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Antioxidant Activity and Characterization of Phenolic Compounds from Bacaba (*Oenocarpus bacaba* Mart.) Fruit by HPLC-DAD-MS^n

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ABSTRACT: The phytochemicals in fruits have been shown to be major bioactive compounds with regard to health benefits. Bacaba (*Oenocarpus bacaba* Mart.) is a native palm fruit from the Brazilian savannah and Amazon rainforest that plays an important role in the diet of rural communities and is also a source of income for poor people. This paper reports the characterization and analyses of phenolics from bacaba fruit extract. The total phenolic content of bacaba fruit amounted to 1759.27 ± 1.01 mg GAE/100 g, the flavonoid content was 1134.32 ± 0.03 mg CTE/100 g, and the anthocyanin content was 34.69 ± 0.00 mg cyn-3-glc/100 g. The antioxidant activity was evaluated through different assays [ORAC, FRAP, DPPH, TEAC, and cellular antioxidant assay (CAA) assays] and revealed a significant antioxidant capacity for bacaba in comparison to the data available in the literature. The assignment of the phenolic compounds using HPLC-DAD-MS^n was based on the evaluation of their UV−vis absorption maxima (λ_{max}) and mass spectral analyses, and 14 compounds were tentatively identified. The results suggest that bacaba fruits are a promising source of phenolics.

KEYWORDS: *Oenocarpus bacaba Mart., antioxidant activity, polyphenols, anthocyanins, HPLC-ESI-MS^n*

INTRODUCTION

The search for antioxidants from natural sources has called attention to compounds that may help to prevent oxidative damage in the body and contribute to the prevention of noncommunicable diseases, and many efforts have been made to identify such compounds.[1] Phenolics are important products of secondary plant metabolism and are characterized by an aromatic backbone with one or more hydroxyl groups. Phenolics often occur bound to mono- and disaccharides and also form complexes with oligosaccharides, lipids, amines, and carboxylic and organic acids. These compounds are used in processed food as natural antioxidants because of their important antioxidant activities. Indeed, with regard to the health benefits for humans, phenolics are believed to play a significant protective role: due to their antioxidant activity, phytochemicals can scavenge free radicals and modulate the activities of enzymes involved in detoxification, oxidation, and reduction processes.[2] Furthermore, phytochemicals can boost the immune system, regulate gene expression and cell signaling, influence cell proliferation and apoptosis, and regulate hormone metabolism.

Brazil is endowed with a remarkable biodiversity, and many edible plants have yet to be discovered. Although many fruits are available, the consumption of such fruits is mainly regional, and commercial utilization is either minimal or absent.[3] The nutritional and health properties of many of these fruits remain unknown, and the associated scientific research is insufficient and inconsequential, ignoring the great nutritional potential of these food sources. In folk medicine, many of these fruits are believed to possess functional properties; however, there are no studies to date investigating the assumed functional properties, such as the antioxidant activity.

Bacaba (*Oenocarpus bacaba* Mart., Arecaceae) is a palm found in the Brazilian Amazon and Cerrado (Brazilian savannah) biomes that produces edible purple-colored berries, which are locally consumed either as a natural juice or processed into drinks, jelly, and ice cream. The investigation of such traditional fruits as bacaba is a relevant topic, as these analyses can reveal the possible functional properties that can add value to the fruit and allow the development of a commercial market for it. Moreover, the nutritional or commercial value that is added through scientific research on the nutritional and health properties of bacaba calls attention to the preservation of the palm. This information also provides additional options for nutritional and dietetic recommendations, taking into account both food practices and cultural issues.

Currently, there are many studies that examine the phenolic compounds of vegetables, fruits, whole grains, and other plants and their antioxidant role in noncommunicable diseases, such as cancer. Many of these studies suggest that phenolic compounds could become healthy food additives for the prevention of chronic diseases.[4] Studies with several common

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fruits and vegetables have already revealed antioxidant and antiproliferative effects on different cell lines. Previous research from our group revealed a relevant antiproliferative activity of a bacaba phenolic extract in HepG2 cells;[5] however, the extract was not characterized. Therefore, the objective of this work was to characterize this bacaba fruit phenolic extract by determining the total contents of phenolics, flavonoids, and anthocyanins and the antioxidant activity.

MATERIALS AND METHODS

Chemicals. All of the chemicals used in the study were of analytical or HPLC grade. Gallic acid, aluminum chloride ($AlCl_3$), (+)-catechin, cyanidin-3-glucoside, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT), and methylene blue were obtained from Sigma-Aldrich (Steinheim, Germany). Folin−Ciocalteu reagent, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), quercetin dihydrate, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein in CAPS solution (20 mM), and Trypan blue solution were obtained from Fluka-Chemie (Buchs, Switzerland). 2,2′-Azobis(2-amidipropane) (ABAP) was purchased from Wako Chemicals, USA, and DMEM/Ham's F-12 was purchased from PromoCell GmbH (Heidelberg, Germany). Fetal calf serum (FCS), penicillin/streptomycin, trypsin/EDTA, and : Hank's balanced salt solution (HBSS; with Ca^{2+}, Mg^{+}, without phenol red) were obtained from PAA Laboratories (Pasching, Austria). The water used in all of the analyses was ultrapure water produced by a Milli-Q system (Millipore Corp., France).

Plant Materials and Sample Preparation. The fruit samples of bacaba (*O. bacaba* Mart.) were acquired from small farmers and collected in Tocantins State, Brazil, during November and December of 2008. The fruits were washed with water to remove debris and dried manually (Figure 1); damaged fruits were excluded. The seeds were discarded, and the samples were lyophilized (LS 3000, Terroni, Brazil), milled in a knife mill (8500 rpm; 12 s) (Retsch, Grindomix GM200, Germany), sealed under vacuum, and stored in the dark in a refrigerator (4 °C) until use (Figure 1). The water content of the original samples was determined by drying at 105 ± 5 °C to a constant weight to allow calculations based on the fresh weight (FW) of the fruits;[6] all of the results refer to the fresh weight of the fruits.

Figure 1. Bacaba (*Oenocarpus bacaba* Mart.): (left) bacaba fruit; (right) vacuum-sealed freeze-dried samples.

Fruit Extract Preparation. According to the methodology of our tests to establish the optimal conditions for the quantitative recovery of the phenolic compounds (data not shown), aqueous acetone (80%; v/v) was used for the extraction of the phenolic compounds from bacaba fruit, as described by Kammerer et al.[7] Briefly, aliquots of 50 mg were placed in conical flasks and extracted with 5 mL of solvent for 2 h under stirring after flushing with argon to prevent oxidation during the extraction. The extracts were centrifuged (10 min, 4000*g*), and the combined supernatants were evaporated to dryness in vacuo at 30 °C. For the HPLC analysis, the residue was dissolved in 10 mL of acidified water (final pH 2.25), whereas the residue was resuspended in PBS^{-} for the additional analyses (final pH 6.35).

Total Phenolics. The polyphenols were quantified using the Folin−Ciocalteu (FC) assay.[8] The total phenolic contents were expressed as milligrams of gallic acid equivalents (GAEq) per gram of fruit FW.

Total Flavonoids. The content of total flavonoids was assessed using the colorimetric aluminum chloride assay described by Sariburun et al. [9] and adapted to microplate measurements in our laboratory. Briefly, 20 μL of $NaNO_2$ solution (10%) was added to 50 μL of catechin or sample dilutions in 96-well plates. After 5 min, 20 μL of $AlCl_3$ (5%, m/v) was added to each well and incubated for 6 min. Subsequently, 100 μL of 1 M NaOH solution was added, and the absorbance was measured at 510 nm using a microplate reader Synergy MX (Biotek, USA). The results were expressed as milligrams of catechin equivalents (CTEq) per 100 g FW.

Total Anthocyanins. The total monomeric anthocyanin content was assessed using the pH-differential method with two buffer systems, potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M), as described by Sellappan et al.[10] and adapted to microplate volumes in our laboratory. Briefly, 20 μL of a standard or sample solution was pipetted into two different 96-well plates and mixed with the respective buffers (180 μL). The absorbances were measured at 510 and 700 nm. Wells containing buffer without the sample solution were used as the blanks. Cyanidin-3-glucoside was used as a standard; therefore, the results were expressed as milligrams of cyanidin-3-glucoside equivalents (cyn-3-glcEq)/100 g FW.[11] The absorbance (A) was calculated according to the following formula: $A = (A_{510\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay procedure using fluorescein as a fluorescence probe was performed according to the methodology developed by Cao and Prior.[12] Briefly, fluorescein in 20 mM CAPS solution, pH 10 (Fluka, St. Louis, MO, USA), was used as the stock solution and diluted in phosphate buffer solution (PBS, pH 7.4) to obtain a working solution of 4 nM. AAPH (the peroxyl radical was generated using 2,2′-azobis(2-amidinopropane) dihydrochloride) was freshly prepared for each assay at a concentration of 15 mM and kept on ice until automatic injection. Trolox solutions were used for the establishing calibration curves (0, 6.25, 12.5, 25, 50, and 100 μM) and were prepared daily from a 100 μM stock solution stored at −80 °C. For each set of measurements, a standard curve was plotted using Trolox as the reference. The analyses were performed using clear-bottom black 96-well plates (Greiner Bio-one Cellstar, Frickenhausen) and an automated plate reader (Synergy MX, Biotek, USA). The exterior wells of the plate were filled with 300 μL of water and preheated to 37 °C to avoid an oscillation of the temperature during the analysis. The sample aliquots (25 μL) were mixed with 200 μL of fluorescein solution (4 nM) and incubated for 30 min at 37 °C in the microplate. AAPH (25 μL) was automatically injected, and the microplate was shaken for 20 s in the fast mode. The fluorescence readings ($\lambda_{excitation}$ = 485 ± 20 nm and $\lambda_{emission}$ = 520 ± 20 nm) were registered each minute over a period of 60 min. All of the samples were analyzed in three different experiments using five wells for each dilution. The antioxidant activity was expressed as micromolar Trolox equivalents (TE) per 100 gFW on the basis of the calculation of the area under the curve (AUC; eq 1) and interpolation to the regression analysis of the Trolox calibration curve.

$$AUC = 0.5 + (f_2/f_1) + (f_3/f_1) + (f_4/f_1) + \ldots + 0.5(f_{60}/f_1) \quad (1)$$

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. This assay was performed as previously described by Re and Pellegrini.[13] Briefly, the radical was formed by mixing 0.5 mL of 20 mM ABTS in phosphate buffer (pH 7.4) with 95 mL of a 2.5 mM ABAP solution. The solution was heated for 15 min at 60 °C and protected from light. Subsequently, 190 μL of the radical solution was added to 10 μL of different dilutions of the fruit extracts (0.01−0.6 mg/mL) and Trolox (5−140 μM) using an automatic injector. The absorbance was monitored at 734 nm for 30 min. All of the samples were analyzed in triplicate for each dilution. The antioxidant activity was expressed as

micromolar TE per 100 g FW on the basis of the calculation of the AUC values and interpolation to the regression analysis of the Trolox calibration curve (eq 2).

$$\mathrm{AUC} = 0.5 + (f_2/f_1) + (f_3/f_1) + (f_4/f_1) + \ldots + 0.5(f_{60}/f_1) \quad (2)$$

Ferric-Reducing Ability of Plasma (FRAP) Assay. The measurement of the reduction of the ferric 2,4,6-tripyridyl-*s*-triazine complex (Fe^{3+}-TPTZ) to its ferrous form (Fe^{2+}-TPTZ) in the presence of antioxidants was performed as previously described.[14,15] Briefly, the FRAP solution was prepared by mixing 30 mM acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3 \cdot 6\ H_2O$ at a ratio of 10:1:1 (v/v/v). $FeSO_4 \cdot 7\ H_2O$ and Trolox were used as the standards to allow further comparisons. A 1.0 mM stock solution of $FeSO_4 \cdot 7\ H_2O$ and a 1.5 mM stock solution of Trolox were used for preparing the different dilutions (0.02−0.14 and 15−50 μM, respectively) for the standard curves, and different dilutions of bacaba (0.5−2.5 mg/mL) were also included to build a curve that allowed quantification in a linear range of detection. A total of 150 μL of FRAP reagent was added to each well, and 20 μL of the standard or sample solutions was added. The absorbance was measured at 593 nm, and a well containing FRAP reagent without the sample was used as the blank. The FRAP values were calculated by the interpolation of the Trolox and $FeSO_4 \cdot 7\ H_2O$ calibration curves.

Free Radical-Scavenging Capacity (DPPH Assay). The free radical-scavenging capacity was analyzed using the DPPH assay in accordance with the method described by Al-Duais et al.[16] and adapted to microplate volumes in our laboratory. A 100 μL aliquot of the standard or diluted sample solution of bacaba (0.01−0.5 mg/mL) was mixed with 100 μL of DPPH (0.3 mM, ethanolic solution). Methanol (200 μL) was used as the control, and 100 μL of ethanol with 100 μL of DPPH was used as the blank. A calibration curve was prepared with different dilutions of Trolox (10, 20, 40, 60, 80, 100, 120, and 140 μM). The absorbance was measured at 510 nm for 30 min[17] and converted to antioxidant activity (AA%). The radical-scavenging activity ($DPPH^{\bullet}$) was calculated according to eq 3

$$\mathrm{AA\%} = 100 - [(A_{\mathrm{sample}} - A_{\mathrm{blank}}) \times 100/A_{\mathrm{control}}] \quad (3)$$

where A_{sample} = the sample absorbance after 30 min of reaction, A_{blank} = the blank absorbance, and A_{control} = the absorbance of the control.

The results of the DPPH reduction in the samples were linearized and interpolated to the standard Trolox curve. Results were obtained from three independent experiments and expressed in millimolar TE per 100 g fruit FW.

Cell Culture of HepG2 Cells. HepG2 cells were cultured in DMEM/Ham's F12 with stable glutamine and supplemented with 10% FCS and 1% P/S at 37 °C in a water-saturated atmosphere with 5% CO_2. The medium was changed every 2 or 3 days, and the cells were subcultured before reaching 80% confluency.

Cellular Antioxidant Activity (CAA) Assay. The CAA assay was performed as described by Wolfe and Liu.[18] Briefly, HepG2 cells were seeded into black 96-well plates (Nunc, Roskilde, enmark) at a density of 6×10^4 cells/well in 100 μL of growth medium without phenol red to avoid undesired background fluorescence. The outer wells of the plates were filled with water to favor temperature stability during the assay. After incubation for 24 h (37 °C; 5% CO_2), the growth medium was removed by gently turning the plate upside down onto a paper towel soaked in 70% ethanol, and the cells were washed with 100 μL of HBSS. The cells were then treated for 1 h with 100 μL of fruit extract (37 °C; 5% CO_2). Different concentrations of the fruit extracts were prepared in a medium containing 25 μM DCFH-DA and devoid of phenol red and FCS. Quercetin was used as a standard compound in the different dilutions in each plate, as suggested by Wolfe and Liu.[18] After 1 h of treatment, the medium was removed, and the cells were washed again with 100 μL of HBSS. Subsequently, 600 μM ABAP was applied to the cells in 100 μL of HBSS. The control triplicates were treated with DCFH-DA and ABAP but did not contain fruit extract, and the blank triplicates were treated only with HBSS without ABAP. The outer wells, which were not used in the beginning, were filled with 300 μL of sterile prewarmed water (37 °C). The fluorescence was measured at 37 °C using a microplate reader (Synergy MX, Biotek, Bad Friedrichshall, Germany) ($\lambda_{\mathrm{excitation}}$ = 485 ± 20 nm and $\lambda_{\mathrm{emission}}$ = 538 ± 20 nm) at 5 min intervals for 90 min. The cells were treated in triplicate for each extract concentration, and control and blank wells were included containing only medium plus DCFH-DA without fruit extract. At least three independent experiments were performed to determine the cellular antioxidant activity. Triplicates were used in each experiment for each extract concentration. After subtraction of the blank, the AUC values for the fluorescence versus time plot were determined to calculate the CAA units for each fruit concentration according to eq 4

$$\mathrm{CAA\ unit} = 1 - (\textstyle\int \mathrm{SA}/\int \mathrm{CA}) \quad (4)$$

where $\int$SA = the area under the sample fluorescence versus time curve and $\int$CA = the area under the control curve.

The median effective dose (EC_{50}) was determined from the median effect plot of $\log_{(f_a/f_u)}$ versus $\log_{(\mathrm{dose})}$, where f_a is the fraction affected (CAA unit) and f_u is the fraction unaffected (1 − CAA unit) by the treatment. The EC_{50} is the concentration at which f_a/f_u = 1, as calculated from the linear regression of the median effect curve; the EC_{50} values are expressed as the mean ± SD for triplicate data obtained for the same experiment. In each experiment, quercetin was used as the standard, and the cellular antioxidant activities for the fruit extract were expressed as micromolar quercetin equivalent (QE) per 100 g of fruit FW and micromolar QE per 100 μmol of phenolics.

Characterization of Phenolic Compounds by HPLC-DAD-MS^n. The availability of phenolic reference substances is limited, and the identification of structurally related plant secondary metabolites solely on the basis of their UV−vis spectra is impossible. Thus, because HPLC coupled to mass spectrometry has proven to be extremely helpful for the peak assignment and further characterization of individual substances, preliminary mass spectrometric analyses were performed using an Esquire 3000+ ion trap system (Bruker, Bremen, Germany) coupled to the HPLC system described below.[19] For this purpose, the residue obtained by freeze-drying was dissolved in 10 mL of water acidified with a diluted HCl solution (pH 3.0). Prior to the phenolic compound enrichment, the solution was adjusted to pH 7.0 and applied to SPE C18 cartridges, and the phenolic acids and flavonoids were recovered by elution with methanol. Preliminary analyses were performed using an Agilent HPLC series 1100 equipped with ChemStation software, a model G1322A degasser, a model G1312 binary gradient pump, a model G1329/G1330A thermoautosampler, a model G1316A column oven, and a model G1315A diode array detector (Agilent, Waldbronn, Germany). Different LC systems for phenolic acid and anthocyanin separation were tested, and the analyses were performed according to the method described by Kammerer et al.[7] Different injection volumes (ranging from 20 to 40 μL) and concentrations of the samples (ranging from 20 to 200 mg/mL) were tested to optimize the separation conditions for the further HPLC analysis using the systems described below.

System I (Anthocyanins). The mobile phase consisted of water/formic acid (95:5, v/v; eluent A) and water/formic acid/methanol (10:10:80, v/v/v; eluent B) using a gradient system. Monitoring was performed at 520 nm at a flow rate of 0.4 mL/min. The total run time was 78 min.

System II (Colorless Phenolics). The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and methanol (50:50, v/v; eluent B). The total run time was 72 min. Simultaneous monitoring was performed at 280 nm and at 320 nm at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

The Brazilian palm fruit bacaba was investigated for its phenolic compounds, antioxidant capacity, and antiproliferative effect on HepG2 cells. As a first step, the water content of the fruit sample was assessed to allow further calculations expressing the results based on the FW. The moisture content amounted to 43.10 ± 1.53%, and the yield of pulp assessed during the

Table 1. Comparison between the Total Phenolic, Flavonoid, and Anthocyanin Contents of Bacaba and Other Fruits Described in the Literature

fruit	scientific name of the plant and cultivar (cv.)	total phenolics (mg GAEq/100 g)	total flavonoids (mg CTEq/100 g)	total anthocyanins (mg cyn-3-glu/100 g)	ref
bacaba	*Oenocarpus bacaba* Mart.	1759.27 ± 1.01	1134.32 ± 0.03	34.69 ± 0.00	
bacuri	*Platonia insignis* Mart.	266.80 ± 3.3	103.8 ± 0.3		20
buriti	*Mauritia vinifera* Mart.	108.1 ± 6.8	71.3 ± 3.6		20
guava	*Psidium guajava* L.	344.9 ± 33.6			46
pequi	*Caryocar villosum* (Aubl.) Pers.	4623.4 ± 102.4	741.2 ± 36.6		20
raspberry	*Rubus idaeus* L. cv. Hollanda Boduru	2062.3 ± 4.1	41.1 ± 0.9	24.3 ± 0.3	9
raspberry	*Rubus idaeus* L. cv. Hollanda Boduru	1822.0 ± 11.9	21.1 ± 0.1	45.6 ± 0.3	21
blackberry water extract	*Rubus fruticosus* L. cv. Jumbo	2445.9 ± 14.8	42.8 ± 0.3	87.1 ± 1.0	21
blackberry methanolic extract		2786.8 ± 21.9	82.2 ± 1.3	52.9 ± 1.1	21
blueberry	*Vaccinium myrtillus* L.	670.9	190.3		47
tucuma	*Astrocaryum acualeatum* G. Mey.	456.8 ± 5.2	433.2 ± 10.4		20

Table 2. Comparison between Bacaba Antioxidant Activity and That of Other Fruits Described in the Literature[a]

sample	scientific name of the plant and cultivar (cv.)	ORAC (μmol TE/100 g)	TEAC (μmol TE/100 g)	FRAP 1 (mmol $FeSO_4 \cdot 7H_2O$ equiv/100 g)	FRAP 2 (mmol TE/100 g)	DPPH (mmol TE/100 g)	ref
açaí (FW)	*Euterpe oleracea* Mart. cv. Allahabad	63000					23
bacaba (FW)	*Oenocarpus bacaba* Mart.	10750.71 ± 1496.51	3294.55 ± 301.55	23.60 ± 0.53	13.44 ± 0.20	34.25 ± 0.20	
blackberry	*Rubus fruticosus* L. cv. Jumbo				17.7		21
blueberry (FW)	*Vaccinium*	6184 ± 775					47
cranberry (FW)	*Vaccinium* L.	9256 ± 138					47
date palm (DW)	*Phoenix dactylifera* L.				3.28		24
guava (FW)	*Psidium guajava* L.	2550 ± 160	3790 ± 340	3333 ± 1.4		3200 ± 510	46
raspberry (FW)	*Rubus idaeus* L.	4765 ± 718			12.7		9, 47
sour cherry (FW)	*Prunus cerasus* L.	1145−1916	2000−2600				48
walnuts (DW)	*Juglans regia* L.					6.1	25

[a]FW, fresh weight; DW, dry weight.

preparation of the plant material by weighing the residues was 29.13%. To optimize the extraction procedure, the total phenolic contents of the samples were assessed using the Folin−Ciocalteu assay after extraction with different dilutions of methanol and acetone (data not shown). The bacaba phenolics were optimally extracted with 80% acetone; therefore, this solvent was used for all further extractions.

Total Phenol, Total Flavonoid, and Total Anthocyanin Contents in Bacaba. The total phenolic content of the bacaba fruit amounted to 1759.27 ± 1.01 mg GAEq/100 g, its flavonoid content was 1134.32 ± 0.03 mg CTEq/100 g, and its anthocyanin value was 34.69 ± 0.00 mg cyn-3-glc/100 g. Bacaba was found to be a rich source of polyphenolic compounds, thus qualifying this fruit as a potential source of phenolics and flavonoids, including anthocyanins. A comparison between the phenolic contents of bacaba and other fruits already described in the literature is provided in Table 1.

Bacaba demonstrated a flavonoid content of 1134 ± 0.03 CTEq mg/100 g, whereas significantly lower values were reported for the pulp of two other native Brazil fruits, both belonging to the Arecaceae family, buriti (*Mauritia vinifera* Mart.) and tucuma (*Astrocaryum acualeatum* G. Mey.) (71.3 ± 3.6 and 433.2 ± 10.4 CTEq mg/100 g FW, respectively). Additionally, bacaba had a higher flavonoid content than that of pequi (*Caryocar villosum* (Aubl.) Pers.) (741.2 ± 36.6 CTEq mg/100 g FW), another Brazilian native fruit belonging to Caryocaraceae.[20]

When compared to the commonly used commercial berries that are recognized for their high amounts of phenolics, our results for bacaba suggest that this fruit may be included in this group. For example, raspberry (*Rubus idaeus* L.) cultivars, such as Hollanda Boduru, showed a total flavonoid content of 41.1 ± 0.9 CTEq mg/100 g FW. Even in comparison to the higher flavonoid content of blackberry (*Rubus fruticosus* L.) cv. Jumbo (82.2 ± 1.3 CTEq mg/100 g FW), bacaba still shows an extremely high content.[21]

Anthocyanins belong to a class of flavonoid compounds that determine the color of orange, red, purple, and blue fruits, vegetables, and flowers. Bacaba exhibited a high anthocyanin content (34.69 ± 0.00 cyn-3-glc mg/100 g FW) when compared to raspberry cv. Hollanda Boduru (24.3 ± 0.3 cyn-3-glu mg/100 g FW).[21] Fruits rich in anthocyanins can be used as natural food colorants in a wide variety of foods and also

have the potential to be used as a functional ingredient for food and pharmaceutical applications.

Antioxidant Activity of Bacaba Phenolic Extracts. Methods to determine antioxidant activity differ with regard to the principle and experimental conditions of the assay.[22] In general, a single assay does not accurately determine all of the groups of antioxidant compounds, particularly in a mixed or complex system, such as fruit matrices. Therefore, different antioxidant assays were applied (ORAC, TEAC, FRAP, DPPH, and CAA) to allow a broader comparison with the other fruits already described in the literature.

The bacaba fruit extract exhibited a considerable antioxidant effect. The ORAC value for bacaba was 10750.71 ± 1496.51 μmol TEq/100 g of fruit, placing this fruit among other fruits with well-described high antioxidant activity, including blueberries, cranberries, and sour cherries, as shown in Table 2. However, none of the samples demonstrated a higher ORAC value than the Brazilian Amazon açaí berry (*Euterpe oleracea* Mart.), which also belongs to the Arecaceae family and is known in the media as a "superfruit" due to its extremely high antioxidant activity. Schauss[23] estimated an ORAC value of 630 μmol TEq/g for açaí. Using the same units, bacaba exhibits an antioxidant activity of 107.5 μmol TEq/g. Similar to the ORAC, the TEAC value revealed a high antioxidant activity for the bacaba phenolic extract (3294.55 ± 301.55 μmol TEq/100 g). However, applying this assay, bacaba did not have a high antioxidant potential when compared to other fruits (e.g., guava), as depicted in Table 2.

For the FRAP assay, $FeSO_4{\cdot}7H_2O$ and Trolox were applied as the standards because both chemicals are commonly utilized to establish calibration curves in this test. The FRAP values for bacaba were 23.60 ± 0.53 mmol $FeSO_4{\cdot}7H_2O$/100 g FW and 13.442 ± 0.206 mmol TEq/100 g FW. Using a DMSO extract of the dry plant, Khanavi et al.[24] indicated that the antioxidant activity of the pulp of date palms (cv. Khenizi), another Arecaceae species that is frequently applied in folk medicine in Iran, amounted to 3.28 mmol $FeSO_4{\cdot}7H_2O$ Eq/100 g FW, which is lower than that of bacaba at 23.60 ± 0.53 mmol $FeSO_4{\cdot}7H_2O$ Eq/100 g FW. Berry fruits are known for their usually high antioxidant values when assessed by different assays. Bacaba showed higher FRAP values than a methanol extract of raspberry cv. Hollanda Boduru (12.7 TEq mmol/100 g FW) or a methanol extract of blackberry cv. Jumbo (17.7 TEq mmol/100 g FW), as described by Sariburun et al.[9]

In the DPPH assay, the radical inactivation was calculated for different sample concentrations and compared to a calibration curve established using Trolox as the standard. In this assay, bacaba displayed a high antioxidant activity (34.251 ± 0.205 mmol TEq/100 g FW) when compared to guava (*Psidium guajava* L.) (3.6 TEq mmol/100 g FW) and walnuts (*Juglans regia* L.) (6.1 TEq mmol/100 g DW).[25]

The antioxidant activity of the phenolic extract of bacaba was also measured using the CAA assay recently designed and proposed by Wolfe and Liu.[18] With regard to the CAA, the bacaba phenolic extract revealed relatively potent antioxidant activities, with an EC_{50} value of 0.7 ± 0.61 mg/mL; pure quercetin showed an EC_{50} value of 4.66 ± 0.61 μM. Compared to the fruits assessed by Wolfe et al., with EC_{50} values of 3.21 ± 0.14 mg/mL for blackberry, 6.77 ± 1.05 mg/mL for wild blueberry, 11.8 ± 0.9 mg/mL for strawberry, and 14.7 ± 0.8 mg/mL for cranberry,[26] bacaba, with its EC_{50} of 0.7 ± 0.61 mg/mL, revealed the lowest EC_{50} value of all and, therefore, appears to have the highest antioxidant activity among these fruits. Wolfe et al.[26] reported the highest antioxidant activity for blackberry, with a CAA value of 154 ± 6.8 mg/mL. This berry was followed by wild blueberry, with a CAA value of 74.1 ± 12.5 mg/mL, cranberry at 33.6 ± 2.0 mg/mL, and strawberry at 42.4 ± 3.3 mg/mL. As the CAA value for bacaba reached 305.2 ± 90.8 mg/mL, bacaba displays the highest CAA value of all these fruits, indicating the highest antioxidant activity. Wolfe and Liu concluded in their work that the CAA values for berries (e.g., wild blueberry, blackberry, strawberry, cranberry) tend to be the highest and that berries are commonly very rich in anthocyanins.[18] According to Wolfe et al.[26] the cellular antioxidant quality (CAQ) measures the cellular antioxidant activity provided by 100 μmol of the phenolics present in the fruit. The CAQ value for bacaba (2.95 ± 0.88 μM QE/100 μmol of phenolics) is comparable to those found for wild blueberry (2.9 μM QE/100 μmol of phenolics) and strawberry (3.0 μM QE/100 μmol of phenolics).[26]

Song et al.[27] reported a positive correlation between the total phenolics in different vegetables and the CAA values. Furthermore, fruits rich in anthocyanins have been demonstrated to exert the highest antioxidant activities,[28] possibly explaining the very high antioxidant activity of bacaba fruit, which, according to our results, is a good source of anthocyanins.

The difference in the antioxidant activity values for the same samples revealed by different assays, even when using the same standard compound, is based on the reaction mechanisms of the methods themselves. Although correlation analyses were not performed, it is implied that the high antioxidant capacities found for bacaba could be based on its high total phenolic, flavonoid, and anthocyanin contents. Furthermore, some factors inherent to scientific research may influence the results, for example, the environmental characteristics, harvesting period, cultivar variability, fruit maturity, and solvent extraction procedures. However, these factors still allow the comparison of the results with the data in the literature. Indeed, comparing the results with other fruits is valuable and provides a clear idea of the status of bacaba within the discussion of the biofunctionality of native fruits in developing countries.

Characterization of Phenolic Compounds of Bacaba by HPLC-DAD-MS^n. The phenolic compounds were separated by RP-HPLC. Ten major compounds were eluted from the HPLC column and were well separated using the chromatographic system applied. The HPLC chromatogram demonstrates the presence of 27 compounds absorbing at 370 nm. The separation of phenolic components of a bacaba extract is presented in Figure 2.

The phenolic fractions were characterized by ESI-MS, and the assignment of the individual non-anthocyanin compounds was based on their main $[M - H]^-$ ions, together with the interpretation of their fragments produced in collision-induced dissociation (CID) experiments. Some compounds were tentatively identified on the basis of the analysis of the pseudomolecular ions and the fragments released in MS^2 and MS^3 experiments. MS^n spectra are very useful for identifying the aglycones of flavonoids, and the analysis of fragmentation patterns is highly diagnostic, allowing the elucidation of structures by comparison with the data in the literature. The MS spectra of the flavonoid glycosides reveal characteristic patterns, depending mainly on the number and nature of the saccharides bound to the aglycone and their linkage type, that is, *C*- or *O*-glycosidic linkage. Many flavonoids display low sensitivity in positive ionization mode MS analyses and are,

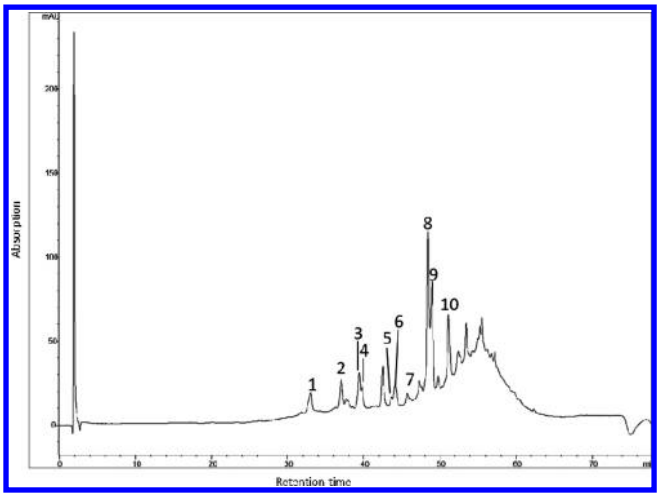

Figure 2. HLPC chromatogram of the phenolics present in a bacaba (*Oenocarpus bacaba* Mart.) extract at a detection wavelength of 370 nm.

therefore, preferably detected in the negative ionization mode.[29] The mass spectrometric data obtained in the present study using the negative ionization mode are presented in Table 3.

Peak 1. The wavelength of the maximum absorption for band I (300−380 nm) was below 355 nm and, thus, is indicative of hydroxyl substitution at the 3-position.[30] The UV spectra showing λ_{max} at 267 and 338 nm are characteristic of apigenin.

In the MS^2 experiment, losses of 120 and 90 Da were observed, thus producing fragments at m/z values of 285, 474, and 503. The fragmentation of *C*-glycosides typically produces $[M - H - 90]^-$ and $[M - H - 120]^-$ ions.[30] The MS^2 analyses of the predominant ion at m/z 593 detected for compound **1** provided the typical fragmentation of a di-*C*-glycosylflavone. In particular, the ions at m/z 503 ($[M - H - 90]^-$) and 473 ($[M - H - 120]^-$, base peak) suggest the occurrence of a 6,8-di-*C*-hexoside. The ions at m/z 353 ([aglycone + 83 Da]) and 383 ([aglycone + 113 Da]) revealed the aglycone to be a trihydroxyflavone, as similarly described by Gil-Izquierdo et al.[31] The precursor ion and major fragment ions corresponding to peak 1 have been previously described; by comparison of these mass spectrometric data with those obtained in the

Table 3. Characterization of the Individual Phenolic Compounds in Bacaba Extracts Using HPLC-DAD-ESI-MS^n

peak	t_R (min)	compd class	compd (tentative identification)	HPLC-DAD UV, λ_{max} (nm)	$[M - H]^-$, m/z	HPLC-ESI (−)-MS^n expt, m/z (% base peak)[a]
1	33.0	*C*-glycoside	vicenin-2 (apigenin-6,8-di-*C*-glycoside 5,7,4′-trihydroxyflavone-6,8-di-*C*-glycoside)[b]	235, 268, 337	593	MS^2 [593]: 473 (100), 353 (39), 503 (37), 383 (20), 354 (17), 285 (15) MS^3 [593 → 473]: 353 (100), 354 (19), 383 (15)
2	37.0	flavonoid, *C*-hexoside	orientin (luteolin-8-*C*-glucoside) or isoorientin (luteolin-6-*C*-glucoside)	348	447	MS^2 [447]: 327 (100), 357 (56), 328 (29)
3	39.4	*C*-glycosylflavone	apigenin-8-*C*-glucoside (vitexin)	340	431	MS^2 [431]: 311 (100), 312 (17), 283 (17), 341 (6) MS^3 [431 → 311]: 283 (100), 312 (37), 118 (27)
4	39.8	isorhamnetin hexoside	isorhamnetin dihexoside or rhamnetin dihexoside	310	639	MS^2 [639]: 315 (100), 477 (53) MS^3 [639 → 315]: 300 (100), 270 (67)
5	43.7	quercetin hexoside	quercetin-3-*O*-hexoside	355	463	MS^2 [463]: 301 (100), 302 (25)
6	44.1	quercetin diglycoside	rutin (quercetin 3-*O*-rutinoside)	265	609	MS^2 [609]: 301 (100), 302 (27), 300 (20), 271 (14), 343 (14)
7	45.7	quercetin glycoside	quercetin-3-*O*-hexoside	260	505	MS^2 [505]: 301 (100), 300 (37), 463 (37)
8	48.5	flavonoid	hexose + deoxyhexose attached to methoxyapigenin methoxyluteolin diosmetin	345	608	MS^2 [607]: 299 (100), 284 (61), 285 (19), 300 (14) MS^3 [607 → 299]: 284 (100), 285 (36)
9	48.9	hexose + deoxyhexose methoxyl function		345	637	MS^2 [637]: 329 (100), 314 (25), 330 (23) MS^3 [637 → 329]: 314 (100), 315 (44), 329 (22)
10	51.1	isorhamnetin glucoside	isorhamnetin acetylhexoside	355	519	MS^2 [519]: 315 (100), 300 (18), 316 (16) MS^3 [519 → 315]: 300 (100), 255 (17)

[a]Based on the fragmentation pattern in mass spectrometric experiments. [b]The tentative identification was obtained by comparing the elution order and pseudomolecular ions $(M - H)^-$ with the data available in the literature.[49]

present study, this compound was tentatively identified as vicenin-2.[31]

Peak 2. Losses of 120 and 90 Da were observed in the MS^2 experiment, corresponding to cross-ring cleavages in the sugar moiety.[32] This fragmentation of the glycoside was responsible for product ions observed at m/z 357 ($[M - H - 90]^-$) and m/z 327 ($[M - H - 120]^-$), and the evaluation of the fragmentation behavior indicates that compound **2** corresponds to a flavone *C*-glycoside, such as isoorientin (luteolin-6-*C*-glucoside) and orientin (luteolin-8-*C*-glucoside), respectively. Although the fragment ions of the parent ion allow the differentiation between C-glycosylation at the 6- and 8-positions, it was impossible in the present study to perform a more detailed characterization of this compound due to the low signal intensities in the fragmentation experiments.[32,33]

Peak 3. Among the compounds having UV spectra characteristic of flavones, two groups of compounds were differentiated on the basis of their MS^n fragmentation: *C*-glycosylflavones and *O*-glycosylflavones. In the MS^2 experiments of the studied *C*-glycosylflavones, losses of 90 and 120 Da were observed, characteristic of a C-glycosylated compound with a hexose linked to the flavone nucleus. The absence of an $[M - H - 18]^-$ ion presumably indicates the position of the C-glycosylation at carbon 8.[34] Additionally, compound **3** revealed an $[M - H]^-$ ion at m/z 431, and its MS/MS spectrum yielded ions at m/z 341 ($[M - H - 90]^-$) and 311 ($[M - H - 120]^-$). Consequently, this component was tentatively assigned as apigenin-8-*C*-glucoside, also named vitexin.[35]

Peak 4. The mass spectrometric experiments of compound **4** in the negative ionization mode produced an $[M - H]^-$ ion at m/z 639 and fragment ions at m/z 477 ($[M - H - \text{hexose}]^-$) and m/z 315 ($[M - H - 2 \times \text{hexose}]^-$). The ion at m/z 315 is characteristic of rhamnetin and isorhamnetin, and the fragmentation pattern indicated that this compound contains two hexose moieties.[36] Therefore, this compound was tentatively identified as either isorhamnetin dihexoside or rhamnetin dihexoside.

Peak 5. The fragmentation pattern reveals this compound to be a quercetin hexoside.[37] In the ESI experiments in the negative ionization mode, quercetin mono- and diglycosides are known to produce the quercetin aglycone at m/z 301, as a result of the loss of the glycosyl moieties.[38] The CID experiments with compound **5** generated the aglycon ion at m/z 301, revealing a loss of 162 Da, together with a fragment ion at m/z 179, which is characteristic of quercetin 3-*O*-glucoside.[39] The same findings were also previously described for quercetin hexoside, with an $[M - H]^-$ ion at m/z 463 and showing a typical loss of 162 Da, resulting in the characteristic fragment ion at m/z 301.[40]

Peak 6. Compound **6** exhibited a pseudomolecular ion at m/z 609 and the loss of the glycosidic moiety, thus releasing the quercetin aglycone ion at m/z 301, equivalent to the loss of a hexose moiety (162 Da) and deoxyhexose moiety (146 Da). The further fragmentation of the fragment ion at m/z 301 produced further fragments at m/z 179 and 151, which are characteristic of quercetin. On the basis of similar mass spectral studies reported in the literature, this compound was tentatively identified as rutin (quercetin 3-*O*-rutinoside).[41]

Peak 7. The mass spectra indicated the presence of a quercetin derivative (aglycone fragment at m/z 301) glycosylated with a hexose and having an acetyl moiety attached to it.[42] The peak was tentatively identified as quercetin-3-*O*-hexoside.[43]

Peak 8. The compound, with a pseudomolecular ion at m/z 607, produced a fragment ion at m/z 299 ($[M - H - 308]^-$) in the MS^2 spectrum, suggesting the loss of a rutinose moiety (flavonoid *O*-rutinoside). Shi et al.[44] found a similar fragmentation pattern for diosmin.[45]

Peak 9. Compound **9** showed a UV spectrum typical of a flavonoid. A characteristic loss of 308 Da was observed in the MS^2 experiment, indicative of a diglycoside composed of a hexose and deoxyhexose moiety.

Peak 10. The HPLC-ESI-MS/MS experiments resulted in the loss of 204 Da, characteristic of an acetyl hexoside (162 + 42 Da). Further fragmentation in the MS^3 experiment showed a further loss of a methoxyl function (15 Da) and an ion at m/z 300, characteristic of an isorhamnetin or rhamnetin backbone unit. A similar spectrum has previously been described for isorhamnetin acetylglucoside.[40]

The separation of the anthocyanins extracted from the bacaba sample is presented in Figure 3: a baseline separation

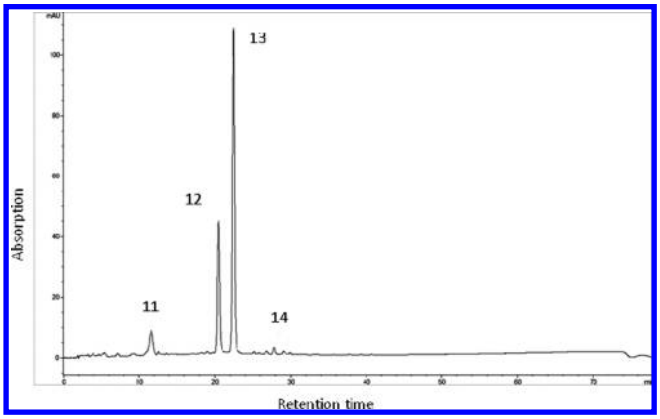

Figure 3. HPLC chromatogram of anthocyanins from a bacaba (*Oenocarpus bacaba* Mart.) sample at a detection wavelength of 520 nm.

was achieved for all of the compounds, and four major peaks were observed in the chromatogram. The UV–vis spectroscopic and mass spectrometric data obtained in the positive ionization mode of anthocyanins are presented in Table 4. All of the compounds were found to be cyanidin glycosides due to the release of the characteristic fragment ion at m/z 287. The mass spectrometric analyses of the four peaks yielded molecular ion peaks (M^+) at m/z 595, 758, 449, 595, and 963, as specified in Table 4.

Peak 11. Compound **1** was assigned as a cyanidin hexoside due to the loss of 162 Da in the MS^2 experiment and the release of an ion at m/z 287 upon further fragmentation.

Peak 12. The fragmentation of compound **2** was characteristic of a cyanidin (m/z 287) aglycone glycosylated with a hexose (162 Da) moiety. Compound **2** was tentatively identified as cyanidin 3-*O*-glucoside.

Peaks 13 and 14. Compounds **3** and **4** revealed fragmentation patterns characteristic of anthocyanins containing hexose and deoxyhexose moieties attached to the cyanidin aglycone, respectively. Compound **3** was tentatively identified as cyanidin-3-*O*-rutinoside.[33]

The characterization of phenolic compounds is important, as these compounds are associated with a range of different health properties. HPLC with diode array and mass spectrometric

Table 4. UV−Vis and Mass Spectrometric Characterization of the Individual Anthocyanins in Bacaba Extracts Using HPLC-DAD-ESI-MS^n

peak	t_R (min)	compd characterization	tentative identification	HPLC-DAD UV, λ_{max} (nm)	$[M]^+$ (m/z)	HPLC-ESI (+)-MS^n expt [a] (% base peak)
11	11.6	cyanidin glycoside		270, 505	758	MS ^{2} [758]: 596
						MS ^{3} [758→596]: 449, 287
12	20.5	cyanidin hexoside	cyanidin 3-*O*-glucoside	270, 505	449	MS ^{2} [449]: 287
13	22.5	cyanidin glycoside	cyanidin-3-*O*-rutinoside	260, 510	595	MS ^{2} [595]: 449, 287
14	27.9	cyanidin glycoside		305, 520	963	MS ^{2} [963→]: 449, 287
						MS ^{3} [967→595]: 449, 287

[a]Based on the HPLC-MS mass fragmentation pattern.

detection has proven to be very useful for the characterization of individual phenolic compounds. In the present study, 10 polyphenols were detected and characterized in bacaba extracts, and, on the basis of their UV spectral data, most of these compounds were characterized as quercetin and rhamnetin derivatives in addition to a range of flavonoids. *O. bacaba* extracts have not been systematically analyzed thus far, and, to the best of our knowledge, the present study represents the first chemical investigation of this plant material. Due to the limited availability of reference compounds, the similarity of the spectral characteristics and the chromatographic behavior allowed only tentative peak assignments. Therefore, mass spectrometric analyses were also performed,[7] and nine compounds were tentatively identified on the basis of a comparison with results previously described in the literature.

In conclusion, the absence of information (phytochemistry and pharmacology) makes any effort to elucidate the chemistry and biological properties of bacaba fruit interesting. The high content of antioxidants compared with other species depicts bacaba as a relevant fruit that should be further investigated, and the evaluation of the antioxidant capacity using cell-free and cell-based assays (CAA) highlights this research over others. Although some compounds were not identified by the HPLC-MS analysis, they are relevant to characterize comprehensively the chromatographic fingerprint, which is a plant-specific trait. From the outcome of the present study, it becomes apparent that bacaba is a potential source of pigments and natural antioxidants that should be considered for future exploitation.

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Notes

The authors declare no competing financial interest.

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