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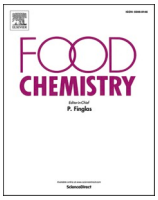

Valorization of natural resources - development of a functional plant-based beverage

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ABSTRACT

Plant-based beverages are gaining attention due to their potential to offer sustainable and health-promoting alternatives to traditional dairy products. This study aimed to develop a dehydrated functional plant-based beverage composed of tigernut tubers (*Cyperus esculentus* L.), mukua pulp (*Adansonia digitata* L.), and thermal water. All matrices and the final beverage were fully characterized in terms of physical properties, nutritional value, chemical parameters, mineral content, and bioactive capacity (antioxidant, antimicrobial, anti-proliferative and hypocholesterolemic effects). Microbiological safety was ensured. To promote zero waste, by-product from the beverage development was characterized for potential applications. The ingredients used to develop the plant-based beverage showed a rich nutritional, chemical and bioactive profile, resulting in a beverage with high levels of unsaturated fatty acids, proteins, and vitamin E. This beverage demonstrated functional potential with antioxidant and hypocholesterolemic effects and no toxicity in *in vitro* screening, positioning it as a promising functional plant-based product with commercial viability.

1. Introduction

There is currently no global, legally standardized definition of "functional foods", as food safety authorities in different regions regulate these foods independently. Functional foods are understood as foods that, beyond basic nutrition, promote health and prevent diseases when included in a balanced diet (Granato et al., 2020; Ye et al., 2018). In this category of functional foods, plant-based beverages are included, although they were originally not part of this sector, as they were primarily consumed as a milk substitute for individuals with lactose intolerance. However, with increased access to information, consumers are making more selective choices, favoring plant-based foods for their health-promoting benefits (antioxidants, anti-inflammatory, antitumor, anticholesterolemic, antimicrobial) (Maleš et al., 2022; Pérez-Rodríguez et al., 2023).

Cyperus esculentus, a member of the Cyperaceae family (Cyperaceae Juss.), is a perennial plant commonly referred to as "junça-amarela", "tigernut" and "chufa". This species produces sweet tubers that are well-documented in the literature for their bioactive properties, including antioxidant, antimicrobial, antibacterial, and anticancer activities (Bezerra et al., 2023). Its tubers are traditionally used in Spain to prepare the plant-based beverage 'horchata' and are considered a healthy food due to their potential to prevent heart disease, control diabetes, improve circulation, and reduce the risk of some cancers (*e.g.*, colon) (Sánchez-Zapata et al., 2012).

Adansonia digitata L. of the mallow family (Malvaceae) is a species native to Africa, commonly known as the "baobab tree." It has a naturally dried, hard-shelled fruit known as "mukua" that is important for feeding rural populations in Africa (Muthai et al., 2017).

This fruit, approved as a 'food ingredient' in the in the United States of America, is considered a superfood due to its rich nutritional profile. Its pulp contains essential minerals, vitamin C, amino acids, dietary

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fiber, and protein, offering benefits such as improved bone health, immune support, digestive health, and muscle maintenance. Additionally, its antioxidant properties help combat oxidative stress, associated with aging and chronic diseases (Chabite et al., 2019; Stadlmayr et al., 2020).

Over the years, thermal waters have gained increasing popularity across various industries. In the food industry, thermal water can serve as an ally due to its minerals' higher bioavailability compared to those in food, leading to better mineral absorption by the body and positively impacting consumer health (Costa-Vieira et al., 2019).

The main objective of this work is to provide the community with a dehydrated functional plant-based beverage that is practical to consume, offering portability and shelf stability by eliminating the need for refrigeration. More than just a milk substitute, this beverage is rich in health-promoting ingredients. To achieve this goal, three natural ingredients were selected (tigernut, mukua, and thermal water) as sources of these beneficial compounds, with the dried form analyzed to ensure its nutritional quality and its impact on the rehydrated product.

2. Materials and methods

2.1. Samples

Dried brown tigernuts labelled BIO, indicating certified organic production free of synthetic pesticides and fertilizers, and originating from Niger were purchased from the Portuguese online natural products market (Naturitas). "The mukua was supplied by a Portuguese agricultural product preparation company (Goldenpuzzle Lda.) linked to Mozambique. Its components (pulp, seeds, and fibers) were manually separated and, along with the thermal water (gasocarbon and bicarbonate) collected from the 'Termas de Chaves' spring (Portugal), stored at room temperature and protected from light."

2.2. Standards and reagents

All chemicals and reagents were obtained from scientific retailers and were of analysis purity, unless for high-performance liquid chromatography (HPLC), which were of HPLC grade.

2.3. Development of the plant-based beverage

The modified methodology described by Neto et al., 2017 and Selma-Royo et al. (2022) was used for the development of the plant tigernut plant-based beverage. Briefly, tubers were exposed to ultraviolet radiation (UVC - 30 min, 3.2 J/cm^2), disinfected with 10 % bleach, and hydrated for 24 h at 4 °C. Afterward, they were washed with running water, and 100 g were ground in 500 mL thermal water containing 7.14 g of mukua pulp. The suspension was subjected to conventional extraction – maceration (simple and efficient process for extracting bioactive compounds) - for 10 min and filtered with a muslin cloth to separate the residue from the aqueous extract. The final plant-based beverage was pasteurized at 73 °C for 10 min and immediately cooled until it reached a temperature of 4 °C. Finally, the plant-based beverage and residue were dehydrated (FreeZone 4.5, Labconco, Kansas City, MO, USA) and stored at room temperature and protected from light.

2.4. Characterization of the different natural matrices (chufa, mukua and thermal water), the plant-based beverage and the residue

2.4.1. Color analysis

The color of the different matrices was determined using a portable colorimeter Konica Minolta CR 400 (Tokyo, Japan), using the standard of the International Commission on Illumination (CIE - L^*, a^* and b^*), D65 illuminant, with 8 mm aperture and 10° observation, following the methodology described by Fernandes et al. (2022). A standard white plate was used as a reference, with reflectance values of $L^* = 93.3 \pm 0.1$, $a^* = 0.46 \pm 0.01$, $b^* = 4.24 \pm 0.2$.

Furthermore, the hue angle and chroma of the different samples were calculated, which represent the tone ($h°$) and saturation of the color (C^*), following equations:

$$h_{ab} = arctan\ (b^*/a^*) \quad (1)$$

$$C^*_{ab} = \sqrt{(a^{*2}) + \left(b^{*2}\right)} \quad (2)$$

The whiteness index (WI) of beverage was calculated following equation:

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \quad (3)$$

2.4.2. Centesimal composition and energetic value

The centesimal composition was determined from analysis of pH, moisture content, energy content, and macronutrient content (crude fat, ash, protein, fiber, and carbohydrate) according to the official AOAC method (AOAC International, 2016). The pH was measured according to the method described by Ueda et al. (2021), a Hanna Instruments HI 902 potentiometer (RI, USA) was used. Moisture content was determined according to AOAC method 925.09 using a moisture analyzer from Adam Equipment (model PBM 163, Oxford, USA). Crude fat was determined according to AOAC method 920.85 by extracting the dry sample with petroleum ether in a Soxhlet apparatus. Ash was estimated by method AOAC 923.03 by incineration in a muffle furnace (Optic Ivymen System, N-8 L, Barcelona, Spain) at 550 ± 15 °C. Protein content (N × 6.25) was determined by the AOAC 920.87 method using the macro-Kjeldahl method. Total dietary fiber (TDF) content was determined by method AOAC 985.29 using the TDF100A kit (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), and the percentage value of total dietary fiber was calculated using the equation:

$$\%TDF = \left(\frac{Reside_{sample} - Protein_{sample} - Ash_{sample} - White}{weight_{sample}}\right) x\ 100 \quad (4)$$

Total carbohydrates were calculated from the difference and energy was estimated using the following equation:

$$Energy = 4\ x\ [protein\ (g) + carbohydrates\ (g)\ ]2\ x\ [dietary\ fibre\ (g)\ ] + 9\ x\ [fat\ (g)\ ] \quad (5)$$

The results were expressed in g per 100 g of each product dry weight (g/100 g dw) and in Kcal and KJ for energy values.

2.4.3. Individual nutrient profile determination

2.4.3.1. Soluble sugars analysis. Soluble sugars were determined according to the method previously described by Ueda et al. (2021). They were analyzed by high-performance liquid chromatography (HPLC) coupled with a refractive index (RI) detector (Knauer, Smartline System 1000, Berlin, Germany), quantified using melezitose as an internal standard (IS) (PanReac AppliChem ITW Reagents Co., Darmstadt, Germany), and identified by chromatographic comparisons with authentic standards. Results were expressed in g/100 g dw.

2.4.3.2. Tocopherols. Tocopherols were determined by the method described by Roriz et al. (2021) using an HPLC system (Knauer, Smartline system 1000) coupled to a fluorescence detector (FP-2020; Jasco, Easton, USA) and programmed for excitation at 290 nm and emission at 330 nm. Quantification was based on IS methodology, and compounds were identified by chromatographic comparisons with commercial standards (α-, β-, γ-, and δ-isoforms, Matreya, Pleasant Gap, PA, USA), using Tocol as the internal standard (Matreya, Pleasant Gap, PA, USA) and Data were analyzed using Clarity 2.4 software. Results were expressed in mg/100 g dw.

2.4.3.3. Individual fatty acids. Fatty acids were determined by a method described by Ueda et al. (2021) using Gas Chromatography (GC) coupled to a flame ionization detector (FID) (DANI 1000, Contone, Switzerland), after a transesterification. Fatty acids were identified by comparing their retention times with peaks from FAME (fatty acids methyl esters) samples using commercial standards (FAME reference standard mixture, standard 47, 885-U, Sigma-Aldrich, St. Louis, MO, USA). Results were recorded and processed using Clarity software (DataApex, Petrzilkova, Czechia) and fatty acids were expressed as a relative percentage.

2.4.4. Mineral profiles

Mineral profiles were prepared following the procedure previously described by Fernandes et al. (2020) using atomic absorption spectrophotometry (AAS) with two detectors, the Pye Unicam PU9100X spectrophotometer (Cambridge, United Kingdom) for potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) and the Perkin Elmer PinAAcle 900 (Waltham, MA, USA) for manganese (Mn), zinc (Zn), iron (Fe) and copper (Cu). A known amount of sample was digested with nitric acid and after cooling, to determine potassium and sodium, the sample was diluted in cesium buffer (Thermo Fisher Scientific Co., Waltham, MS, USA) (1:10 mL), to for calcium and magnesium, a lanthanum solution (ThermoFisher Scientific Co., Waltham, MS, USA) (10 g/L) was used and for manganese and copper, magnesium nitrate was used. Pure analytical solutions were used to determine the elements by comparison. The results were expressed in mg/100 g dw.

2.4.5. Bioactive evaluation

Bioactivities were performed on an extract of the samples obtained by dynamic maceration with 80 % hydroethanolic solution for a period of 1 h at room temperature. The extract solutions were filtered (Whatman paper No. 4) and the residues were re-extracted under the same conditions. The extract solutions were concentrated under reduced pressure (Rotavapor Büchi R-210, Flawil, Switzerland) at 40 °C and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) for subsequent analyses.

2.4.5.1. Antioxidant activity. The antioxidant activity of the different matrices was studied according to the methodology described by Derbassi et al. (2022). Two complementary assays were performed: the thiobarbituric acid reactive substance (TBARS) assay, to assess its ability to prevent oxidative stress in lipid-rich environments, and the Cellular Antioxidant Assay (CAA), to determine its efficacy in mitigating oxidative stress at the cellular level. These assays are crucial to validate the beverage's potential as a functional food with health-promoting properties.

I) TBARS assay

The TBARS assay was performed with pig brains from a local slaughterhouse dissolved in Tris-HCl buffer (20 mM, pH = 7.4), and employing $FeSO_4$ (10 mmol L^{-1}) and ascorbic acid (0.1 mmol L^{-1}) at concentrations selected based on established protocols. These concentrations ensure consistent induction of oxidative stress and enable comparative evaluation of antioxidant activities. To 200 µL of sample solutions were added 100 µL of $FeSO_4$, 100 µL of ascorbic acid, and 100 µL of the supernatant of brain tissue homogenate. Two blanks were used as negative controls: one with Tris-HCl buffer and another with deionized water. A positive control was included using Trolox, a standard antioxidant compound. After one hour of incubation at 37.5 °C, 500 µL trichloroacetic acid (28 % *w/v*) and 380 µL thiobarbituric acid (TBA, 2 % *w/v*) were added, followed by incubation for 20 min at 80 °C. The absorbance of the samples was measured at 532 nm. Results are expressed in EC_{50} mg/mL values, which means the sample concentration responsible for 50 % of lipid peroxidation.

II) Cellular antioxidant assay

For the cellular antioxidant assay, used a murine macrophage cell line (RAW264.7) obtained from ECAAC - European Collection of Authenticated Cell Cultures. After cultivation in DMEM medium containing 10 % FBS, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL), cells were incubated with antioxidant compounds and 2,2′-azobis(2- methylpropionamidine) dihydrochloride (AAPH), to determine intracellular ROS. The fluorescent marker used was 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Quercetin was used as a positive control, and dichlorohydroflurescein and DMEM culture medium were used as a negative control. The modified methodology described by Wolfe and Rui (2007) was used for the measurements. CAA was quantified by examining the percent reduction in fluorescence and was calculated using the following equation:

$$CAA = \%reduction = 1 - AUCsample/AUCcontrol\ x\ 100 \quad (6)$$

2.4.5.2. Antimicrobial activity. The antimicrobial activity of the samples was evaluated using the microdilution method according to the modified methodology described by Almeida et al. (2022). The antimicrobial capacity of the samples was tested against food bacteria, clinical bacteria, and fungi. The selection of microorganisms aimed to evaluate the antimicrobial activity of the samples against pathogens of relevance in food safety (food bacteria), clinical settings (clinical bacteria), and potential food contamination by fungi capable of producing mycotoxins. Eight food bacteria were used, including five gram-negative bacteria (*Enterobacter Cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium* and *Yersinia enterocolitica*) and three gram-positive bacteria (*Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*). Of the eight clinical bacteria used, five were gram-negative (*E. coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *P. aeruginosa*) and three gram-positive (*Enterococcus faecalis*, *L. monocytogenes* and *MRSA*). Two fungi of the genus *Aspergillus* (*A. brasiliensis* and *A. fumigatus*) were used. Results are presented in minimum inhibitory concentration (MIC, mg/mL - lowest concentration of extract that inhibits bacterial growth) and minimum bactericidal/fungicidal concentration (MBC/MFC, mg/mL - lowest concentration of extract that kills bacteria or fungi).

2.4.5.3. Antiproliferative activity. The cytotoxic and hepatotoxic potential of the samples was evaluated by applying the sulforhodamine B assay according to the procedure described by Barros et al. (2013) using ellipticine as a positive control (Sigma-Aldrich, St. Louis, MO, USA) and a cell suspension without sample as a negative control. Cytotoxic activity was evaluated against three human tumor cell lines, namely gastric adenocarcinoma (AGS), breast cancer (MCF-7) and non-small cell lung carcinoma (NCI-H460), purchased from the Leibniz-Institute DSMZ – Collection German Microorganisms and Cell Cultures GmbH. For toxicity, a non-tumor African green monkey kidney cell line (VERO) was used, purchased from the European Collection of Authenticated Cell Cultures - ECACC. Results were expressed in GI50 (µg/mL), which is the extract concentration necessary to inhibit 50 % of cell proliferation.

2.4.5.4. In vitro cholesterol absorption. The hypocholesterolemic potential of the samples (plant-based beverage, mukua pulp, tigernut tubers and residue) was determined through an *in vitro* cellular transport assay in Human colorectal adenocarcinoma cell line CaCo2 following the modified methodology previously described by Gil-Ramírez et al. (2014). CaCo2 obtained from the Leibniz-Institute DSMZ – Collection German Microorganisms and Cell Cultures GmbH. Briefly, the CaCo2 cell line was maintained in RPMI-1640 medium containing fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) and was incubated at 37 °C with a humidified atmosphere containing 5 % CO_2. Afterwards, the cells were placed onto a 44 cm^2 insert membrane with 0.4 µm pore size at a density of $5*10^5$ cell

per insert. The culture medium was replaced every 3 days and cells were allowed to differentiate for 21 days before further experimental procedures. The integrity of the cell layer was evaluated by measuring the transepithelial electrical resistance (TEER) (EVOM2; World Precision Instruments, Sarasota, FL). Only inserts with values above 200 Ω were utilized. The samples were applied to CaCo2 cell monolayers at the subtoxic concentrations (400 μg/mL) in 975 μL of RPMI-1640 medium without FBS at the upper compartment. After the microplate was incubated at 37 °C with 5 % CO_2 for 1 h. The solutions from the upper and lower compartments and the cell monolayer obtained on the membrane were collected and cholesterol concentrations were analyzed using the colorimetric method using the Total Cholesterol & Cholesteryl Ester Colorimetric Assay Kit (Abcam, ab282928).

Cholesterol concentration (C, ng/ μL) is calculated following the equation:

$$C = B/V \times D \quad (7)$$

where B is the amount of cholesterol in the sample well from the standard curve, V is the sample volume added into the reaction well (μl) and D is the sample dilution factor.

2.4.6. Microbial load

The microbial load of dried tigernuts, fresh and dehydrated plant-based beverage and freeze-dried residues was evaluated. For this purpose, selective media were used for the growth and counting's of the following microorganisms: total aerobic mesophiles, *B. cereus*, coliforms, molds, and yeasts. The procedure described by the International Organization for Standardization, 2003 was used to prepare the samples (International Organization for Standardization, 2003). Briefly, a mass of 1 g of each sample (in triplicate) was mixed with 9 mL of peptone water and 2 serial dilutions were made (10^{-2} to 10^{-3}).

To count total aerobic mesophiles (ISO 4833-2:2013) and coliforms (ISO 4832:2006), the incorporation technique was used, that is, 1 mL of each dilution was placed in a Petri dish and 15 mL of Plate Count Agar (PCA) medium (total aerobic mesophiles) and Violet Red Bile Lactose Agar (VRBLA) medium (coliforms) were added. The procedure was carried out in duplicate. After solidification of the medium, the plates were incubated at 30 °C in the inverted position. Colonies of total aerobic mesophiles were counted after 72 h of incubation on plates containing 15 to 300 colonies (Limit of Quantification (LOQ) = 1 log (Colony Forming Units) CFU per g). The coliforms colonies count was carried out after 48 h of incubation on plates containing between 10 and 150 colonies (LOQ = 1 log CFU/g). Regarding the verification of the growth of *Bacillus cereus* (ISO 7932:2004), molds and yeasts (ISO 21527-1/2:2008), the spread plate technique was used, in which 0.2 mL of the dilution was spread on a plate containing 15 mL of *Bacillus Cereus* Agar (PEMBA) (*B. cereus*) or Dichloran Rose Bengal Chloramphenicol (DRBC) (molds and yeasts) using a spreader. PEMBA plates were incubated in the inverted position at 30 °C for 24 h, counting only the plates with 15 to 150 colonies (LOQ = 1.7 log CFU /g). DRBC plates were incubated on the upright position at 25 °C for 5 days, counting all plates with less than 150 colonies (LOQ = 1.7 log CFU/g). Yeast and mold counts were performed after 3 and 5 days of incubation, respectively.

3. Results and discussion

3.1. Color

The color of the different matrices was measured according to the CIELab spatial system, and the results are shown in Table 1. For a better understanding of the color parameter, the color saturation (C^*), which indicates the intensity or purity of the color, and the tone in degrees (h°), which describes the hue angle (*e.g.*, red, yellow, green, or blue), were calculated; the results are also represented in Table 1.

The color of the mukua pulp is mentioned by other authors, but as far as it was possible to verify, there are no published studies related to the measurement of the color of the mukua pulp using a CIELab space system (Odoom, 2021). Thus, this study contributes to the physical characterization of mukua pulp, with color values in CIELab of $L^* = 70.2 \pm 0.1$, $a^* = 6.04 \pm 0.01$ and $b^* = 26.31 \pm 0.02$. It was possible to verify the saturation of the color of the mukua pulp ($C^* = 26.99 \pm 0.02$), which means that this fruit has a bright color, with a tone between red and yellow (0 to 90°), represented with a h° of 77°, which corresponds a hue in the yellow range. The vibrant yellow color suggests potential applications in the food industry, where it could be used as a natural colorant to enhance the aesthetic appeal of products such as beverages, desserts, or sauces. The yellow hues may also contribute positively to consumer perception. Additionally, the yellow coloration might indicate the presence of bioactive compounds like carotenoids, which could support the development of functional foods or nutraceuticals. These attributes highlight the potential versatility of mukua pulp in various applications (Nabi et al., 2023).

Table 1
Averages of color parameters L^*, a^*, b^*, C^* and h° (in degrees °) (mean ± SD).

	CIE Lab color			C^*	h°
	L^*	a^*	b^*		
Mukua pulp	70.2 ± 0.1	6.04 ± 0.01	26.31 ± 0.02	26.99 ± 0.02	77.07 ± 0.02
Tigernut tuber	73 ± 1	2.2 ± 0.2	23.9 ± 0.6	24.0 ± 0.5	85 ± 1
Dehydrated plant-based beverage	82.3 ± 0.1	0.28 ± 0.01	22.2 ± 0.3	22.2 ± 0.3	89.28 ± 0.05
Residues	78 ± 2	1.2 ± 0.3	20 ± 1	20 ± 1	87 ± 1

In terms of color, there are three phenotypes of tigernuts, yellow, brown, and black (Maduka et al., 2018). As in this study, Codina-Torrella et al. (2015) determined the color of brown tigernut tubers from different areas (including Nigeria), however the results are not consistent with those of in this work ($L^* = 73 \pm 1$, $a^* = 2.2 \pm 0.2$ and $b^* = 23.9 \pm 0.6$), since the authors characterized the surface of the tubers and here the tubers are characterized as a whole, subjected to a crushing process. The tubers used in these studies were light in color and shades very close to yellow, as expected.

For the plant-based beverage, the CIELab coordinates were $L^* = 82.3 \pm 0.1$, $a^* = 0.28 \pm 0.01$, $b^* = 22.2 \pm 0.3$, the hue is in the yellow range with an angle of almost 90°. Badejo et al. (2020) used the CIELab system (with calculation of C^* and h°), to characterize a fresh beverage of tigernut with 2.5 % mukua pulp. In their study, the authors determine different color values than those presented in this work, as this difference is probably due to the fact that the concentration of mukua pulp in the beverages is not the same and the fact that a dehydration process is applied. Thus, the authors characterize a slightly lighter beverage, with reddish and less yellow tones ($L^* = 73.62 \pm 0.03$, $a^* = 2.22 \pm 0.02$ and $b^* = 2.22 \pm 0.02$), with lower color saturation (lower color intensity) but in the yellow range just like the beverage developed in the present study, although it comes closest to the yellow hue. As far as could verify, there are no published studies on the color of dehydrated plant-based beverages based on tigernut tubers with mukua pulp.

The determination of the color of the residue resulted from the development of the tigernut plant-based beverage with mukua pulp is consistent with the results of Sánchez-Zapata et al. (2009), although the residue obtained in the present study is slightly lighter, with less red and more yellow tones, with a color saturation of 20 ± 1 and close to 90° in the yellow range.

In addition, the whiteness index (*WI*) of the beverage was calculated, as it is a very relevant parameter for plant-based beverages, traditionally inspired by the appearance of milk (*WI* = 81.9). In this study, the beverage showed a *WI* of 71.6 ± 0.2. While the whiteness of milk is often associated with purity and freshness, products with different *WI* values

are increasingly being developed, appealing to consumers seeking innovative options (McClements, 2020). To our knowledge, no studies have been published on the *WI* of dehydrated tigenut plant-based beverage with mukua pulp. However, Codina-Torrella et al. (2018) investigated the influence of ultra-high-pressure homogenization on traditional 'horchata' formulations, reporting a *WI* of 77.19 ± 0.22. The higher *WI* observed in their study may be attributed to the absence of mukua pulp, whose yellowish coloration likely contributed to the lower *WI* of the beverage developed in this study.

3.2. Centesimal composition and energetic value

The proximate composition of the mukua pulp, the tigernut tubers, the dehydrated plant-based beverage and the residues from the development of the beverage was determined by analyzing the pH, moisture, energy and macronutrient content (crude fat, ash, protein, fiber and carbohydrate). The pH value of the thermal water was also determined, and the results are presented in Table 2, as are the results of the proximate composition. The thermal water used in this study has a pH of 7.30 ± 0.02 and is considered alkaline water according to A. Silva et al. (2020). The pH of the developed plant-based beverage is 6.26 ± 0.02, which is consistent with the results obtained by Djomdi Hamadou et al. (2020) for the beverage made from tigernut tubers (pH = 6.8). Achieving a pH value within this range is important for maintaining the palatability of the beverage, as it ensures a mild and pleasant flavor, which is desirable in plant-based beverages, and contributes to the stability and overall sensory appeal of the product.

However, this beverage was developed with a known amount of mukua pulp, which has a high acidity due to its high citric acid content (8.73 ± 0.03 g/100 g dw) and it was expected that the pH of the developed plant-based beverage would be much more acidic, as author Badejo et al. (2020) described in the study in which he added 2.5 % mukua pulp to the beverage made from tigernut tubers and obtained a pH of 4.79 ± 0.01. Probably the minerals contained in thermal water do not increase the acidity of the beverage, as it had tested the development of the same beverage with distilled water (pH = 7.84) and obtained a pH of 4.50 ± 0.01. This pH control is a great advantage for the acceptability of this beverage by the consumer. The interesting nutrient content of the plant-based beverage results from the combination of the natural matrices used, mukua pulp and tigernut tubers, which have a high protein content and a low available carbohydrate content. The high protein levels distinguish it from many conventional plant-based beverages, providing a promising alternative for consumers seeking functional and nutrient-dense options. Although the crude fat content of the tubers, the beverage and the residue is somewhat high, next point of this article shows that approximately 65 % of this fat corresponds to monounsaturated fatty acids, which are described as beneficial for the consumer's health.

The consumption of 100 g of a dehydrated plant-based beverage, which corresponds to approximately 1 L of beverage, provides 486 ± 4 Kcal of energy. Almost all the total dietary fiber from tubers and mukua is lost during the production of the plant-based beverage, as it remains in the solid residues. This is because most dietary fiber is insoluble and is retained in the solids removed during the filtration or extraction process. As a result, it is not possible to quantify the fiber content in the final beverage using the method employed in this study. However, after filtering the beverage, high-fiber residue is obtained that can be used as food.

The centesimal composition of mukua, tigernut tubers and residues has already described by other authors who, although expressing the results in units far removed from those of the present work, nevertheless reveal some differences in macronutrient content, possibly due to the geographical location and the state of maturity of the matrices at the time of harvesting (Aguilar et al., 2015; Asogwa et al., 2021). The results obtained for the dehydrated tigernut plant-based beverage cannot be compared with the results of other authors because, to our knowledge, no studies on the dehydrated tigernut plant-based beverage have yet been published, so this study represents an innovation in this field. However, a study by Pérez-Rodríguez et al. (2023) on plant-based beverages compared the labels of various products and reported that tigernut beverages typically have low protein and carbohydrate contents, which contrasts with the findings of this study. Although the results are not directly comparable, it can be inferred that the incorporation of mukua pulp positively influences these parameters.

Table 2
Centesimal composition of the different matrices (mean ± SD).

	Thermal water	Mukua	Tigernut	Plant-based beverage	Residue
pH	7.30 ± 0.02			6.26 ± 0.02	
Moisture (%)		11.9 ± 0.3	6.8 ± 0.3	5.4 ± 0.5	20 ± 2
Crude fat (g/100 g dw)		1.8 ± 0.1	19.8 ± 0.4	21 ± 2	25.2 ± 0.1
Ahs (g/100 g dw)		5.81 ± 0.08	1.6 ± 0.3	4.7 ± 0.8	1.50 ± 0.05
Protein (g/100 g dw)		2.7 ± 0.3	5.4 ± 0.2	7.5 ± 0.1	5.2 ± 0.5
Fiber (%TDF)		57 ± 1	43 ± 1	0	38 ± 2
Available Carbohydrate (g/100 g dw)		33 ± 1	30 ± 2	67 ± 2	30 ± 3
Energy (kcal/100 g dw)		272 ± 3	407 ± 3	486 ± 4	444 ± 5

3.3. Individual nutrient profile determination

The individual nutritional profile of the mukua pulp, tigernut tubers, plant-based beverage and residues was determined by identifying and quantifying of free sugars, tocopherols, and fatty acids; the results are shown in Table 3.

As far as free sugars are concerned, 3 sugar molecules were identified in small quantities in the mukua pulp and consequently in the beverage and in the residues, while the tigernuts contain only sucrose, and in a relatively high quantity. All sugars contained in the developed plant-based beverage are natural. Sucrose is the predominant sugar, with 27 ± 1 g per 100 g of dehydrated beverage, which corresponds to approximately 1 L of fresh beverage when rehydrated. The natural sugar content, particularly the presence of sucrose, could potentially contribute to the sensory appeal and acceptance of the product by consumers, although further sensory studies are needed to confirm this. The sugars present in mukua pulp and tigernut tubers were previously described by M. L. Silva et al. (2023) – 7.0 g fructose, 7.9 g glucose and 1.7 g sucrose - and by Sánchez-Zapata et al. (2012) – 13.03 g sucrose – respectively. The results differ slightly from those obtained in this work, which may be due to variations in the maturity of the matrices, as sugar accumulation is influenced by the ripeness stage. Further studies are needed to confirm this hypothesis. Aguilar et al. (2015) give the percentage of sugar the tigernut beverage and in the residues without mentioning which types of sugar are present.

The plant-based beverage contains 6.7 ± 0.6 mg/100 g dw of tocopherols, divided into two isoforms, alpha and beta, derived from the tigernut tuber, as the mukua pulp has a low proportion of vitamin E in the alpha-tocopherol isoform (0.023 ± 0.004 mg/100 g dw), which also applies to the residue. As far as we are aware, only one published study has described the tocopherol content of mukua pulp (Vinha et al., 2024). The authors identified two isoforms of tocopherol, alpha-tocopherol (0.54 ± 0.03 mg/100 g dw) and beta-tocopherol (0.0509 ± 0.0001 mg/100 g dw). The differences in the results of the two studies may be attributed to the geographical origin of the fruits, as the authors used fruits from Angola. In addition, the absence of detection of beta-

Table 3
Individual nutrient profile of mukua pulp, tigernut tubers, plant-based beverage, and residues (mean ± SD).

	Mukua	Tigernut	Plant-based beverage	Residue
Soluble sugars (g/100 g dw)	4.53 ± 0.01	16 ± 1	29 ± 1	8.3 ± 0.1
Fructose	2.3 ± 0.2		1.14 ± 0.04	0.28 ± 0.03
Glucose	1.8 ± 0.1		1.2 ± 0.1	0.25 ± 0.01
Sucrose	0.48 ± 0.01	16 ± 1	27 ± 1	7.8 ± 0.1
Tocopherols (mg/100 g dw)	0.023 ± 0.004	6.1 ± 0.8	6.7 ± 0.6	6.1 ± 0.2
Alpha	0.023 ± 0.004	4.2 ± 0.5	4.8 ± 0.3	4.23 ± 0.07
Beta		1.9 ± 0.3	1.8 ± 0.3	1.9 ± 0.2
Individual fatty acids (%)				
C16:0	10.3 ± 0.5	16.3 ± 0.3	16.4 ± 0.3	15.8 ± 0.4
C16:1	0.29 ± 0.03	0.29 ± 0.02	0.31 ± 0.01	0.30 ± 0.01
C17:0	0.25 ± 0.02			
C18:0	3.5 ± 0.3	6.3 ± 0.1	8.3 ± 0.6	5.2 ± 0.1
C18:1n9c	81 ± 1	66.5 ± 0.2	65 ± 1	68.1 ± 0.2
C18:2n6c	3.53 ± 0.06	8.77 ± 0.06	8.3 ± 0.2	8.811 ± 0.004
C18:3n3	0.356 ± 0.003	0.22 ± 0.01	0.210 ± 0.002	0.214 ± 0.004
C20:0	0.331 ± 0.005	0.671 ± 0.005	0.66 ± 0.01	0.64 ± 0.02
C20:1	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.253 ± 0.001
C20:2		0.245 ± 0.002	0.45 ± 0.04	0.20 ± 0.02
C22:0	0.321 ± 0.02	0.23 ± 0.02	0.21 ± 0.01	0.23 ± 0.02
C24:0		0.36 ± 0.03	0.22 ± 0.01	0.23 ± 0.02
SFA	15 ± 1	23.8 ± 0.1	26 ± 1	22.1 ± 0.2
MUFA	81 ± 1	67.0 ± 0.2	65 ± 1	68.7 ± 0.2
PUFA	3.9 ± 0.1	9.24 ± 0.04	9.0 ± 0.1	9.228 ± 0.008

tocopherol in our study may be due to the robustness of the analytical method used by the authors, which is probably more sensitive than the one used in this work. For tigernut tubers, there are few studies demonstrating the presence of vitamin E without resorting to the characterization of the extracted oil. However, Hernández-Olivas et al. (2022) describes the composition of tigernut tuber flour and reports the presence of 34 ± 4 μg of alpha-tocopherol per g of flour, although the results presented by the authors are superior to those presented in this paper, here two isoforms of tocopherols (alpha and beta). The same authors quantified vitamin E in tigernut plant-based beverage, but the quantitative results cannot be compared as in this paper it was present the results for a dehydrated beverage and the authors for a liquid beverage. As far as it can see, this is also an innovative study, as it is the first publication on the identification and quantification of tocopherols from solid residues resulting from the development of a tigernut plant-based beverage. This study is important as it highlights the potential of this waste as a source of natural antioxidants that can be reused for applications in food, dietary supplements, nutraceuticals or cosmetics, contributing to the valorization and sustainability of waste.

In both mukua pulp and tigernut tubers, oleic acid (C18:1n9c - omega-9) is the most abundant compound, followed by palmitic acid (C16:0) in much lower percentages, so that monounsaturated fatty acids (associated with disease prevention) are present in greater quantities in these matrices. The same is true for the plant-based beverage and the residues, which means that the pasteurization temperature has no influence on the degradation of these compounds.

The fatty acids are described in the literature for the mukua seeds without referring to the fatty acids present in the pulp. The results presented in this paper for the fatty acids in the tigernut tuber and in the plant-based beverage are in agreement with those of Sánchez-Zapata et al. (2012). The identification of the fatty acids in the residue resulting from the development of the vegetable beverage has been described by Razola-Díaz et al. (2022).

3.4. Mineral profiles

Mineral characterization of the different matrices was carried out and the results are presented in Table 4 and are expressed in mg of mineral per 100 g dry weight of sample.

As expected, the thermal water used to develop the plant-based beverage contains a variety of minerals, with sodium (3854 ± 253 mg/100 g dw) being the most abundant macro-mineral, followed by potassium (2600 ± 211 mg/100 g dw). Other macro-minerals (calcium and magnesium) and micro-minerals (iron, manganese, copper, and zinc) were also detected. Although sodium intake is associated with hypertension, sodium, when consumed in a controlled manner, has beneficial effects on the proper maintenance of electrolyte and fluid balance and nerve transition. The consumption of potassium-containing foods is often associated with athletes due to its effect on the balance of muscle contractions, but its benefits go further: it also contributes to the balance of fluids and nerve transmission, as well as the adequate maintenance of blood pressure (Gharibzahedi & Jafari, 2017). The composition of thermal waters is not uniform, it varies according to the geological environment of their underground source, and therefore the mineral comparison with other thermal waters is less relevant.

The mukua pulp had contain a high mineral content, with potassium (20,597 ± 866 mg/100 g dw) being the mineral present in the greatest quantity, followed by magnesium (3319 ± 72 mg/100 g dw) and calcium (2820 ± 64 mg/100 g dw). Consuming 100 g of mukua pulp provides the European Union's recommended daily dose (RDD) of potassium (2000 mg/day), calcium (800 mg/day), magnesium (375 mg/day) and manganese (2 mg/day) (Commission of European Communities, 2008). Calcium is one of the macro-minerals with the most health benefits for the consumer. It plays an important role in the maintenance of bones and teeth, the functioning of the nervous system, the immune system, blood clotting, the regulation of blood pressure and even contributes to muscle relaxation (Gharibzahedi & Jafari, 2017). It is equally important to consume magnesium, as this mineral is essential for the formation of proteins, nerve and muscle activity, the immune system, and the prevention of colds (Gharibzahedi & Jafari, 2017; Quattrini et al., 2016). The mineral composition determined in this work agrees with that given by Asogwa et al. (2021) in her review article on the fruits

Table 4
Mineral profile of the matrices under study. Results expressed in mg/100 g dw (mean ± SD).

	Thermal water	Mukua	Tigernut	Plant-based beverage	Residue
[K]	2600 ± 211	20,597 ± 866	641 ± 61	1262 ± 32	424 ± 2
[Na]	3854 ± 253	20 ± 2	16 ± 1	567 ± 16	157 ± 15
[Ca]	426 ± 15	2820 ± 64	20 ± 2	41 ± 2	27 ± 1
[Mg]	168 ± 30	3319 ± 72	87 ± 1	133 ± 2	66.6 ± 0.3
[Fe]	8.4 ± 0.4	1.58 ± 0.01	2.9 ± 0.2	2.88 ± 0.03	2.64 ± 0.02
[Mn]	1.0 ± 0.1	2.0 ± 0.1	0.9 ± 0.3	1.1 ± 0.2	0.84 ± 0.04
[Cu]	0.00059 ± 0.00007	0.63 ± 0.02	0.63 ± 0.02	0.81 ± 0.04	0.67 ± 0.02
[Zn]	1.0 ± 0.1	0.95 ± 0.06	5.5 ± 0.1	6 ± 1	5.3 ± 0.1

of *Adansonia digitata* L., although the potassium (2010–2390 mg/100 g), calcium (257–370 mg/100 g) and magnesium (126–179 mg/100 g) values described by the authors are lower than those determined in this work. When comparing the mineral content of the mukua pulp with other fruits that are considered rich in certain minerals, it is found that the mukua has a higher potassium content than avocado (507 mg/100 g), a higher calcium content than milk (120 mg/100 g) and a higher magnesium content than pumpkin seeds (527 mg/100 g) (Dotto & Chacha, 2020; Ford et al., 2023; Scholz-Ahrens et al., 2020).

Regarding the mineral content of tigernut tubers, potassium (641 ± 61 mg/100 g dw) was the most abundant macro-mineral, followed by magnesium (87 ± 1 mg/100 g dw), while zinc (5.5 ± 0.1 mg/100 g dw) was the micro-mineral in the greatest quantity. Zinc (RDD = 10 mg/day) is an important microelement for the structure of some enzymes, for the production of proteins and genetic material, plays an important role in taste, smell and digestion and also contributes to wound healing and immune system health (Commission of European Communities, 2008; Gharibzahedi & Jafari, 2017). Nina et al. (2019) studied the mineral composition of the three tigernut phenotypes, and although the mineral content differs from that determined in this work, the authors describe potassium (120 mg/100 g) as the mineral with the highest content. As expected, the plant-based beverage developed contains a similar mineral profile to the ingredients used in its production. Thus, sodium (1262 ± 32 mg/100 g dw) is the macroelement present in the greatest quantity, followed by potassium (567 ± 16 mg/100 g dw) and magnesium (133 ± 2 mg/100 g dw), and zinc (6 ± 1 mg/100 g dw) is the most abundant microelement. Badejo et al. (2020) investigated the mineral profile of a tigernut beverage with mukua pulp, and although the results obtained by the authors differ from those of this work, it can be stated that potassium (86 μg/mL) is also present in significant quantities in beverage developed by the authors. Comparing the results obtained in this work with those described for "Horchata" with protected designation of origin (PDO), it can be seen that there is a relevant difference in the mineral content between the beverages, with the beverage developed in this work being the product that provides the highest mineral content (potassium: horchata PDO = 30 mg/100 g fw, plant-based beverage = 122 mg/100 g fw; sodium: "Horchata" PDO = 30 mg/100 g fw, plant-based beverage = 55 mg/100 g fw; magnesium: horchata PDO = 6 mg/100 g fw, plant-based beverage = 13 mg/100 g fw and zinc: horchata PDO = 0.2 mg/100 g fw, plant-based beverage = 0.6 mg/100 g fw) (Boeting et al., 2010).

As with the plant-based beverage, potassium (424 ± 2 mg/100 g dw) is the mineral with the largest amount in the beverage residue followed by sodium (157 ± 15 mg/100 g dw) and magnesium (66.6 ± 0.3 mg/100 g dw). As far as it can see, there are still no scientific studies on the mineral characterization of solid residues from the development of tigernut plant-based beverage.

3.5. Bioactive evaluation

3.5.1. Antioxidant activity

The antioxidant capacity of the different matrices was evaluated using the TBARS and CAA assays; the results are shown in Table 5.

Regarding the ability to inhibit lipid peroxidation of the different matrices tested by inhibiting the formation of thiobarbituric and malondialdehyde complexes, mukua pulp is the natural ingredient with the highest antioxidant potential (EC_{50} = 0.0230 ± 0.0001 mg/mL), followed by thermal water (EC_{50} = 0.80 ± 0.03 mg/mL) and tigernut tuber (EC_{50} = 2.8 ± 0.3 mg/mL).

Table 5
Antioxidant capacity of different matrices tested in two assays (TBARS and CAA). Results expressed in EC_{50} mg/mL (TBARS) and in % (CAA).

	Thermal water	Mukua	Tigernut	Plant-based beverage	Residue
TBARS (EC_{50} mg/mL)	0.80 ± 0.03	0.0230 ± 0.0001	2.8 ± 0.3	7.72 ± 0.07	0.95 ± 0.04
CAA (%)	0	32	0	0	0

EC_{50} - sample concentration responsible for 50 % of lipid peroxidation.

Interestingly, the plant-based beverage has a lower antioxidant capacity than the final residues of the beverage development process, which is probably due to the pasteurization process (73 °C for 10 min) to which the beverage is subjected as the degradation of heat-labile compounds with antioxidant capacity occurs. The residue also has a better antioxidant potential (EC_{50} = 0.95 ± 0.04 mg/mL) than the tigernut tuber, which can be explained by the fact that the residue is a mixture of tubers with mukua pulp and thermal water. These results are very interesting as they add value to the waste, transforming it into a potential food product that is not only rich in nutrients but also has a high antioxidant potential. The CAA antioxidant assay is a very sensitive test that measures the ability of compounds to inhibit the formation of fluorescent dichlorofluorescein by peroxyl radicals in a murine macrophage cell line. In the present study, only the mukua pulp presented an antioxidant capacity with a value of 32 %. Different studies have mentioned that natural mineral waters can be associated with antioxidant potential, but there are few studies to support this fact. Therefore, the present study is important for the scientific community as it proves through a biochemical method that mineral water used in this study has antioxidant potential. Firouzi et al. (2022) demonstrated the antioxidant capacity of mineral colloids from thermal water by a spectrophotometric method (DPPH). Several authors have characterized the pulp of mukua at the bioactive level and tried to confirm the traditional beliefs associated with this fruit. Braca et al. (2018) demonstrated the antioxidant potential of mukua pulp from different geographical areas using four different chemical methods (DPPH - 392.22 ± 28.13 mg TE/g; ABTS - 799.44 ± 35.96 mg TE/g; FRAP - 458.50 ± 23.41 mg TE/g; BCB - 68.83 ± 0.38 % AA), and although it is not possible to compare the results obtained in the present study with those described by the authors, it is possible to conclude that the mukua pulp has a high antioxidant capacity. The studies available in the literature on the biological activities of tigernuts prove their antioxidant potential, however they refer to chemical methods, which makes it difficult to compare the results (Bezerra et al., 2023). Plant-based beverage made from tigernut tuber have taken up a lot of space on the market and there are more and more studies on the potential benefits of their consumption for consumer health. Badejo et al. (2020) investigated the influence of the addition of mukua pulp to a processed plant-based beverage made from tigernuts, with antioxidant activity being the biological activity studied among the various parameters analyzed. The authors found that while the plant-based beverage with tigernut alone had antioxidant activity, the beverage with tigernut and mukua had greater antioxidant potential. Again, the quantitative results cannot be compared with the results of this work, as the authors used only three chemical assays (DPPH, ABTS and FRAP) to determine the antioxidant activity of the beverages. The residues from the development of the plant-based beverage showed antioxidant activity as previously described by other authors, however they use different methods to those used in this work and therefore it cannot compare the results (Roselló-Soto et al., 2018).

3.5.2. Antimicrobial activity

The antimicrobial activity has been tested against food and clinical isolates, and Tables 6 and 7 show the MIC, MBC and MFC values (mg/mL) values for each matrix (maximum concentration tested – 10 mg/mL) and the positive controls used.

In general, the matrices studied do not have high antimicrobial capacity, but thermal water and mukua pulp inhibit bacterial growth the most. The food bacteria most susceptible to thermal water were *E. coli* and *Y. enterocolitica* (MIC = 2.5 mg/mL), and among clinical bacteria, the strain *E. faecalis* (MIC = 2.5 mg/mL) was the most sensitive to thermal water. The thermal water did not show a bactericidal activity

Table 6
Antibacterial activity of different matrices against a panel of food and clinical bacteria. Results are expressed in MIC and MBC (mg/mL).

	Thermal water		Mukua		Tigernut		Plant-based beverage		Residue		Streptomicin		Methicilin		Ampicillin	
Food bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																
E. cloacae	10	>10	10	>10	10	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
E.coli	2.5	>10	10	>10	>10	>10	>10	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
P. aeruginosa	5	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
S. enterica	>10	>10	5	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Y. enterocolitica	2.5	>10	0.3	5	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																
B. cereus	5	>10	10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
L. monocytogenes	10	>10	1.25	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
S. aureus	10	>10	1.25	10	>10	>10	>10	>10	>10	>10	0.007	0.007	0.007	0.007	0.15	0.15
Clinical bacteria											Ampicillin		Imipenem		Vancomycin	
Gram-negative bacteria																
E. coli	10	>10	n.t.	n.t.	>10	>10	>10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
K. pneumoniae	10	>10	>10	>10	10	>10	>10	>10	10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
M. morganii	10	>10	5	>10	>10	>10	>10	>10	>10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
P. mirabilis	10	>10	>10	>10	>10	>10	>10	>10	>10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
P. aeruginosa	10	>10	n.t.	n.t.	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria																
E. faecalis	2.5	>10	0.6	>10	>10	>10	>10	>10	>10	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
L. monocytogenes	10	>10	n.t.	n.t.	>10	>10	>10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	10	>10	n.t.	n.t.	2.5	>10	>10	>10	2.5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

Table 7
Antifungal activity of different matrices. Results are expressed in MIC and MFC (mg/mL).

		Thermal water	Mukua		Tigernut		Plant-based beverage		Residue		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. brasiliensis	10	10	>10	>10	10	>10	>10	>10	10	>10	0.06	0.125
A. fumigatus	10	>10	>10	>10	10	>10	>10	>10	10	>10	0.5	1

for any of the bacteria tested at the highest concentration tested (MIC >10 mg/mL). In addition, the thermal water shoed an antifungal effect against the fungi tested, but only against *A. brasiliensis* (MFC = 10 mg/mL).

Mukua pulp flour has a high bacteriostatic capacity against *Y. enterocolitica*, *L. monocytogenes*, *S. aureus* and *E. faecalis* with MIC values of 0.3, 1.25, 1.25 and 0.6 mg/mL, respectively, and also has a bactericidal capacity against *Y. enterocolitica* (MBC = 5 mg/mL) and *S. aureus* (MBC = 10 mg/mL). On the contrary, the mukua pulp doesn't have the capacity to inhibit the growth of the tested fungi, nor does it have a fungicidal effect.

Regarding the antimicrobial activity of the last ingredient of the formulation developed, it was found that although tigernuts have low antibacterial and antifungal activity, the strains *E. cloacae*, *K. pneumoniae*, *MRSA*, *A. brasiliensis* and *fumigatus* are susceptible to this tuber, with stronger growth inhibition in *MRSA* (MIC = 2.5 mg/mL), which is a strain of Methicillin-Resistant *S. aureus*. This tuber has neither a bactericidal nor a fungicidal capacity against the microorganisms tested at the maximum concentration tested. These results corroborate those presented by (Oliveira et al., 2020., Thompson et al., 2023. and Nwosu et al., 2022), in which the authors demonstrate the antibacterial capacity of thermal water, mukua pulp and tigernut tuber, respectively, in their studies.

The tigernut plant-based beverage only demonstrates bacteriostatic activity against *E. cloacae* with an MIC of 10 mg/mL, without exhibiting antifungal activity. The residue resulting from the development of the plant-based beverage has the same antimicrobial activity as mukua pulp against *E. cloacae* (MIC = 10 mg/mL), *K. pneumoniae* (MIC = 10 mg/mL), *MRSA* (MIC = 10 mg/mL), *A. brasiliensis* and *fumigatus* (MIC = 10 mg/mL). These results were already expected, because although the beverage is made from ingredients with antimicrobial activity, it undergoes a pasteurization process in which some compounds responsible for the bioactive potential of the different ingredients may be lost due to temperature degradation. This study is innovative as it determines the antimicrobial capacity of the tigernut plant-based beverage and its residues as, to our knowledge, no tests of the antimicrobial capacity of these matrices have been published to date.

3.5.3. Antiproliferative activity

The cytotoxic capacity of the different matrices tested was evaluated against three tumor cell lines, revealing that only the mukua pulp exhibited antitumor activity against the cell lines tested, with better inhibition of cell proliferation of the AGS line (GI_{50} = 92 ± 1 μg/mL). The toxicity of the matrices in a non-tumor cell line was also evaluated and it was found that at the maximum concentration tested (400 μg/mL) none of the matrices inhibited cell proliferation of non-tumor cells. These results are consistent with those of previous studies, such as the study conducted by Firouzi et al. (2022), in which the authors demonstrate that mineral colloids (obtained from thermal water) are safe compounds by evaluating their effect on liver and kidney tissue of normal rats; in the study carried out by Suliman and El-Hddad (2023), the authors verify that the fruit of *Adansonia digitata* L. inhibits cell proliferation in the HeLa cell line (cervical cancer cell line), and the authors Tshikalange et al. (2017) prove that the pulp of mukua has no toxicity in the cells of human kidneys; in the bibliographical work carried out by Bezerra et al. (2023) several studies are showing that tigernut

tuber not toxic *in vivo*, which indicates that the products made from this tuber (*e.g.* plant-based beverage) are safe for consumption. Regarding the toxicity of the tigernut plant-based beverage and its solid residues, as far as it have been able to verify, there are no published studies demonstrating that these products do not exhibit toxicity to non-tumor cells, therefore, this work is a pioneer on this topic.

3.5.4. *In vitro cholesterol absorption*

In an initial screening, the potential hypocholesterolemic effect of methanolic extracts of mukua pulp, tigernut tubers, plant-based beverage, and residue was investigated using a colorimetric method in a cell assay. To understand whether cholesterol and extract compounds compete for absorption through the cellular CaCo2 barrier, absorbance was measured at three different time points (T0, T4 and T24 h) and in two wells (Top - above the cellular barrier, Low- after the cellular barrier). The results are presented graphically in Fig. 1.

At time zero, the cholesterol present on top of the cell barrier (12 ng/mL) corresponds to 100 % of the added cholesterol in all samples. After a 4 h test in which the cholesterol and the samples were in contact with the cell barrier at 37 °C, different behaviors were observed in terms of cholesterol absorption. Of the ingredients tested, mukua pulp extract showed a greater capacity to inhibit cholesterol absorption after 4 h, with a higher percentage of cholesterol detected above the barrier (Top – 43 %, Low – 26 %). In contrast, the tigernut tuber extract showed a higher percentage of cholesterol below the barrier after 4 h (Top – 26 %, Low – 28 %). For both the plant-based beverage and the residue, it is observed that there is a higher percentage of cholesterol above the barrier after 4 hours (Top – 25 %, Low – 22 %; Top – 23 %, Low – 17 %), as its compounds can compete with cholesterol during absorption.

The percentage of cholesterol was also determined after 24 h. In contrast to the results obtained after 4 h of testing, the percentage of cholesterol after 24 h in low are higher for the mukua extract (Top – 48 %, Low – 51 %).

For the tigernut tuber extract, the percentage of cholesterol above the barrier is equal to the percentage below the barrier (Top – 26 %, Low – 26 %). And for both the plant-based beverage and the residue, the percentage of cholesterol above the barrier still appears to be higher (Top – 31 %, Low – 24 %; Top – 29 %, Low – 27 %).

In general, after 4 h of testing, only the tigernut tubers showed a higher percentage of cholesterol below the cell barrier, and after 24 h, only the mukua pulp extract showed a higher percentage of cholesterol below the cell barrier.

The results after 4 h of testing suggest that the mukua extract may contain a greater amount of compounds with the ability to bind to proteins and prevent greater absorption of cholesterol through the cell barrier, as it contains a higher percentage of cholesterol in the upper layer of the barrier compared to the other samples tested. It is also clear that after 4 h, both the extract from the plant-based beverage and the residue have the potential to inhibit cholesterol absorption, as the percentage found above the cell barrier is slightly higher than that found in the lower layer of the cholesterol barrier, suggesting that there may be a limitation of cholesterol absorption due to the presence of compounds that bind to proteins and other molecules. Although a higher percentage of cholesterol is detected in the lower layer of the tigernut tuber extract after 4 h of testing, the percentage of cholesterol detected in the upper layer is slightly higher than in the other samples. These percentage differences can probably be explained by the fact that cholesterol is not detectable in the cell membrane (organization of lipid microdomains, distribution of receptors, fluidity of the two layers and integrity of the membrane) with the tests used, suggesting that further studies are needed to justify these results (Lee & Bensinger, 2022).

After a test duration of 24 h, the results are not as anticipated. Although the cholesterol content in the layer above the cell barrier is higher than after a test duration of 4 h, this outcome could be attributed to potential cellular degradation. It is worth noting that cells can synthesize cholesterol, but only in low quantities.

To gain a better understanding of this phenomenon, specific methods would need to be employed (Craig et al., 2024). Different authors mention that the mukua pulp and the tigernut tuber have been determined to have hypocholesterolemic potential in animals. Abdelbagi et al. (2023) confirms with a meta-analysis on the influence of mukua on the lipid profile of rats fed a high-fat diet that the inclusion of mukua in the diet of these animals contributes significantly to the reduction of cholesterol, triglycerides, and LDL in the blood. Mahmod and Sabry (2021) investigated the potential benefits of tigernut tuber consumption on cholesterol levels in animals with hypercholesterolemia and concluded that supplementation with 10, 15 or 25 % tigernut significantly reduced serum total cholesterol, triglycerides, and LDL. As far as we are aware, no studies have been published to date demonstrating the hypocholesterolemic potential of beverages made from tigernuts or their

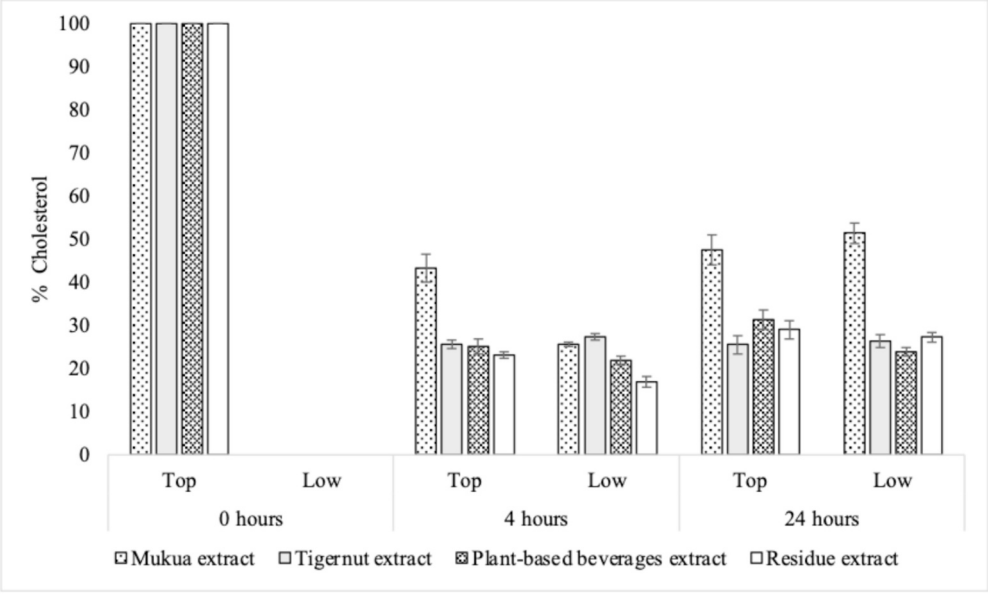

Fig. 1. Graphic representation of the potential hypocholesterolemic effect of extracts of mukua pulp, tigernut tubers, plant-based beverage, and residue. Results expressed in percentage of cholesterol (%).

by-products, which underlines the importance and relevance of this study.

All these results are only a first screening that allows to understand if there is a hypothesis that the tested extracts, including the plant-based beverage, could be hypocholesterolemic.

3.6. Microbial load

In this study, the microbial load of dried tigernut tuber, fresh plant-based beverage and dehydrated beverage, as well as the dehydrated residue flour resulting from the beverage was verified. The presence of total aerobic mesophiles, *Bacillus cereus*, coliforms, yeasts and molds was determined, and the results, expressed in log10 CFU/g of product, are presented in Fig. 2.

Regarding the microbiological quality of the beverages, only the presence of total aerobic mesophiles was detected (liquid beverage: 3.25 ± 0.06 log10 CFU/g; dehydrated beverage: 2.6 ± 0.1 log10 CFU/g), with the dehydrated beverage being the product with the lowest count, demonstrating that the dehydration process is a potential factor responsible for the reduction of viable bacteria in this product, since the absence of water reduces the proliferation of bacteria and increases the useful life of the products. The residue showed a high microbial load compared to the other matrices, with total aerobic mesophiles (5.17 ± 0.04 log10 CFU/g), coliforms (4.7 ± 0.1 log10 CFU/g) and yeasts (4.95 ± 0.05 log10 CFU/g) being detected. Only aerobic mesophiles (2.5 ± 0.1 log10 CFU/g) were detected in the tigernut tuber, probably because the whole, dry tuber was used. None of the samples analyzed showed counts of *B. cereus* and molds.

These results show that the disinfection treatment of the tigernut tubers and the pasteurization process applied in this study are adequate to ensure the food safety of the developed plant-based beverage. Although the presence of total aerobic mesophiles was detected, these microorganisms are below the legally permitted levels (pasteurized natural "horchata" - max. 5.40 log10 CFU/g; liquid beverage in the study – 3.25 ± 0.06 log10 CFU/g; dehydrated beverage in the study - 2.6 ± 0.1 log10 CFU/g) (Real Decreto, 1988). Furthermore, the results obtained in this work corroborate those obtained by Codina-Torrella et al. (2018). In their study on the microbiological stabilization of tigernut beverages by application of high pressure, the authors compared the microbial load of a beverage without preservation treatment with that of three beverages subjected to pasteurization or high pressure, and obtained in the pasteurized beverage the presence of 2.78 ± 0.25 log CFU/mL of aerobic mesophiles.

Regarding the microbial load of the residue, this by-product contains high levels of microorganisms, as expected, which is consistent with the results obtained by Sánchez-Zapata et al. (2009) in the microbiological characterization of solid waste from the development of "horchatas", assuming that the residues to be consumed fresh must undergo a thermal process (*e.g.*, pasteurization), but when consumed as alternative flour, the microbiological load is no longer a problem, since the cooking temperature of flour products supposes destruction of the microbial strains. A low microbial load was expected in the tubers, as they were analyzed dry and whole after their acquisition, that is, only the microbiological quality of the surface of the unpeeled tubers was assessed. Ugbo et al. (2022) also studied the microbiological quality of tigernuts and concluded that dried and washed tubers can have up to 2.8×10^3 CFU/mL (3.45 log10 CFU/mL) of aerobic bacteria, which shows that the microbial loads in the tubers depend on other factors other than the matrix, such as the geographical area or type of cultivation.

4. Nutritional and health claims

The information on food labels is one of the most important factors in the consumer's choice of products, but the mandatory information on the label can be very complex for some consumers with less knowledge of the food sector. Therefore, labeling products with nutritional claims (where available) is a great benefit for consumers as it helps them to make the right choice of products (Miller & Cassady, 2015). With the results obtained in this work, it was possible to associate some nutritional claims with the developed dehydrated plant-based beverage, according to EFSA and through the application of REGULATION (EC) No 1924/2006. Namely "WITH NO ADDED SUGAR" and "CONTAINS NATURALLY OCCURRING SUGARS" to inform the consumer that the sugar indicated on the label comes from the natural ingredients used. It was possible to associate the claim "LOW SODIUM" with the dehydrated plant-based beverage developed to inform the consumer that although the beverage is made with thermal water with high sodium concentrations, the same sodium concentration is not present in the final product (European Parliament, 2006).

As with the plant-based beverage, the residues may also contain nutritional claims, in addition to those attributed to the beverage, the residues may also contain the claim "HIGH FIBER" as it provides more than 3 g of fiber per 100 kcal (European Parliament, 2006).

Health claims on a food label that are permitted in the European Union are formulated in accordance with REGULATION (EU) No 432/2012. However, health claims are a very complex issue and difficult to

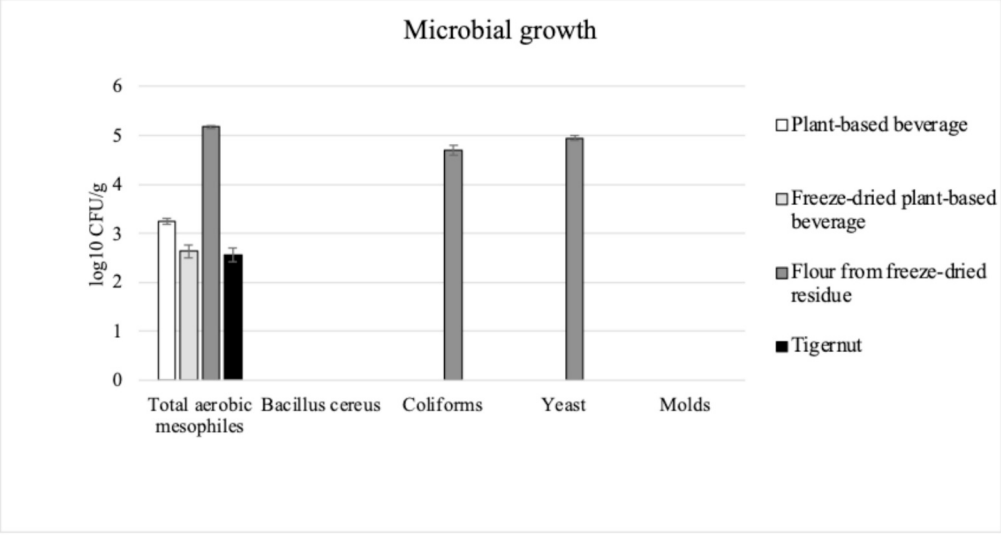

Fig. 2. Graphic representation of the microbial load present in the fresh, freeze-dried plant-based beverage, in the residues and in the tigernut tuber.

comply with for non-fortified products. After analyzing the possible claims that could be applied to the developed plant-based beverage, it was found that the beverage can bear the health claim related to the consumption of carbohydrates "Carbohydrates contribute to the maintenance of normal brain function", but the label must also include the information that the beneficial effect is obtained with a daily dose of 130 g of carbohydrates from all sources (the developed beverage provides 97 g of carbohydrates per 100 g of fresh beverage). As mentioned above, this beverage may contain the nutrition claim of low sodium content and consequently be given the health claim "Reducing consumption of sodium contributes to the maintenance of normal blood pressure" (European Commission, 2013). However, it no longer contains any health claims, although it contains high levels of various health-promoting compounds. An example of this is the health claim associated with oleic acid "Replacing saturated the diet with unsaturated fats contributes to the main tenancy of normal blood cholesterol levels. Oleic acid is an unsaturated fat" that can only be used if at least 70 % of fats contained in the product come from unsaturated fats and the unsaturated fats provide at least 20 % of the energy value. The beverage developed in this work fulfils the first condition, but the total fat content provides only 21 % of the energy, which corresponds to 15.5 %. unsaturated fats (European Commission, 2010, 2013).

5. Conclusions

The development of food products rich in compounds beneficial to health is a current topic of great importance to the food industry. Thus, this work aimed to provide the food industry with a dehydrated plant-based beverage developed with natural ingredients. The transformation of the three natural ingredients into a plant-based beverage resulted in a dehydrated product with an interesting nutritional content. Although 21 % of its energy is provided by fat, more than 70 % of the fat is unsaturated and its consumption is associated with health benefits for the consumer. This product also contributes to the daily intake of vitamin E and minerals, such as potassium and magnesium. One of the biggest challenges in producing beverage based on tigernut tubers is the natural presence of microorganisms. In this work, the tubers were subjected to different sterilization processes and the plant-based beverage was also subjected to a pasteurization process, resulting in a beverage with low concentrations of colony forming units, which in addition to being within the parameters required for food security are still much lower values, going from 10^5 to 10^2. Furthermore, it was possible to present a flour with a high content of nutrients and minerals and rich in fiber, as an alternative to wheat flour, through the recovery of residue from the development of the plant-based beverage, contributing to the goal of zero waste.

In the future, sensory analyses will be carried out to understand the acceptability of the dehydrated plant-based beverage and flour by the consumer.

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CRediT authorship contribution statement

Filipa A. Fernandes: Writing – original draft, Methodology, Investigation, Formal analysis. **Custódio Roriz:** Writing – original draft, Methodology, Investigation. **Ricardo C. Calhelha:** Writing – review & editing, Investigation. **Paula Rodrigues:** Writing – review & editing, Writing – original draft, Validation, Methodology. **Tânia C.S.P. Pires:** Writing – original draft, Methodology, Investigation. **Miguel A. Prieto:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Isabel C.F.R. Ferreira:** Writing – review & editing, Validation, Conceptualization. **Lillian Barros:** Writing – review & editing, Validation, Methodology, Conceptualization. **Sandrina A. Heleno:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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