are compounds formed by triterpenoids or steroidal aglycones and a carbohydrate moiety by ester or ether linkages. They exist in diverse classes of plants, predominantly legumes, roots, and selected medicinal herbs. Their existence in food products has been considered to be harmful if consumed often, hence, their continued use in diets may compromise nutrient absorption [51]. Conversely, it has been claimed that they could also be beneficial since they display ability to lower plasma cholesterol, they have anticancer activity, and they may act as an inhibitor of viral replication. It is not yet clear, though, whether the net effect in the diet would be negative [35]. Saponins have been reported to possess some bioactive components which are responsible for metabolic and potential health benefits, treatment of various ailments, such as inflammation and fatigue [15]. They also provide energy, improve cognitive function and erectile dysfunction in men, and act on the central nervous system of humans to provide therapeutic effects.

The Alkaloid contents (mg/g) of the samples were significantly (P = .05) different ranging from 24.89 (P100) to 29.05 mg/g (EP15), with the CP sample (27.78 mg/g) higher than the laboratory control sample. The variation in the alkaloid contents of both control samples might be due to differences in the varieties, genetic compositions and environmental factors of the cassava used. The observed trend in the alkaloid contents was an increase with increased enrichment levels of AYBS. Oxalates in the flour blends were also significantly (P = .05) different ranging from 1.71 (P100) to 3.23 mg/g (EP15), with both the commercial and laboratory controls not significantly (P = .05) different. The results for phytochemicals components indicated that enrichment of *Pupuru* flour blends with AYBS at 5%, 10% and 15% reduced cyanide and tannin which could be detrimental to consumers but increased the phytates, saponins, alkaloids and oxalates that might be of beneficial health implications.

3.3 Functional Properties of *Pupuru* Flour Blends

The functional properties of spontaneously fermented *Pupuru* flour blends are as shown in Table 3. Foaming capacity (FC) is a property of protein in food samples which could be a benefit in their solubility, capacity to incorporate air for swelling, thus, giving the end product a honeycomb structure as desired in baked products and ice cream [52, 53, 54]. The ability of food materials to foam vary with the types of protein, solubility and additional factors [55]. Good foaming ability is a function of the flexible protein molecule that could reduce surface tension, conversely, poor foaming ability is due to highly ordered globular protein which is relatively difficult to denature by heat [56]. The FC of spontaneously fermented *Pupuru* flour blends ranged significantly (P = .05) from 3.28 in P100 to 4.92% for both EP5 and EP 15 while CP had 9.84% which was twice the highest value. These values were higher than those reported by [57, 58] for African breadfruit and Kidney bean/wheat flour blends, respectively. High emulsion capacity of food products indicates better flavour retention, mouth feel and taste [59] and flours with good ECs will be useful for preparation of comminuted meat products and their analogues [53]. Emulsification Capacity (EC) varied between 54.00 (P100) and 61.22% (EP15) with 47.06% in CP which was lower than the laboratory-prepared control (P100). These values were higher than those reported by [57, 58 for African breadfruit kernel and seed and Kidney bean/wheat flour blends, respectively. Disparity in EC of flour blends has been reported to be as a result of differences in the globular protein contents [60]. The ability to absorb water is a very vital property of flours and starches used in food preparations [61]. Water Absorption Capacity (WAC) of a food suggests the quantity of water accessible for gelatinisation and is an indication of the amount of water retained in its protein matrix [31, 54]. WAC also shows the level of granular integrity and defines the weakness of associative forces between the starch granules, which permits more accessible molecular surfaces for binding with water molecules [62]. WAC indicates some product characteristics such as bulkiness, consistency, moistness, starch retrogradation and staling [63]. The (WAC) of the *Pupuru* flour blends ranged significantly (P = .05) from 3.88 g/ml in EP10 and EP15 to 4.07 g/ml in P100 and EP5, while the CP had 1.92 g/ml. These values were higher than those reported by [57, 58, 64] in studies on African breadfruit kernel and seed; Kidney bean/wheat; and smoked-dried *Pupuru* flour blends, respectively. The trend was a significant (P = .05) reduction in WAC with increased percentage of substitution with AYB seed flour, which might be as a result of low availability of polar amino acids which have been reported as the primary sites for water interaction of proteins [65, 53]. The ability of food materials to absorb water is occasionally ascribed to its proteins content [30]. Increase in WAC in food systems permits food processors to manipulate the functional properties of doughs [66]. The values obtained in this study were desirable and could imply that the protein quality of the AYB seed flour used was good and able to bind a large quantity of water as reported by [67]. Oil absorption capacity (OAC) is a pointer to the rate at which protein adheres to fat in food formulations [56, 54]. Fat serves as a flavour retainer and improves the mouthfeel of food products [68]. Flours with high oil absorption capacities are known as excellent meat extenders [53]. The OAC of the spontaneously fermented *Pupuru* flour blends differed significantly (P = .05) ranging between 1.94 (EP15) and 2.14 g/ml (P100, EP5 and EP10) with 1.92 g/ml for CP. These values were lower than those reported by [61] for starch from improved cassava cultivars and 3.80% reported by [69] for Jack bean starch.

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Table 4. Functional Properties of spontaneously fermented *Pupuru* flour blends and the commercial control sample

Parameters	CP	P100	EP5	EP10	EP15

Foaming capacity (%)	9.84±0.13[a]	3.28±0.17[d]	4.92±0.02[c]	4.92±0.01[c]	4.92±0.59[c]
Emulsification (%)	47.06±0.70[e]	54.00±0.99[d]	56.86±0.31[c]	56.86±0.89[c]	61.22±1.11[ab]
Water Absorption Capacity (g/ml)	1.92±0.03[e]	4.07±1.89[a]	4.07±0.64[ab]	3.88±0.48[c]	3.88±0.53[c]
Oil Absorption Capacity (g/ml)	2.02±0.11[a]	2.14±0.09[a]	2.14±0.09[a]	2.14±0.01[b]	1.94±0.11[c]
Swelling Index (g/ml)	2.78±0.02[b]	3.29±0.01[a]	2.89±0.53[b]	2.88±0.01[b]	2.58±0.03[c]
Packed Bulk Density (g/ml)	0.76±0.14[a]	0.77±0.96[a]	0.77±0.73[a]	0.72±0.31[b]	0.72±0.78[b]
Loose Bulk Density (g/ml)	0.52±0.05[bc]	0.53±0.11[c]	0.56±0.09[b]	0.56±0.09[b]	0.56±0.01[b]
Dispersibility (%)	47.18±0.13[ab]	47.14±0.03[a]	42.86±0.05[c]	42.85±0.53[c]	45.71±0.01[b]
Least Gelation (%)	0.80±0.21[a]	1.00±0.00[d]	1.00±0.00[d]	1.20±0.00[c]	1.40±0.00[b]

Mean ± SD with different superscripts in the same column are significantly different (P = .05), n = 3. Key: CP = Commercial *Pupuru* Sample; P100 = Laboratory 100% *Pupuru*; EP5 = 5% AYB-enriched *Pupuru;* EP10 = 10% AYB-enriched *Pupuru*; EP15 = 15% AYB-enriched *Pupuru*.

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Swelling Index (SI) of a starchy food is the degree of the ability of starch to imbibe water and swell [61] The swelling indices of the spontaneously fermented *Pupuru* flour blends varied significantly (P = .05) from 3.08 in EP15 and 3.79 g/ml in P100 with CP having 2.78 g/ml. There was no significant difference (P = .05) among the swelling indices of CP, EP5 and EP10 but the P100 was significantly higher (P = .05) and EP15, significantly lower (P = .05). The values obtained in this study were lower than those (5.49 to 6.92 %.) reported by Ikegwu et al. [61] in a study that converted different cassava cultivars into starch. The lower results might be due to the inclusion of AYB seed in the flour blends, as well as various preliminary treatments such as fermentation, toasting and drying that have been applied to the flour blends in this study. The lower swelling indices also suggested a more highly ordered arrangement in their granules, as reported that swelling index of granules depicts the magnitude of associative forces within them [70], hence, the higher the swelling index, the lower the associative forces within a starchy food system. A significant decrease (P = .05) in swelling index of the P100 was observed from 3.79 to 3.08-3.39 for the AYB seed-enriched samples, which might have been as a result of the presence of lipids in the AYB seed which might have reduced the swelling capacity of the *Pupuru* flour particles, as reported by [71]. This trend was in agreement with the findings of [72, 73] and the reduced swelling index with increased percentage inclusion of AYB seed might be attributed to the reduced starch component in the enriched samples which could have reduced the absorption of water. The reduced swelling capacity was attributed to a high fat content

which might have reduced the ability of a mixture of wheat and peanut flours to bind water [74]. Swelling capacity has been implicated for a greater volume and more feeling of satiety per unit weight of fermented cassava products to consumers while a swelling index of a minimum of 3.0 has been recommended as preference of consumers [75]. Bulk Density (BD) of a flour is a function of its particle size and is inversely proportional to bulk densities (Loose and bulk) [55]. BD indicates heaviness, greater compactness and is imperative in determining the packaging requirements and material handling of flours [76]. The structure of starch polymers influences bulk density, thus, a loose structure of starch polymers could result in low bulk density [77]. Packed bulk densities (g/ml) of *Pupuru* flour blends varied from 0.72 (EP10 and EP15) to 0.77 g/ml (P100 and EP5), while CP had 0.76 g/ml. Loose bulk densities of the *Pupuru* flour blends ranged between 0.53 (P100) and 0.56 g/ml (EP5, EP10 and EP15) while CP had 0.52 g/ml which was not significantly different (P = .05) from P100 but was significantly (P = .05) lower than all the AYB seed-enriched samples (EP5, EP10 and EP15). These results indicated that the inclusion of AYB seed increased the loose bulk density of the *Pupuru* flour blends. There were no significant differences (P = .05) in the Packed and Loosed bulk densities of the control and the enriched samples *Pupuru* flour blends, as observed by [73] in a study on Soy-Melon Gari production. The result might have been due to the starch content of the flour blends which tends to make the mixture less bulky and lighter [66, 73]. However, the packed bulk densities were consistently higher than the Loosed bulk densities, implying that more quantity of the enriched *Pupuru* flour blends can be packed better than the same specific volume of the control sample, as reported by [78]. Dispersibility of a flour is its ability to reconstitute in water. The higher it is, the better the reconstitution of the flour in water [31] but the dispersibility value for flour samples during the storage is usually relatively high [62], hence, the ease to reconstitute and produce doughs with fine consistencies when mixed with water. The dispersibility of the *Pupuru* flour blends varied significantly (P = .05) from 47.14 in P100 to 45.71% in EP15. There was no significant difference (P = .05) between the CP and P100; EP5 and EP10 but EP15 was significantly (P = .05) higher than all the others. These values were lower than those reported (69 – 86%) for orange flesh sweet potato-sorghum-soy flour blend [77]. Gelling ability of a food product is predisposed by the nature of its inherent proteins, starch and gums, and their interaction during heat treatment [79]. Least gelation properties of the *Pupuru* flour blends ranged from 0.80 (CP) to 1.40% (EP15). There were significant differences (P = .05) among all the samples, CP (0.80%) was significantly (P = .05) lower than P100 (1.00), with EP5 and EP10 were 1.20% which were significantly (P = .05) lower than EP15 (1.40%). The low least gelation values could be attributed to probable development of intermolecular hydrogen bonds between amylose molecules and other proteins present in the cooled gel samples [80]. The rate of gelatinisation of starch is determined by the rate of starch granule swelling [81]. In a study that enriched *Gari* with Soy-Melon observed a higher gel strength in the control sample and reported that protein enriched-samples contained relatively lower number of starch granules as compared to the unenriched samples [73].

Comment [ASA13]: Do not start a sentence with an acronym.

CONCLUSION

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This study showed that the utilisation of African yam bean (*Sphenostylis stenocarpa*) seed (AYBS) to enrich *Pupuru* increased its In-vitro antioxidant capacity and phytochemical constituents, reduced the toxic cyanide content enhanced the functional properties. Thus, its suitability for consumption as a nutraceutical to scavenge free radicals, delay aging process, prevent or combat cardiovascular and degenerating diseases, and enhance overall health. The results is an indication that these combinations of *Pupuru* flour blends will lead significantly to expansion of African yam bean (*Sphenostylis stenocarpa*) seed use, thus, lessening its potential extinction but generating income to local farmers.

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Comment [ASA15]: Uniformity in references must be seen

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Comment [ASA18]: Uniformity in references must be seen Small letter

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