American Journal of Medicine and Health Care (AJMHC)

VOLUME 1 ISSUE 1 (2026)

PUBLISHED BY
E-PALLI PUBLISHERS, DELAWARE, USA

American Journal of
Medicine and Health Care (AJMHC)

Volume 1 Issue 1, Year 2026
https://journals.e-palli.com/home/index.php/ajmhc

Assessment of Phytochemicals, Antinutritional Compositions and Antioxidant activity of Added Sugar Free Bread Made with Provitamin A-Enriched Cassava/Wheat Composite Flour

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Article Information

Received: January 05, 2026

Accepted: March 02, 2026

Published: June 17, 2026

Keywords

Antioxidants, Beta Cells Dysfunction, Diabetes Mellitus, Oxidative Stress, Vitamin A Cassava

ABSTRACT

Oxidative stress is a major contributing factor in cellular dysfunction triggering the initiation of chronic diseases such as diabetes and cancer. This study investigated the antinutritional compositions and in vitro antioxidative effects of milk flavored bread baked using vitamin A-biofortified cassava flour (CAF)/wheat (WTF) composite flour. The improved yellow provitamin A-biofortified cassava variant (IBA154810) were collected from International Institute of Tropical Agriculture (IITA) Nigeria. The tubers were processed into fine flour. Wheat flour was replaced with the gluten-free cassava flour to produced bread samples in ratio CAF/WTF: 0/100, 20/80, 40/60, 80/20 and 100/0. Phytochemical screenings of composite flour breads revealed the presence of alkaloids; oxalate Saponins, Flavonoids, tannin, and phytate. The antinutritional compositions of the composite breads were low and may not warrant any significant toxicological concern. The Phytate/Fe (0.38±0.05 - 0.98±0.05), Phytate/Zn (0.23±0.04 - 0.75±0.05), Phytate/Ca (0.00±0.00 - 0.02±0.00), and [Ca][Phytate]/Zn (0.26±0.05 - 0.65±0.04) molar ratios indicated good minerals bioavailability. Total β-carotenoids (RAE)µg/g ranged between (0.05±0.01 - 2.78±0.10). Bread samples antioxidant activity against ABTS, DPPH, NO and OH free radicals ranged from (3.04-9.94 mMTE/100g), (36.40-60.63%), (37.71-58.69%) and (56.78-71.09%). The total phenol (19.96±0.05 - 38.21±0.02mgGAE/g), total flavonoids (0.25±0.03 - 4.88±0.16) , FRAP (5.10±0.03 - 6.69±0.00AAE/g) and Fe2+ Chelation ability (14.85±0.08 - 52.18±0.11%) of the bread samples increases with CAF substitution. The results revealed that the provitamin A-enriched composite bread samples possess good antioxidant activities which could help the body neutralize harmful free radicals, reduce oxidative stress and minimize the risk of developing diabetes and other chronic diseases.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder in which the body is unable to produce or efficiently utilize insulin hormone that pancreas beta-cells produce to regulate blood glucose levels, resulting to constantly elevated blood glucose level in sufferers (Deepti *et al.,* 2017). Globally, this silently killing disease is projected to increase significantly according to the International Diabetes Federation (IDF, 2021 and Saeedi *et al.,* 2019). A practical approach to lower high blood sugar is to prevent the absorption of carbohydrates after food intake. However, most alpha–glucosidase inhibitors and most anti-diabetic drugs have been reported to be costly with severe side effects thereby necessitating a need for affordable, safe and effective alternative therapy especially among developing countries (Gong *et al.,* 2020). Recently, oxidative stress and vitamin A deficiency have been discovered to be a pivotal factor during development and complications of type-2 diabetes, indicating their roles in the integrity of insulin producing β-cells and poor glucose metabolism (Khalique *et al.,* 2022; Osman *et al.,* 2024 & Zhou *et al.,* 2021), Carotenoids are important due to their conversion to vitamin A and ability to act as an antioxidant which helps prevent most chronic diseases (Bohn, 2017; Fu *et al.,* 2017; Young & Lowe, 2018). Cassava (Manihot esculenta Crantz) is an important food crops consumed as a major staple food in Africa (Droppelmann *et al.,* 2018). Furthermore, cassava has been reported as a food with a low glycemic index and significant antioxidant activity(Coker *et al.,* 2023; Ilona *et al.,* 2017). Vitamin A-biofortified cassava would therefore provide a multifaceted approach to tackling diabetes and its attendant cardiovascular diseases. Therefore, the vitamin A in the vitamin A-enriched cassava/wheat composite bread is thus central to hypoglycemic, antioxidant effects and improved insulin sensitivity. Therefore, this study sought to evaluate the in vitro antioxidant potentials of vitamin A-enriched cassava- wheat composite bread as a dietary intervention to tackle oxidative stress in type-2 diabetes

LITERATURE REVIEW

Oxidative stress stemming from overproduction of free radicals as been known to be a significant factor in the development and progression of diabetes and its associated cardiovascular diseases. These reactive oxygen species (ROS) which include the hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), superoxide radical (O_2•-) and other reactive molecules are byproducts of

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normal cellular metabolism (Caturano *et al.,* 2023). They are important for physiological cell signaling at typical concentrations (Miao *et al.,* 2009; Pacher, 2007) however, increased production of ROS can result to severe oxidative damage in various biomolecules such as proteins, lipids, DNA and other cellular components engendering various disease conditions (Therese *et al.,* 2025; Farber, 1994). This condition along with the incapability of the body's antioxidants system to quench these highly reactive molecules contributes to Beta-cell dysfunction, apoptosis, insulin resistance, and consequently impairing glucose uptake into cells (Badawi, 2010; Darenskaya *et al.,* 2021; Forman & Zhang 2021;). Elevated oxidative stress further triggers development of serious vascular complications, including nephropathy, retinopathy, resulting to vision impairment and blindness in severe cases (Yousef, 2023). Therefore, the deteriorating effects of oxidative stress in numerous disease pathological processes are deeply rooted (Caturano *et al.,* 2023; Moskovitz *et al.,* 2002; Halliwell & Gutteridge, 1999). Although the body has active enzymatic neutralizing mechanisms (thioredoxin reductase (TrxR), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase(GR), and peroxiredoxin (Prx) (Vilchis-Landeros *et al.,* 2024) which protect it against oxidative stress. However, the efficiency of these mechanisms declines progressively with factors like aging, Mitochondria dysfunction, infection and other contributing factors (Maldonado *et al.,* 2023; Zhao *et al.,* 2021), thereby necessitating the need to supply the body with frequent antioxidants to counteract the effects of harmful radicals. Currently, as the global prevalence of diabetes increases rapidly with no definitive cure despite previous pharmacological interventions, there is need to shift from glucose centered approach towards maintenance of healthy pancreatic beta-cells remedy via dietary remedy (Yoshifumi, 2020). The inhibition of digestive enzymes (α–amylase and α–glucosidase), vaso-constriction (Angiotensin-I converting enzyme (ACE) and cholesterol synthesis [3-hyroxy-3-methylglutaryl-CoA (HMG-CoA) reductase] and antioxidant mechanisms have been suggested as a useful approach for the management/prevention of type-2 diabetes and hypertension. Conventional drugs targeting one or more of these therapeutic mechanisms are available in the market. However, the cost of these synthetic drugs and their associated side effects has heightened demands for alternate effective and affordable therapy especially among developing nations (Chaudhary & Tyagi, 2018). Provitamin A carotenoids, known as scavenger antioxidants, belong to a group of non-enzymatic antioxidants that work together with antioxidant enzymes to counteract the beginning of the oxidation chain (Vilchis-Landeros *et al.,* 2024). They contribute to the elimination of ROS by activating Nuclear factor erythroid 2-related factor 2 (Nrf2) which regulate the expression of genes involved in protecting the cells against inflammation, detoxification of foreign chemicals and cellular resistance to oxidants (Qiang Ma, 2013). They possess beneficial effects on human health such as the enhancement of immune response and reduction of the risk of degenerative diseases such as macular degeneration, cardiovascular diseases, cancer, and cataract (Udensi *et al.,* 2022; Adefegha, 2018). The action of carotenoids against diseases has been linked to their potent antioxidant property, specifically, their ability to quench singlet oxygen and interact with free radicals (Palozza & Krinsky, 1992). This ability to neutralize ROS has been attributed to their conjugated double bond system with optimal efficiency found in carotenoids with nine or more conjugated double bonds according to Foote *et al.,* (1970). Cassava (Manihot esculenta Crantz) which is the third most important food source in the tropic has been reported as a staple food with low glycemic index, significant antioxidant activity and low incident of diabetes among ardent consumers (Ilona *et al.,* 2017). Also, recent studies are beginning to unravel the roles of vitamin A in blood glucose control, insulin production, and the pancreatic beta-cell integrity (Khalique *et al.,* 2022; Amisten *et al.,* 2017; Zhou *et al.,* 2021). Therefore, this research work assessed the Phytochemicals, Antinutritional Compositions and in vitro Antioxidant activity of breads produced with Vitamin A-Enriched Cassava/Wheat Composite flour.

MATERIALS AND METHODS

Materials

Atomic Absorption Spectrophotometer (Buck Scientific, Model 235), UV-vis spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, UK), pH monitoring was carried out using AMT 12 MICROPROCESSOR PH/MV/TEMPERATURE METER (Bench-Top pH/Mv Meter), Haier Thermocool Refrigerator, electro-heating standing temperature air dry oven (JINOTECH, DHG-0A), Gas oven, Thiobarbituricacid (TBA) was obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA). Except stated otherwise, all other chemicals and reagents were of analytical grades and the water used was glass distilled.

Sample Collection Preparation

A year old IBA154810 pro-vitamin A-enriched cassava roots were collected from International Institute of Tropical Agriculture, Oyo State Nigeria. All wheat bread flour and other bread ingredients (yeast, margarine, milk flavour, preservative, whole milk, salt and eggs were bought from Akure Ultramodern market, Ondo state Nigeria. The cassava roots were peeled, washed, grated and dried in hydrator at 60°C for 5 hours.

Afterwards, it was ground, sieved through a 500µm analytical sieve and packaged. The bread was prepared by mixing the CAF with WTF in the ratio of (CAF/WTF) as follows: 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0. Each flour blends were mixed with aspartame 0.02%, yeast 1%, whole milk 2%, salt 1.6%, egg albumin 2%, margarine 2% and preservative 0.06% with the composite flour samples respectively, after which water was added and mixed to get homogenous dough using an electric mixer. It was cut into equal pieces, manually shaped and placed in a greased baking pan. The dough was baked at $220^{o}C$ for 25 minutes following 1hour proofing (Oguntuase *et al.,* 2019). The breads samples were cooled and packaged.

Phytochemical Screenings

The phytochemical screenings for phytates, tannins, flavonoids, steroids, terpenoids, Anthraquinone, saponins, alkaloids, were conducted using standard procedures stated in Amoo *et al.,* (2023).

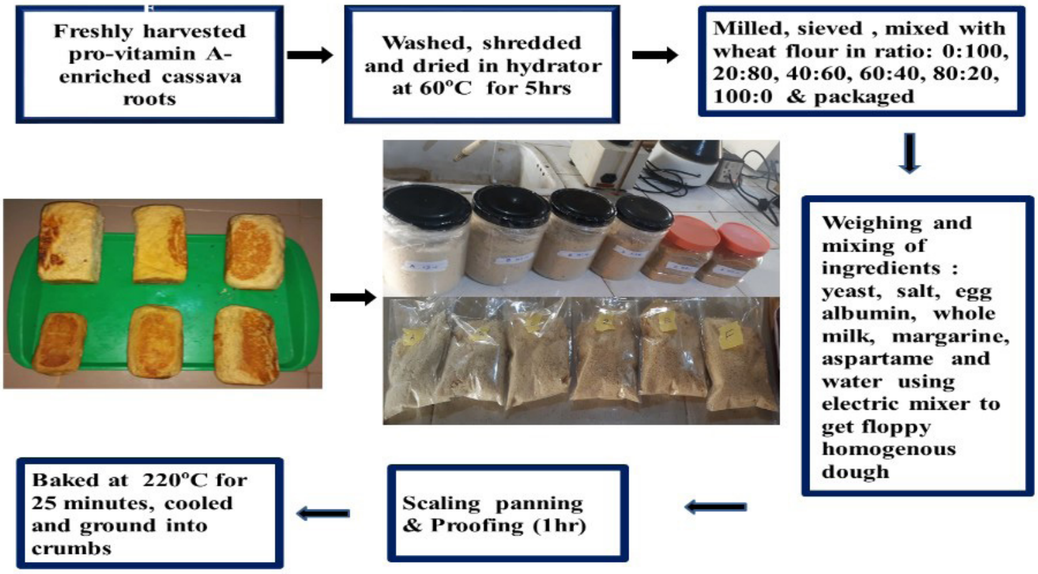

Figure 1: Flow chart of bread production

Determination of Antinutrients in the Composite Bread Samples

Determination of Tannin

Determination of Tannins was carried out according to the procedure of Makkar and Goodchild (1996). 0.2g of finely ground bread sample was weighed into a 50ml sample bottle. 10ml of 70% aqueous acetone was added and covered. The bottle was put in an ice bath shaker and shaken for 2hours at $30^{o}C$. Each solution was centrifuge and the supernatant store in ice. 0.2ml of each solution was pipetted into the test tube followed by 0.8ml of distilled. Standard tannin acid solutions were prepared from a 0.5mg/ml of the stock and the solution made up to 1ml with distilled water. 0.5ml of Folin-ciocateau reagent was added to both sample and standard followed by 2.5ml of 20% Na_2CO_3 the solution was then vortexed and allow to incubate for 40minutes at room temperature, its absorbance was read at 725nm against a reagent blank concentration of the same solution.

Determination of Phytate

4grams of the bread crumbs was soaked in 100ml of 2% HCl for 3hrs and then filter through a No 1 Whatman filter paper. 25ml of the filtrate was taken and placed in a conical flask. 5ml of 0.3% of ammonium thiocyanate solution was added as indicator. After which 53.5 of distil water was added to give it the proper acidity and titrated against 0.00566g per ml of standard iron (iii) chloride solution that contain about 0.00195g of iron per ml until a brownish yellow colouration persist for 5min (Ola & Oboh, 2000).

$\text{Phytate (\%)} = (V \times 0.00195 \times 1.19)/2 \times 100$

Where: v = Volume of standard iron (iii) chloride solution used for titration

Determination of Oxalate

Total oxalates were determined according to the procedure of (Ologbo *et al.,* 2000). 1.0 gram of the bread crumbs sample was weighed and 75ml of 0.75 M H_2SO_4 solution was added. The mixture was carefully stirred intermediately with magnetic stirrer for one hour and filtered using whatman No1 filter paper. 25 ml of the filtrate was collected and filtered hot $90^{o}C$ against 0.05M $KMNO_4$ solution till a faint pink colour appeared that persisted for at least 30 minutes. Then the amount of oxalate in each sample was then calculated by:

$\text{Oxalate (mg/g)} = (Vt \times 0.9004)/(W)$

Where:

Vt = volume of 0.05M KMnO4 used for titration;

W = weight of sample.

Determination of Saponins

The saponins contents were determined following the procedure stated by Amoo *et al.,* (2025). 100ml of 20% (v/v) aqueous ethanol was added to 2.0g each of the bread samples in a conical flask. The samples were heated over a hot water bath for 4 hours at 55oC with constant shaking. The mixtures were filtered and the residues re-extracted with 100ml of 20% aqueous ethanol. The combined extracts of each sample were concentrated to 40ml over water bath at $90^{o}C$. The concentrate was transferred to a 250ml separating funnel and 250ml of diethyl ether was added, shaken vigorously and allowed to separate. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. The combined aqueous phases were extracted using 60 ml of n-butanol. The combined n-butanol extracts (upper portion) was washed twice with 10ml of 5% aqueous extract NaCl. Finally, the remaining solution was heated over hot water bath to evaporate to dryness and then dried in the oven at 105OC to constant weight. The saponins content was calculated as percentage as shown;

Saponins (%) = $S_r/S \times 100$
Where: S_r = Weight of saponins residue;
S = Weight of bread sample

Determination of Alkaloids

5.0 grams of the bread crumbs was placed in a 250ml beaker and 200 ml of 10 % (v/v) ethanolic acid in ethanol was added. The mixture was kept for 4hrs at 25^{o}C. Afterwards, it was filtered and the filtrate concentrated to a quarter of its original volume. Concentrated NH_4OH was added drop-wise until precipitation was completed. The mixture was allowed to settle and the precipitate collected on a previously dried and weighed filter paper. The residue was washed with dilute NH_4OH. The precipitate, alkaloid, was dried and weighed. The percentage alkaloid was calculated by difference according to the method of Trease and Evans (1989);

Alkaloids (%) =$(W_2\text{-}W_1)/W \times 100$
Where:
W1= Weight of dried empty filter paper
W2 = Weight of filter + alkaloids residue
W = Weight of bread sample

Estimation of Mineral Content and Bioavailability of Zinc and Calcium

The mineral contents (Fe, Na, Cu, K, Ca, Zn, and Mg) contents were determined using Atomic Absorption Spectrophotometer (Buck Scientific Model 235) was determined as described by AOAC, (2012). The mole of phytate and minerals were determined by dividing the weight of phytate and minerals with its atomic weight respectively (phytate: 660g/mol; Zn: 65g/mol; Ca: 40 g/mol). The molar ratio between phytate and mineral was obtained after dividing the mole of phytate with the mole of minerals according to Gemede (2020). Calculation of (phytate): (Zn) molar ratio, (Ca):(phytate) molar ratio, and (Ca):(phytate)/(Zn) molar ratios was used as the indication in the prediction of bioavailability of Zn in the bread samples.

Evaluation of in Vitro Antioxidant Activity

Preparation of Acetone-Aqueous Extracts of Bread Samples

10g of the bread crumps samples were extracted in 100ml of acetone-water mixture (70% v/v). The extracts were incubated and agitated at 100 rpm for 48 hours. Then, the extracts were vacuum-filtered and kept at 4 ^{0}C for analysis.

Determination of Total Phenol Content

The phenolic contents were determined using Folin-Ciocateau reagent and expressed as Gallic Acid Equivalents (GAE) (Singleton *et al.,* 1999). The bread extracts were diluted with methanol by taking 3ml of methanol and 1ml of crude extract solution. To the sample solution, 1ml of 5-fold diluted Folin Ciocalteu's reagent was added. The contents were mixed well, kept for 5 minutes at room temperature followed by the addition of 1ml of 10 % aqueous sodium carbonate. After incubation at room temperature for one hour for colour development, the absorbance was read at 760nm (Shimadzu UV-1650 PC Shimadzu Corporation, Kyoto, Japan) against blank. Gallic acid (100-1000 mg/mL) was used to construct the calibration curve. Results were calculated as Gallic acid equivalent (mg/g) of samples. The determination was done in triplicates and concentrations of phenolic compounds were calculated from obtained standard Gallic acid calibration graph.

Determination of Total Flavonoids

The total flavonoids content of the bread extracts was determined according to the Spectrophotometric method developed by Bao (2005). 0.2ml of bread extract was added to 0.3ml of 5% $NaNO_3$ at zero time. After 5min, 0.6ml of 10% $AlCl_3$ was added and after 6min, 2ml of 1M NaOH was added to the mixture followed by the addition of 2.1ml of distilled water. Absorbance was read at 510nm against the reagent blank and Flavonoids content was expressed as mg quercetin equivalent.

Determination of Ferric Reducing Property

The reducing property of the bread extracts was determined by (Pulido *et al.,* 2000), 0.25ml of extract was mixed with 0.25ml of 200mM of Sodium phosphate buffer (pH 6.6) and 0.25ml of 1%KFC. The mixture was incubated at 50^{o}C for 20min, thereafter 0.25ml of 10% trichloroacetic acid was added and centrifuged at 2000rpm for 10minutes, 1ml of the supernatant was mixed with 1ml distilled water and 0.1% of $FeCl_3$ and the absorbance read at 700nm.

Determination of 2, 2-Diphenyl-1-Picrylhydrazyl Radical (DPPH) Free Radical Scavenging Activity

Free radical scavenging activities of the bread extracts were determined using a stable DPPH method developed by Brand-Williams *et al.,* (1995). DPPH which is a free radical of violent colour was scavenged by antioxidants in the food matrix and turn it to yellow colour from violet which was proportional to the radical scavenging activity of the bread. The assay contained 1ml of 0.1mM DPPH in methanol and varying concentrations of extracts (50-1000 ug/ml) methanol and standards in the same solvent and made up to 3.5ml with methanol. The contents were mixed quickly and incubated for 30min at 30°C in a water bath. The degree of reduction of absorbance was recorded in UV-Vis spectrophotometer at 517nm. The percentage of scavenging activity was calculated as:

Percentage DPPH inhibition (%) =$(A_C - A_S)/A_C \times 100$

Where:

A_c = Absorbance of control (without sample);

A_s= Absorbance of sample.

2, 2’-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Radical Scavenging Ability

The ABTS scavenging ability of the bread extract was determined according to the method describe by Re *et al.,* (1999). The ABTS radical was generated by reacting an (7mM) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM/l, final conc.) in the dark for 16hours. After ABTS· radical formation, the absorbance at 734 nm was then corrected to 0.700 using ethanol. 0.2ml of the appropriate dilution of the bread aqueous extract was then added to 2.0ml of ABTS solution and the absorbance was read at 732nm after 15mins. The TROLOX equivalent antioxidant capacity was subsequently calculated from calibration curve.

Nitric Oxide Radical (NO.) Scavenging Assay

Nitric oxide (NO.) generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci *et al.,* (1994). 0.5ml of the reaction mixture containing SNP (5mM) in phosphate buffered saline (pH 7.3), with the bread extract at different concentrations, was incubated at 25°C for 180min in front of a visible polychromatic light source (25 Watt tungsten lamp). The NO radical thus generated interacted with oxygen to produce the nitrite ion (NO_2–) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% Naphthylethylenediamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546nm. The percentage NO generation was estimated by comparing the absorbance of the control and those of the reaction mixture containing test sample using the below formula:

NO (%) = (Absorbance of control - Absorbance of test sample)/(Absorbance of control)×100

Determination of Fe 2+ Chelation

The ability of the bread extracts to chelate ferrous ions was estimated according to the method of Dinis *et al.,* (1994). Briefly, 50 µl of 2 mM FeCl2 was added to 1 ml of different concentrations of the bread extracts (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. Percentage inhibition was calculated using the below equation:

Percentage inhibition (%) = (Absorbance of control - Absorbance of test sample)/(Absorbance of control)×100

•OH radical scavenging activity

Hydrogen peroxide undergoes Fenton reaction to release the hydroxyl radical in the presence of iron. Hydroxyl radical scavenging activity is based on the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with thiobarbituric acid (TBA) to give a yellow colour which absorbs at 532 nm. Briefly, Hydroxyl radicals were generated by the reaction;

Fenton reaction Fe^{2+} + H_2O_2→OH- + HO•

The Fenton reaction system (Fe3+-ascorbate-EDTA-H_2O_2) is mixture consisted of 0.2 ml FeSO4·7H2O (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml 2-deoxyribose (10 mM) mixed with 1.2 ml phosphate buffer (0.1 M, pH 7.4). Afterwards, the bread extracts at (0.2 ml, 1 mM and 2 mM) were added to the Fenton reaction mixture followed by 0.2ml H_2O_2 (10 mM) and incubated at 37°C for 4hours. after which 1 ml of 2.8% trichloroacetic acid (TCA) and 1 ml of TBA (1%) were added and placed in a boiling water bath for 10mins. for colour development. The resultant mixture was brought to room temperature, centrifuged at1000 × g for 3 min and the absorbance read at 532 nm against a blank solution as stated by Chung *et al.,* (1997)

OH Scavenging activity (%) = (Absorbance of control - Absorbance of test sample)/(bsorbance of control)×100

Statistical Analysis

The data obtained from the procedures was collated and analyzed using Statistical Package of Social Science (SPSS) version 21.0. For the Invivo study, data analysis and graph construction were performed using the software GraphPad Prism version 8.02 (GraphPad Prism Software, Inc). The results were analyzed by two-way Analysis of Variance (ANOVA), followed by Tukey’s multiple comparisons test, and data are presented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Phytochemical Screening

Plant derived foods consist of several beneficial phytochemicals (Ibrahim *et al.,* 2023). The phytochemical screening of the pro-vitamin A- enriched cassava/wheat composite flour bread samples as depicted in Table 1 revealed the presence of alkaloids, oxalate Saponins, Flavonoids, tannin, and phytate. The screenings conducted on the control sample (100WFB) were only positive for oxalate, alkaloids and phytate. The presence of terpenoids was only observed in 80CABand 100CAB. All the bread samples showed the presence of alkaloids oxalate and phytate. Phytochemicals are protective bioactive chemicals with anti-carcinogenic, anti-mutagenic, anti-inflammatory, and anti-oxidant properties (Amoo *et al.,* 2025).Alkaloids are classes of plant phytochemicals that have therapeutic and antimicrobial properties (Okwu, 2004). Phytates and oxalates are known to adversely affect mineral bioavailability by forming insoluble salts with zinc, calcium, and iron, thus preventing their utilization. Flavonoids are potent water soluble antioxidants that prevent oxidative cell damage and offer protection against different levels of carcinogenesis (Okwu, 2004). According to (Amira *et al.,* 2017) saponins have been known to exhibit potential therapeutic advantage and are theorized as an alternative hypoglycemic medication for diabetes management. Alkaloids display antimicrobial and anti-parasitic properties, act as narcotics, can alter DNA, have an important role in the immune systems, and treat cardiovascular and metabolic disorders, inflammation, infectious diseases, and miscellaneous problems (Aniszewski, 2015). Tannins are dietary phytochemicals that are responsible for the astringent taste of foods and drinks (Chikezie *et al.,* 2008). The presence of tannins can cause browning in fresh foods and processed products, but some tannin possesses beneficial biological properties (Eleazu *et al.,* 2014).

Table 1: Phytochemical screening of the composite bread samples

Samples	**100 WTB**	**20CAB**	**40CAB**	**60CAB**	**80CAB**	**100CAB**
Steroids	-	-	-	-	-	-
Oxalate	+	+	+	+	+	+
Terpenoids	-	-	-	-	+	+
Phytate	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-
Flavonoids	-	+	+	+	+	+
Saponins	-	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Tannin	-	+	+	+	+	+

KEY: + = Present, ++ = abundantly present, - = absent

WTB = 100% wheat flour bread (control), 20CAB = 20% provitamin A-enriched cassava flour + 80% wheat flour bread, 40CAB = 40% provitamin A-enriched cassava flour + 60% wheat flour bread, CAB = 60% pro-vitamin A-enriched cassava flour + 40% wheat flour bread, 80CAB = 80% provitamin A-enriched cassava flour + 20% wheat flour bread, 100 CAB = 100% provitamin A-enriched cassava flour bread.

Antinutritional Composition of Pro-Vitamin A-Enriched Cassava/Wheat Flour Composite Bread Samples

The antinutrients content of pro-vitamin A- enriched cassava-wheat flour composite bread samples are presented in Table 2. The tannin content range between (0.87—2.81 mg/g), Tannins are high-molecular-weight (>500), water-soluble phenolic chemicals that can precipitate protein, particularly pepsin (Adamczyk *et al.,* 2017).Daily consumption of diet containing up to 1.5 – 2.5 g of tannin is considered safe (Sharma *et al.,* 2019). Therefore, the tannin level found in this study should not have any harmful effects associated with chemical. The Phytate concentrations of the bread samples ranges between (5.77-14.32 mg/g) and all the bread samples varied significantly ($p < 0.05$) compared to the control 100WTB. In nutritional studies, phytic acid has been known to be the most potent antinutritional factor in foods which contribute to reduced essential minerals absorption, (Grases *et al.,* 2017). The lower phytate contents of the CAF/WTF composite bread samples suggest that the minerals present may be much more bioavailable compared to the control. The oxalate level range between (0.09—0.19 mg/g), the oxalate content of 40CAB, 60CAB, 80CAB and 100CAB are not significantly different ($p<0.05$) but higher compared to the control sample.

Table 2: Antinutritional composition of provitamin A-enriched cassava/wheat flour composite breads

Sample	**Oxalate (mg/g)**	**Tannin (mg/g) TAE**	**Phytate (mg/g)**	**%Alkaloids**	**% Saponins**
100WTB (control)	$0.09^{b}\pm0.00$	$0.93^{e}\pm0.09$	$14.32^{a}\pm0.71$	$0.65^{d}\pm0.11$	$0.02^{a}\pm0.00$
20CAB	$0.09^{b}\pm0.00$	$0.87^{e}\pm0.01$	$9.98^{b}\pm0.02$	$1.10^{c}\pm0.04$	$0.05^{a}\pm0.01$
40CAB	$0.18^{a}\pm0.01$	$1.76^{d}\pm0.02$	$8.97^{b}\pm0.19$	$1.17b^{c}\pm0.11$	$0.11^{a}\pm0.02$
60CAB	$0.18^{a}\pm0.00$	$2.01^{c}\pm0.01$	$6.59^{c}\pm0.00$	$1.29^{ab}\pm0.02$	$0.09^{a}\pm0.09$
80CAB	$0.19^{a}\pm0.02$	$2.45^{b}\pm0.05$	$6.76^{c}\pm0.01$	$1.35^{a}\pm0.01$	$0.13^{a}\pm0.09$
100CAB	$0.18^{a}\pm0.00$	$2.81^{a}\pm0.03$	$5.77^{c}\pm1.16$	$1.16^{a}\pm0.26$	$0.17^{a}\pm0.19$

Note: The Mean± SD values in the same row with varied superscript are significantly different ($p < 0.05$), n = 3.

Keys: WTB = 100% wheat flour bread (control), 20CAB = 20% provitamin A-enriched cassava flour + 80% wheat flour bread, 40CAB = 40% provitamin A-enriched cassava flour + 60% wheat flour bread, CAB = 60% pro-vitamin A-enriched cassava flour + 40% wheat flour bread, 80CAB = 80% provitamin A-enriched cassava flour + 20% wheat flour bread, 100 CAB = 100% provitamin A-enriched cassava flour bread

The Saponins content of all the bread samples are not significantly different (p<0.05).The values range between (0.02-0.17%). Saponins are known to bind to some essential minerals and vitamins to create insoluble mineral complexes, thereby reducing their bioavailability (Samtiya *et al.,* 2020). Previous findings showed that low level of saponins in foods are not detrimental to health, but higher quantities in diet can be poisonous at about ≥150 mg/kg body weight (Samtiya *et al.,* 2020). The low level of oxalates and saponins in the bread samples analysed indicates that the minerals and vitamins that these antinutrients typically form complexes with are bioavailable in the bread samples. The alkaloid content increased with cassava flour addition and ranged from 0.65 to 1.35 %. The alkaloid content of the bread samples fall within the range reported by Eleazu *et al.,* (2014) for cassava bread products. Thehydrogen cyanide content ranges between (0.03-0.11mg/kg). The hydrogen cyanide concentrations of the bread samples investigated as shown in Figure 2 are within the acceptable HCN level of 10 mg/kg body weight recommended by FAO/WHO (2005) for safe cassava food products thereby lessening the severe fear of food poisoning reports due to cyanide intake from cassava products (Gregory *et al.,* 2023).

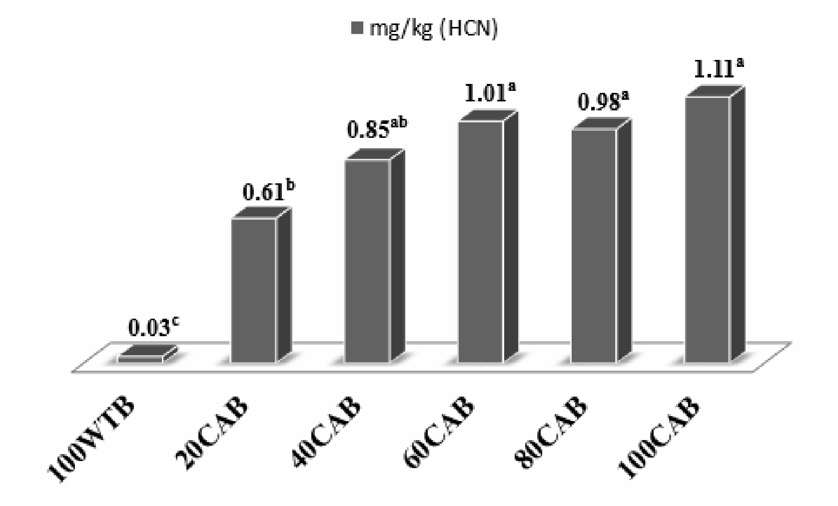

Figure 2: Hydrogen cyanide content of the bread samples

Note: The Mean±SD values in the same row with varied superscript are significantly different ($p < 0.05$), n= 3.

Keys: WTB = 100% wheat flour bread (control), 20CAB = 20% provitamin A-enriched cassava flour + 80% wheat flour bread, 40CAB = 40% provitamin A-enriched cassava flour + 60% wheat flour bread, CAB = 60% pro-vitamin A-enriched cassava flour + 40% wheat flour bread, 80CAB = 80% provitamin A-enriched cassava flour + 20% wheat flour bread, 100 CAB = 100% provitamin A-enriched cassava flour bread.

Estimation of Mineral Bioavailability

The bread samples were analysed for mineral content (iron, calcium, zinc, sodium, magnesium and potassium) as well as phytate / (Zn and Ca) molar ratios. The mineral content (ppm) of the bread samples ranged as follows: Na (39.70-53.56) K (48.11-93.19), and Ca (32.80-44.20), while Mg, Zn, Fe, were 30.64-45.07, 1.50-1.85, and 0.87-1.7 respectively as shown in Table 3. The mineral Zn, Mg, Na, and Ca were significantly ($p < 0.05$) higher in CAF/WTF composite flour bread samples than the 100WFB control sample. Potassium was the most abundance of all the minerals. Phytate, also known as inositol hexakisphosphate, is a phosphorus containing compound that can bind with minerals and reduce mineral absorption (Norhaizan & Ain, 2009). It has been known to inhibit mineral bioavailability owing to its ability to chelate essential minerals in biological system, mostly divalent ones (Fe, Mg, Zn, and Ca) (Ages *et al.,* 1999). Its structure has high density of negatively charged phosphate groups which promote its ability to form stable complexes with mineral ions resulting to poor intestinal absorption (Walter *et al.,* 2002). The Phytate/Zinc, phytate/calcium, and Phytate/iron [Ca][Phytate]/Zn molar ratios ranged from (0.23-0.75), (0.00-0.02) and (0.35-0.98) respectively. The molar ratios of phytate to Zinc, calcium and iron of all bread samples were < 1.00, indicating that the bioavailability of Zn, iron and calcium is not likely to be affected by phytate content in bread samples indicating good bioavailability of the minerals (Fergusson *et al.,* 1988; Norhaizan & Ain, 2009;). According to Oboh and Amusan (2009), the molar ratio of [Ca] [phytate]/[Zn] was discovered to be a preferred quantification for the determination of Zinc bioavailability as compared to [Phytate]/[Zn]molar ratio. The calculated [Ca][Phytate]/Zn molar ratios of the composite bread samples ranging from (0.26-0.39) were below 0.5 mol/kg compared to the (control) 100WTB which is considered reasonable values for Zn bioavailability (Fergusson *et al.,* 1988). The retinol activity equivalents (RAE) of the bread samples are higher compared to values reported by Awoyale *et al.,* (2018), which could be attributed to higher provitamin A content of the new vitamin A-biofortified cassava variant used for the study as well level of exposure during processing (Britton, 1996).

Table 3: Micronutrient composition, bioavailability of Zn, Ca, Fe and vitamin content of the pro-vitamin A- enriched cassava/ wheat flour composite bread samples (ppm)

Sample	**100 WTB**	**20CAB**	**40CAB**	**60CAB**	**80CAB**	**100CAB**
Mineral content						
Sodium (Na)	$46.52^{d} \pm 0.03$	$40.12^{f} \pm 0.02$	$48.50^{c} \pm 0.01$	$39.70^{f} \pm 0.30$	$51.33^{b} \pm 0.50$	$53.56^{a} \pm 0.05$
Potassium (K)	$93.17^{a} \pm 0.30$	$67.20^{e} \pm 0.32$	$80.40^{c} \pm 0.09$	$78.83^{d} \pm 0.28$	$75.89^{e} \pm 0.01$	$48.11^{g} \pm 0.19$
Calcium (Ca)	35.03e±0.02	$32.80^{f} \pm 0.01$	$41.00^{b} \pm 1.00$	$37.67^{d} \pm 0.20$	$39.18^{c} \pm 0.20$	$44.20^{a} \pm 0.00$
Magnesium (Mg)	$30.64^{f} \pm 0.05$	$35.00^{d} \pm 0.01$	$35.12^{e} \pm 0.02$	$45.07^{a} \pm 0.11$	$40.71^{c} \pm 0.01$	$42.76^{b} \pm 0.06$
Iron (Fe)	$1.21^{bcd} \pm 0.01$	$0.94^{cd} \pm 0.03$	$0.87^{d} \pm 0.00$	$1.70^{a} \pm 0.31$	$1.53^{ab} \pm 0.02$	$1.27^{bcd} \pm 0.05$
Zinc (Zn)	$1.85^{d} \pm 0.01$	$2.10^{bcd} \pm 0.01$	$2.32^{ab} \pm 0.02$	$1.50^{e} \pm 0.30$	$2.14^{bc} \pm 0.03$	$2.54^{a} \pm 0.22$
Phytate/mineral molar ratio						
Phytate / Fe	$0.98^{a} \pm 0.05$	$0.88^{a} \pm 0.02$	$0.87^{a} \pm 0.01$	$0.35^{b} \pm 0.08$	$0.37^{b} \pm 0.00$	$0.38^{b} \pm 0.05$
Phytate / Ca	$0.02^{a} \pm 0.00$	$0.02^{b} \pm 0.00$	$0.01^{c} \pm 0.00$	$0.01^{cd} \pm 0.01$	$0.01^{cd} \pm 0.00$	$0.00^{d} \pm 0.00$
Phytate / Zn	$0.75^{a} \pm 0.05$	$0.47^{b} \pm 0.00$	$0.38^{bc} \pm 0.00$	$0.42^{bc} \pm 0.09$	$0.31^{cd} \pm 0.00$	$0.23^{d} \pm 0.04$
[Ca][Phytate]/Zn	$0.65^{a} \pm 0.04$	$0.38^{b} \pm 0.00$	$0.39^{b} \pm 0.01$	$0.39^{b} \pm 0.08$	$0.30^{c} \pm 0.00$	$0.26^{d} \pm 0.05$
Total β-carotenoids (µg/g)	$0.19^{f} \pm 0.01$	$3.43^{e} \pm 0.06$	$5.91^{d} \pm 0.01$	$6.60^{c} \pm 0.08$	$8.37^{b} \pm 0.07$	$10.29^{a}6 \pm 0.10$
β-carotenoids (RAE) (µg/g)	$0.05^{f} \pm 0.01$	$0.92^{e} \pm 0.06$	$1.60^{d} \pm 0.01$	$1.78^{c} \pm 0.08$	$2.26^{b} \pm 0.07$	$2.78^{a} \pm 0.10$

Note: The Mean± SD values in the same row with varied superscript are significantly different ($p < 0.05$), n = 3.

Keys: WTB = 100% wheat flour bread (control), 20CAB = 20% provitamin A-enriched cassava flour + 80% wheat flour bread, 40CAB = 40% provitamin A-enriched cassava flour + 60% wheat flour bread, CAB = 60% pro-vitamin A-enriched cassava flour + 40% wheat flour bread, 80CAB = 80% provitamin A-enriched cassava flour + 20% wheat flour bread, 100 CAB = 100% provitamin A-enriched cassava flour bread.

In Vitro Antioxidant Activities of Provitamin A-Enriched Cassava/Wheat Composite Bread Samples

The results of the antioxidant activities of pro-vitamin A-enriched cassava-wheat flour composite bread samples are shown in Table 4. The level of total phenolic compounds of a food matrix has been related to its antioxidant activities (Amoo *et al.,* 2025). The concentration of total phenolic compounds varied significantly ($p<0.05$) in the bread samples, ranging from 19.96-38.21 mg GAE g−1 bread extract. The values obtained for Total Phenol (mg/g GAE) in this study are higher compared to the values reported by Oguntuase *et al.,* (2022) for wheat-bambara groundnut bread samples. The Flavonoids content ranged from 0.25-4.88mg QE g−1 of bread extract. Flavonoids possess a broad spectrum of chemical and biological activities, including radical-scavenging properties. One of the most well-known effects of Flavonoids on carbohydrate metabolism is the inhibition of digestive enzymes (α-glucosidase and α-amylase), responsible for the metabolism of dietary carbohydrates to glucose (Bahadoran *et al.,* 2013). The increasing phenolic and Flavonoids content of the bread samples is an indication that the CAF contains higher content of phenol and Flavonoids than WTF. The antioxidant activity of the bread samples against ABTS, DPPH, NO and OH- free radicals ranged from (3.04-9.94 mM TE/100g), (36.40-60.63%), (37.71-58.69%) and (56.78-71.09%) respectively. Antioxidants in the food sample react with DPPH• free radicals, thereby reducing the concentration based on the number of their available hydroxyl groups. Therefore, the absorption at 517 nm is proportional to the amount of residual DPPH• (Juan *et al.,* 2005). It is visually noticeable as a discolouration from purple to yellow. The Antioxidant activity of the formulated bread samples was significantly ($p < 0.05$) higher against DPPH compared to the control. Antioxidants carry out their protective roles on cells in one of the ways either by neutralizing/scavenging free radicals produced in the cells or by preventing the production of free radicals via chelating transition metal composition (Alia *et al.,* 2003; Amic *et al.,* 2003). The Ferric reducing antioxidant properties (FRAP) of the bread samples is a measure of its reducing ability to transform Fe^{3+} - Fe^{2+} (Amoo *et al.,* 2025) and an important index that indicates its antioxidant potential ranged from (5.10-6.82mg AAE g−1). Fe^{3+} reduction is commonly used to indicate electron donating activity mechanism of phenolic antioxidant activity which also correlate with other antioxidant properties (Ayoade *et al.,* 2015).The Fe^{2+} chelation varied from (13.82-52.18%). The reaction of Ferrozine with ferrous ions to form a red coloured ferrozine-Fe^{2+} is interrupted in the presence of coexisting chelators and as a result the red color of the complex is decreased. Therefore, the chelating activity of the CAF/WTF bread samples can be determined by measuring the rate of color reduction. The metal Chelating agents are effective secondary antioxidants due to their ability to reduce the redox potential and stabilize the oxidized form of the metal ion (Gordon and Hudson, 1990). The iron binding capacity of the CAF/WTF bread samples in this study suggests the presence of polyphenols that have potent iron chelating capacity. The Fe2+ chelation ability and FRAP of the experimental composite bread samples increases with CAF inclusion and higher compared to 100WFB control sample.

Table 4: Invitro Antioxidant Potential of pro-vitamin A- enriched cassava – wheat flour composite bread samples

Sample	**100 WTB**	**20CAB**	**40CAB**	**60CAB**	**80CAB**	**100CAB**
Total Phenol (mg GAE /g)	$19.96^{d}\pm0.05$	$20.81^{d}\pm 0.02$	$38.21^{a}\pm0.02$	$36.08^{b}\pm0.00$	$32.63^{c}\pm0.03$	$33.66^{c}\pm1.49$
Total Flavonoids (mg QE /g)	$0.25^{d}\pm0.03$	$0.26^{d}\pm0.00$	$0.51^{c}\pm0.02$	$0.48^{c}\pm0.03$	$4.10^{b}\pm0.02$	$4.88^{a}\pm0.16$
FRAP(mg AAE /g)	$5.10^{a}\pm0.03$	$5.74^{c}\pm0.05$	$6.66^{b}\pm0.00$	$6.64^{b}\pm0.02$	$6.82^{a}\pm0.01$	$6.69^{b}\pm0.00$
NO %	$58.69^{a}\pm0.01$	$57.36^{b}\pm0.02$	39.08e±0.09	$37.71^{f}\pm0.00$	$48.86^{d}\pm0.02$	$50.16^{c}\pm0.00$
OH %	$71.09^{a}\pm0.13$	$69.24^{b}\pm0.01$	$60.12^{d}\pm0.01$	$61.21^{c}\pm0.00$	$58.56^{e}\pm0.12$	$56.78^{f}\pm0.00$
DPPH %	$40.91^{e}\pm0.00$	$36.40^{f}\pm0.06$	$56.84^{c}\pm0.04$	$53.64^{d}\pm0.00$	$58.65^{b}\pm0.02$	$60.63^{a}\pm0.00$
Fe^{2+} Chelation (%)	14.85±0.08	13.82±0.14	23.61±0.07	30.93±0.04	50.87±0.06	52.18±0.11
ABTS (mM TE/100g)	$9.94^{a}\pm0.27$	$9.62^{b}\pm 0.01$	8.17d ± 0.04	$8.76^{c}\pm0.00$	$3.04^{f}\pm0.03$	$3.59^{e}\pm 0.03$

Note: The Mean± SD values in the same row with varied superscript are significantly different ($p < 0.05$), n = 3.

Keys: WTB = 100% wheat flour bread (control), 20CAB = 20% provitamin A-enriched cassava flour + 80% wheat flour bread, 40CAB = 40% provitamin A-enriched cassava flour + 60% wheat flour bread, CAB = 60% pro-vitamin A-enriched cassava flour + 40% wheat flour bread, 80CAB = 80% provitamin A-enriched cassava flour + 20% wheat flour bread, 100 CAB = 100% provitamin A-enriched cassava flour bread.

CONCLUSION

The data generated from this study show that the pro-vitamin A-enriched cassava/wheat composite flour bread has beneficial dietary phenolic phytochemicals, free radical scavenging activity, and antioxidative effects which increases with the cassava flour supplementation. The antinutrient compositions and the cyanogenic potential of the composite bread samples are low indicating good minerals availability, palatability and its safety for human consumption. As bread is a common staple food, the pro-vitamin A-enriched cassava/wheat composite bread could offer a cheap, effective and preventive dietary means of managing type 2 diabetes and its associated cardiovascular diseases which could help alleviate the problem of high cost and side effects of synthetic antidiabetic drugs. However, a comprehensive invivo experimental study is still needed to ascertain its antihyperglycemic efficacy in diabetic subjects.

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