Chemical characterization, antioxidant and antiatherogenic activity of anthocyanin-rich extract from *Euterpe edulis* Mart. in mice

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Summary

This study aimed to assess the influence of organic solvents on the extraction of anthocyanins from Juçara palm fruit and to evaluate the freeze-dried Juçara extract regarding the reduction of cardiovascular risk in mice with apolipoprotein E (ApoE$^{-/-}$) knockout. Anthocyanin content, fatty acids profile and antioxidant activity of Juçara extract were analysed by ultra performance liquid chromatography, gas chromatography and 2,2-diphenyl-1-picryl-hydrazyl assay, respectively. The antiatherogenic effect was assessed in vivo. The extraction with ethanol and citric acid resulted in the highest anthocyanin concentration (1 249.74 mg·l^{-1}). The freeze-dried Juçara extract contained 9.52 g·kg^{-1} of cyanidin-3-O-glucoside and 16.31 g·kg^{-1} of cyanidin-3-O-rutinoside. The lipid profile was 27.1% palmitic acid, 33.0% oleic acid, 32.9% linoleic acid and 1.8% linolenic acid. Mice groups which received Juçara extract presented decreased activities of catalase and superoxide dismutase vs. positive control group ($p < 0.05$) in vitro; and also presented lower values of total cholesterol, low-density lipoprotein (LDL) and glucose, as well as the ratios of total cholesterol/high-density lipoprotein (HDL) and LDL/HDL in vivo. The consumption of Juçara extract alone did not affect the size of atherosclerotic plaques in ApoE$^{-/-}$ mice. The Juçara extract presented antioxidant activity but did not show anti-atherogenic effect in vivo.

Keywords

Euterpe edulis Mart.; anthocyanin; extraction; cardiovascular disease

Euterpe edulis Mart. is mainly found in the Atlantic Forest of Brazil. It is also known as Juçara palm. The plant is currently threatened with extinction due to intensive extraction of the palm heart. The fruits of Juçara palm have an intense purple colour due to the high content of anthocyanins, which is higher than in other tropical fruits such as acerola (*Malpighia emarginata)*, jambolão (*Syzygium cumini)* or guajiru (*Chrysobalanus icaco*) [1]. *Euterpe edulis* fruits are a natural source of antioxidants and recent studies reported the presence of bioactive compounds such as anthocyanins, quercitin, rutin and phenolic acids [2–4].

Anthocyanins are important plant pigments, which belong to a phenolic compounds class collectively called flavonoids [5]. The anthocyanin phenolic structure can confer antioxidant activity through the donation or transfer of electrons from hydrogen atoms. Various studies suggested that high consumption of natural antioxidant-rich foods can increase plasma antioxidant capacity and reduce the risk of some cancers, heart disease and stroke [6–10].

The growing interest in the use of natural compounds in foods in order to replace artificial ones has led to the development of several methods for the extraction of bioactive compounds. The choice of the extraction method as well as the choice of

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the solvent to be used in anthocyanin extraction is important in order to obtain the compounds of interest present in the natural extract. The polar character of anthocyanins makes these compounds soluble in polar solvents such as methanol, ethanol and water [11]. The flavylium cation form of anthocyanins can be stabilized in an acidic medium. Furthermore, the efficiency of anthocyanin extraction depends on a number of factors, including the ratio of the solvent volume to the raw material mass, as well as the type of solvent and acidulant agent used.

Antioxidants, such as anthocyanins present in the diet, can potentially be involved in the prevention of atherosclerosis by inhibiting low-density lipoprotein (LDL) oxidation, reducing its atherogenicity, and consequently the risk of coronary artery disease [12]. Mice deficient in apolipoprotein E (ApoE) contain the entire spectrum of lesions similar to those observed during human atherogenesis. Therefore, this is a useful model for a study that evaluates the various factors that control or influence atherosclerotic plaque development, including the use of anthocyanin-rich foods, which have a great antioxidant potential.

The objective of this study was to determine the most efficient method to obtain an anthocyanin-rich extract of Juçara from the Atlantic Forest (*Euterpe edulis* Mart.), as well as to evaluate its chemical composition, and its antioxidant and antiatherogenic activities both in vivo and in vitro.

MATERIALS AND METHODS

Fruit harvesting and preparation of the Juçara pulp

Ripe *E. edulis* fruits were collected in a remaining area of the Atlantic Forest, located in the southeast of the Minas Gerais state, Brazil, called "Zona da Mata" (20°45' latitude (S), 42°51' longitude (W)). After harvesting, the fruits were threshed and transported to the Nutrition and Health Department at Federal University of Viçosa (Viçosa, Minas Gerais, Brazil) in airtight plastic bags and were immediately submitted to processing. The steps after fruit gathering that preceded the process of anthocyanin extraction were:

a) pre-wash: the fruits were dipped in water to remove dirt;
b) softening: immersion of the fruits in drinking water at 40 °C for a period of 20 min to facilitate fruit pulping [13];
c) wash with chlorinated water: immersion of the fruits in a solution containing active chloride (20–50 $mg \cdot kg^{-1}$) for about 30 min;
d) removal of the excess chloride: the fruits were washed by spraying with clean water;
e) removing the excess water and weighing: the fruits were dried on a paper towel and weighed in plastic containers.

For the anthocyanin extraction, 100 g of fruit were separated and used for extraction with 50 ml of solvent in a 2:1 ratio (fruit:solvent). The fruits and the test-solvent were homogenized in a circular mixer (Arno, São Paulo, Brazil) for 4 min. The homogenate samples were then sieved, pulped, filtered through cotton and the volume was transferred to a 250 ml volumetric flask. The volume was adjusted with the test-solvent itself. The solution was stored in amber bottles at –15 °C until anthocyanin analysis. The whole process was performed in the dark due to the photosensitivity of anthocyanins. The seeds were separated, washed and dried for subsequent weighing and correlated with the gross weight of the fruit.

Optimization of the extraction conditions

The extraction process was performed in batches, in 2 × 5 factorial experiments, using two different acidulant agents combined with five different extractor solvents, followed by monitoring of the total anthocyanin concentration in order to optimize the extraction conditions and to develop an efficient method. Different combinations of 95% ethanol (S1), 80% ethanol (S2), 70% ethanol (S3), 60% ethanol (S4) and water (S5) with the addition of the acidulants 0.1% hydrochloric acid (A1) and 0.3% citric acid (A2) were tested to investigate the influence of the solvent on anthocyanin extraction. The 10 extractions were repeated in triplicate. The anthocyanin concentration was determined by the procedure of differential pH measurement described in the following section.

Once the most efficient method to obtain an anthocyanin-rich Juçara pulp was determined, a new harvest of Juçara fruit was performed in order to submit the pulp to the lyophilization process using a Liotop LP510 freeze dryer (Liobras, São Carlos, Brazil) for future analyses. In this process, 4.678 kg of Juçara pulp was used and 0.701 kg of freeze-dried Juçara extract was obtained, which corresponds to 15% of the weight of the Juçara pulp.

Analysis of anthocyanins

Total anthocyanin content determination

The total anthocyanin content present in the Juçara pulp and in the freeze-dried Juçara extract

were determined spectrophotometrically using the pH differential absorbance method as described by GIUSTI and WROLSTAD [14]. The absorbance of the Juçara samples was measured at 510 nm and 700 nm in buffers at pH 1.0 (0.025 mol·l^{-1} potassium chloride adjusted with HCl) and pH 4.5 (0.4 mol·l^{-1} sodium acetate adjusted with HCl). The samples were left in an ultrasonicator (Hielscher, Ringwood, New Jersey, USA) for 30 min. After this period, the samples were filtered, poured into a 50 ml volumetric flask and the volume was diluted first with the corresponding buffer. Each flask was allowed to stand for 30 min, protected from light and heat. The analyses were performed in triplicate for each sample.

The difference of the absorptivity values at pH 1.0 and pH 4.5 was directly proportional to the concentration of anthocyanins:

$$A = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}} \qquad (1)$$

The calculation was based on cyanidin-3-glucoside with a molecular mass (*MM*) of 449.2 g·mol^{-1}, molar absorptivity (ε) of 26 900 l·mol^{-1}cm^{-1} and the corresponding dilution (*D*). Anthocyanin concentration was expressed as milligrams of cyanidin-3-glucoside equivalents per litre of pulp or extract.

Determination of anthocyanins by HPLC-DAD

Identification and quantification of anthocyanins from the freeze-dried Juçara extract were performed by high performance liquid chromatography – diode array detection (HPLC-DAD), using model SCL 10AT VP (Shimadzu, Kyoto, Japan). The chromatographic analysis proposed by SCHAUSS et al. [15] was done with some modifications. Separations were performed using a Phenomenex Gemini RP-18 (5 μm; 250 mm × 4.6 mm) column with a Phenomenex ODS guard column (C18, 5 μm; 4 mm × 3 mm), both from Phenomenex (Torrance, California, USA). The mobile phases consisted of 89% ultrapure water acidified with formic acid to pH 2.0 and 11% acetonitrile (v/v) (phase A), and 100% acetonitrile (phase B) run at 1 ml·min^{-1}. The injection volume was 50 μl. The run time was 45 min, and the detector readings were taken at 520 nm. The gradient at the pumps was: 0% B from 0 min to 20 min; 50% B (linear gradient) from 20 min to 22 min, 50% B from 22 min to 27 min, 0% B (linear gradient) from 27 min to 29 min, and 0% B from 29 min to 45 min.

The freeze-dried Juçara extract (0.01 g) was resuspended in 5 ml of 1% HCl aqueous solution and filtered through a Millipore Nylon membrane filter (pore size, 0.2 μm; Millipore, Billerica, Massachusetts, USA). Cyanidin-3-O-glucoside (Sigma-Aldrich, St. Louis, Missouri, USA) and cyanidin-3-O-rutinoside (Sigma-Aldrich) were used as the external standards for anthocyanins. Their molar absorptivities were 26 900 l·mol^{-1}cm^{-1} and 28 840 l·mol^{-1}cm^{-1}, respectively. Identification and quantification of anthocyanins were based on their spectral characteristics and retention time, as compared to authentic standards.

Determination of anthocyanins by UPLC–ESI/MS

The qualitative analysis of the anthocyanin compounds from the freeze-dried Juçara extract was performed using ultra performance liquid chromatography coupled to a mass spectrometer equipped with an electrospray ionization source (UPLC-ESI-MS). Triple quadrupole mass spectrometer, model 6430 (Agilent Technologies, Waldbronn, Germany), was operated in positive ion mode under the following conditions: capillary voltage, 4.0 kV; solvation flow, 13 l·min^{-1}; nitrogen gas nebulization, 241316.5 Pa; source temperature, 340 °C; fragmentor, 135 V, dwell time, 200 s; MS-MS scan from 100 m/z to 500 m/z. Collision energies from 20 eV to 25 eV for the transitions 465 > 303 (delfinidin-3-glucoside), 609 > 301 (peonidin-3-rutinoside), 449 > 287 (cyanidin-3-glucoside), 595 > 287 (cyanidin-3-rutinoside) and 433 > 271 (pelargonidin-3-glucoside) were used to analyse the products of ionization. The chromatographic assays were performed using UPLC, model 1290 Infinity System (Agilent Technologies). The separation of anthocyanins was conducted using an Acclaim 120, C18 reverse phase column (5 μm, 150 mm × 2.1 mm; Dionex, Sunnyvale, California, USA) with a C18 pre-column (Dionex) at 25 °C. The mobile phase consisted of 1% formic acid in water (v/v) (mobile phase A) and 1% formic acid in acetonitrile (v/v) (mobile phase B) at a flow rate of 0.2 ml·min^{-1}. The linear gradient of phase B was increased from 3% to 30% from 0 min to 40 min, 30% to 100% from 40 min to 45 min, and decreased from 100% to 3% from 45 min to 50 min. The anthocyanins were monitored at 310 nm and 520 nm.

Physico-chemical characterization

Chemical composition of the freeze-dried Juçara extract

The contents of moisture, ash, lipids, proteins and saccharides of the freeze-dried Juçara extract were determined according to the analytical techniques described by the Association of Official Analytical Chemists [16]. The determination of minerals (iron, calcium, copper, magnesium, zinc and manganese) was per-

formed by flame atomic absorption spectrometry (SpectrAA 220FS; Varian, Melbourne, Australia). Sodium and potassium were determined by flame photometry (Corning 400, Corning Medical, Medfield, Massachusetts, USA). Phosphorus was determined by a photocolorimetric method using BEL1105 photometer (BEL Photonics, Osasco, Brazil).

Lipid profile of the freeze-dried Juçara extract

Lipid extraction was performed according to the methodology of Folch et al. [17]. Saponification and esterification were performed according to Hartman and Lago [18]. The analysis of fatty acid methyl esters was performed by gas chromatography, class model GC-17A (Shimadzu) with a 100 m × 0.25 mm diameter fused silica SP-2560 (polysiloxane biscyanopropyl) column and a flame ionization detector. The vaporizer temperature was 250 °C and the detector temperature was 260 °C. The carrier gas used was nitrogen at 20 cm·s^{-1} at 175 °C. The division of the sample on the injector was 1 : 50, 1 μl of solution being injected. The peaks were identified by comparison of retention times with known standard methyl esters (FAME mix; Supelco, St. Louis, Missouri, USA) and quantified by area integration.

Analysis of carotenoids

The carotenoids α-carotene, β-carotene, lycopene and β-cryptoxanthin were extracted according to the method described by Rodriguez et al. [19] with modifications. The quantification was performed using an HPLC-DAD system according to methodology described by Pinheiro Sant'Ana et al. [20]. Separations were conducted using the Phenomenex Gemini RP-18 (5 μm; 250 mm × 4.6 mm) with a Phenomenex ODS guard column (C18, 5 μm; 4 mm × 3 mm). The mobile phase was methanol : ethyl acetate : acetonitrile (70 : 20 : 10, v/v/v), with a flow rate of 1.7 ml·min^{-1} and a run time of 10 min. The detection wavelength was 450 nm. Three repetitions were used for extraction and analysis.

Antioxidant activity in vitro

The antioxidant activity of the freeze-dried Juçara extract in vitro was determined by the DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay, according to the Brand-Williams method [21]. Solutions from freeze-dried Juçara extract were prepared at concentrations ranging from 0.1 mg·ml^{-1} to 25.0 mg·ml^{-1}, as recommended by Rufino et al. [22]. A 0.1 ml aliquot of each solution was added to 3.9 ml of DPPH solution (0.06 mmol·l^{-1}; Sigma-Aldrich) dissolved in methanol and allowed to stand for 30 min in the dark. The absorbance was recorded at 515 nm. The mean concentration of DPPH in the reaction was obtained based on the calibration curve by linear regression. The DPPH percentage was calculated by the following equation, using DPPH solution as the control.

$$DPPH = [Abs_C - (Abs_S - Abs_B)/Abs_C] \times 100 \quad (2)$$

where $DPPH$ is percentage of DPPH capture; Abs_C is absorbance of control; Abs_S is absorbance of sample; Abs_B is absorbance of blank.

Animals and experimental design

A total of 40 apolipoprotein E deficient (Apo E$^{-/-}$) adult mice and 10 C57BL/6 adult mice at 14 weeks of age were used. The animals were kept in an environment with a 12 : 12 h light-dark cycle, temperature of (21 ± 2) °C, and 60–70% relative humidity. The experimental protocol was approved by the Ethics Committee of the Department of Veterinary Medicine, Federal University of Viçosa, No. 56/2010. The animals were divided into five groups:

- G1 – negative control (C57Bl/6, n = 10), received American Institute of Nutrition (AIN-93M) Rodent Diets [23];
- G2 – positive control (ApoE knockout, n = 10) received AIN-93M,
- G3 – (Apo E knockout, n = 10) received the AIN-93M diet with 2% freeze-dried Juçara extract;
- G4 – (Apo E knockout, n = 10) received the AIN-93M diet with 6% freeze-dried Juçara extract;
- G5 – (Apo knockout E, n = 10) received AIN-M93 with 50 mg·kg^{-1}d^{-1} of simvastatin (Galena, Campinas, São Paulo, Brazil). The surfactant Tween 80 (Sigma-Aldrich) in powder form was added to simvastatin in the diet in order to obtain better drug absorption.

Euthanasia was performed after 75 days.

Antioxidant activity in vivo

The liver was used for the in vivo antioxidant activity analysis of the freeze-dried Juçara extract present in the diet, through the enzymatic activity of catalase (CAT) and superoxide dismutase (SOD), according to the methodology described below.

Catalase activity was determined by the hydrogen peroxide decay rate at 240 nm using spectrophotometer Multiskan Go (Thermo, West Palm Beach, Florida, USA), according to Aebi [24].

Superoxide dismutase (SOD) activity was de-

termined based on the ability of SOD to remove O_2^-, according to DIETERICH et al. [25] adapted by using microplate reader Asys UVM 340 (Biochron, Holliston, Massachusetts, USA) at 570 nm. The protein content was measured according to the method described by LOWRY et al. [26].

Biochemical analyses of blood parameters in ApoE$^{-/-}$ mice

Analyses were performed using Bioclin/Quibasa kits (Quibasa Quimica Basica – Bioclin, Belo Horizonte, Brazil) and Cobas Mira Plus instrument (Roche Diagnostics, Rotkreuz, Switzerland), in the clinical laboratory of the Division of Health, Federal University of Viçosa.

The following parameters were measured in mouse serum: total cholesterol (by enzymatic-colorimetric method, peroxidase), HDL (by enzymatic-colorimetric method, direct), triglycerides (by enzymatic-colorimetric method, peroxidase), glucose (by enzymatic-colorimetric method, glucose oxidase), creatinine (by colorimetric method, modified Jaffé), aspartate aminotransferase (AST; by UV kinetic method), and alanine aminotransferase (ALT; by UV kinetic method). LDL levels were calculated using the Friedewald equation [27].

Determination of atherosclerotic plaque size

Lipid deposition in the aortic arch and in the thoracic aorta were measured using en face analysis with Sudan IV dyes [28]. At euthanasia, the aortas were dissected and all the adventitia from the aortic valve to the iliac bifurcation were carefully removed and immediately stored in 10% formalin. After that, the aortas were opened longitudinally and fixed for 12 h in a formalin saccharose solution (4% paraformaldehyde, 5% saccharose, 20 mmol·l^{-1} butylated hydroxytoluene (BHT), 2 mmol·l^{-1} EDTA, pH 7.4) at 4 °C. Afterwards, the aortas were placed in a 70% ethanol solution for 5 min. Subsequently, they were stained in a solution containing 0.5% Sudan IV, 35% ethanol and 50% acetone for 10 min under agitation, and then bleached in an 80% ethanol solution for 5 min. The stained aortas were photographed using an 8.1 megapixel digital camera (Canon, Tokyo, Japan) with controlled distance, zoom and luminosity. The analyses were performed using the Image-Pro Plus software package (Media Cybernetics, Rockville, Maryland, USA). Pixels were converted into square centimeters using a standard microscopic scale. The sum of the atherosclerotic lesion area (where lipid accumulation was seen) was calculated by the software, and the results were expressed in square centimeters. To ensure that there were no differences in the total size of the aorta between animals, this area was also measured.

Statistical analyses

The statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, Illinois, USA). The frequency (mean, median, standard deviation, minimum, maximum) of all groups was determined. The Kolmogorov-Smirnov test was used to check the data symmetry and equality of variance (normal distribution). Analysis of variance (ANOVA) was used for comparisons involving three or more groups, and Tukey's test at 5 % of probability was used when necessary. The Kruskal-Wallis test was used when the ANOVA test was contraindicated (non-normal distribution).

RESULTS AND DISCUSSION

Influence of the extractor solvent on the recovery of anthocyanins

The obtained Juçara extracts had variable concentrations of anthocyanins, as determined by spectrometry (Fig. 1). The three best solvent combinations to obtain an anthocyanin-rich Juçara pulp were those with citric acid added to 95% ethanol (S1A2), 80% ethanol (S2A2), and 70% ethanol (S3A2). These results were probably due to the acidulant and preservative action of this agent. The mixture of 80% ethanol and 0.3% citric acid was selected to obtain an anthocyanin-rich pulp. Spectrophotometric determinations of the total anthocyanin concentration obtained using

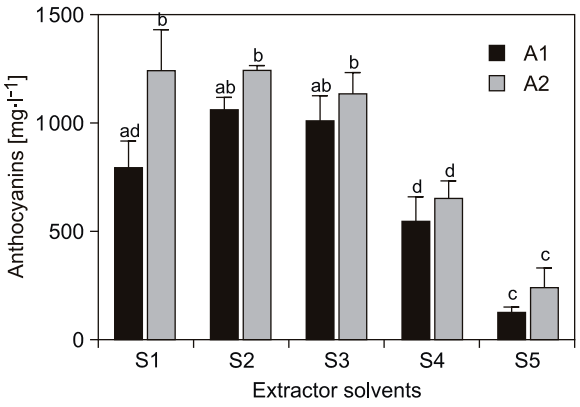

Fig. 1. Efficiency of the solvents for the extraction of anthocyanins from Juçara extract.

Means followed by the same letter in the bars are not different based on Tukey's test, $p > 0.05$.
S1 – 95% ethanol, S2 – 80% ethanol, S3 – 70% ethanol, S4 – 60% ethanol, S5 – water, A1 – 0.1% HCl, A2 – 0.3% citric acid.

the solvent mixture S2A2 was 1249.74 mg·l^{-1} of pulp. All the extraction solutions prepared in this experiment had pH lower than 4.0, ranging from 1.72 to 3.44, to ensure the stability of the anthocyanin molecules. In a study performed by Borges et al. [29] researchers tested different solvents to extract anthocyanins from *E. edulis* and observed better results for anthocyanins concentration using the solvent methanol + 1.5 mol·l^{-1} HCl.

Quantification of anthocyanins

The total anthocyanin content present in the freeze-dried Juçara extract was 24.714 g·kg^{-1} of extract. Borges et al. [2] showed that five samples of Juçara palm trees of the species *E. edulis*, from five different regions of Santa Catarina, presented different anthocyanin contents ranging between 148.40–4099 mg·kg^{-1} fruit. These values are very different for the same fruit from different regions.

The profiles of anthocyanins in the freeze-dried Juçara extract assessed by HPLC-DAD are presented in Fig. 2. The total anthocyanin content (cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside) in freeze-dried Juçara extract was 25.83 g·kg^{-1}, the cyanidin-3-O-glucoside content in freeze-dried Juçara extract was 9.52 g·kg^{-1} and the cyanidin-3-O-rutinoside content in freeze-dried Juçara extract was 16.31 g·kg^{-1} (peak 2). Compared to these results, other studies found lower anthocyanin contents for *E. edulis*, from (0.856 ± 0.005) g to (4.099 ± 2.33) g of cyanidin-3-glucoside per kilogram of fresh matter [3, 4]. These differences could be partly due to differences in the growing conditions such as altitude, the fruiting period of palm and the intensity of luminosity, as well as the ripeness stage of the fruit [2, 3]. Del Pozo-Insfran et al. [30] quantified anthocyanins from *Euterpe oleracea* by HPLC and found cyanidin-3-O-glucoside and perlagonidin-3-O-glucoside with the respective values of (1040 ± 58.2) mg·l^{-1} and (74.4 ± 2.90) mg·l^{-1} (fresh pulp basis). They did not find cyanidin-3-O-rutinoside. Pompeu et al. [31] obtained anthocyanins yield similar to our study regarding fruits of *E. oleracea* from the north, i.e. 62.0% of cyanidin-3-O-rutinoside and 38.0% of cyanidin-3-O-glucoside, whereas in our study the results were 63.1% and 36.9%, respectively.

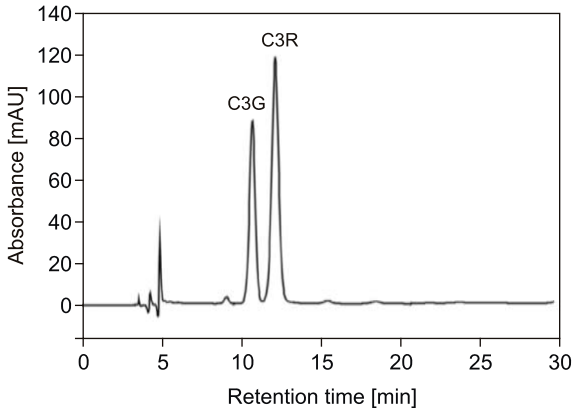

Fig. 2. HPLC chromatogram of anthocyanins from the freeze-dried Juçara extract.

C3G – cyanidin-3-O-glucoside, C3R – cyanidin-3-O-rutinoside.

Complementary to HPLC/DAD analyses, the HPLC-ESI-MS methodology also identified cyanidin-3-glucoside (37.8%) and cyanidin-3-rutinoside (53.0%) as major anthocyanins in frozen pulp of *E. edulis,* accounting for approx. 90% together. The other 10% corresponded to other minor anthocyanins: cyanidin-3-sambunoside (2.8%), peonidin-3-rutinoside (0.4%), pelargonidin-3-rutinoside (5.7%) and delphinidin-3-glucoside (0.4%; Tab. 1). The results of this study are in concordance with data reported by Brito et al. [1]. These authors also found cyanidin-3-glucoside (52.9%) and cyanidin-3-rutinoside (45.9%) as predominant anthocyanins in fruits of *E. edulis*, followed by cyanidin-3-sambunoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside and cyanidin-3-raminoside. Additionally, many researchers studying the fruits of *E. oleracea* also identified cyanidin-3-rutinoside and cyanidin-3-glucoside as the main anthocyanins present in the fruit [15, 30, 32–36]. Schauss et al. [15] also found small amounts of cyanidin-3-sambubioside, peonidin-3-glucoside and peonidin-3-rutinoside in *E. oleracea*.

The freeze-dried Juçara extract was found to have higher total anthocyanins content compared to other anthocyanin-rich fruits, such as blueberries (0.8–2.6 g cyanidin-3-glucoside per kilogram fresh matter), and blackberries (1.2–1.5 g cyanidin-3-glucoside per kilogram fresh matter) [37].

Physico-chemical characterization

Chemical composition

The proximate chemical composition of the freeze-dried Juçara extract was: 6.6% protein, 16.8% lipids, 67.8% saccharides and 2.4% ash. Borges et al. [2] reported that *E. edulis* fruits had large variations in the lipid (18.5% to 44.1%), protein (5.1% to 8.2%) and ash (from 1.6% to 3.3%) content, depending on the season and place where the harvest was done. Schauss et al. [15] found 8.1% protein, 32.5% lipids and 52.2% saccharides. Menezes et al. [38] reported 4.9% moisture, 3.7% ash, 8.1% protein, 40.8% lipids and 42.5% saccha-

Tab. 1. Anthocyanins identified by HPLC-ESI-MS in the freeze-dried pulp of *E. edulis* fruits.

Compounds	Retention time [min]	λ_{max} [nm]	Precursor ion $[M^+H]^+$ [m/z]	Product ions MS/MS [m/z]	Relative composition [%]
Cyanidin-3-glucoside	18.83	520	449	287	37.8
Cyanidin-3-rutinoside	19.35	520	595	287, 449	53.0
Cyanidin-3-sambunoside	17.98	310	581	287	2.8
Peonidin-3-rutinoside	21.87	520	609	301, 463	0.4
Pelargonidin-3-glucoside	21.01	520	433	271	5.7
Delfinidin-3-glucoside	19.89	520	465	303	0.4

λ_{max} – maximum absorption wavelength.

rides in a freeze-dried *E. oleraceae* pulp.

The mineral composition of the freeze-dried Juçara extract (in milligrams per kilogram) was: 267 mg phosphorus, 8922 mg potassium, 1000 mg calcium, 980 mg magnesium, 1493 mg sodium, 20.7 mg zinc; 52 mg iron, 239 mg manganese and 11.1 mg copper. The results of this study show lower calcium contents and higher iron contents than those determined in other studies with *E. oleracea* [15, 38] and *E. edulis* Mart. [13].

The lipid profile of the freeze-dried Juçara extract was 27.1% palmitic acid (C16:0), 34.0% oleic acid (C18:1, omega 9), 32.9% linoleic acid (C18:2, omega 6) and 1.8% linolenic acid (C18:3, omega 3). The lipid profile of the freeze-dried Juçara extract obtained in this study was different from those found by MENEZES et al. [38] for *E. oleracea*. BORGES et al. [2] showed that the highest proportion of fatty acids was comprised of oleic acid (from 44.2% to 55.6%) and linoleic acid (from 18.2% to 25.4%). SILVA et al. [13] detected 34.4% palmitic acid, 2.6% palmitoleic acid, 36.0% oleic acid and 8.0% linolenic acid in Juçara pulp. Regarding the high incidence of mono- and polyunsaturated fatty acids, it is worth noting the importance of these fatty acids in the prevention of risk factors for cardiovascular disease, such as dyslipidemia, obesity and even diabetes mellitus. It is known that a high ratio of oleic and linoleic/linolenic fatty acids may prevent the incidence of these diseases.

The freeze-dried Juçara extract contained 0.0239 mg of β-carotene per kilogram of extract (Fig. 3), which represents 39830-fold tolerable upper intake level (UI) of vitamin A per kilogram of freeze-dried Juçara extract. This result is higher than that found by SCHAUSS et al. [15] of 10002 UI of vitamin A per kilogram of the freeze-dried Juçara extract.

Antioxidant activity in vitro

The concentration of Juçara extract required to reduce the initial concentration of DPPH radical by 50% (EC_{50}) was 42.72 mg·l^{-1}. This result can also be expressed as 3.55 g freeze-dried Juçara extract per gram DPPH, considering that 0.01203 g of DPPH was used. BORGES et al. [2] measured the antioxidant capacity of five fruits of *E. edulis* palm using the DPPH assay and found EC_{50} values varying from 0.85 g to 4.83 g Juçara extract per gram DPPH. In another study, BORGES et al. [3] also reported similar results for antioxidant activity, finding (59.9 ± 3.4)% inhibition of DPPH in 40.99 mg cyanidin-3-glucoside per gram fresh matter of *E. edulis.* BICUDO et al. [4] found that the antioxidant activity of Juçara fruit increased through ripening. The authors observed that the phenolic compounds in the fruit and their antioxidant activity were influenced by the place where the palm grew. Part of the DPPH-connected information in the literature could not be compared with the results of this study because the results were expressed in other units (such as micromoles of Trolox equivalent per gram of dry matter).

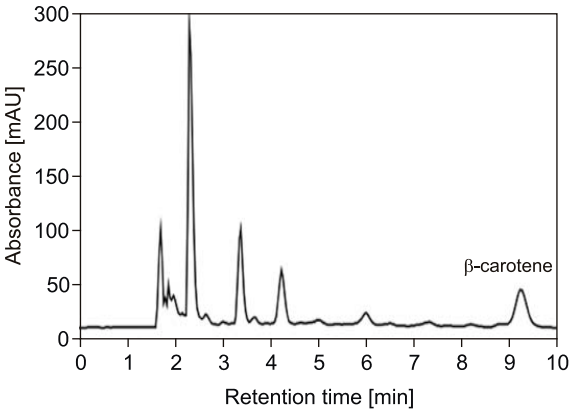

Fig. 3. HPLC chromatogram of carotenoids from the freeze-dried Juçara extract, indicating presence of β-carotene.

In vivo study

Biochemical blood parameters of the experimental animals

The results of the biochemical parameter assessments are shown in Tab. 2. Groups G3, G4 and G5 showed a statistically significant reduction in LDL, and the ratios of total cholesterol/HDL and LDL/HDL, which are indicators of cardiovascular risk, when compared to the G2 group. Therefore, the groups that consumed a diet with the addition of freeze-dried Juçara extract had a reduced cardiovascular risk when compared with the control group, which did not ingest this diet. This fact can be explained by the evidence that phenolic compounds may also inhibit the intestinal absorption of dietary lipids by forming complexes with biomolecules and thus interfere with the process of emulsification, solubilization and hydrolysis of micelles [39–41]. However, there was no significant difference between the groups with an Juçara-based diet and the G2 control groups for triglycerides and HDL. Groups G3, G4 and G5 showed a significant reduction in glucose when compared to the G2 group. However, there was no significant difference between the groups regarding the enzymes used as markers of hepatotoxicity (AST and ALT), as well as for the marker used for nephrotoxicity (creatinine). Miyazaki et al. [42] used an anthocyanin-rich extract from purple sweet potato in a ApoE$^{-/-}$ model and also found no significant differences in HDL cholesterol, triglycerides, AST and ALT between the treatment and the control groups. The G5 group, to which the drug simvastatin was administered, presented an unexpected effect in reduction of glucose in the plasma. Sweany et al. [43] indicated an improvement in glycemic control in patients treated with simvastatin. The exact mechanism of the reduction of glucose levels by statins is not yet known. Thus, it is important to emphasize the importance of studies on glucose homeostasis in the mechanism of action of this class of drugs.

The results from this study demonstrate a beneficial effect of adding freeze-dried Juçara extract to the mouse diet. The groups who consumed the Juçara extract presented lower values of total cholesterol, LDL and glucose. Furthermore, there was no evidence of possible hepatotoxic or nephrotoxic effects.

Atherosclerotic lesions in the aorta

There was no significant difference ($p > 0.05$) in the total area of the aortas between the animal groups by one-way ANOVA. After the confirmation of the similar areas of the aorta, it was possible to analyse the areas of atherosclerotic plaques and to relate them to the lesion areas (Tab. 3). The reduction in atherosclerotic areas was not significant ($p > 0.05$) in all groups. However, some groups fed with freeze-dried Juçara extract added to the diet presented lesions that were visually less evolved. In the present study, the consumption of the freeze-dried Juçara extract from *E. edulis* alone did not affect the size of atherosclerotic plaques in ApoE$^{-/-}$ mice. In contrast to our findings, Xia et al. [44] reported that an anthocyanin-rich extract from black rice reduced the size of advanced atherosclerotic plaques by 18% after 20 weeks of intervention in 30 week-old ApoE$^{-/-}$ mice. Such differences may be related not only

Tab. 2. Biochemical parameters of mouse sera.

Parameter	Positive control (G2)	2% Juçara (G3)	6% Juçara (G4)	Simvastatin (G5)
Glucose [mmol·l^{-1}]	7.892 ± 2.549^{a}	5.178 ± 0.078^{b}	5.211 ± 0.829^{b}	5.305 ± 0.975^{b}
Total cholesterol [mmol·l^{-1}]	10.753 ± 1.327^{a}	8.756 ± 0.927^{bc}	7.580 ± 0.975^{c}	9.494 ± 1.583^{ab}
HDL [mmol·l^{-1}]	0.751 ± 0.093^{a}	0.764 ± 0.095^{a}	0.673 ± 0.034^{a}	0.986 ± 0.277^{b}
LDL [mmol·l^{-1}]	9.619 ± 1.325^{a}	7.573 ± 0.880^{b}	6.529 ± 0.978^{b}	7.975 ± 1.517^{b}
Triglycerides [mmol·l^{-1}]	0.836 ± 0,113^{a}	0.915 ± 0.333^{a}	0.823 ± 0.205^{a}	1.162 ± 0.284^{a}
Cholesterol/HDL	0.378 ± 0.083^{a}	0.299 ± 0.036^{b}	0.293 ± 0.048^{b}	0.259 ± 0.058^{b}
LDL/HDL	0.339 ± 0.080^{a}	0.259 ± 0.036^{b}	0.252 ± 0.047^{b}	0.218 ± 0.218^{b}
AST [μkat·l^{-1}]	1.900 ± 0.450^{a}	2.336 ± 0.778^{a}	1.940 ± 0.465^{a}	2.102 ± 0.788^{a}
ALT [μkat·l^{-1}]	0.370 ± 0.125^{a}	0.644 ± 0.330^{a}	0.557 ± 0.331^{a}	0.551 ± 0.229^{a}
Creatinine [μmol·l^{-1}]	122.87 ± 68.92^{a}	64.53 ± 43.31^{a}	71.60 ± 34.47^{a}	98.12 ± 73.37^{a}

Means followed by the same letter in the same row are not different based on Tukey's test, $p > 0.05$.
HDL – high density lipoprotein, LDL – low density lipoprotein, AST – aspartate aminotransferase, ALT – alanine aminotransferase.
G2 – mice groups that received AIN-93M diet, G3 – mice groups that received AIN-93M diet with 2% freeze-dried Juçara extract, G4 – mice groups that received AIN-93M diet with 6% freeze-dried Juçara extract, G5 – mice groups that received AIN-M93 diet with 50 mg·kg^{-1}d^{-1} of simvastatin.

to the age of the animals (21 weeks vs 30 weeks) and lesion stage (stable plaque vs vulnerable plaque), but also to treatment duration (12 weeks vs 20 weeks). Studies using 20-week interventions led to different results [44, 45]. Studies showed no correlation between the levels of total cholesterol in the plasma and the atheromatous lesions in ApoE$^{-/-}$ mice. Despite the good results obtained in this study regarding the lipid profile, no significant ($p > 0.05$) reduction in the atheromatous plaques was observed in ApoE$^{-/-}$ animals.

Tab. 3. Total area of the aorta and percentage of lesioned area in mice groups.

Mice groups	Total area of the aorta [cm^2]	Lesioned area [%]
Negative control (G1)	0.168 ± 0.02	–
Positive control (G2)	0.193 ± 0.01	10.3 ± 1.91
2% Juçara (G3)	0.171 ± 0.03	7.9 ± 5.01
6% Juçara (G4)	0.178 ± 0.02	8.4 ± 3.84
Simvastatin (G5)	0.165 ± 0.02	6.6 ± 5.09

Data were expressed as mean with standard deviation. There was no significant difference between the groups by one-way ANOVA.
G1 – mice groups (C57Bl) that received AIN-93M diet, G2 – mice groups that received AIN-93M diet, G3 – mice groups that received AIN-93M diet with 2% freeze-dried Juçara extract, G4 – mice groups that received AIN-93M diet with 6% freeze-dried Juçara extract, G5 – mice groups that received AIN-M93 diet with 50 mg·kg^{-1}d^{-1} of simvastatin.

Enzymatic activity: catalase and superoxide dismutase in the liver

The enzymes catalase (CAT) and superoxide dismutase (SOD) showed greater activity in the positive control (G2) group than in the negative control (G1), which pointed to differences in stress inherent to the ApoE knockout model. The results obtained for the groups that received 2% and 6% of Juçara extract showed a significant decrease ($p < 0.05$) in CAT and SOD activities compared with the positive control (G2) group. Therefore, the freeze-dried Juçara extract added to the diet supplied, in part, the antioxidant activity of these liver enzymes. The Fig. 4 shows the variation in CAT and SOD activities between the groups after treatment with the freeze-dried Juçara extract and simvastatin. Hassimotto et al. [46] showed that even a small amount of anthocyanins given to rats was sufficient to increase the plasma antioxidant capacity. Lichtenthahler et al. [35] showed that the two main anthocyanins, cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, extracted from eleven commercial and non-commercial pulps from *E. oleracea*, showed good antioxidant capacity against peroxyl radicals, peroxynitrite and hydroxyl radicals. Tsuda et al. [47] reported that feeding rats with cyanidin-3-O-glucoside significantly reduced the antioxidant activity of enzymes in vivo. According to the authors, when administered orally, cyanidin-3-O-glucoside is absorbed in the gut and is distributed through the bloodstream to the tissue. In the tissue, this anthocyanin and its metabolites can react with reactive oxygen species, thereby reducing hepatic tissue damage.

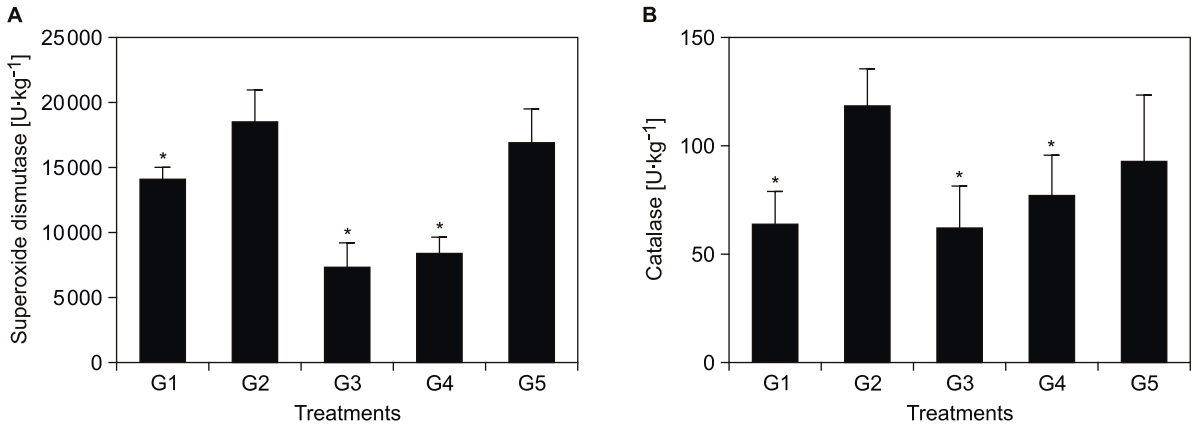

Fig. 4. Enzymatic activity in mice groups treated with Juçara extract and simvastatin.

A – Superoxide dismutase activity, B – Catalase activity.
Activities of enzymes are expressed per kilogram of protein. * – Means with significant difference based on Tukey's test, $p < 0.05$.
G1 – mice groups (C57Bl) that received AIN-93M diet, G2 – mice groups that received AIN-93M diet, G3 – mice groups that received AIN-93M diet with 2% freeze-dried Juçara extract, G4 – mice groups that received AIN-93M diet with 6% freeze-dried Juçara extract, G5 – mice groups that received AIN-M93 diet with 50 mg·kg^{-1}d^{-1} of simvastatin.

CONCLUSION

The results of this study show the antioxidant potential of Juçara extract and demonstrate that one of the most efficient methods for anthocyanin extraction is the combination of 80% ethanol with 0.3% citric acid, which provided an anthocyanin concentration of 1249.74 $mg \cdot l^{-1}$ of Juçara extract. The predominant anthocyanins were cyanidin-3-O-glucoside (9.52 $g \cdot kg^{-1}$ of freeze-dried Juçara extract) and cyanidin-3-O-rutinoside (16.31 $g \cdot kg^{-1}$ of freeze-dried Juçara extract). The indicators of cardiovascular risk, such as LDL, the ratios of total cholesterol/HDL and LDL/HDL, showed a reduction ($p < 0.05$) in $ApoE^{-/-}$ mice that received diet with addition of Juçara extract, compared to the control group. However, no reduction of atherosclerotic plaque areas was observed in the groups fed with freeze-dried Juçara extract added to the diet in a 10-weeks experiment. The hepatotoxicity and nephrotoxicity markers did not show a significant difference between the mouse groups in this experimental model. However, the in vivo antioxidant activity of SOD and CAT decreased in the mice groups given the Juçara diet. These results demonstrate that enzyme activity was suppressed by the presence of natural antioxidants from Juçara after absorption and metabolism in the groups that received 2% or 6% freeze-dried Juçara extract. These findings suggest that the anthocyanins in Juçara extract can protect against damage related to oxidative stress in mice. Regarding the high content of mono- and polyunsaturated fatty acids, these results encourage us to continue investigations, mainly with the aim of elucidating new mechanisms of action, which may strengthen the involvement of anthocyanins in the reduction of the atherosclerotic process. These data can also reinforce the use of Juçara as a functional food with beneficial effects.

Acknowledgments

The authors would like to thank the National Council for Scientific and Technological Development – CNPq (Brasilia, Distrito Federal, Brazil), the Coordination of Improvement of Higher Level Personnel – CAPES (Brasilia, Distrito Federal, Brazil) and the Foundation for Research Support of Minas Gerais – FAPEMIG (Belo Horizonte, Minas Gerais, Brazil) for financially supporting this project. The authors also thank to Biomolecules Center at Federal University of Viçosa for providing LC-MS.

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Received 23 May 2014; revised 5 August 2014; accepted 2 September 2014; published online 17 March 2015.