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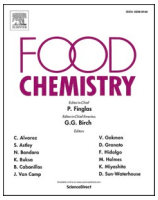

Systemic characterization of pupunha (*Bactris gasipaes*) flour with views of polyphenol content on cytotoxicity and protein *in vitro* digestion

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ABSTRACT

Although the Amazonian fruit pupunha *(Bactris gasipaes)* is rich in phenolic compounds, it is rarely consumed. Application could be expanded by transforming it into products with a longer shelf life. Therefore, the objective of this study was to produce pupunha flour (PF) from fruits harvested at different locations, characterize the flours using various physicochemical analyses and spectroscopic techniques, and evaluate its cytotoxic effects and *in vitro* protein digestion. PF were classified using principal component analysis and some dependencies on origin were detected. PF impaired protein digestion *in vitro*, probably due to the high content of phenolic compounds. The cytotoxicity results in L929 cells showed that the flours had cytotoxic potential. Therefore, it could be concluded that PF presented interesting physicochemical properties for its application in food development. However, the negative and undesirable effects on protein *in vitro* digestion and L929 cell viability indicated a potential risk for its use.

1. Introduction

The Amazon region is home to many fruits that have high nutritional potentials, including Pupunha (*Bactris gasipaes*), Bacaba (*Oenocarpus bacana*), Buriti (*Mauritia flexuosa*), and Inaja (*Attalea maripa*) (Santos et al., 2015). These fruits are rich in macronutrients and phytonutrients such as carotenoids, sterols, and fatty acids. They are also characterized by a high content in phenolic compounds. For example, total phenolic contents of 17.59, 2.81, 0.65 and 0.45 mg AGE/g has been reported in the literature for Bacaba, Buriti, Pupunha and Inaja, respectively (Contreras-Calderón et al., 2011; Finco et al., 2012; Santos et al., 2015), whereas for pulp conventional fruits such as banana (*Musa acuminata; Gonja*), mango (*Mangifera indica L.*; *Tommy Atkins*), melon (*Cucumis melo L.*) and avocado (*Persea americana*) contents are 0.51, 0.50, 0.07 and 0.04 mg AGE/g, respectively (Ahmed et al., 2021; Brat et al., 2006). However, the commercial use of Amazonian fruits has not been explored in detail, despite their potential socioeconomic impact, which can strengthen local/regional economies.

To reach this objective, the production of flours made from these fruits would offer the possibility to incorporate them into products with longer shelf life and greater added value. This is particularly the case for pupunha, for which Rojas-Garbanzo et al. (2012) found that its content in phenolic compounds was preserved throughout the drying process to produce flour. More recently, it has been demonstrated that pupunha flour was suitable to prepare cakes (Martínez-Girón et al., 2017) and cookies with a high carotenoid concentration (Silva Ribeiro et al., 2021).

Despite the advantages, including phytochemical compounds, especially antioxidant and antimicrobial activities (Araujo et al., 2021), and the potential use of pupunha flour in some products demonstrated by the cited literature, there is a lack on in-depth studies characterizing broadly the pupunha flour in physicochemical terms. Such data would help to guide possible applications in food development, and specify their potential benefits and limitations. The presence of polyphenols may represent some health and nutrition benefits, but also hazards, depending on their nature and concentration. For example, the tea polyphenol EGCG at 400 μM has a clear detrimental effect on HT29 cell

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viability (D'Agostino et al., 2012), and a similar negative effect was found for bitter melon seed in L929 cells (Andrade et al., 2022). Furthermore, He, Lv and Yao (2007) and Quesada et al. (1996) found that food phenolic compounds may inhibit enzymes during the digestive process. In the case of proteases, this could lead to reduced protein digestibility.

Additionally, the variability of characteristics depending on the location where the fruit is harvested has been insufficiently documented (Yuyama et al., 2003). Before considering a wide dissemination and use of pupunha flour, more in-depth research that considers the regional variability of the raw material is required. Therefore, the first objective of the present study was to produce flours from fruits collected in different areas of the Amazon region and characterize them through various physicochemical methods (thermal behavior, spectroscopic techniques) in order to generate a more systematic database. Second, the safety and nutritional aspects were investigated by evaluating the cytotoxic effects of the material produced on L929 cells, as established by ISO 10993-5 (2009), and its effect on protein digestion when incorporated into a dairy food matrix.

2. Material and methods

2.1. Material

Pupunha fruits were harvested from the states of Acre and Rondônia (Brazil). After receiving the fruits, they were inspected for any mechanical damage and/or dirt, and then sanitized in sodium hypochlorite solution (100 ppm) for 15 min. After this process, they were selected considering the uniformity of size and color. The fruits were used in their entirety (pulp and peel) for flour production. Table 1 shows the location of fruit harvests.

Pupunha flours (PF) were produced by freeze-drying the fruits (Terroni, LC1500, São Carlos, Brazil) for a period of 48 h. The drying time was determined based on preliminary tests. At the end of the drying process, the samples were ground in a knife mill (Marconi, MA340, Piracicaba, Brazil), and a 16 *mesh* sieve was used to standardize the granulometry. After sieving, the flours were stored at −18 °C.

2.2. Methods

2.2.1. Proximate composition

The moisture, crude protein, ash, lipid, carbohydrate, and crude fiber contents of the flours were determined according to the methodologies defined by the AOAC (1980) and AOAC (1985). The results were expressed in relation to dry matter (DM).

2.2.2. Elemental composition

The concentrations of sulfur, phosphorus, and boron were analyzed using a colorimetric spectrophotometer (Femto, 600 Soft, Brazil); potassium was analyzed using a flame photometer (Micronal, B462, Brazil); and calcium, iron, magnesium, copper, zinc, and manganese were analyzed using an atomic absorption spectrophotometer (Varian, Fast Sequential AA240FS, USA).

Table 1
Harvest location and identification of the six pupunha fruit samples used for the manufacture of the flours.

Location of Pupunha Fruit Harvest			
Samples	Latitude	Longitude	City (State) Brazil
PF – 01	10° 0′ 25″ South	67° 50′ 44″ West	Rio Branco (Acre)
PF – 02	9° 35′ 33″ South	67° 32′ 26″ West	Porto Acre (Acre)
PF – 03	9° 56′ 44″ South	67° 10′ 35″ West	Rio Branco (Acre)
PF – 04	9° 45′ 15″ South	66° 36′ 48″ West	Nova Califórnia (Rondônia)
PF – 05	9° 46′ 14″ South	66° 21′ 22″ West	Extrema (Rondônia)
PF – 06	8° 46′ 3″ South	63° 52′ 12″ West	Porto Velho (Rondônia)

PF: pupunha flour.

2.2.3. Preparation of the phenolic extract

Free phenolic compounds were extracted as described by Santos et al. (2015) with some modifications. Initially, 20 mL of water/methanol (50:50, v/v) were added to tubes containing 5 g of flour for the first extraction step. The mixtures were then homogenized (300 rpm; 1 h) in a shaker (Marconi, MA 420, Piracicaba, Brazil) and centrifuged (Eppendorf, 5430R, Germany) at 7830 rpm at 15 °C for 10 min. The supernatant was then collected and placed in an amber bottle. Subsequently, 20 mL of water/acetone (30:70, v/v) were added to the residue from the previous step and subjected to the same agitation and centrifugation rates. Finally, the methanol and acetone extracts were combined and stored at −18 °C.

2.2.4. Phenolic content and antioxidant potential analysis

Total phenolic content was quantified using the Folin-Ciocalteau method according to Singleton, Orthofer and Lamuela-Raventós (1999). 0.5 mL aliquots of the diluted extract solutions were added to a tube containing the Folin-Ciocalteau reagent (2.5 mL). Then, 2.0 mL of sodium carbonate solution (7.5 %) was added to each tube, which was homogenized (IKA, Vortex 1 V1, Staufen, Germany), and the solution was kept at rest for another 2 h. Absorbance readings were performed on a Spectrum One spectrophotometer (Perkin-Elmer, Waltham, MA, USA) at 740 nm, and the values were converted into mg of gallic acid/g of flour using a gallic acid standard curve.

The antioxidant potential was measured using the ferric reducing antioxidant power (FRAP), radial capture 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and oxygen radical absorption capacity (ORAC) methods, all performed in triplicate. The first method, FRAP, was performed following the methodology of Benzie and Strain (1996), with adaptations. Initially, an aliquot (0.1 mL) of the extract was placed in a tube and then 2.9 mL of the FRAP reagent solution was added. The mixture was incubated in a thermostat bath at 37 °C for 30 min (Marconi, MA 159, Piracicaba, Brazil). The samples were then read at 593 nm using a spectrophotometer (Perkin-Elmer, Waltham, MA, USA). The final result was expressed as Trolox equivalent (TE) in μMolTE. g^{-1}.

The analysis by the $ABTS^{\bullet+}$ radical followed the methodology proposed by Larrauri, Rupérez and Saura-Calixto (1997) with modifications. Initially, 0.3 mL of extract was added to 3 mL of the $ABTS^{\bullet+}$ radical, homogenized (IKA, Vortex 1 V1, Staufen, Germany) and, after 6 min of mixing, the reading was performed in a spectrophotometer at 734 nm (Perkin-Elmer, Waltham, MA, USA), using Trolox as a standard.

The antioxidant potential was determined using the ORAC method according to Ou, Hampsch-Woodill and Prior (2001). First, 25 μL of the extract was added to 150 μL of fluorescein and incubated in a microplate for 10 min at 37 °C in a spectrofluorometer (BMG Labtech, FLUOstar OPTIMA, Germany). Subsequently, 25 μL AAPPH was added to each well. Fluorescein reduction was measured for 120 min at 528 nm, with excitation at 485 nm (BMG Labtech, FLUOstar OPTIMA, Germany). Trolox was used as an external standard, and the results were expressed in μMol TE. $g\text{-}^{1}$.

2.2.5. Principal component analysis (PCA)

PCA was used as a qualitative method to observe the variation between chemical samples, grouping and/or separating them depending on the similarity of proximate composition data, total phenolic compounds content and antioxidant activity. Data were processed using Origin software (version 2019b, OriginLab Corporation, Northampton, MA, USA).

2.2.6. Fourier transform infrared spectroscopy

FTIR measurements were carried out using a Vertex 70 Fourier Bruker spectrometer (Bruker, Ettlingen, Germany) with a coupled accessory to measure the attenuated total reflectance (ATR) using a wavenumber of 4 $cm\text{-}^{1}$ and 32 scans.

2.2.7. Differential scanning calorimetry (DSC)

The thermoanalytical behavior of the pupunha flour samples was analyzed in duplicate by DSC, in the range of 0 to 200 °C, using a heating ramp of 10 °C/min and a continuous flow of non-oxidative N_2 atmosphere of 50 mL/min. The initial transition temperature (Ti), peak transition temperature (Tp) and enthalpy of reaction (Δh) were determined by DSC Q100 equipment using TAV4 software (TA Instruments, New Castle, PA, USA).

2.2.8. X-ray diffractometry (XRD)

The XRD analyses were conducted using a MiniFlex 300 benchtop diffractometer (Rigaku, Tokyo, Japan). The samples were subjected to Cu-Ka monochromatic UV/ionizing radiation (λ = 0.1542 nm) under a voltage of 30 kV, current of 10 mA, angular range of 3-40° (θ-2θ) and with a step of 0.01°. The relative crystallinity (RC) and relative crystal size (L_{HKL}) referring to the 15.3°, 17.1°, 18.4°, 21.5°, and 23.1° crystal planes were calculated as described by Valencia et al. (2015) and Villas-Boas et al. (2020), respectively, using PAN-analytical™ X'pert high score Plus and PeakFit v. 4.12.

2.2.9. Solid state ^{13}C nuclear magnetic resonance (NMR) spectroscopy

High-resolution solid state ^{13}C NMR spectra were acquired using a Bruker® Avance 400 spectrometer (υ = 100.5 MHz for ^{13}C and 400.0 MHz for ^{1}H), coupled to a 4.0 mm CPMAS (cross polarization pulse sequence and magic angle sample spinning) probe at 12 KHz. The samples were packed into zirconia rotors. The 90° pulse duration were 2.5 μs and 5.0 μs for ^{1}H and ^{13}C nuclei, respectively, 5 s recycle delay (d1), 1024 transients, acquisition time (Aq) of ~ 25 μs and ^{1}H–^{13}C decoupling frequency of 70 kHz. The chemical shift was calibrated using the methyl peak of hexamethylbenzene (HMB) at 17.3 ppm. All spectra were obtained from the Fourier transform and processing of 2000 complex points (Facchinatto et al., 2020). Crystallinity values and double helix content were calculated by deconvolution of the spectra from Voigt functions, according to the procedure described by Villas-Boas et al. (2020), using PeakFit software v. 4.12.

2.2.10. Cytotoxicity of pupunha flour

The phenolic extract of pupunha flour was prepared with 15 g of pupunha flour diluted in 300 mL of water/acetone (70:30, v/v) and water/methanol (50:50, v/v). The mixture was allowed to rest for 48 h at room temperature in a dark place. Afterwards, the solution was filtered in a vacuum system and the solvents used for the extraction were removed by distillation under reduced pressure (Fisatom, mod.802, Brazil) for approximately 50 min at 55 °C. At the end of rotaevaporation, the aqueous fraction containing phenolic compounds was frozen and lyophilized for 48 h (Terroni, LC1500, São Carlos, Brazil) to obtain the pure phenolic fraction of the pupunha flour.

The cytotoxicity of the phenolic extracts from pupunha flour was determined according to the standard method ISO 10993-5 (2009). Initially, 100 μL/well of commercial cells (L929) was plated in triplicate, at a concentration of 5×10^3 cells/well in 96 wells plates (Corning, NY, USA) containing DMEM-F12 culture medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; ThermoFisher, Carlsbad, CA), 10 % fetal bovine serum (InvitrogenTM, Carlsbad, CA), and 1 % antibiotics (InvitrogenTM, Carlsbad, CA). The plates were incubated at 37 °C in 5 % CO_2 for 24 h (Incubator 3111, ThermoScientific, Massachusetts, USA). The phenolic extract obtained from pupunha flour was diluted in the culture medium at 0.000128, 0.00064, 0.0032, 0.016, 0.08, 0.4, 2, and 10 mg/mL, and the cells were incubated again under the described conditions.

After 24 h, cell viability was analyzed using an MTT colorimetric assay (Thyazolyl Blue Tetrazolium Bromide – Sigma Aldrich, Saint Louis, USA). The cytotoxicity index (IC_{50}) was determined using the GraphPad Prism 9.2 software, and the analyses performed in triplicate.

2.2.11. Pupunha flour incorporation into a yogurt: effects on in vitro protein digestion

The impact of Pupunha flour on protein digestion in a model product (stirred yogurt) was evaluated using the INFOGEST *in vitro* digestion protocol with adaptations (Brodkorb et al., 2019). Yogurts without flour (control) or with 25 % Pupunha flour incorporated into the formulation were evaluated. Pupunha flour (PF-02) was used, and the digestion was performed in triplicate.

Briefly, 5 g of yogurt was homogenized with 5 mL of commercial saliva (pooled saliva; LeeBiosolutions, Maryland Heights, USA). After 2 min at 37 °C under agitation using a magnetic stirring plate (MIXdrive 6, 2^{mag}, Munich, Germany) immersed into a water bath (Optima T100, Grant Instruments ltd, Cambridgeshire, United Kingdom), the gastric phase of digestion was initiated: 8 mL of simulated gastric fluid (SGF) were added to the mixture and the pH was adjusted to 3.0 with 1 M HCl. Thereafter, 5 μL of $CaCl_2$, 1 mL of pepsin (final concentration 2000U/mL) and distilled water were added to adjust the volume to 10 mL. Gastric digestion was performed at 37 °C with agitation for 60 min.

The intestinal phase was started by adding 8.5 mL of simulated intestinal fluid (SIF), and the pH was adjusted to 7.0 using 2 M NaOH. After, 2.5 mL of bile solution (final concentration 10 mM of bile salts), 40 μL of $CaCl_2$, and 5 mL of pancreatin (final concentration: 100 U/mL based on trypsin activity) were added, and the final volume was adjusted to 40 mL using distilled water. The reaction mixture was incubated at 37 °C with agitation for 1 h until the end of digestion.

To monitor the release of free NH_2 groups induced by the enzymatic hydrolysis of proteins, samples were collected at 5, 15, 30, and 60 min of both the gastric and intestinal phases of digestion. The free NH_2 groups were measured using the O-phthaldehyde (OPA) method adapted from Church et al. (1985). Then, 100 mL of reagent was prepared with 2.5 mL of OPA (10 mg/mL in ethanol), 2.5 mL of 20 % SDS, 50 μL of β-mercaptoethanol and 95 mL of 20 mM sodium tetraborate. 96-well UV clear plates and a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, USA) were used to measure the absorbance at 340 nm after 10 min of contact with regular agitation between the OPA reagent (100 μL) and diluted samples (50 μL). Each sample was measured in triplicate, and the number of α-amino groups released during hydrolysis was estimated based on the difference in the mean absorption at 340 nm between the hydrolyzed and non-hydrolyzed samples, using L-methionine for the calibration curve (0–2 mM).

2.2.12. Statistical analysis

Statistical analyses were performed with at least three replicates. Results were expressed as mean value ± standard deviation and statistically evaluated as the difference between means using Duncan's test at 95 % confidence interval using SAS software (Version 9.2, SAS, Inc.) for centesimal and elemental composition, antioxidant activity, thermal properties, and digestion analyses.

3. Results and discussion

3.1. Proximate composition

The pupunha flour had a high concentration of carbohydrates (average of 86.35 g/100 g of dry matter (DM)), followed by lipids (average of 8.61 g/ 100 g of DM), proteins (average of 6.34 g/ 100 g of DM), and fibers (average of 2.92 g/100 g of DM) (Table 2). These values were close to those reported by Rojas-Garbanzo et al. (2012) for pupunha flour.

The centesimal composition results also showed that the harvest location significantly influenced the composition of the produced pupunha flour. The concentration of carbohydrates, lipids, protein and fiber varied between, 82.4 and 92.0 g/100 g of DM, 4.6 and 11.2 g/100 g of DM, 4.83 and 9.70 g/100 g of DM and 1.7 and 5.4 g/100 g of DM, respectively (Table 2).

Yuyama et al. (2003) studied the fruit of Pupunha (*Bactris gasipaes*)

Table 2
Characterization of the chemical composition, elemental composition, phenolic compound content, antioxidant properties, thermal properties and crystallinity by XRD and NMR techniques for the six elaborated pupunha flours.

Analyze		Pupunha flour (PF)						
		PF − 01	PF − 02	PF − 03	PF − 04	PF − 05	PF − 06	Average
Chemical composition	Water content (g/100 g of flour)	$6.41^{b} \pm 0.4$	$5.22^{c} \pm 0.1$	$6.49^{b} \pm 0.4$	$8.01^{a} \pm 0.2$	$4.85^{c} \pm 0.5$	$7.17^{b} \pm 0.3$	**6.36 ± 1.2**
	Protein (g/100 g of DM*)	$6.09^{b} \pm 0.1$	$4.83^{e} \pm 0.01$	$5.70^{c} \pm 0.1$	$6.16^{b} \pm 0.01$	$5.56^{d} \pm 0.01$	$9.70^{a} \pm 0.1$	**6.34 ± 1.7**
	Glycidic fraction (g/100 g of DM)	$11.15^{a} \pm 0.01$	$4.65^{d} \pm 0.6$	$9.10^{b} \pm 0.01$	$8.40^{b} \pm 0.2$	$11.23^{a} \pm 0.2$	$7.15^{c} \pm 0.1$	**8.61 ± 2.5**
	Fiber (g/100 g of DM)	$1.69^{d} \pm 0$	$2.08^{cd} \pm 0$	$2.45^{c} \pm 0.3$	$4.05^{b} \pm 0.2$	$1.93^{cd} \pm 0.1$	$5.36^{a} \pm 0.2$	**2.92 ± 1.5**
	Carbohydrate (g/100 g of DM)	$85.20^{c} \pm 0.6$	$91.99^{a} \pm 0.7$	$87.25^{b} \pm 0.1$	$87.37^{b} \pm 0.3$	$83.91^{d} \pm 0.1$	$82.38^{e} \pm 0.3$	**86.35 ± 3.4**
	Ash (g/100 g of DM)	$2.28^{b} \pm 0.1$	$1.67^{d} \pm 0$	$1.99^{c} \pm 0$	$2.04^{c} \pm 0$	$2.22^{b} \pm 0.1$	$2.58^{a} \pm 0.1$	**2.13 ± 0.3**
Elemental composition	N (g/kg)	$8.7^{b} \pm 0.03$	$7.1^{c} \pm 0.15$	$8.9^{b} \pm 0.67$	$9.3^{b} \pm 0.43$	$9.0^{b} \pm 0.26$	$13.4^{a} \pm 0.14$	**9.3 ± 0.23**
	P (g/kg)	$0.44^{bc} \pm 0.01$	$0.39^{c} \pm 0.02$	$0.56^{a} \pm 0.06$	$0.41^{c} \pm 0.02$	$0.63^{a} \pm 0.12$	$0.54^{ab} \pm 0.03$	**0.5 ± 0.04**
	K (g/kg)	$8.5^{b} \pm 0.37$	$6.0^{d} \pm 0.06$	$13.0^{a} \pm 0.58$	$7.2^{c} \pm 0.06$	$13.3^{a} \pm 0.95$	$8.9^{b} \pm 0.15$	**9.5 ± 0.35**
	Ca (g/kg)	$17.8^{a} \pm 2.16$	$17.7^{a} \pm 4.62$	$2.3^{c} \pm 0.17$	$21.5^{a} \pm 4.98$	$2.3^{c} \pm 0.06$	$8.5^{b} \pm 0.42$	**11.7 ± 2.25**
	Mg (g/kg)	$0.7^{b} \pm 0.09$	$0.4^{cd} \pm 0.03$	$0.5^{c} \pm 0.06$	$0.8^{b} \pm 0.09$	$0.3^{d} \pm 0.1$	$1.2^{a} \pm 0.07$	**0.6 ± 0.03**
	S (g/kg)	$0.4^{b} \pm 0.01$	$0.3^{b} \pm 0.17$	$1.9^{a} \pm 0.35$	$0.2^{b} \pm 0.04$	$2.2^{a} \pm 0.06$	$0.4^{b} \pm 0.17$	**0.9 ± 0.13**
	Cu (mg/kg)	$22.9^{ab} \pm 2.69$	$23.6^{ab} \pm 5.22$	$16.4^{ab} \pm 6.42$	$25.0^{a} \pm 3.03$	$21.9^{ab} \pm 6.53$	$15.0^{b} \pm 2.29$	**20.8 ± 1.93**
	Fe (mg/kg)	$68.6^{c} \pm 4.10$	$40.2^{d} \pm 7.02$	$312.1^{b} \pm 17.04$	$68.2^{c} \pm 6.74$	$442.6^{a} \pm 14.50$	$58.9^{c} \pm 4.60$	**165.1 ± 5.43**
	Mn (mg/kg)	$13.9^{d} \pm 0.87$	$9.6^{e} \pm 0.20$	$24.9^{a} \pm 0.85$	$19.4^{c} \pm 1.66$	$10.0^{e} \pm 0.28$	$21.6^{b} \pm 0.46$	**16.6 ± 0.54**
	Zn (mg/kg)	$31.4^{c} \pm 0.55$	$29.4^{c} \pm 0.75$	$44.3^{a} \pm 1.23$	$30.2^{c} \pm 1.66$	$37.9^{b} \pm 1.34$	$38.9^{b} \pm 1.54$	**35.8 ± 0.44**
Antioxidant properties	Total Phenolics (mgAGE**/g of DM)	$1.84^{cb} \pm 0.06$	$2.52^{a} \pm 0.03$	$1.94^{b} \pm 0.04$	$1.96^{b} \pm 0.03$	$1.73^{c} \pm 0.15$	$2.64^{a} \pm 0.21$	**2.11 ± 0.38**
	FRAP (μMol TE*** / g of DM)	$0.23^{c} \pm 0.01$	$0.35^{a} \pm 0.02$	$0.24^{c} \pm 0.01$	$0.19^{d} \pm 0.02$	$0.27^{b} \pm 0.01$	$0.22^{c} \pm 0.02$	**0.25 ± 0.05**
	ABTS (μMol TE/ g of DM)	$13.3^{c} \pm 0.5$	$18.4^{ab} \pm 1.3$	$16.2^{b} \pm 1.1$	$17.1^{b} \pm 0.9$	$16.8^{b} \pm 2.4$	$20.0^{a} \pm 3.3$	**16.9 ± 2.25**
	ORAC (μMol TE/ g of DM)	$35.21^{cb} \pm 4.4$	$38.43^{b} \pm 6.4$	$28.93^{d} \pm 5.1$	$34.65^{cb} \pm 2.9$	$30.18^{cd} \pm 2.4$	$50.38^{a} \pm 4.8$	**36.30 ± 7.74**
Thermal properties	T_0 (°C)	$67.99^{a} \pm 1.44$	$64.21^{a} \pm 1.93$	$66.31^{a} \pm 3.88$	$66.08^{a} \pm 4.46$	$69.70^{a} \pm 4.45$	$69.47^{a} \pm 5.64$	**67.29 ± 2.14**
	T_p (°C)	$113.16^{a} \pm 3.00$	$109.99^{a} \pm 1.70$	$111.51^{a} \pm 3.33$	$111.11^{a} \pm 2.23$	$111.12^{a} \pm 1.87$	$114.96^{a} \pm 1.87$	**111.97 ± 1.78**
	T_f (°C)	$176.19^{a} \pm 1.21$	$174.02^{a} \pm 9.28$	$176.31^{a} \pm 5.60$	$177.27^{a} \pm 4.98$	$159.77^{a} \pm 4.97$	$168.18^{a} \pm 12.04$	**171.95 ± 6.81**
	Δ_H (J/g)	$123.34^{a} \pm 37.54$	$140.0^{a} \pm 31.67$	$117.69^{a} \pm 31.07$	$145.92^{a} \pm 61.01$	$107.62^{a} \pm 40.95$	$89.89^{a} \pm 9.27$	**120.74 ± 20.71**
XRD	Relative crystallinity (%)	34.1	36.7	32.9	37.3	35.4	34.3	**35.11 ± 1.3**
	****L_{HKL} 15.3°	6.0	5.8	5.9	5.7	5.5	5.7	**5.76 ± 0.2**
	17.1°	6.2	5.4	5.5	6.5	6.3	6.3	**6.03 ± 0.5**
	18.4°	5.0	4.7	4.8	6.9	5.9	5.9	**5.53 ± 0.9**
	21.5°	6.4	6.7	6.3	8.1	7.1	6.4	**6.83 ± 0.7**
	23.1°	4.6	4.6	4.6	4.6	4.5	4.5	**4.56 ± 0.06**
NMR	Relative crystallinity (%)	51.1	37.1	48.5	50.5	46.2	44.2	**46.26 ± 5.2**
	Double Helix (%)	61.3	51.5	60.0	61.1	60.9	66.5	**60.21 ± 4.85**

* DM, dry matter; ** GAE, gallic acid equivalent; *** TE, trolox equivalent; and ****L_{HKL}, relative crystal size; Means with equal letters on the same column line do not differ significantly by Duncan's test ($p > 0.05$).

from different regions of Brazilian Amazonian states (Pará and Amazonas) and Peru, and observed differences in the centesimal composition of the fruits with respect to harvest locality. These results show that the different pupunha cultivation conditions (rainfall index, type of climate, soil quality, luminosity, and other conditions) affect the centesimal composition of its fruits and, consequently, the derived food products that use it.

3.2. Elemental composition

The pupunha flour collected in the states of Acre (PF-01 to PF-03) and Rondônia (PF-04 to PF-06) showed significant differences in the concentrations of all macro and microminerals analyzed (Table 2).

Rojas-Garbanzo et al. (2012) previously reported calcium levels of 0.26 g/kg for pupunha flour, which is much lower than the average found in the present study (11.7 g/kg). Furthermore, the levels of micronutrients (iron, copper, manganese, and zinc) were lower than those found in this study. The differences found between the samples, such as pupunha flour samples, for the results obtained in the literature are probably caused by soil characteristics in each harvesting location, the type of management, and the fertilization used (Yuyama et al., 2003).

In relation to the daily amounts recommended by the National Research Council (1989), the daily intakes of potassium, manganese and iron are, respectively, 2000, 2–5 and 10 mg, respectively. Therefore, a portion of 250 g of food completely prepared with the pupunha flours could already supply 100 % of this demand. This result contrasts with that found by Yuyama et al. (2003), who studied pupunha flour and reported a maximum contribution of 12, 6 and 5 % of the recommended daily intake of potassium, iron, and manganese, respectively.

3.3. Phenolic content and antioxidant potential analysis

The pupunha flours showed an average concentration of total phenolic compounds of 2.11 mg GAE/g of DM (Table 2). In comparison with the available literature, regardless of cultivation location, the results found were superior to those found by Rojas-Garbanzo et al. (2012) (0.63 mg GAE/g of DM) and Santos et al. (2015) (0.30 mg GAE/g of DM) for pupunha flour. Such discrepancies may result from the different environmental conditions at the harvest locations or from differences in flour processing and phenolic compound extraction methods.

When compared to other flours, the studied samples had an average of phenolic compounds concentration higher and/or comparable to that found in bean (2.88 mg catechin equivalent CE/g of DM), rice (0.90 mgCE/ g of DM) (Arribas et al., 2019), guava (0.84 mg GAE/g) (Alves & Perrone, 2015) and macaúba pulp (2.62 mg GAE/g) (Andrade et al., 2020). Thus, the results for pupunha flour demonstrate the potential of flour as a source of phenolic compounds for dietary intake.

Considering the average values of the antioxidant activity of the pupunha flour samples, obtained by the FRAP (0.25 μmol TE/g of DM) and ABST (16.9 μmol TE/g of DM) methods (Table 2), it is observed that these were lower and higher, respectively, than those found by Contreras-Calderón et al. (2011) for crushed pupunha pulp (FRAP – 3.98 μmol TE/g; ABTS – 14.1 μmol TE/g). The results obtained by the ORAC method (32.10 μmol TE/g of DM) were similar to those found by Rojas-Garbanzo et al. (2012) for pupunha flour. These results show that regardless of the phenolic content, the presence of these compounds is not indicative of antioxidant activity. As previously mentioned, the differences between the techniques, especially in the extraction stage, and the nature of the phenolic compounds, may restrict the antioxidant potential of raw materials.

In addition, similar to the results of centesimal composition, the phenolic compounds and antioxidant activity of pupunha flour were also significantly affected by the location of the harvested fruits or fruit ripeness.

3.4. Principal component analysis (PCA)

A principal component analysis was performed on the six pupunha flour samples, using the concentrations of 21 elements (centesimal and elemental compositions, phenolic content and antioxidant capacity). Based on the eigenvalues, the first three principal components (PC) explained 88.78 % of the total variance. The loadings of the most significant variables in the first three main components and the variances explained by each component are presented in Supplementary Material 1. Fig. 1 shows the score and load graphs for the combination of PC1 (39.98 %) and PC2 (34.63 %).

It is possible to observe that samples PF-03, PF-05, and PF-06 contained the largest components of PC1. In addition, the phenolic compound content had a higher positive correlation with the antioxidant potential when estimated with the ABTS method than with the other tested methods, as revealed by the shorter distance, and hence a more significant relationship, between corresponding data points. This finding confirms that different methods of measuring antioxidant capacity yield discordant results.

Finally, PCA revealed that the samples were discriminated according to their physicochemical characteristics, despite the fact that samples were collected from nearby regions. This may be linked mainly to two factors: the environmental characteristics of each harvesting location and the ripening stage of each fruit lot.

3.5. Fourier transform infrared spectroscopy

Fig. 2a shows the FT-IR spectra of the PF samples, where the characteristic bands of polysaccharides and lipids are predominant in the spectra. The signals between 2900 and 2850 cm^{-1} are due to C—H stretching, in large quantities in the saturated fatty acid chain, and the signal around 1730 cm^{-1} is characteristic of the stretching of the C═O bond of the ester carboxyl (Osiro et al., 2011).

The most intense band of the spectra in Fig. 2a between 1000 and 1100 cm^{-1} is attributed to the stretching of the C—O bond of saccharides and polysaccharides, such as pectin, cellulose, and hemicellulose, which are part of the cell wall composition of fruits and vegetables. There is at approximately 3300 cm^{-1} in these spectra, which corresponds to the symmetrical stretching of the O—H bonds of these compounds (Osiro et al., 2011). Furthermore, the bands at approximately 1630 cm^{-1} can be visualized due to the asymmetric stretching of the COO– and O—H groups, which are also attributed to the polysaccharides (Valencia et al., 2015).

Due to the high concentration of lipids and carbohydrates in the samples, FT-IR spectra cannot be used to identify other constituents, such as proteins and phenolic compounds; therefore, characteristic signals cannot be attributed to these functional groups.

An analysis of the band intensities between 3000 and 2800 cm^{-1} indicate that samples PF-01 and PF-05 had the highest amount of lipids,

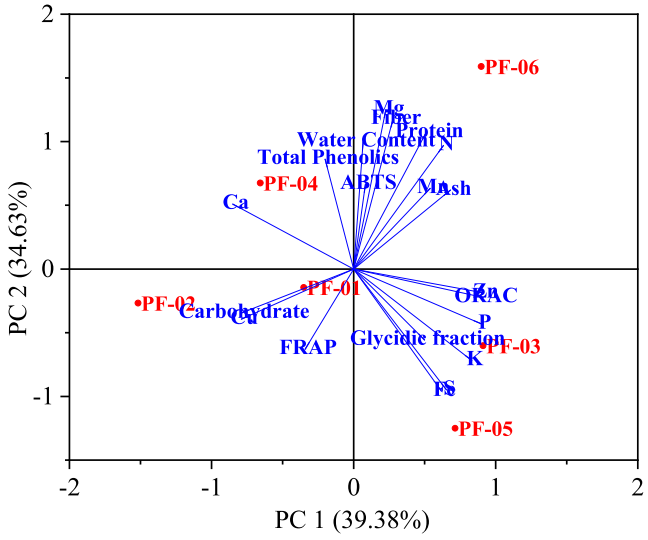


Fig. 1. Principal component analysis for the parameters of centesimal and elemental composition, phenolic concentration and antioxidant activity of pupunha flours harvested in different locations; bi-plot of loadings and scores of the analyses.

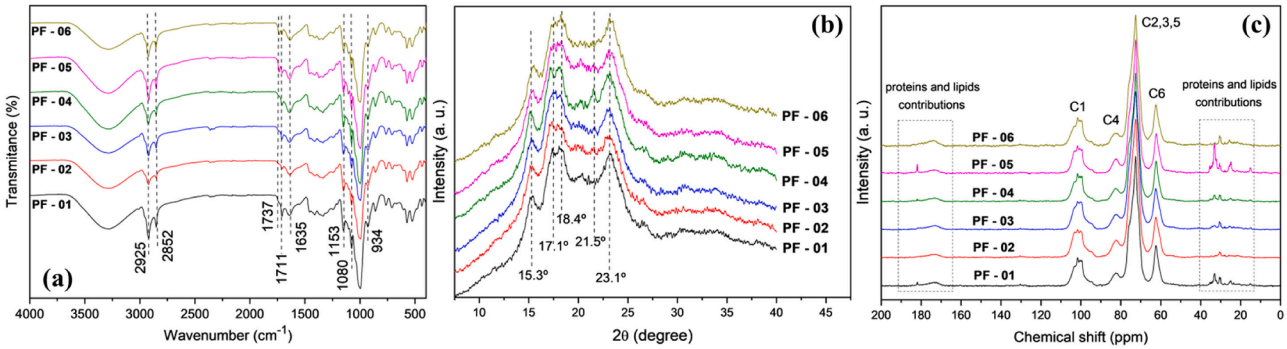


Fig. 2. (a): FTIR spectra of the six pupunha flour (PF) samples with the identification of the main vibrational bands; (b): X-ray diffractograms of the six pupunha flour (PF) samples with the identification of the main crystalline planes; (c): structural composition of the six pupunha flour (PF) samples identified by solid-state ^{13}C CPMAS spectra.

and sample PF-02 had the smallest amount of this nutrient, which is in agreement with the analysis of centesimal composition (Table 2). Thus, as the FTIR analysis identified qualitative differences between the PF samples, mainly in the polysaccharides and lipids contents, this method could be considered very valuable to quickly investigate and classify the levels of these specific chemical compounds.

3.6. Differential scanning calorimetry (DSC)

Table 2 lists the thermodynamic parameters of pupunha flour. The thermograms revealed only one endothermic peak for all samples, with mean T_0, Tp and Δ_H of 67.29 °C, 111.97 °C and 120.74 J/g, respectively. In general, the results for the different PF samples did not show any significant differences. Studies evaluating the thermal properties of starch from Pupunha (Felisberto et al., 2020; Neto et al., 2017; Valencia et al., 2015) have reported lower values than those reported in the present study. This difference can be attributed to the possible protective action of the other constituents of the flour (lipids, carbohydrates, proteins, fibers, etc.).

The endothermic behavior of the samples can be attributed to the fact that in heterogeneous food systems the thermal curves can come from reactions associated with several components, therefore, the reaction enthalpy is dependent on the compounds that are subject to thermal transitions. Thus, enthalpy values may be largely related to lipids and carbohydrates. On the other hand, Neto et al., (2017) suggested that the observed peaks are the result of an order–disorder phase transition of the starch (carbohydrate) under heating during a temperature range (gelatinization) characteristic of the starch source. According to the authors, the amount of amylopectin is one of the factors that determines the enthalpy of the sample, since this structure hinders water accessibility and causes a greater amplitude between the initial and final transition temperatures and, consequently, a slow gelatinization of more crystalline regions. Another hypothesis is that the reaction enthalpy values may be directly related to the non-denaturing nature of the protein found in the samples during flour processing. According to Hermansson (1979), when protein denaturation occurs during processing, there is little or no endothermic peak during the DSC analysis. Therefore, as flour production was carried out by a cold and vacuum process, it can be suggested that the proteins in the samples did not denature.

3.7. X-ray diffractometry (XRD)

All samples showed similar diffraction profiles, with crystalline planes centered near 2θ at 15.3°, 17.1°, 18.4° and 23.1° (Fig. 2b), which are normally attributed to type C semicrystalline starches (Dome et al., 2020). A peak at 21.5° was also observed for only one sample, PF-04, and therefore, could not be attributed to the general characteristics of pupunha flour. Similar diffraction patterns have been found in other studies involving pupunha starch (Felisberto et al., 2020; Neto et al., 2017). The type C pattern concentrates on the characteristics of types A (normal and waxy cereals) and B (some tubers and corns with high amylose content); thus the matrices with this type of pattern have distinct characteristics (Felisberto et al., 2020). Among them, a higher concentration of resistant starch than the other types can serve as substrates for the large intestine microbiota.

The mean relative crystallinity (RC) value of the PF samples was 35.1 ± 1.3 %, relatively higher than that achieved by Valencia et al. (2015), while the mean values of relative crystal size (L_{HKL}) were 5.8 ± 0.1, 6.0 ± 0.4, 5.5 ± 0.7, 6.8 ± 0.5 and 4.6 ± 0.04 nm (Table 2), calculated in Supplementary Material (Figure S1). Factors such as the variable composition of other macronutrients in flour can considerably affect RC values. In the present study, the low variability of RC and L_{HKL} serves as an indication of the low relevance of these parameters to geographic casualties. Thus, the PFs seemed to preserve a uniform composition in relation to the crystallinity pattern of the starches, with little dependence on the region of origin.

3.8. Solid state ^{13}C NMR

Fig. 2c shows the solid state ^{13}C CPMAS spectra of the PF samples, together with the attribution of the different types of carbons in the crystallized starch molecules. The spectra of all samples reveal a profile that largely belongs to pupunha starch, which is widely observed in flour samples from other plant species. These results reinforce the finding that the crystalline starch structure shows little variation between the samples, in agreement with the XRD diffraction patterns. It was also verified that, despite the proximity of spectral profiles between the signals of C1 and C4 carbons, their deconvolution treatment (Figure S2) resulted in slightly different RC values and double helix compositions between the samples (Table 2). Generally, these parameters tend to be proportional because the relative amount of helical structures tends to maintain the spatial regularity of the macromolecular domains and, therefore, the crystalline ordering of the chains. It is important to emphasize that although the interpretation converged because of the close relationship of the CR values by different techniques (XRD and NMR), they do not refer to the same physical origin: XRD observes long-range ordering, while NMR observes short-range ordering (Facchinatto et al., 2020). As both methods provide complementary information about the general morphology of the samples, it is expected that the XRD values are relatively lower as they involve structures other than starch, such as proteins and lipids. According to NMR, however, the morphology of the starch can be followed independently of the other components, since these components do not interfere with the C1 and C4 signals, as indicated in Fig. 2c.

As shown in Table 2, the PF-02 sample showed the highest

amorphous characteristic (lowest CR value) calculated by the NMR spectra, while PF-03 showed the lowest CR value calculated by the XRD diffraction patterns, demonstrating that the NMR and XRD CR results are different in terms of both order of magnitude and sample ranking.

3.9. In vitro cytotoxicity

The pupunha flour extracts were non-cytotoxic up to a concentration of 0.4 mg/mL (Fig. 3). According to ISO 10993-5 (2009) a reduction in cell viability of < 70 % of the blank indicates cytotoxic potential and, complementing, Andrade et al., (2022) demonstrated that extract of interest that induce a 50 % reduction in cell viability (IC_{50}) below 1 mg/mL demonstrate cytotoxic activity. Thus, the cytotoxic potential of pupunha is not negligible, since the IC_{50} was 0.940 mg/ mL.

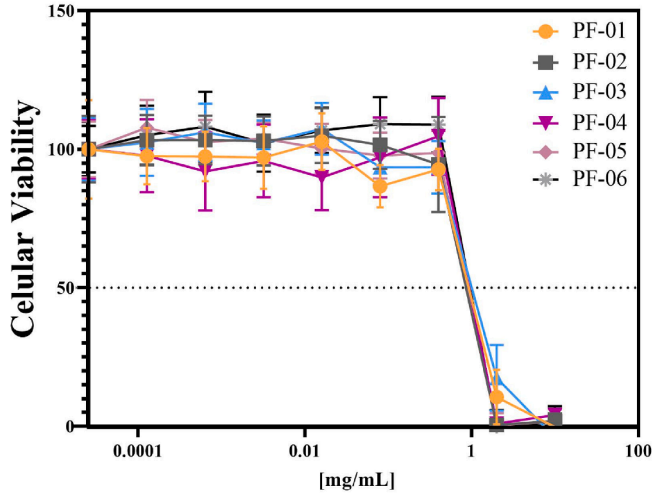
In a recent study, it was shown that passion fruit leaf methanolic extract had no toxic effect on L929 cell, but exerted a strong metabolic inhibitory effect on cancer cells (HeLA; 10.83 μg/mL) (Sisin et al., 2017). Other studies have also found that phenolic extracts from fruits have inhibitory effects on other types of cancer cells, such as HepG2, HeLA, AGS, and SW620 (Custódio et al., 2011; Navarro et al., 2019).

On the other hand, Okonogi et al. (2007) demonstrated that pomegranate peel extract stimulated the metabolic activity of human healthy tissue (PBMC) and Caco-2 (human colon adenoma) cells, therefore, recommending the controlled use of this extract as a supplement, food, and/or drug for humans. In another study, proposed by Ampasavate, Okonogi and Anuchapreeda (2010), mangosteen peels were shown to contain an agent with cytotoxic potential in PBMC cells.

Overall, our present results, together with previous reports on phenolic fruit extracts, show that phenolic extracts can exert different effects on different types of cells and, therefore, their use must be cautiously monitored. Thus, notwithstanding their health-promoting properties, as already reported in the previous sections, phenolic compounds need to be studied in greater depth, since their use in food supplements has increased, and there is no legislation that regulates the maximum concentration allowed.

3.10. Pupunha flour incorporation into a yogurt: effects on in vitro protein digestion

Fig. 4 presents the *in vitro* protein digestion results, as inferred from the release of free NH_2 groups, for yogurt with or without incorporated pupunha flour (25 %; w/w). The results obtained with the control yogurt (Y) are typical of what is classically observed during *in vitro* digestion of foods: a small extent of protein hydrolysis during the gastric phase followed by more rapid and extensive reaction during the small intestinal phase (Brodkorb et al., 2019). In the presence of PF in yogurt (Y + PF), a clear inhibition of protein digestion was observed, particularly in the intestinal phase, as the release of NH_2 groups in the reaction medium was significantly lower than that in the control yogurt (Y).



PF-01	PF-02	PF-03	PF-04	PF-05	PF-06
0.8779	0.8587	1.006	0.9610	0.8848	1.052

Fig. 3. Results of cytotoxicity assay using the phenolic extract of pupunha flour (PF) samples; values highlighted for each PF batch refer to the results found for the IC_{50} index of the extracts.

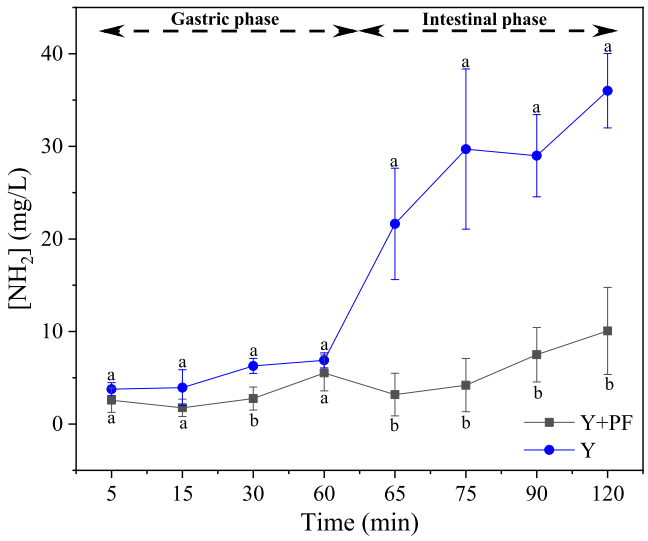


Fig. 4. Concentrations of free NH_2 groups during digestion of the control yogurt (Y) and yogurt containing 25 % (w/w) pupunha flour (Y + PF); Means with equal letters at a given time do not differ significantly, according to Duncan's test ($p > 0.05$).

It is unknown whether polyphenols in pupunha flour or some compounds inhibit the digestive enzymatic action. Rojas-Garbanzo et al. (2011) reported the presence of an anti-nutritional factor in pupunha flour but did not identify it. An earlier study reported that the aqueous extract of pupunha flour inhibited the action of proteolytic enzymes and attributed this effect to a low molecular weight component (Kroneberg et al., 1983).

The observed inhibition could also be explained, at least part, by the direct influence of phenolic compounds on digestive proteases. Although this topic has been extensively discussed in the available literature, the conclusions are still contradictory. Tagliazucchi, Verzelloni and Conte (2005) studied the effect of some beverages and isolated phenolic compounds on pepsin activity during pork digestion, and found that the isolated phenolic compounds favored the digestion process of the evaluated food matrix. The authors also reported that dealcoholized red wine, green tea, and pure phenolics EGCG, resveratrol, catechin, and quercetin could increase the concentration of peptides during the digestion of pork.

In contrast, Lamothe et al. (2014) demonstrated that green tea polyphenols can inhibit protease activity and lead to a lower rate of proteolysis during digestion of three distinct dairy products (milk, yogurt, and cheese). Other reports, mostly related to the presence of tannins, have suggested that phenolics act as protein binding agents that limit digestion (He et al., 2007; Quesada et al., 1996). The effect of three phenolic acids (p-cumalic acid, caffeic acid, gallic acid and chlorogenic acid) on the digestion of β-conglycinine (7S), an important soy protein, was evaluated, showing that there was no positive or negative effect on protein digestion (Gan et al., 2016). Finally, a recent review concluded that there is no consensus on the influence of tannins on protein digestibility, since there is evidence that such molecules can denature substrates and facilitate the action of proteases, but can also bind to substrates and prevent access to the catalytic site of digestive enzymes (Morzel et al., 2022). Therefore, it appears that the impact of

polyphenols on protein digestion is highly dependent on their nature, structure, and concentration and may also differ according to the studied food.

Based on evidence found by other authors, it is suggested that the tannins in pupunha flour may act as anti-nutritional factors, although the literature related to the fruit and/or pupunha flour has not identified the presence of these compounds (Felisberto et al., 2020; Kroneberg et al., 1983; Martínez-Girón et al., 2017; Rojas-Garbanzo et al., 2011, 2012).

4. Conclusion

Information about unconventional under-utilized fruit, as pupunha, as well as the development of new forms of use with longer and more efficient shelf life, such as flour, has allowed the development of products with health appeals. The present study provides data on many different physicochemical characteristics of pupunha flour, which will contribute to the literature on this scarcely studied product. In addition, this study is the first to demonstrate that the phenolic extract of pupunha flour may pose risks to cellular metabolism as well as to the gastrointestinal digestion of yogurt proteins, suggesting that the amount of flour incorporated into the food should be carefully considered. In addition, although the flours showed significant differences in relation to centesimal composition, elemental composition, and phenolic compound contents, they all provided similar results to the cytotoxicity test. This reinforces the idea that attention must be given to the rampant use of foods with functional appeal related to only one aspect, such as antioxidant capacity.

There is a need for new studies that evaluate the different ways to obtain and process flour to verify whether such conditions can positively impact the quality parameters, mainly the cytotoxic aspects and the impact on protein digestion, as well as to verify its effect on the total phenolic content. As emphasized in other analyses in this study, pupunha flour has technological properties that can vary depending the harvest location; thus, it can be applied to a multitude of products.

CRediT authorship contribution statement

Y.J.S. Santos: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **W.M. Facchinatto:** Methodology, Validation, Formal analysis. **A.L. Rochetti:** Methodology, Formal analysis, Writing – review & editing. **R.A. Carvalho:** Methodology, Validation, Writing – review & editing. **S. Le Feunteun:** Methodology, Validation, Writing – review & editing. **H. Fukumasu:** Methodology, Validation, Writing – review & editing. **M. Morzel:** Methodology, Validation, Writing – review & editing. **L.A. Colnago:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision. **F.M. Vanin:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision, Visualization, Project administration, Funding acquisition.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134888.

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