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Original article

Obtaining and characterization of anthocyanins from *Euterpe oleracea* (açaí) dry extract for nutraceutical and food preparations

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ABSTRACT

The main constituents of the *Euterpe oleracea* Mart., Arecaceae, fruits (açaí) are anthocyanins. This paper aimed to standardize the extraction process and characterize an anthocyanin-rich dry extract obtained from this fruit. A 2^3 full factorial design was used. The volumes of ethanol 92% and acetic acid and the extraction time were used as factors. Total solids and anthocyanins content were used as feedback. The dry extract was obtained by freeze-drying. The content of anthocyanins was determined spectrophotometrically. Fourier Transform Infrared Spectroscopy, Differential Scanning Calorimeter, Thermogravimetry, Scanning Electron Microscopy, and Atomic Absorption Spectrometry were used for characterizingthe dry extract. The DPPH method was used for evaluating radical scavenging activity. The extraction conditions were established. The most influent factor was the volume of acetic acid. The dry extract moisture content was equal to 1.39 ± 0.25%, the evaporation residue 97.25 ± 1.28%, total ashes 0.62 ± 0.12%, and the anthocyanin content was 61.75 ± 3.28%. The elemental composition shows the presence of manganese 4.85 ppm, iron 1.62 ppm, zinc 0.05, copper 1.38 ppm, calcium 1.01 ppm, cadmium 0.003 ppm, nickel 0.37 ppm, and lead 0.38 ppm. The dried extract IC_{50} estimated by the radical scavenging assay with DPPH was 31.25 ± 2.31 ppm. The optimal extraction conditions were: the volume of ethanol 92%: 400 ml; volume of acetic acid: 75 ml; an extraction time: 4 h.

Introduction

Euterpe oleracea Mart., Arecaceae, is a Brazilian palm tree popularly known as açai. The fruit pulp of this species has been used for years as a dietary complement. A wide range of health-promoting and therapeutic benefits has been attributed to the fruit (Heinrich et al., 2011). Inhabitants from the North and Northeast of Brazil reported the ethnomedical use of the *E. oleracea* fruit pulp to prevent flu, fever, and pain (Matheus et al., 2006). The dark green oil from the fruit is an effective anti-diarrheal, while the grated fruit peel is topically used for skin ulcers (Schauss et al., 2006a). A potent antioxidant effect of *E. oleracea* fruits extract was associated with their chemical composition (Schauss et al., 2006b).

Anthocyanins are phenolic compounds presenting certain instability, making the extraction, storage, and processing complex procedures. Anthocyanins are sensitive to factors like temperature, light, and pH (Tonon et al., 2010). These compounds are usually extracted with polar solvents as water, acetone, or ethanol acidified with strong acids (Revilla et al., 1998). Thus, it was reported the phenolic extraction from these fruits using ethanol 70% and hydrochloric acids as the solvent (Pompeu et al., 2009). Nonetheless, mineral acids (i.e., HCl) can cause hydrolysis of the pendant acyl groups degrading the anthocyanin contain (Liazid et al., 2007). To avoid this, a more concentrated ethanol solution (less polarity) and, a weaker acid (e.g., acetic or phosphoric acid) have to be used (Bobbio et al., 2000).

Although this palm tree has attracted considerable attention because of its medicinal properties and a potent antioxidant activity, all beverages and dietary supplements on the market are prepared with the fruit pulp (Schauss et al., 2006a, 2006b; Schauss, 2016). Up to our knowledge, there is no report attempting to pro-

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duce and standardize an anthocyanin-rich extract from this plant fruit for its utilization in pharmaceuticals, nutraceutical and/or food preparations. Thus, systematic studies for developing a simple method for obtaining and characterizing an anthocyanins-rich extract from the fruits of *E. oleracea* are really needed. This aim of this paper was to establish optimal conditions for preparing an anthocyanin dry extract and its physicochemical characterization for its use in pharmaceutical, nutraceutical, and food preparations.

Material and methods

Vegetal material

Ripe fruits of açaí (*Euterpe oleracea* Mart., Arecaceae) were collected in the morning, in the Recanto Santa Clara, located at—0º57′37.4″N 51º16′06.4″W, Mazagão City, Amapá State, Brazil. A voucher of the species was deposited in the Herbarium of the Research Institute of Amapá, Brazil (HAMAB) with the number 10777.

Chemicals

Absolute ethanol and concentrated acetic acid were purchased from (Sigma–Aldrich®, USA), the absolute ethanol was diluted to 92% concentration. The other chemicals were pure for analysis purchased from Alphatec® (Brazil).

Design of experiments for the extraction process

For the extraction, a preliminary assay was made using ethanol 92% (600, 700, 800, and 900 ml), a less polar solvent than those used by other authors (Pompeu et al., 2009), acidified with 50 ml of pure acetic acid, a weak acid (Bobbio et al., 2000). A dynamic maceration was made for 10 h, evaluating the total solids and anthocyanin contain every 1 h. The process was made at room temperature to preserve the anthocyanin integrity. Up to 6 h, the total solids extracted in all solutions and the anthocyanin contain increased reasonably; however, after this time, both parameters remained practically constant (Data not shown). After that, an experimental design was made, using a 2^3 full factorial design with two central points (10 runs). It was studied the effect of three independent variables: volume of ethanol 92% (400–500 ml), a volume of concentrated acetic acid (25–75 ml) and the extraction time (4–6 h) over the response variables (total solids (%), and total anthocyanins (%)). For evaluating the effectiveness of the maceration process, three replicates (30 runs) of the experimental set were made. All runs in a design matrix of 2^3 full factorial designs were randomized. The experimental matrix (actual and coded factors) is presented in Table 1.

Multiple regression, the first-degree model was used to express the responses (y) as a function of all three factors:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where β_0, β_i, and $\beta_{i,j}$ represents the average effect, main effects, and two-way interactions effects, respectively (Montgomery and George, 2003). Design-Expert version 6.0 was used for performing the experimental design and the data analysis.

Extraction process

A proper amount of ethanol 92% and acetic acid was poured into an erlenmeyer flask (1000 ml) according to the experimental run. The ripe fruit (100 g) were poured into the erlenmeyer with the solution. The flasks were coated with aluminum paper to protect the extract from light. The extraction was made by maceration during the time described in Table 1 for each experiment. The flasks were stirring all the time by using an automatic shaker (Nova Etica, Brazil). After that, the extracts were filtered by Whatman number one filter paper, and the filtrate was preserved from light in an amber flask. All processes were made at room temperature ($25 \pm 2\,^{\circ}C$).

Total solids

Total solids were determined by the gravimetric method. The liquid extract (5 ml) was placed in a porcelain crucible, and the mass was determined in an analytical balance (0,0001 g, Acculab, Sartorius, Germany). Afterwards the crucible with the sample was placed in a water bath at 100 °C until almost the liquid present was removed. Then, the crucible was placed in an oven for 1 h at 105 °C. After this period, the crucible with the residue was placed in a desiccator at room temperature, to be weighed subsequently. The crucible was placed back in the oven following the same procedure and after one hour is weighed again. The process continues until two consecutive weight of the crucible with the residue not differ more than 20 mg. The assay was performed in triplicate. The Total solids was calculated by dividing the residual mass in the crucible by the initial mass of extract, multiplied by 100.

Anthocyanins content

The pH differential method for determining monomeric anthocyanin was used. In a volumetric flask (50 ml), were added 10 ml of buffer solution (pH 1, potassium chloride 0.025 M, Sigma, USA). Posteriorly, 1 ml of liquid extract (or 10 mg of dry extract) was added. The volume was completed by using the same buffer solution. In another flask with the same capacity, 10 ml of buffer solution (pH 4.5; sodium acetate 0.4 M, Sigma Co., USA) and 1 ml of the liquid extract was added. The volume was completed using a buffer solution of pH 4.5. Both flasks were gently stirred manually, and let it in repose for 5 min, protected from light. Afterward, both solutions were filtered through a Whatman paper. After that, the absorbance of both samples was recorded using a spectrophotometer (Shimadzu, Japan) at 520 nm and, 700 nm. The total content of anthocyanins (AT) was calculated as cyanidin-3-glucoside (mg/l) equivalents, using the following expression:

$$AT(mg/ml) = A * 834.9 \quad (2)$$

where $A = (A_{520nm} - A_{700nm})_{pH=1.0} - (A_{520nm} - A_{700nm})_{pH=4.5}$; and 834.9 is a constant term including the molar absorptivity of the cyanidin-3-glucoside ($26900\ mol^{-1}.cm^{-1}$), the molecular weight of cyanidin-3-glucoside ($449.2\ g.mol^{-1}$), factor dilution, the path length of the cell (1 cm) and the weight correction factor. The result was expressed as cyanidin-3-glucoside (AOAC, 2006).

Freeze drying process

The liquid extract optimized was concentrated in a vacuum rotatory evaporator (Ika-Werke, Switzerland) at a temperature below 35 °C until the alcohol was evaporated. Then, the concentrated extract was frozen at −20 °C, and posteriorly was freeze-dried during 72 h in a freeze dryer (LS 3000, Terroni, Brazil). After this time the powder was poured in a desiccator for 24 h.

pH evaluation

The pH of 1 g of dry extract dissolved in 15 ml of deionized water was measured. The pH-meter was calibrated using buffer solutions

Table 1
Experimental matrix for 2^3 full factorial experimental design for the extraction process with the real and coded factors.

Run	Ethanol 92% (ml)		Acetic Acid (ml)		Extraction time (h)	
	Actual	Coded	Actual	Coded	Actual	Coded
1	400.00	(−1)	25.00	(−1)	4.00	(−1)
2	400.00	(−1)	25.00	(−1)	6.00	(+1)
3	400.00	(−1)	75.00	(+1)	4.00	(−1)
4	400.00	(−1)	75.00	(+1)	6.00	(+1)
5	450.00	(0)	50.00	(0)	5.00	(0)
6	450.00	(0)	50.00	(0)	5.00	(0)
7	500.00	(+1)	25.00	(−1)	6.00	(+1)
8	500.00	(+1)	25.00	(−1)	4.00	(−1)
9	500.00	(+1)	75.00	(+1)	6.00	(+1)
10	500.00	(+1)	75.00	(+1)	4.00	(−1)

of pH 4 and pH 7 (Alphatec, Brazil). Measurements were performed in triplicate at 25 °C.

Moisture content

The moisture content of the dry extract was determined by the gravimetric method according to USP, in a gravimetric balance (Sartorius, SA 325. Germany) (United States Pharmacopeial Convention, 2012). Measures were made in triplicate. The mean and standard deviation were reported.

Total ashes

Total ashes were determined using the gravimetric method (United States Pharmacopeial Convention, 2012). The mass of dried extract (1 g) was weighed and poured in a porcelain crucible. Samples, spread it in the bottom of the crucible, were incinerated in a furnace with a gradual temperature increase as 200 °C for 30 min, 400 °C for 60 min and 600 °C for 90 min. Posteriorly, crucibles were poured into a desiccator at room temperature. After that, the crucibles were weighted, and the total ash content calculated.

Mineral content

The presence of minerals in natural extracts plays an important role in their *in vivo* antioxidant activity. For this reason, the mineral content of the dry extract was determined. The analysis was made by Atomic Absorption Spectrometry (Quimis, AA6300, Shimadzu, Japan) using an automatic sampler (ASC6100) with a FAAS optimizer. Ashes obtained in the ash analysis were dissolved in concentrated nitric acid and transferred into a 50 ml volumetric flask. The volume was completed with distilled water. Sample solutions were directly injected on the equipment, and the mineral concentration was determined using appropriates calibration curves. The analysis for calcium, iron, manganese, zinc, copper, nickel, cadmium, aluminum, and lead were made in triplicate (Argota et al., 2014).

HPLC-UV fingerprint

The HPLC-UV profile of the dry extract was obtained using an HPLC-UV Shimadzu, Class-VP (Japan) equipped with a high-pressure pump (LC-20 AT); automatic sampler (SIL-20AC) and UV detector SPD 20A. A column C-18 Agilent at 30 °C with 0.5 ml/min of flow was used. It was used as a gradient program with two mobile phases. As phase A, methanol: water:acetic acid (14:85:1) and as phase B, acetonitrile:acetic acid (99:1) were used. The process initiates with a relationship 95:5 (A:B) and ending with relationship 80:20 (A:B). The run time was 15 min. The wavelength used was 520 nm. The standard cyanidin-3-*O*-glucoside was obtained from Sigma–Aldrich®, USA (Datt, 2012)

Fourier transforms infrared spectroscopy

Fourier Transforms Infrared Spectroscopy (FTIR) analysis was made in an infrared spectrometer (Shimadzu, Japan) recording the spectrum between 4000 cm^{-1} and 450 cm^{-1}, with a resolution of 1 cm^{-1} and accumulation of 100 scans (scan rate 0.5 cm^{-1}/s). As a blank was used potassium bromide (Datt, 2012).

Thermal analysis

Thermo analytical curves were obtained in module, simultaneously, in a TG/DSC equipment TA - Instruments, Model 2960, operating under the following conditions: heating rate of 5 °C/min in a nitrogen atmosphere with flow of 50 ml/min using an alumina crucible (α-Al_2O_3) containing approximately 8–10 mg of sample at a temperature range of 25–300 °C (Fernandes et al., 2013).

Scanning Electron microscopy

Surface microphotographs of the dry extract were obtained using a Scanning Electron Microscope (Tabletop 3030Plus (Hitachi, Japan)) using 15 kV with 100× of magnificent.

Determination of the residual acetic acid on the extract

The detection of the acetic acid residual in the final dry extract was performed by HPLC (Shimadzu, Japan) as described in United States Pharmacopeial Convention (2012). Thermo Hypercarb 5 μ, 150 × 4.6 mm column at room temperature; 0.1% phosphoric acid as the mobile phase and diluent, flow rate 1 ml/min; run time 5 min. for the standard and 30 min for the sample; detector wavelength at 205 nm.

DPPH radical-scavenging

The antioxidant potential of these solutions was assessed by the method of inhibition of the radical DDPH described by Blois (1958), and the data were normalized using the natural logarithm of the concentrations of the extract solutions. Subsequently, a linear regression between the logarithms of the concentrations and percentages of inhibition of DPPH was performed. Then, DPPH IC_{50} was determined by a linear regression analysis by using the StatGraphics Centurion, StatEase Co. MA, USA.

Data analysis

The responses were analyzed by using Design Expert software (Version 6.0.1, Stat-Ease, Inc. Minneapolis, USA). To evaluate if the model describes adequately the behavior of the responses, an analysis of variances was made. It was selected the model in which the *p*-value of the ANOVA test was significant ($p < 0.05$), and the

Table 2
Results of the experimental matrix obtaining for the anthocyanin extraction process.Anthocyanin content (Square root linear model) obtained from the experimental design.

Run	Ethanol 92%(ml)	Acetic acid(ml)	Time(h)	Total solids (%)	Anthocyanins(mg/L)
1	400.00	25.00	4.00	1.30 ± 0.34	43.67 ± 3.26
2	400.00	25.00	6.00	2.00 ± 0.26	45.71 ± 2.36
3	400.00	75.00	4.00	2.35 ± 0.18	61.75 ± 3.28
4	400.00	75.00	6.00	2.70 ± 0.31	66.28 ± 2.71
5	450.00	50.00	5.00	2.03 ± 0.41	48.76 ± 1.69
6	450.00	50.00	5.00	2.15 ± 0.23	58.21 ± 4.66
7	500.00	25.00	6.00	1.45 ± 0.11	36.15 ± 2.57
8	500.00	25.00	4.00	1.35 ± 0.54	46.88 ± 2.37
9	500.00	75.00	6.00	2.65 ± 0.33	66.53 ± 1.28
10	500.00	75.00	4.00	2.74 ± 0.39	65.57 ± 3.10

All results are expressed as the mean ± SD, n = 3.

Table 3
Analysis of variance for total solids (first-degree factorial model) and anthocyanin content (Square root linear model) obtained from the experimental design.

Source	Total solids		Anthocyanins content	
	F-value	p-value	F-value	p-value
Model type	First-degree polynomial		Square root linear	
Model significance	123.01	0.0011*	11.35	0.0114*
A: Volume of alcohol	0.86	0.4230	0.06	0.8151
B: Volume of acid	630.09	0.0001*	33.89	0.0021*
C: extraction time	37.59	0.0087*	0.11	0.7561
A: B interaction	23.60	0.0167*	–	–
A:C interaction	36.18	0.0092*	–	–
B:C interaction	9.75	0.0523	–	–
Lack of Fit	0.28	0.8014	0.55	0.7511
Adjusted R^2	0.9850		0.7952	
Predicted R^2	0.9156		0.4880	

* Significant, $p < 0.05$.

adjusted R^2 value was major than 0.75 (Montgomery and George, 2003). To select the better extraction condition, it was used the desirability function by using the Design Expert Software v.6.0.

Results

Design analysis

The experimental matrix outcome of the extraction process is shown in Table 2. The analysis of variance for the responses adjusted model is shown in Table 3.

Total solids was adjusted to a first-degree polynomial model ($p < 0.05$, ANOVA test). The lack of fit test was not statistically significant. The adjusted R^2 was 0.9850 and predicted R^2 was equal to 0.9156 with PRESS (predicted residual error sum of squares) equal to 0.23. All the factors and interactions were significant ($p < 0.05$), except the factor volume of ethanol and the interaction volume of acid-extraction time, both with $p > 0.05$ (Table 3).

Fig. 1 presents the response surface plots obtained for both responses. The factor volume of ethanol 92% practically no affects the total solids content (Fig. 1A), while the volume of acetic acid shows a sharp effect on total solids. The higher the volume of acetic acid the higher the total solids extracted. As the extraction time increased, a little increase in total solid was observed.

Anthocyanins content was fitted to a square root linear model ($p < 0.05$, Table 3). Only the term volume of acetic acid was significant ($p < 0.05$). No interactions among the factors were detected. The lack of fit test was no statistically significant. The adjusted R^2 was equal to0.7952; the predicted R^2 was 0.4880 with a high PRESS value of 858.43.

The factor volume of ethanol practically no affects the anthocyanins extraction (Fig. 1B). On the contrary, as the volume of acetic acid increase, a marked enlargement in anthocyanin contains was observed. In the same way for total solids, the extraction time has practically no affects the anthocyanin content.

Selecting the optimal conditions for the extraction process

In the sake to obtain a rich anthocyanin extract, the optimal conditions favoring the increase of the anthocyanin content in the minor time were selected (Data not showed). Solutions offered by the desirability function was already included in the experimental design as run number 3 (D = 1.00) and, run 4 (D = 1.00). As it, it was selected the run number 3 because of the extraction time was minor (4 h, Table 1). For testing the predictability of the adjusted models, three replicates using the conditions volume of ethanol 400 ml, volume of acetic acid 75 ml, and a extraction time of 4 h (run 3) were made. A *t*-test was done to compare the predicted and observed results (Table 4).

Dry extract characterization

Physical properties and elemental composition

The moisture content of the dry extract was 1.39 ± 0.25%, the evaporation residue 97.25 ± 1.28%, total ashes 0.62 ± 0.12%, and the anthocyanin content 61.75 ± 3.28 mg/l. The elemental composition evaluated by AAS showed the presence of manganese 4.85 ppm, iron 1.62 ppm, zinc0.05, copper 1.38 ppm, calcium 1.01 ppm, cadmium 0.003 ppm, nickel 0.37 ppm, and lead 0.38 ppm.

FTIR

The dry extract FTIR fingerprint is shown in Fig. 2. The spectrum shows a band at 3362.07 cm^{-1} associated with the stretching vibration of O—H bonds. At 2924.21 cm^{-1} was observed a characteristic band of the C–H stretching vibration and at 1753.67 cm^{-1} the characteristics stretching vibration of the ester carbonyl group were

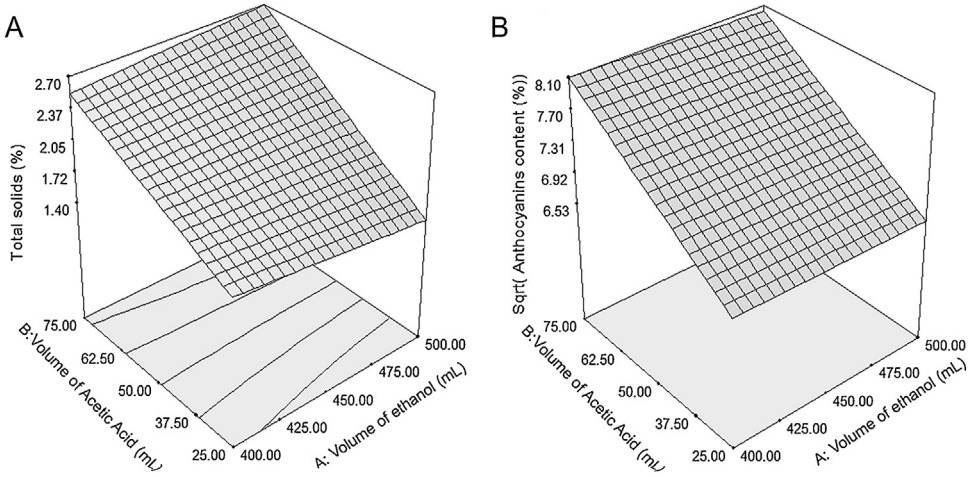


Fig. 1. The response surface plots for total solids (A) and anthocyanin contain (B).

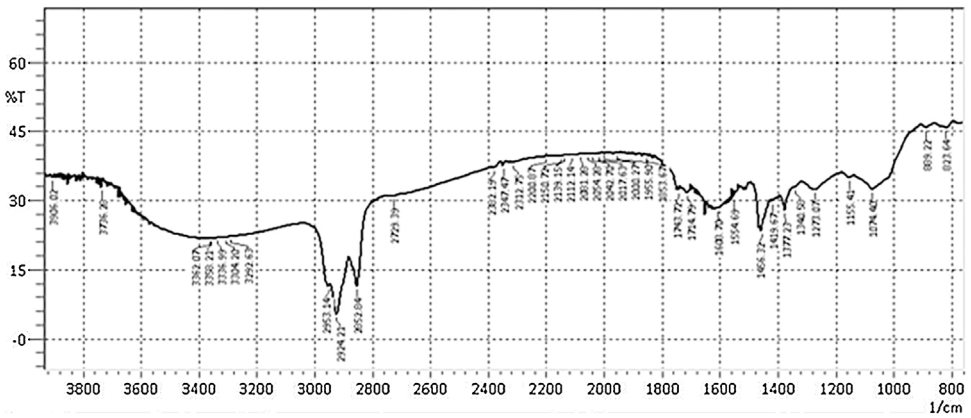


Fig. 2. FTIR spectra of the dry anthocyanin extract.

Table 4
Comparison between the observed and predicted values of both response variables.

Response	Observed	Predicted	t-test; p-value
Total solids (%)	2.37 ± 0.17	2.31	t = 2.33, p = 0.0798
Anthocyanins content (mg/ml)	67.23 ± 2.55	66.06	t= -0.82, p = 0.4573
	n = 3		

observed. A band at 1608.70 cm^{-1} corresponding to C—C of aromatic rings stretching vibration. In the same way, it was observed a band at 1512.25 cm^{-1} corresponding to the axial deformation of the C=C bond in aromatic rings. The spectrum also showed a band at 1440.88 cm^{-1} related to the deformation of C–H bonds and, another band at 1026.17 cm^{-1} corresponding to the C–O bonds stretching of phenol. Other bands were observed between 1456-1419 cm^{-1}; 1377-1340 cm^{-1} and 1155-889 cm^{-1}.

HPLC–fingerprint

The HPLC-UV fingerprint of the dry anthocyanin extract is shown in Fig. 3. In the HPLC condition used it was observed four defined peaks, at 5.175, 6.348, 6.786 and 7.712 min of retention time. The fourth peak was identified as cyanidin 3-*O*-glucoside (by retention time compared to standard and co-elution). The UV spectra show a characteristic peak of anthocyanins at 281 nm and 523.71 nm.

DSC/TG analysis

In Fig. 4 can observe the loss of mass variation of the lyophilized extract as a function of temperature while the sample was subjected to a progressive increase of temperature (5 °C/min.) between 25 and up to 300 °C. Two thermal events were recorded. The first occurred between 26-119.2 °C and the second at temperatures between 119–300 °C. The DSC curve showed two endothermic events at temperatures between 26–139 °C.

Macroscopic and microscopic evaluation

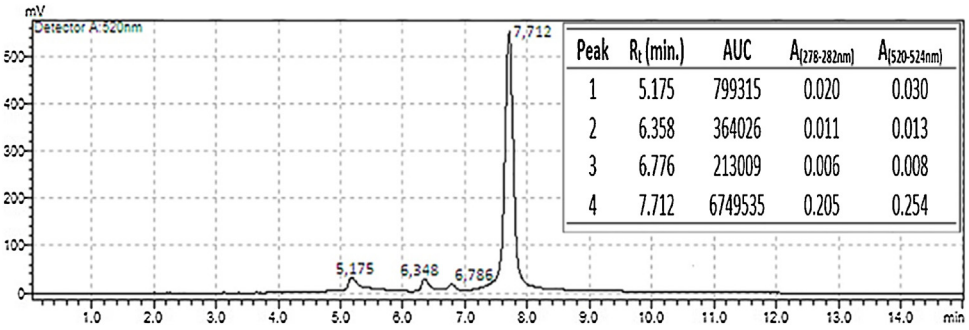
A microphotograph of the anthocyanins dry extract is shown in Fig. 5. The extract appears as a gross and porous powder with some laminar particles of different sizes.

Residual solvent

The acetic acid residual remained in the final dry extract was 25 ± 2.33 ppm.

Radical-scavenging

The dried extract IC_{50} estimated by the radical scavenging assay with DPPH was ±2.31 ppm.



Peak	R_t (min.)	AUC	$A_{(278-282nm)}$	$A_{(520-524nm)}$
1	5.175	799315	0.020	0.030
2	6.358	364026	0.011	0.013
3	6.776	213009	0.006	0.008
4	7.712	6749535	0.205	0.254

Fig. 3. HPLC-UV fingerprint of the anthocyanins dry extract.

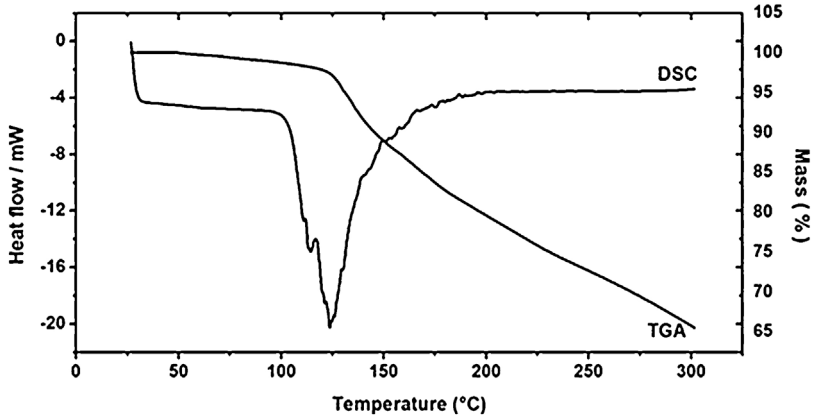


Fig. 4. DSC and TGA analyses of the anthocyanins dry extracts.

Discussion

In the market, products of açaí are manufactured using the whole pulpor the pulp previously lyophilized. Although it is accepted the wide range of health and therapeutic benefits of the açaí, there is no evidence scientifically founded about the exceptional health supplement that this palm tree is (Heinrich et al., 2011). Researchers have been paid particular attention to the antioxidant activity showed by this plant fruits. Nonetheless, results obtained are not conclusive and contradictory (Heinrich et al., 2011) due to the presence of a high number of other metabolites like fatty acid, anthraquinones, carbohydrates and the presence of considerable amount of fibers (Schauss, 2015).

The fruit pulp of açaí was used as an antioxidant for stabilizing yogurt. The assay was wrong because the açaí pulp increased monounsaturated and polyunsaturated fatty acid contents and the physicochemical characteristics of yogurt were altered (Spiritu et al., 2010). In this regard, the use of anthocyanins extracted from the açaí fruit pulp seems to be a promissory solution for pharmaceutical and food preparations, because of simple composition and facility for quality control.

In the extraction processes, if the response variability is well explained by the variation of the factors, it is possible to describe the response using a mathematical model. The statistic adjusted R^2 is a mathematical descriptor of the extension in which the response is explained by the model (Montgomery and George, 2003). Adjusted R^2 compares the explanatory power of the fitted models containing different numbers of predictors. Adjusted R^2 value increases, only, if a new term included improves the model more than would be expected by chance. On the other side, Predicted R^2 indicates how well the fitted model predicts the responses for new observations. The predicted R^2 provides crucial information because when is apart more than 0.3 from the adjusted R^2 imply that the model can be used for prediction. The PRESS statistic is a form of cross-validation in which the lower the PRESS value, the better the descriptive response of the model. A non-significant lack of fit is a measure of the model robustness (Gabrielson et al., 2002; Montgomery and George, 2003).

One of the most critical parameters in an extraction process is the amount of soluble solids extracted. As was previously noted, the volume of alcohol practically no affected the amount of the extracted solids, probably because in all experiments the amount of this solvent was enough to dissolve all available solids in the fruit. This statement seems to be valid, considering that, in a preliminary experiment using the same amount of fruit, and volumes of acidified ethanol (92%) of 600, 700, 800, and 900 ml, the total solids extracted was not statistically different (Data not reported). On the contrary, as the amount of acetic acid raised up the contain of total solids increased, probably due to the affinity of the anthocyanins for an acid medium, as well as the other relative acidic substances as amino acids, some peptides, and proteins that can also be extracted by this solvent (Dai and Mumper, 2010).

The extraction time is a critical factor that must be studied before using the extraction process on a larger scale (Sharapin, 2000). In this work, the extraction time showed a small influence on total solids extracted. To this regard, in the sake to optimize this factor, in a previous experiment was observed that after 6 h under dynamic maceration, regardless the fruit mass and the solvent volume, the anthocyanins content remained without statistical differences. For this reason, minor's volumes were evaluated during the standardization (400–500 ml). It seems to be that, the amount of the soluble substances present in the fruit was rapidly extracted at the beginning of the process and just a little amount was slowly solubilized after 6 h of the extraction.

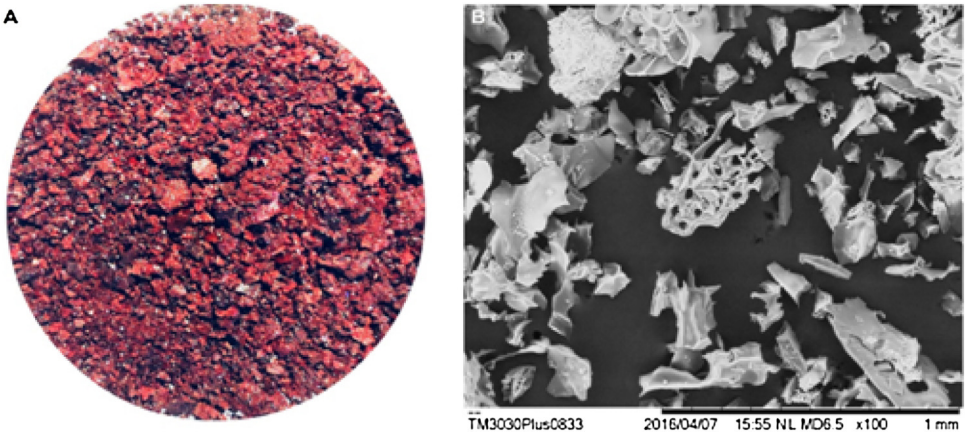


Fig. 5. Macroscopic (A) and microscopic (by SEM) aspect (B) of the dry anthocyanin extract.

As the factor Volume of ethanol and the interaction volume of acid-extraction time were no significant ($p < 0.05$; Table 3), the first-degree polynomial model for this response can be expressed as:

$$\text{Total solids (\%)} = 2.07 + 0.54 * B + 0.13 * C + 0.10 * A * B\text{-}0.13 * A * C \quad (5)$$

where A: volume of ethanol, B: volume of acetic acid, and C: extraction time.

The above expression allows navigating the design space for making predictions because a good agreement between adjusted R^2 (0.9959) and predicted R^2 (0.9858) (Gabrielson et al., 2002), the non-significant lack of fit, and the low PRESS value (0.2376). All these statistics suggest that the total solids variability is well explained by the fitted model. Anthocyanins are the most abundant polyphenol in the açaí fruit pulp (Schauss et al., 2006a). Among polyphenols, anthocyanins are considered the most active compounds, contributing to the high antioxidant activity of this plant extract (Pompeu et al., 2009).

Structurally, anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts, being composed of an aglycon moiety called anthocyanidin and carbohydrate residues (glucose, rhamnose, xylose, galactose, arabinose, rutinose) (Grayer, 1988). At low pH, anthocyanins are predominantly present in the flavylium cation form, giving a reddish color to aqueous solutions (Cheminat and Brouillard, 1986). Flavylium cation is stable in acid solutions, being the only groups of phenolic compounds that can be favored by increasing the acidity of the extracted solution. For this reason, mineral and organic acids are used for anthocyanins extraction (Revilla et al., 1998). However, mineral acid can hydrolyze the acyl groups of anthocyanins, losing stability and activity. In this work, it was observed, a strong effect of the volume of acetic acid in the anthocyanin content. This occurs, probably, because the oxygen atom in the aromatic ring of anthocyanins has a high electronic density allowing to accept a proton (H^+) from the acetic acid, getting a positive charge (cation flavylium). Thus, anthocyanins are dissolved preferably, to the rest of the phenolic substances, that are usually extracted using a more basic and polar solution (Revilla et al., 1998; Dai and Mumper, 2010).

Anthocyanins content did not show statistically significant differences regardless of the extraction time ($p > 0.05$, table 5). This may be explained by the fact that glycosylated anthocyanins in the açaí fruit are localized in the most external region of the fruits (Rogez, 2000), and as the volume and acidity of the solvent are adequate, anthocyanins are extracted in the first moments of the extraction, and just a little amount is removed later. As the volume of acetic acid was the factor statistically significant, the mathematical equation of the model can be expressed as:

$$\text{Sqrt (Anthocyanins content) mg/100 ml} = 54.07 + 10.96 * B \quad (6)$$

Where B is the volume of acetic acid. Even though the Adjusted R^2 of the model was good (Table 5) the different from Predicted R^2 was above 0.3, and the model appears not good for predictions.

In a study reporting the optimization of the phenolic extraction from açaí fruits, the temperature (29.5, 45.0, and 60.5 °C), extraction time (1–8 h) and a solution of hydrochloric acid (0.04 mol/l) in ethanol (70%) were used as factors. They observed that the increase in the temperature from 29.5 °C to 60.5 °C improved the phenolic extraction, but the anthocyanins extraction was reduced. They also reported no statistically significant differences of the extraction time (Pompeu et al., 2009). The anthocyanins content in this study was minor, probably, because the use of a more polar solvent, favored the extraction of the more polar phenolics. Contrarily, the presence of the flavylium cation in anthocyanins reduce the affinity for polar solvents limiting its extraction. On the other hand, the use of mineral acid as hydrochloric acid tends to form salts, increasing the polarity of the anthocyanin structure causing hydrolysis (Revilla et al., 1998; Dai and Mumper, 2010).

These facts, joined to prolonged extraction times using temperatures over 30 °C, diminish the anthocyanins stability, and reduce its solubility in ethanol. Probably by this reason, the extraction for a long time diminished the anthocyanins extraction and the extraction time was no statistically significant. Differently, in our work, the amount of anthocyanins extracted was superior, because of the experimental conditions favoring the anthocyanins solubilizing and extraction. The use of a weak acid, a less polar ethanol solution (92%), and a process developed at room temperatures increased the solubility and the stability of anthocyanins. Nonetheless, in the study of Pompeu et al. (2009) the purpose of the extraction was not anthocyanins but the phenolic compounds in general. It is important because, apart from our work, there is no report about the extraction and characterization of an anthocyanin-rich extract from the açaí fruit.

Selecting the optimal conditions for an extractive process is a complex task depending on many factors. First, depending on the quality of the vegetal material and the quality of the collection process and preparation; and secondly depend on the extraction condition as the temperature, solvent type, extraction time, stirring, light protection etc. The application of the desirability function allowed selecting the better conditions for the extraction process. The run number 3 was selected as the optimal conditions for anthocyanin extraction establishing a volume of ethanol of 400 ml, a

volume of acetic acid 75 ml, and the extraction time of 4 h. It was made a comparison between the predicted values and the values observed experimentally (Table 4). Despite the observed value for total solids (2.37 ± 0.17%) was statistically different (t = 2.33, p = 0.0798) from the predicted value (2.31%), the observed value was better than the predicted by the software. On the other hands, for anthocyanin contain, no statistically significant differences (t = −0.82, p = 0.4573) between the observed (67.23 ± 2.55 mg/ml) and predicted value (66 mg/ml) was observed, which is a sign of the good predictive capability of the adjusted model (Montgomery and George, 2003). The optimal conditions expressed in run 3, were used for the preparation of the anthocyanins liquid extracts to be dried by freeze-drying.

The moisture content is a critical parameter in vegetal materials. A vegetal extract with low humidity is necessary to maintain the chemical and microbiological stability (Sharapin, 2000). A well dry extract diminishes the risk of microbiological contamination and reduces the occurrence of redox reactions. Anthocyanins are sensitive to factors like temperature, light, pH, oxygen and, others (Tonon et al., 2010). For this reason, the drying of the liquid extract was made using a freeze dryer to preserve the chemical and pharmacological properties and the physicochemical and microbiological stability.

The anthocyanins dry extract was characterized by using different techniques. The moisture content of the extract was according to the lyophilized extracts with values under 2% (Sharapin, 2000). The extract shows a low content of ashes (0.62%) with a rich mineral composition, including Mn, Fe, Cu, and Ni. All of these elements are essential cofactors of the four isoenzymes of the superoxide dismutase (SOD) that can contribute to the in vivo antioxidant activity of this extract. Also, they contain zinc, calcium, and traces of lead and cadmium. The sum of the total mineral content of the dry anthocyanin extract is minor than 20 μg/g, and this value is the limit for heavy metals (United States Pharmacopeial Convention, 2012).

The HPLC-UV fingerprint of the dry anthocyanin extract shows four defined peaks, at 5.17, 6.35, 6.79 and 7.71 min (cyanidin 3-*O*-glucoside) of retention time, in the used experimental condition (Fig. 2). An analytic method for determining the major constituents of açaí fruits was developed by using UPLC/HPLC of a mixture of the standard of anthocyanins. In both techniques, the order of elution was cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, pelargonidin-3-*O*-glucoside, cyanidin and pelargonidin (Avula et al., 2010).

Due to the eight conjugated double bonds (chromophore) carrying a positive charge, anthocyanins are intensely colored under acidic conditions. The maximum absorption in the visible range for anthocyanins is usually between 502–528 nm. The nature of sugar substitution has no relevant effect on the spectrum. The other maximum absorption band falls in the UV range between 270 and 282 nm (Stój et al., 2006; Gouvêa et al., 2012). The UV spectra obtained for each one of the peaks obtained by HPLC for the dry extract show the characteristic peaks of the anthocyanins at 278–282 nm and 520–524 nm.

The FTIR spectra obtained for the extract (Fig. 2) showed all characteristic bands of anthocyanins by those observed by other authors for different anthocyanin extracts and a standard of anthocyanin-3-*O*-glycosidic (Ahmed et al., 2014; Teixeira-Neto et al. (2009)). Others bands observed at 1456–1419; 1377–1340 and 1155–889 cm^{-1} are associated with monosaccharide such as glucose and galactose that are commonly present in the structure of anthocyanins (Kacurakova and Mathlouthi, 2007).

In TGA analysis, the first thermal event recorded at temperatures between 26–119 °C showed (Fig. 3) a weight loss of 1.34% (0,108 mg), corresponding to loss of surface water as well as the volatile substances that can be present. After the dehydration of the extract, the decomposition of the organic components of the sample at temperatures between 119–216 °C (−2.6637 mg, 32.88%) was recorded. The wide endothermic event observed in DSC analysis from 26 °C to 139 °C with a maximum peak at 123.71 °C (−2,55 J, −283.04 J/g) is characteristic of water release processes and posterior thermal decomposition of the organic material.

The freeze-drying process renders a porous and light material as it observes in the SEM microphotograph (Fig. 5). A wide range of particle sizes, characteristic of the lyophilized products (Thi and Hwang, 2016) was observed. As the majority of the lyophilized materials, a porous consistency is due to the pass of the sublimated water. Porous solid materials are desirables for solid preparation as tablets and capsules because porosity improves the disintegration and dissolution process. Residual solvents, in pharmaceuticals, are defined as volatile organic chemicals that are used or produced in the manufacture of drug substances or excipients, or the preparation of drug and nutraceutical products (United States Pharmacopeial Convention, 2012). Because of the residual solvents do not provide any benefit, they should be removed, to the extent possible, to meet ingredient and product specifications, good manufacturing practices, or other quality-based requirements. As in the extraction was used acetic acid (A class 3 residual solvent, USP), is essential to demonstrate that the final anthocyanin dry extract was substantially free of acetic acid or held below the USP limit requirements of 5000 ppm. The acetic acid content in the final product was 25 ± 2.33 ppm. This value agrees with the standard of the international health authorities (United States Pharmacopeial Convention, 2012) and could be used safely in pharmaceutical or nutraceutical preparations.

From a nutritional and pharmaceutical perspective, it is important to evaluate the antioxidant potential of phytochemicals in plant materials and their extracts (Schauss, 2016).

Usually, more than one technique is used for antioxidant characterization because plant extracts and derivatives have more than one antioxidant mechanism of action. As the dry extract could be used for nutraceutical and pharmaceutical formulations, in this work the evaluation of the radical scavenging activity was made, a way for evaluating the potential antioxidant activity of this product after the extraction and processing.

The dry extract antioxidant activity shows a good radical scavenging capacity with IC_{50} of 31.25 ± 2.31 ppm. This value is better than the other antioxidant vegetal extract as the fluid extract of *Tamarindus indica* (IC_{50} for DPPH assay 44.36 ± 3.72 mg/ml) (Escalona-Arranz et al., 2015). The presence of anthocyanins and probably other phenolic compounds in the dry extract can justify the excellent antioxidant properties observed.

The identity, pureness, and quality of vegetal extracts must be established, and when is possible, must be compared with reference material (United States Pharmacopeial Convention, 2012). The thermal behavior, FTIR (Datt, 2012; Petenatti et al., 2014), and HPLC–UV fingerprint (Waksmundzka and Sherma, 2011; Jothy et al., 2013; Cortes et al., 2013) are used for plant materials and plant extracts characterization with or without the use of standard for comparison, because provide unique characteristics of each product. In this work, the combined use of these techniques allowed a complete physicochemical characterization of the dry extract from the açaí fruits. This characterization will allow, in the future, its use in processes of quality control for including the dry extract as an active ingredient in pharmaceutical formulations.

Authors contribution

HRS, DCA, ALP., JRRA, MBS, AVTLTS, JOCSJ. were responsible for investigation, methodology, and validation. AMF and HOC were responsible for data curation and formal analysis. JCTC was respon-

sible for the supervision, writing - original draft and writing - review and editing.

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Ethical statement

I declare that in the execution of the manuscript "Obtaining and characterization of anthocyanins from *Euterpe oleracea* (açaí) dry extract for nutraceutical and food preparations.", no animals were used, and all procedures were conducted within the ethical standards.

Conflicts of interest

The authors declare that they have no competing interests.

Authors contribution

HRS, DCA, ALP., JRRA, MBS, AVTLTS, JOCSJ. were responsible for investigation, methodology, and validation. AMF and HOC were responsible for data curation and formal analysis. JCTC was responsible for the supervision, writing - original draft and writing - review and editing.

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