

The powerful *in vitro* bioactivity of *Euterpe oleracea* Mart. seeds and related phenolic compounds

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Abstract

The *Euterpe oleracea* Mart. (açai) is a plant from the Amazon region, classified as "super fruit" because of its various functional properties. However, limited investigation has been performed on açai by-products, such as seeds. Therefore, the aim of this work was to characterize the phenolic compounds of the aqueous extract of açai seeds and further evaluate its bioactivity (antioxidant and cytotoxic activities). Only proanthocyanidins were detected, being a B-type (epi)catechin tetramer the most abundant; however, procyanidin trimers were the most predominant form. Açai seeds extract revealed a high antioxidant (EC_{50} ranging from 3.6 to 19.4 $\mu\text{g/mL}$) and cytotoxic activity, being more effective in the cervical carcinoma cell line (HeLa; $GI_{50} = 18 \mu\text{g/mL}$); it did not show toxicity for non-tumor cells. Açai seeds are considered a waste and could have an added economic benefit, through the extraction of natural antioxidants, particularly proanthocyanidins, that could find applications in food and pharmaceutical industries.

Keywords: *Euterpe oleracea*; Phenolic characterization; Antioxidant properties; Antitumor activity.

1. Introduction

The *Euterpe oleracea* Mart. is a plant in the Amazon region popularly known as açai and has high nutritional and functional value. It is currently classified as "super fruit" because of its antioxidant capacity, anti-inflammatory effects, hypocholesterolemic and antitumor activity (Stoner, 2009; Costa et al., 2012; Kang et al., 2012; Souza, 2012).

Most of the beneficial effects of açai are attributed to the phenolic compounds present in the fruit, being rich in anthocyanins, but also in proanthocyanidins that are present in the monomeric form (catechin and epicatechin) or oligomeric procyanidins (Kang et al., 2011). The antioxidant mechanism of flavonoids involves the direct elimination or extinction of reactive oxygen species and also inhibition of oxidative enzymes that generate these species (Kang et al., 2012).

Kang et al. (2011) identified five flavonoids in the pulp of açai and concluded that velutin acts as a modulator of NF- κ B, which plays a central role in the innate immune response in humans and chronic inflammatory conditions. Furthermore, the authors state that these flavonoids have anti-inflammatory activity capable of acting on both the proliferative inflammation as exudative phase, and thus playing an important role in protecting against atherosclerosis. Moreover, Rocha et al. (2007) suggest that diet rich in polyphenols can participate in a preventive way against cardiovascular disease, an effect from the antioxidant activity that increases the bioavailability of nitric oxide or hypotensive and vasodilatory properties of these compounds.

Other studies confirm the ability of açai pulp in inhibiting the production of reactive oxygen species (ROS) and activity of cyclooxygenase 1 and 2, in addition to inducing dependent endothelial vasodilation in rats. Furthermore, *in vivo* studies with healthy volunteers demonstrated the influence of açai pulp, in the considerable increase of antioxidants in plasma (Schauss et al, 2006; Rocha et al., 2007; Mertens-Talcott et al.,

2008). [Mulabagal and Calderon \(2012\)](#) conducted a study with açai powder, identifying bioactive compounds such as fatty acids, anthocyanins and other polyphenols used in dietary supplements.

Nevertheless, the studies available in literature focus on the fruit and limited investigation has been performed on açai seeds. [Silva et al. \(2014\)](#) conducted a study with the açai fruit including the seeds, evaluating the cytotoxic effects in malignant cell lines and concluded that the seeds present a higher polyphenols content compared to the pulp being its antimutagenic potential was also higher. The same authors have warned of the need for further studies to characterize the compounds responsible for these effects. Therefore, the aim of this study was to fully characterize the phenolic composition of açai seeds and further evaluate the antioxidant properties and cytotoxic effects.

2. Materials and methods

2.1. Samples and extracts preparation

The fruits of *Euterpe oleracea* Mart. (local name: açai) were purchased in January 2014 in a local supermarket of the city Belém - Pará – Brazil. For preparation of the extracts, the epicarp and endocarp were removed and the seeds were macerated with distilled and deionized Milli-Q water for 30 minutes at 25°C. The samples were centrifuged for 30 min at 50000 g at 4 °C (Thermo Scientific - HeraeusMultifuge X1R, MA,USA). The supernatant was collected and submitted to lyophilization for 96 hours at -101°C, 23mHg (Liotop modelo K105, SP, Brazil).

2.2. Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). The phenolic compound standards were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Foetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St Louis, MO USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Characterization in phenolic compounds

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C₁₈, 3 µm (4.6 mm × 150 mm) column thermostatted at 25 °C was used. The solvents used were: (A) 2.5% acetic acid in water, (B) HPLC-grade acetonitrile. The elution gradient established was 0% B for 5 min, from 0 to 10% B for 35 min, from 10 to 14.5 % B for 5 min, from 14.5 to 19% B for 10 min, from 19% to 55% B for 10 min, isocratic 80% B for 3 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -40 V, entrance potential (EP) -7 V, collision energy (CE) -20V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -40 V, EP -10 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between m/z 100 and 1800 (Guimarães et al., 2013).

The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (1-100 µg/ml) for each available phenolic standard: (+)-catechin ($y=131.65x+4.1144$; $R^2=1$) and (-)-epicatechin ($y=150.77x-16.092$; $R^2=1$). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in mg per g of extract.

2.4. Evaluation of bioactive properties

2.4.1. *General.* Aqueous extracts (prepared according with the previous section) were re-dissolved in water (1 mg/mL for antioxidant activity evaluation and 8 mg/mL for cytotoxicity screening). The final solutions were further diluted to different concentrations to be submitted to the following *in vitro* assays.

2.4.2. *Antioxidant activity.* DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{\text{DPPH}} - A_{\text{S}}) / A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+} , measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β -carotene bleaching was evaluated through the β -carotene/linoleate assay; the neutralization of linoleate free radicals avoids β -carotene bleaching, which is measured by the formula: $(\beta\text{-carotene absorbance after 2h of assay} / \text{initial absorbance}) \times 100$. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B) / A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2013). Trolox was used as positive control and the results were expressed as EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) in $\mu\text{g per mL}$ of extract.

2.4.3. Cytotoxicity in human tumor cell lines. Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460 HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO₂. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. Sulforhodamine B assay was performed according to a procedure previously described by the authors ([Barros et al., 2013](#)). Ellipticine was used as positive control and the results were expressed as GI₅₀ values (sample concentration that inhibited 50% of the net cell growth) in µg per mL.

2.4.4. Cytotoxicity in non-tumor liver cells primary culture. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure established by the authors ([Abreu et al., 2011](#)); it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Ellipticine was used as positive control and the results were expressed as GI₅₀ values (sample concentration that inhibited 50% of the net cell growth) in µg per mL.

3. Results and Discussion

3.1. Characterization in phenolic compounds

The HPLC phenolic profiles of *Euterpe oleracea* seeds extract was recorded at 280 nm, and shown in **Figure 1**. Compound characteristics and tentative identities are presented in **Table 1**. Twenty-two compounds were detected being all assigned as proanthocyanidins. These compounds were assigned based on their pseudomolecular ions and MS² fragmentation patterns, characterised by the formation of product ions from the cleavage of the interflavan bond and retro-Diels-Alder (RDA) and heterocyclic ring fissions (HRF) of the elementary flavan-3-ol units ([Friedrich et al., 2000](#); [Gu et al., 2003](#); [Dias et al., 2015](#)). Mass spectra do not allow, however, establishing the position of the linkage between flavanol units (*i.e.*, C4-C8 or C4-C6) nor differentiating between isomeric catechins (*e.g.*, catechin/epicatechin).

Compounds **9** and **17** were positively identified as (+)-catechin and (-)-epicatechin according to their retention time, mass and UV-vis characteristics by comparison with commercial standards. (+)-Catechin and (-)-epicatechin have been frequently reported in fruits of *Euterpe* species ([Pacheco-Palencia et al., 2009](#)).

Compounds **3**, **5**, **6**, **11**, **14**, **20** and **22** presented the same pseudomolecular ion [M-H]⁻ at *m/z* 577 and MS² fragmentation patterns coherent with B-type (epi)catechin dimers (*i.e.*, (epi)catechin units with C4-C8 or C4-C6 interflavan linkages). Characteristic product ions were observed at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152-18 mu), attributable to the HRF, RDA and further loss of water from an (epi)catechin unit, and at *m/z* 289 and 287, that could be associated to the fragments corresponding to the lower and upper (epi)catechin unit, respectively. Similarly, compounds **2**, **10**, **12** and **19** (pseudomolecular ions [M-H]⁻ at *m/z* 865), compounds **1**, **4**, **8**, **16**, **18** and **21** (pseudomolecular ions [M-H]⁻ at *m/z* 1153) and compounds **7**, **13** and **15** (pseudomolecular ions [M-H]⁻ at *m/z* 1441) can be assigned as B-type (epi)catechin trimmers and tetramers, and pentamers respectively. In all cases, fragmentation patterns

are coherent with those expected for these types of compounds, *i.e.*, similar to those observed for proanthocyanidins dimers but with additional fragments from the alternative cleavages of different interflavan bonds. Proanthocyanidins dimers and trimers have been reported in fruits of *Euterpe* species (Pacheco-Palencia et al., 2009). B-type (epi)catechin tetramer (compound **4**) was the most abundant proanthocyanidin and procyanidin trimers were the most predominate form (**Table 1**). In fruits there are several studies reporting the phenolic composition, mostly identifying phenolic acids, flavones, flavonols and anthocyanidins (Pacheco-Palencia et al., 2009; Kang et al., 2011; Gordon et al., 2012; Mulabagal & Calderón, 2012). As far as we have seen, Pacheco-Palencia et al. (2009) are the only authors reporting the presence of proanthocyanidins in fruits of açai. To our knowledge this is the first report describing the phenolic profile of açai seeds.

3.2. Bioactive properties

Four assays including DPPH radical scavenging activity, reducing power, inhibition of β -carotene bleaching and lipid peroxidation inhibition measured by the decrease in thiobarbituric acid reactive substances (TBARS) were applied to evaluate the antioxidant effects of açai aqueous extracts (**Table 2**). The reduction of the purple stable free radical DPPH to the yellow hydrazine is performed by trapping the unpaired electron, and the degree of discoloration indicating the scavenging activity of the samples (Amarowicz et al., 2004; Antolovich et al., 2002). The EC₅₀ value, obtained for this assay, for seeds of açai was 8.8 μ g/mL. The antioxidant activity of a constituent can be directly related to its reducing power, being this assay a reliable method to evaluate the antioxidant capacities of various compounds. High absorbance values at 690 nm indicate high reducing power. As shown in **Table 2**, açai extract presented an EC₅₀

value of 19.4 $\mu\text{g/mL}$. β -Carotene bleaching inhibition measures the capacity that an antioxidant can neutralize any free radical that is formed in the system (e.g, free radical linoleate), inhibiting the discoloration of β -carotene (Amarowicz et al., 2004). To further characterize the antioxidant ability, the β -carotene bleaching inhibition and TBARS assays were performed revealing the following EC_{50} values 107 and 3.6 $\mu\text{g/mL}$, respectively. The TBARS assay measures the malondialdehyde (MDA) formed from the oxidation of unsaturated fatty acids, afterwards the MDA reacts with thiobarbituric acid (TBA) to form a pink pigment (TBARS), the formation of TBARS in brain homogenates is a consequence of lipid peroxidation (Ng et al., 2000). Therefore, the low EC_{50} values (Table 2) obtained for this assay in the presence of açai seeds extract are very promising.

There are some reports that evaluate the antioxidant activities of açai fruits, but they cannot be compared to our results, due to the different antioxidant activity assays applied or the different expression of the results (Pacheco-Palencia et al., 2009; Kang et al., 2011 and 2012; Gordon et al., 2012). Nevertheless, to our knowledge there are no studies regarding antioxidant activity of açai seeds.

Açai seeds extract showed inhibitory effect on the growth of different human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and was more effective for cervical carcinoma cell line (HeLa, $\text{GI}_{50} = 18 \mu\text{g/mL}$) (Table 2). Furthermore, this extract did not show toxicity to non-tumor cells (primary cultures of liver cells, PLP2). To our knowledge, Silva et al. (2014), was the only report found in literature that performed cytotoxic effects using cell lines derived from breast and colorectal adenocarcinomas (Caco-2, HT-29, MDA-MB-468 and MCF-7) with different parts of açai, including its seeds. These authors concluded that an ethanol/water seed extract was significantly more effective against the MCF-7 cell line than total fruit and bark extracts.

Nevertheless, they refer that further studies are needed to identify the compounds responsible for this cytotoxic activity.

Overall, açai seeds are considered a waste and could have an added economic benefit, through the extraction of natural antioxidants, particularly in proanthocyanidins. The antioxidant and cytotoxic capacity of the seeds extract can be strongly dependent on the phenolic profile. However, it cannot be ruled out that other non-phenolic compounds might be involved in the antioxidant activity. In this study, we reported for the first time, the phenolic composition, antioxidant and cytotoxic properties of seeds of açai that can have potential applications in the food and pharmaceutical industry.

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS² fragment ions (in brackets, relative abundances), tentative identification and quantification of phenolic compounds in *Euterpe oleracea* seeds extract (mean \pm SD).

Compound	Rt (min)	λ_{max} (nm)	Molecular ion [M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification	Quantification (mg/g)
1	13.7	282	1153	865(15),863(15),577(8),575(10),289(8),287(18)	B-type (epi)catechin tetramer	4.1 \pm 0.2
2	14.2	280	865	739(9),713(13),695(10),577(30),575(13),425(14),407(29),289(5),287(22)	B-type (epi)catechin trimer	12.9 \pm 0.2
3	14.6	278	577	451(18),425(48),407(100),289(49),287(11)	B-type (epi)catechin dimer	11.5 \pm 0.6
4	14.9	280	1153	865(8),863(12),577(14),575(10),289(5),287(24)	B-type (epi)catechin tetramer	14.8 \pm 0.4
5	15.4	282	577	451(19),425(65),407(100),289(56),287(20)	B-type (epi)catechin dimer	3.6 \pm 0.7
6	16.1	280	577	451(17),425(33),407(100),289(83),287(21)	B-type (epi)catechin dimer	2.6 \pm 0.1
7	16.9	280	1441	865(36),287(14)	B-type (epi)catechin pentamer	10.8 \pm 0.1
8	17.1	280	1153	865(9),863(12),577(29),575(23),289(10),287(18)	B-type (epi)catechin tetramer	4.1 \pm 0.2
9	17.8	278	289	245(93),203(82),187(21),161(30),137(44)	(+)-Catechin	10.6 \pm 0.2
10	18.5	280	865	739(5),713(7),695(12),577(15),575(15),425(10),407(31),289(12),287(28)	B-type (epi)catechin trimer	6.9 \pm 0.3
11	19.4	282	577	451(22),425(34),407(100),289(45),287(7)	B-type (epi)catechin dimer	6.0 \pm 0.1
12	20.5	278	865	739(7),713(10),695(7),577(11),575(16),407(32),289(47),287(32)	B-type (epi)catechin trimer	7.0 \pm 0.4
13	21.2	280	1441	1153(5),865(10),577(5),289(8),287(6)	B-type (epi)catechin pentamer	6.7 \pm 0.1
14	22.2	280	577	451(26),425(64),407(100),289(70),287(11)	B-type (epi)catechin dimer	8.5 \pm 0.1
15	23.2	282	1441	1153(7),865(13),577(28),289(35),287(20)	B-type (epi)catechin pentamer	8.9 \pm 0.2
16	24.3	282	1153	865(4),863(7),577(5),575(4),289(3),287(12)	B-type (epi)catechin tetramer	6.8 \pm 0.1
17	26.1	280	289	245(77),203(52),187(16),161(34),137(24)	(-)-Epicatechin	8.6 \pm 0.1
18	28.1	282	1153	865(3),863(14),577(7),575(5),289(14),287(5)	B-type (epi)catechin tetramer	4.7 \pm 0.1
19	31.8	280	865	739(4),713(12),695(11),577(18),575(11),407(22),289(4),287(25)	B-type (epi)catechin trimer	6.1 \pm 0.2

20	33.4	278	577	451(30),425(90),407(80),289(100),287(30)	B-type (epi)catechin dimer	3.5 ± 0.1	
21	34.9	280	1153	865(21),863(7),577(14),575(21),425(14),289(21),287(42)	B-type (epi)catechin tetramer	5.0 ± 0.2	
22	35.8	282	577	451(8),425(54),407(92),289(62),287(8)	B-type (epi)catechin dimer	4.0 ± 0.1	
						Procyanidin monomer	19.1 ± 0.1
						Procyanidin dimer	39.8 ± 0.4
						Procyanidin trimer	41.8 ± 0.1
						Procyanidin tetramer	39.6 ± 0.1
						Procyanidin pentamer	17.5 ± 0.1
						Total proanthocyanidins	158 ± 1

Table 2. Antioxidant and citotoxicity of *Euterpe oleracea* seeds extract (mean \pm SD).

<i>Euterpe oleracea</i>	
Antioxidant activity (EC₅₀, $\mu\text{g/mL}$)	
DPPH scavenging activity	8.8 \pm 0.24
Reducing power	19.4 \pm 0.3
β -carotene bleaching inhibition	107 \pm 1
TBARS inhibition	3.6 \pm 0.1
Cytotoxicity to tumor cell lines (GI₅₀ values, $\mu\text{g/mL}$)	
MCF-7 (breast carcinoma)	72 \pm 3
NCI-H460 (non-small cell lung cancer)	22 \pm 1
HeLa (cervical carcinoma)	18 \pm 3
HepG2 (hepatocellular carcinoma)	24 \pm 1
Cytotoxicity to non-tumor cells (GI₅₀ values, $\mu\text{g/mL}$)	
PLP2	125 \pm 9

The antioxidant activity was expressed as EC₅₀ values, what means that higher values correspond to lower reducing power or antioxidant potential. EC₅₀: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC₅₀ values: 41 $\mu\text{g/mL}$ (reducing power), 42 $\mu\text{g/mL}$ (DPPH scavenging activity), 18 $\mu\text{g/mL}$ (β -carotene bleaching inhibition) and 23 $\mu\text{g/mL}$ (TBARS inhibition). GI₅₀ values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. Ellipticine GI₅₀ values: 1.21 $\mu\text{g/mL}$ (MCF-7), 1.03 $\mu\text{g/mL}$ (NCI-H460), 0.91 $\mu\text{g/mL}$ (HeLa), 1.10 $\mu\text{g/mL}$ (HepG2) and 2.29 (PLP2).

