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ORIGINAL PAPER

Anthocyanins, Phenolic Acids and Antioxidant Properties of Juçara Fruits (*Euterpe edulis* M.) Along the On-tree Ripening Process

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Abstract Juçara (*Euterpe edulis* M.) fruits are an interesting source of phenolic compounds, mainly anthocyanins, making them valuable to the food and pharmaceutical industries. Juçara fruits were harvested along the on-tree ripening process between March and June as practiced in Paraná state, Brazil and examined for their total anthocyanin content (TAC), total phenolic content (TPC), total phenolic acid (TPA) and total antioxidant capacity (TAA). Overall, TAC increased (91.52–236.19 mg cyanidin-3-glucoside equivalent/100 g dm) whereas TPC (81.69–49.09 mg GAE/g dm) and TPA (44.27–30.95 mg/100 g dm) decreased during ripening of juçara fruits. Use of tandem mass spectrometry allowed the identification of cyanidin-3,5-diglucoside, peonidin-3-glucoside and peonidin-3-rutinoside for the first time in juçara fruits. The analysis of the phenolic acids by HPLC-MS/MS indicated the presence of gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, sinapinic and ferulic acids. The high antioxidant capacity using DPPH radical scavenging capacity (655.89–745.32 μmol TE/g dm) and ORAC assays (1088.10–2071.55 μmol TE/g dm) showed that juçara fruits have potential as a source of novel natural antioxidants for disease prevention and health promotion, and also as natural food additives for developing new functional food products.

Keywords Juçara · *Euterpe edulis* M · Fruit ripening · Antioxidant capacity · Mass spectrometry

Introduction

Juçara (*Euterpe edulis* Martius, Arecaceae) is a palm tree widely distributed in the Atlantic Forest. It is found mainly in the Brazilian states of the Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais and Bahia [1]. Juçara palm tree produces a non-climacteric round fruit which grows in bunches and has a pericarp covering a hard seed. The berries are small with a diameter of about 1 to 1.5 cm and the seed constituting 85 % of the fruit. During ripening, the epicarp evolves from green to dark purple or almost black. Juçara is usually used in the form of pulp or juice, whereby the pericarp is macerated and mixed with different amounts of water and separated from seeds. The process results in a liquid that is creamy with an intense dark purple color and characteristic flavor.

Berry fruits may be one of the components of healthy diets because they contain a wide variety of phenolic compounds. The phenolics contribute to the protection against degenerative diseases, and their effects on health have been mostly attributed to their antioxidant properties [2, 3]. Anthocyanins are an important group of phenolics in juçara fruits; they are responsible for their pigmentation. Moreover, anthocyanins are interesting for their use as natural water-soluble colorants

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thereby reducing the use of synthetic colorants in foods [4]. As reported by several authors [5–8], anthocyanins and antioxidant activity have been quantified in juçara fruits. However, no reports are available on the levels of phenolic compounds and antioxidant activity during ripening of juçara fruit. In general, the ripening process of fruits involves biochemical and metabolic changes of primary and secondary compounds, resulting in nutritional, palatable and potentially health-promoting phytochemicals. Since fruit maturation has impact on its commercialization and on human nutrition, it is important to investigate changes in bioactive compounds occurring during maturation of juçara fruits. Thus, the aim of this study was to evaluate anthocyanins, phenolic acids, and total antioxidant capacity of juçara fruits (*Euterpe edulis*) along the on-tree ripening process.

Materials and Methods

Plant Materials Juçara fruits were harvested at the experimental station of the Agricultural Research Institute of Paraná at Antonina City, Parana, Brazil. Fruits were harvested every two weeks between March and June 2012 and sorted into three stages of maturation according to external colour. Fruits of each harvesting stage (HS) were processed separately, following the specifications that are usually applied by the industry. After reception, the fruits were selected, washed, weighed, and sanitized in chlorinated water. The subsequent maceration step consisted of steeping the fruits in hot water (50 °C) for 30 min to facilitate the separation of the exo- and mesocarp from the seeds. This separation was carried out in a juçara specific extractor by adding water in a proportion of 0.6 l/kg fruit. Then, the obtained pulp was freeze-dried, milled to pass through 0.5 mm screen and stored at −20 °C prior to extraction. Samples were defatted with hexane (3×10 ml) using sonication for 30 min and centrifuged at 8,500 × *g* ($RC6^+$ Sorvall Instruments, Newtown, CT, USA) for 15 min at 20 °C and the moisture content of samples was determined by AOAC official method 925.10 [9] before extraction of compounds.

Preparation of Extracts for Analysis Defatted juçara powder (1 g) was extracted three times with a total of 15 mL methanol: 1M HCl (85:15, v/v) under dark conditions using a mechanical shaker (G-33, New Brunswick Scientific, Enfield, CT, USA) at room temperature, and then centrifuged at 3,500 × *g* ($RC6^+$, Sorvall Instruments, Newtown, USA) for 30 min at 12 °C. The supernatant was separated and used as a crude extract to determine total anthocyanin content, total phenolic content, and total antioxidant capacity, and also for the determination of anthocyanin by high-performance liquid chromatography coupled with Q-TOF mass spectrometry (HPLC-MS/MS). Defatted juçara powder were subjected to alkaline and acid hydrolysis for determination of their phenolic acid composition by HPLC-MS/MS according to a previous method [10].

Determination of Total Anthocyanin Content (TAC) and Total Phenolic Content (TPC) TAC was determined by using a spectrophotometric pH differential method [11] and the results were expressed as milligrams of cyanidin-3-glucoside equivalents (mg C3G) per 100 g dry matter (dm). TPC was measured using a colorimetric Folin-Ciocalteau method [12]. Gallic acid was used as a standard and results expressed as milligrams of gallic acid equivalents (mg GAE) per g dry matter (dm).

Radical Scavenging Assays The DPPH radical scavenging capacity of crude extracts was measured according to a method previously reported [13]. The oxygen radical absorbance capacity (ORAC) of crude extract was measured following the method described by Huang et al. [14]. The results were expressed as μmoles of Trolox equivalents (TE) per g dry matter (dm).

HPLC-MS/MS Determination The chromatographic separation of anthocyanins was carried on a HPLC (Waters 2695) system equipped with a photodiode array detector (Waters 996) and autosampler (Waters 717 plus) (Waters Corporation, Milford, MA, USA). The analytical column used was a Gemini 5 μm RP-18 (150 mm×4.6 mm) (Phenomenex, Torrance, CA, USA). A 10-μl volume of each sample crude extract was injected for analysis. The anthocyanin composition was eluted with a gradient mobile phase consisting of A (0.1 % formic acid in water) and B (0.1 % formic acid in methanol). The flow rate was 0.5 mL/min. A linear gradient was programmed as follows: 0–5 min, 10 % B; 5–8 min, 10–15 % B; 8–10 min, 15–20 % B; 10–13 min, 20–25 % B; 13–18 min, 25–30 % B; 18–25 min, 30–35 % B; 25–30 min, 35–45 % B; 30–33 min, 45–60 % B; 33–35 min, 60–95 % B; 35–42 min, 95 % B; 42–44 min, 95–10 % B; 44–50 min, 10 % B. The quadrupole time-of-flight mass spectrometer (Q-TOF-MS) (Micromass, Waters Corp., Milford, MA, USA) was calibrated by using sodium iodide for positive mode through the mass range of 100–1500. MS parameters were set as follows: capillary voltage: 2100 V, sample cone voltage: 30 V, source temperature: 120 °C, desolvation temperature: 250 °C, desolvation gas (nitrogen gas) flow rate: 900 l/h. The MS/MS spectra were acquired by using collision energy of 30 V.

The HPLC–MS/MS system for analysis of phenolic acids was the same model as used above for anthocyanin analyses. However, a Symmetry 5 μm C-18 column (250 mm×4.6 mm)

was used to separate the phenolic acids. The mobile phase consisted of A (1 % acetic acid in water) and B (1 % acetic acid in acetonitrile), and the flow rate was 0.7 ml/min. The gradient was as follows: isocratic elution: 0–5 min, 5 % B, linear gradient: 5–17 min, 5–15 % B, linear gradient: 17–40 min, 15–20 % B, linear gradient: at 40–60 min, 20–50 % B, isocratic elution: 60–65 min, 50 % B, linear gradient: at 65–67 min, 50–5 % B, and isocratic elution: 67–70 min, 5 % B before the next injection. A T-split was used to reduce the flow before the sample was introduced to the mass spectrometer. The contents of phenolic acids were quantified using external calibration curves. The Q-TOF-MS was calibrated by using sodium iodide for negative mode through the mass range of 100–1000. MS parameters were set as follows: capillary voltage: 700 V, sample cone voltage: 30 V, source temperature: 150 °C, desolvation temperature: 300 °C, desolvation gas (nitrogen gas) flow rate: 900 l/h. The MS/MS spectra were acquired by using collision energy of 20 V.

Statistical Analysis All the experiments were performed in triplicate, and the results were reported as mean ± SD (standard deviation). Data were subjected to one-way analysis of variance for comparison of means using SAS Software 9.3 version (SAS Institute Inc., Cary, NC, USA) and significant differences were calculated according to Tukey test at the 5 % level. Correlations among data obtained were calculated using Pearson's correlation coefficient (r).

Results and Discussion

Total Phenolic Content (TPC) Phenolic acids and anthocyanins were the major phenolic compounds analyzed in this study. The TPC of juçara fruits from different harvesting stages are shown in Table 1. Fruit TPC was 81.69 mg GAE/g dm at the harvesting stage 1 (HS1) and decreased to 49.09 mg GAE/g dm at the end of the ripening period (HS6), although there were non-linear changes to HS3 and HS4. This result was, probably, influenced by environmental and physiological factors. Meteorological data (Online Resource 1) provided by Simepar Technological Institute, Curitiba, Paraná, Brazil, corresponding to the experimental period indicated a continuous increase in the rainfall registered from the onset of the experiment (average monthly rainfall of 3.19 mm in March, 4.46 mm in April, 6.64 mm in May and 8.66 mm in June). Thus, the rainfall may have diluted the cellular juice and attenuated the TPC of juçara fruits. On the other hand, TPC can be influenced by anthocyanin synthesis since it usually decreases as anthocyanin accumulates [15].

Antioxidant Capacity There is growing interest in the possibility that consuming a diet rich in antioxidants may reduce the risks of many common chronic diseases [16]. The TAA of juçara fruits measured using DPPH radical scavenging capacity and ORAC assays are shown in Table 1. DPPH ranged from 655.89 to 745.32 μmol TE/g dm. Among the harvesting stages, HS5 had the highest DPPH radical scavenging activity whereas HS2 and HS4 were similar but differed significantly from HS3 and HS6. The ORAC values ranged from 2071.55 to 1088.10 μmol TE/g dm (Table 1). Fruits from HS1 had the lowest ORAC values. No significant difference in ORAC values was observed for HS4 and HS5. The antioxidant activities measured by DPPH and ORAC assays did not show the same trend from HS1 to HS6, due to the variations in response by the antioxidant constituents of the juçara fruit to the radical sources (peroxyl radical or DPPH radical) in the assays [17]. Juçara pulp was found to have a relatively high antioxidant capacity with respect to other anthocyanin rich fruits such blackberries [18] and açai [19]. Overall, the TAA of juçara fruits increased significantly with maturation while TPC decreased. A negative correlation ($-0.4 \leq r \leq -0.7$) was found between TPC and antioxidant capacity using DPPH and ORAC assays. Although the extract of HS1 showed the highest TPC, it did not exhibit the highest TAA. The same levels of phenolics

Table 1 Total phenolic content and antioxidant capacity by DPPH radical scavenging capacity (DPPH) and ORAC assays in juçara fruits at six harvesting stages

	Total phenolic content (mg GAE/g dm)	Antioxidant capacity (μmol TE/g dm)	
		DPPH	ORAC
HS1: March 29th—immature violet	81.69 ± 2.16^{A}	655.89 ± 5.50^{D}	1088.10 ± 10.26^{E}
HS2: April 12th—mature purple	67.31 ± 0.88^{B}	726.30 ± 8.70^{B}	1178.48 ± 15.93^{D}
HS3: April 26th—mature purple	54.53 ± 3.80^{C}	709.51 ± 4.53^{C}	1564.20 ± 13.37^{B}
HS4: May 10th—mature purple	55.37 ± 2.42^{C}	724.92 ± 1.35^{B}	1266.36 ± 25.40^{C}
HS5: May 24th—ripe dark purple	52.12 ± 1.54^{C}	745.32 ± 5.32^{A}	1285.09 ± 22.59^{C}
HS6: June 7th—ripe dark purple	49.09 ± 3.32^{C}	703.32 ± 1.21^{C}	2071.55 ± 37.03^{A}

Values in the same column with different letters are significantly different ($p<0.05$)

Table 2 Total anthocyanin content (TAC) and anthocyanin composition in juçara fruits at six harvesting stages

	$[M+H]^+$ product ion m/z	HS1	HS2	HS3	HS4	HS5	HS6
TAC[a]	–	91.52±3.46^{E}	95.67±2.89^{E}	122.68±1.14^{C}	113.56±2.26^{D}	236.19±3.88^{A}	210.42±1.75^{B}
C3,5dG[a]	611/287	0.17±0.01^{B}	0.17±0.02^{B}	0.19±0.01^{B}	0.11±0.01^{C}	0.18±0.01^{B}	0.33±0.02^{A}
C3G[a]	449/287	29.09±0.17^{E}	33.94±0.21^{D}	30.51±0.64^{E}	42.95±0.15^{C}	85.61±0.51^{B}	108.97±1.07^{A}
C3R[b]	595/287	43.92±0.24^{E}	42.77±0.68^{E}	74.26±0.67^{D}	76.59±0.82^{C}	137.27±1.22^{A}	87.08±0.81^{B}
P3R[a]	579/271	nd	0.04±0.01^{A}	0.06±0.01^{A}	nd	nd	0.07±0.03^{A}
PN3G[a]	463/301	0.33±0.02^{B}	0.38±0.01^{B}	0.42±0.01^{B}	1.27±0.06^{A}	1.21±0.02^{A}	1.24±0.03^{A}
PN3R[a]	609/301	0.47±0.01^{C}	0.55±0.03^{C}	0.71±0.04^{B}	0.46±0.01^{C}	0.81±0.05^{AB}	0.83±0.06^{A}

[a] The results are represented as mg C3G/100 g dm; [b] The results are represented as mg C3R/100 g dm; nd: Not significant quantity; Values in the same row with different letters are significantly different ($p<0.05$)

do not necessarily correspond to the same antioxidant responses, due to the fact that antioxidant properties of single compounds within a group can vary remarkably [20]. Our results suggest that anthocyanins may be a major contributor to the TAA in juçara fruits in addition to other phytochemicals.

Total Anthocyanin Content (TAC) and Anthocyanin Composition TAC of juçara fruits at six harvesting stages is shown in Table 2 ranging from 91.52 to 236.19 mg C3G equivalent/100 g dm. Fruits from HS5 had the highest TAC and fruits from HS3 showed higher TAC than that of fruits from HS4 ($p<0.05$), which suggests a transient accumulation of anthocyanins in juçara fruits. Juçara palm tree grows in open fields under sun irradiation and temperature effects; moreover, fruits located in the external parts of the bunch receive more sunlight than those located under the shade. Thus, the variations in TAC observed among the harvesting stages can be attributed to genetic factors and environmental conditions as growth temperature and light intensity [21]. The results showed a positive correlation ($0.4\leq r\leq 0.7$) between TAC and DPPH and ORAC activities for juçara fruits. Anthocyanin composition as detected by HPLC-QTOF-MS/MS is displayed in Table 2 and Fig. 1a. Two main peaks were detected comprising cyanidin-3-glucoside (C3G) (RT=21.65 min) and cyanidin-3-rutinoside (C3R) (RT=23.03 min). Low levels of cyanidin-3,5-diglucoside (C3,5dG) (RT=20.73 min), pelargonidin-3-rutinoside (P3R) (RT=24.47 min), peonidin-3-glucoside (PN3G) (RT=25.17 min), and peonidin-3-rutinoside (PN3R) (RT=26.40 min) were also found in juçara fruits. C3,5dG, PN3G and PN3R were identified in juçara fruits for the first time. C3G ranged from 29.09 to 108.97 mg/100 g dm and C3R ranged from 42.77 to 137.27 mg/100 g dm. C3R was significantly higher than C3G at HS5, and C3G was significantly higher than C3R at HS6. Brito et al. [5] identified six anthocyanins in juçara fruits from São Paulo state, Brazil;

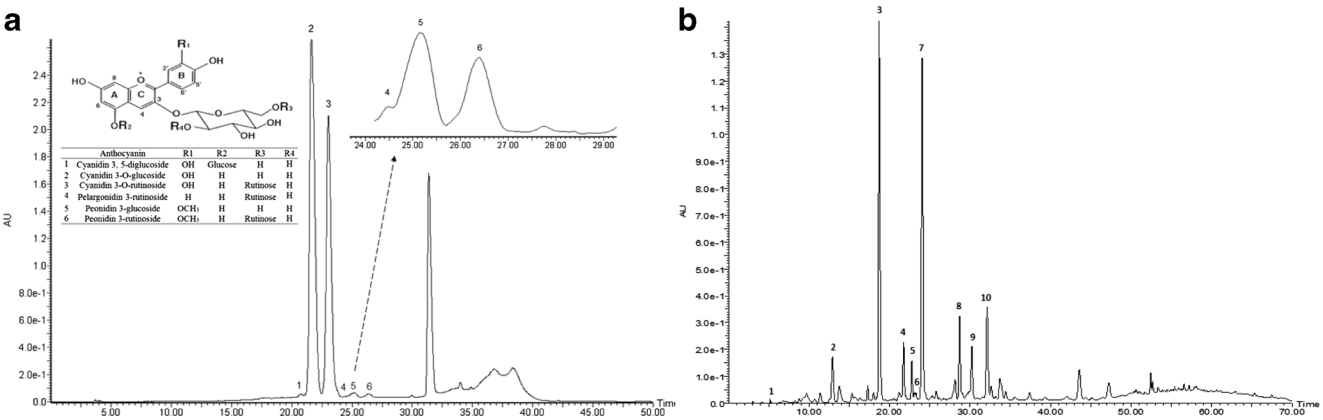

Fig. 1 **a** The chromatogram of anthocyanins at HS6 (detected at 520 nm), compounds were identified as follow: 1A = cyanidin 3, 5-diglucoside, 2A = cyanidin 3-O-glucoside, 3A = cyanidin 3-O-rutinoside, 4A = pelargonidin 3-rutinoside, 5A = peonidin 3-glucoside, 6A = peonidin 3-rutinoside. **b** The chromatogram of phenolic acids at HS6 (detected at 280 nm), compounds were identified as follow: 1B = gallic acid, 2B = protocatechuic acid, 3B = *p*-hydroxibenzoic acid, 4B = vanillic acid, 5B = chlorogenic acid, 6B = caffeic acid, 7B = syringic acid, 8B = *p*-coumaric acid, 9B = sinapinic acid, 10B = ferulic acid

Table 3 The phenolic acids composition in juçara fruits at six harvesting stage

	$[M–H]^-$ product ion m/z	HS1[a]	HS2[a]	HS3[a]	HS4[a]	HS5[a]	HS6[a]
Gallic acid	169/125	0.41 ± 0.02^{DE}	0.65 ± 0.08^{BC}	0.98 ± 0.11^{A}	0.75 ± 0.03^{B}	0.53 ± 0.05^{CD}	0.31 ± 0.02^{EF}
Protocatechuic acid	154/109	2.59 ± 0.14^{C}	2.64 ± 0.17^{C}	2.54 ± 0.13^{C}	3.20 ± 0.11^{B}	3.73 ± 0.23^{A}	3.80 ± 0.18^{A}
p-hydroxybenzoic acid	137/93	14.75 ± 0.21^{A}	14.72 ± 0.19^{A}	10.29 ± 0.11^{B}	9.92 ± 0.08^{B}	7.16 ± 0.14^{C}	6.47 ± 0.18^{D}
Vanillic acid	167/152	2.96 ± 0.12^{C}	2.91 ± 0.14^{C}	2.66 ± 0.18^{C}	3.49 ± 0.21^{B}	3.88 ± 0.11^{AB}	3.97 ± 0.09^{A}
Chlorogenic acid	353/191	2.51 ± 0.06^{A}	2.48 ± 0.10^{A}	1.85 ± 0.08^{B}	1.65 ± 0.09^{B}	1.31 ± 0.01^{C}	1.33 ± 0.02^{C}
Caffeic acid	179/135	0.85 ± 0.02^{A}	0.84 ± 0.03^{A}	0.59 ± 0.02^{B}	0.38 ± 0.01^{C}	0.36 ± 0.02^{C}	0.21 ± 0.01^{D}
Syringic acid	197/182	9.84 ± 0.15^{A}	8.59 ± 0.13^{B}	7.52 ± 0.09^{C}	7.55 ± 0.14^{C}	6.57 ± 0.11^{D}	4.70 ± 0.12^{E}
p-coumaric acid	163/119	3.11 ± 0.18^{A}	2.82 ± 0.13^{A}	2.40 ± 0.12^{B}	2.28 ± 0.06^{B}	1.35 ± 0.02^{C}	1.03 ± 0.01^{D}
Sinapinic acid	222/178	2.61 ± 0.11^{CD}	2.79 ± 0.14^{BC}	2.44 ± 0.07^{D}	2.99 ± 0.05^{AB}	3.16 ± 0.12^{A}	3.22 ± 0.09^{A}
Ferulic acid	193/149	4.62 ± 0.13^{B}	3.54 ± 0.12^{C}	3.63 ± 0.08^{C}	4.52 ± 0.09^{B}	5.61 ± 0.15^{A}	5.92 ± 0.11^{A}
Total		44.27 ± 1.17^{A}	41.99 ± 1.24^{A}	34.92 ± 1.02^{BC}	36.74 ± 0.79^{B}	33.67 ± 0.98^{CD}	30.95 ± 0.86^{D}

[a] The results are represented as mg/100 g dm; Values in the same row with different letters are significantly different ($p<0.05$)

three of them (cyanidin 3-sambubioside, pelargonidin 3-glucoside and cyanidin 3-rhamnoside) were different to those found in our study, suggesting the influence of planting location, even different metabolic pathways between fruits cultivated in São Paulo and Paraná states.

Total Phenolic Acids (TPA) The phenolic acids can be divided into two classes, derived from benzoic and cinnamic acids, respectively. In this study, hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic) were quantified in substantially higher amounts than hydroxycinnamic acids (chlorogenic, caffeic, *p*-coumaric, sinapinic and ferulic) (Table 3, Fig. 1b) but their concentrations decreased during ripening with the exception of protocatechuic and vanillic acids. TPA of juçara fruits decreased from HS1 to HS6 (44.27–30.95 mg/100 g dm). Some phenolic acids are bound to the cell walls and their subsequent decrease throughout the maturation is due to polymerization, oxidation and conjugation reactions [22]. In general, *p*-hydroxybenzoic acid was the predominant form of phenolic acids (6.47–14.75 mg/100 g dm) for all harvesting stages. Syringic and ferulic acids, respectively, were the second and third major phenolic acids in juçara fruits. A negative correlation ($-0.5\leq r\leq -0.8$) was found between TPA content and DPPH and ORAC values. The QTOF-MS/MS was used to confirm the identity of the gallic (RT= 5.80 min), protocatechuic (RT=13.10 min), *p*-hydroxybenzoic (RT=18.80 min), vanillic (RT= 21.80 min), chlorogenic (RT=22.90 min), caffeic (RT= 23.20 min), syringic (RT=24.30 min), *p*-coumaric (RT= 28.40 min), sinapinic (RT=30.10 min) and ferulic acids (RT=32.30 min) (Table 3, Fig. 1b). In a recent study, the juçara extract showed to have protective effect against TBH-induced oxidative stress in the Vero cells in a dose-dependent manner [23]. However, toxicological evaluation of juçara in human, animal and cell culture models indicated that the pulp of *Euterpe edulis* contains compounds with the capacity to induce mutagenicity and clastogenic/aneugenic effects [24].

Conclusions

The quantification and identification of phenolic compounds in juçara fruits along the on-tree ripening process were evaluated by various chemical assays for the first time. It was observed that TAC and TAA increase through ripening. The results suggest that juçara fruits offer higher levels of potentially health-promoting compounds from late May to early June, the optimal harvesting stage according to this study. The demonstration of high anthocyanin content and high antioxidant activity confirms juçara fruit as promising and an excellent source of dietary phytochemicals.

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Conflict of Interest None.

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