Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(1):268-276

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Identification of Phenolic Compounds by LC/MS-MS and Antioxidant and Anti Tyrosinase Activities of the *Attalea speciosa* Mart. ex Spreng. Mesocarp

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ABSTRACT

This paper describes the antioxidant and anti-tyrosinase activity and chemical composition of the mesocarp of Attalea speciosa Mart. ex Spreng fruits (babassu, family Arecaceae). The total phenolic content determined with the Folin-Ciocalteu reagent was higher in the ethyl acetate fraction, hydroalcoholic fraction and hydroalcoholic extract. Antioxidant activity was assayed by the in vitro DPPH, ABTS, and FRAP assays. The highest antioxidant activity was observed for the ethyl acetate fraction which also has showed the highest levels of proanthocyanidins in the vanillin-HCl assay, which may be related to its greater inhibitory activity of mushroom tyrosinase. Analysis by HPLC-MS/MS has revealed the presence of nine compounds identified as (epi)catechin monomers and their oligomers. The presence of these compounds, which were identified for the first time in babassu mesocarp, is possibly related to the antioxidant and anti-tyrosinase activity of this palm tree.

Keywords: Babassu; Electrospray mass spectrometry; Catechins; Antioxidant activity; Tyrosinase inhibitory activity

Abbreviations: HEB: hydroalcoholic extract of babassu; HF: hexane fraction; CF: chloroform fraction**;** EAF: ethyl acetate fraction; HAF: hydroalcoholic fraction; DPPH: 2,2-diphenyl-1-picrylhydrazyl**;** ABTS: 2,2′-azino-bis(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP: ferric reducing antioxidant power; HPLC: high-performance liquid chromatography**;** ESI: electrospray ionization**;** MS: mass spectrometry**;** MS/MS: tandem mass spectrometry**;** TPC: total phenolic content; CP: concentration of proanthocyanidins

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INTRODUCTION

Attalea speciosa Mart. ex Spreng. (synonym *Orbignya phalerata* Mart.), a palm tree of the Arecaceae commonly known as babassu (Figure 1), is a native species widely distributed in Brazil, especially in the mid-northern region (Pará, Maranhão, Piauí, Tocantins, Mato Grosso, and Goiás). This plant occupies about 18 million hectares and represents an extractive resource of economic and social importance [1].

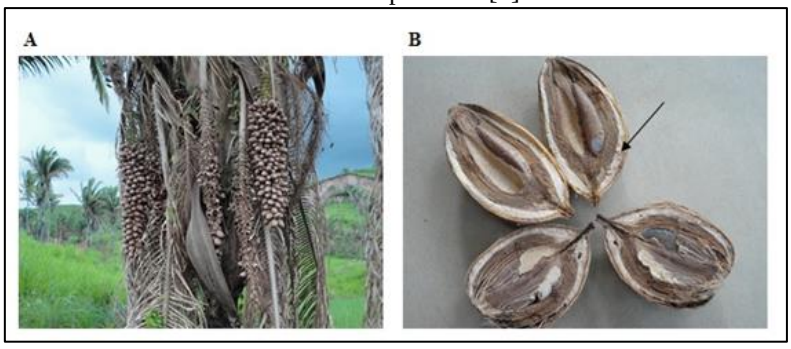

Figure 1: A. *Attalea speciosa* Mart. ex Spreng. (babassu); B. Detail of the mesocarp of the fruit (arrow)

Babassu has been exploited in different economic activities ranging from the generation of energy to its use as a food source [2]. Particularly important is the potential of the babassu fruit, which consists of the epicarp, mesocarp, endocarp and kernels, as a human food (fleshy kernel, flour, nut, milk, oils, and fatty acids) [3]. Ethnobotanical studies have shown that 68% of coconut breakers use products derived from babassu for therapeutic purposes [4], especially the mesocarp which is used to treat gastritis, dysmenorrhea, constipation, colitis, obesity, arthritis, tumors, inflammation and skin diseases [4,5].

Pharmacological studies have demonstrated differents functional properties of babassu that explain its use in folk medicine, such as anti-inflammatory activity;[5] antiulcerogenic activity [6]; antithyroid activity [7]; antithrombotic activity [8]; antifungal activity [9]; antimicrobial activity [10]; healing wound properties [11]; antitumor activity [12]; adjuvant in immune responses [13], and immunomodulatory activity [14]. No toxicity of the mesocarp has been observed in *in vivo* studies conducted by Barroqueiro et al. [15] and Silva et al. [16].

The babassu mesocarp contains calcium, potassium, magnesium and phosphorus.[17] It is a flour consisting of approximately 50% starch [18], 5.38% proteins and 4.02% lipids [19], in addition to phenolic compounds [20], indicating its potential for dietary inclusion.

Despite of biological activities attributed to the mesocarp of the babassu fruit and its possible functional properties, antioxidant and tyrosinase inhibitory activities and chemical composition have not been reported. Therefore, the objective of the present study was to identify and quantify phenolic compounds of the mesocarp of *Attalea speciosa* fruits and evaluate their bioactivities to provide scientific evidence to full utilization of the fruit in the industrial development.

MATERIALS AND METHODS

Reagents and standards

Ethanol, methanol, hexane, chloroform, ethyl acetate, formic acid, hydrochloric acid, acetic acid, sodium carbonate, and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). All chemicals used in the study were of analytical or HPLC grade. Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, iron(III) chloride hexahydrate, iron(II) sulfate heptahydrate, gallic acid, catechin, mushroom tyrosinase, L-tyrosine and 3,4-dihydroxyphenylalanine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used in this experiment was purified in a Millipore Milli-Q apparatus (Purelab UHQ-PS, Elga).

Plant material

The mesocarp of *Attalea speciosa* fruits was collected at Arari, Maranhão, Brazil (3º27'13" S latitude and 44º46'48" W longitude).

Sample extraction and fractionation

The mesocarp fruit was obtained by mechanical breaking and dried in an air circulating oven at 40 ºC and then triturated with a knife mill. The mesocarp powder (1000 g) was extracted by maceration with 4000 mL of a mixture of ethanol:water (70:30, v/v) for 48 h. The extract was filtered and concentrated to a small volume at 40 ºC in a rotary evaporator under vacuum, obtaining the hydroalcoholic extract of babassu (HEB).

The babassu extract (HEB, 10 g) was dissolved in 100 mL methanol:water (70:30, v/v) by stirring. The solution was subjected to fractionation by liquid-liquid using hexane, chloroform and ethyl acetate. Each fraction was concentrated to a small volume at 40 ºC in a rotary evaporator under vacuum, obtaining the hexane (HF), chloroform (CF), ethyl acetate (EAF), and hydroalcoholic fraction (HAF).

Determination of Total Phenolic Content (TPC)

The TPC of the HEB and fractions of the mesocarp was determined with Folin-Ciocalteu reagent and 20% sodium carbonate [21]. The reaction mixture was kept in the dark for 2 h at room temperature, and absorbance was then measured at 760 nm in a Lambda 35 UV-Vis spectrophotometer (PerkinElmer). The TPC was calculated from the calibration curve constructed with standard solutions of gallic acid (1.0-30.0 μg/mL) (r^2 = 0.998) and is expressed as milligrams of gallic acid equivalents per gram of babassu mesocarp (mg GAE/g). All analyses were conducted in triplicate.

DPPH$^{\bullet}$ radical scavenging activity assay

The antioxidant activity of the of the HEB and fractions of the mesocarp was evaluated using the DPPH free radical scavenging assay as described by Brand-Williams et al. [22] with modifications. The methanolic solutions of each sample concentration (5.0-100.0 μg/mL) were added to a methanol solution of DPPH (40.0 μg/mL). After 30 min of reaction at room temperature in the dark, the absorbance of each solution was read at 517 nm in a Lambda 35 UV-Vis spectrophotometer (PerkinElmer). DPPH solution was used as control and methanol was used as blank. Standards of

Trolox and catechin were treated under the same conditions. The percent inhibition was calculated using the following formula:

$$DPPH^{\bullet} \text{ scavenging activity (\%)} = 100 - [(A_{sample} - A_{blank}) \times 100/A_{control}],$$

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

The IC_{50} value (μg/mL), which corresponds to the concentration of the extract necessary to scavenge 50% of radicals, was calculated. All analyses were conducted in triplicate.

$ABTS^{\bullet+}$ radical cation scavenging activity assay

The antioxidant activity of the HEB and fractions of the mesocarp was also evaluated by the $ABTS^{\bullet+}$ method (2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid) as described by Re et al. [23] with modifications. For formation of the ABTS radical, 7 mM $ABTS^{\bullet+}$ solution was mixed with 2.45 mM potassium persulfate solution and the mixture was stored in the dark for 16 h. The radical was diluted in ethanol PA to an absorbance of 0.700 ± 0.020 at 734 nm. Three different dilutions of the samples (1.0–6.0 μg/mL) were then added to 3.0 mL of ABTS radical in the dark. Absorbance was read in a Lambda 35 UV-Vis spectrophotometer (PerkinElmer) after 6 min of reaction using ethanol as blank. Standards of Trolox and catechin were treated under the same conditions. The percent inhibition was calculated using the following formula:

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = 100 - [(A_{sample} - A_{blank}) \times 100/A_{control}],$$

Where A_{sample} = absorbance of the sample after 6 min of reaction, A_{blank} = absorbance of the blank, and $A_{control}$ = absorbance of the control.

The IC_{50} value (μg/mL), which corresponds to the concentration of the extract necessary to scavenge 50% of radicals, was calculated. All analyses were conducted in triplicate.

Ferric Reducing Antioxidant Power (FRAP) assay

The method described by Benzie and Strain [24], with some modifications, was used to determine the antioxidant activity based on iron reduction using the FRAP assay. FRAP measures the ferric-reducing ability of a sample in acid medium (pH 3.6), forming an intense blue color as the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form. FRAP reagent was prepared immediately before analysis by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2.5 mL $FeCl_3 \cdot 6\ H_2O$ (20 mM) in aqueous solution. Different concentrations of 100 μL of the samples (1.0–100.0 μg/mL) were added to 300 μL distilled water and 3.0 mL of FRAP reagent, and the mixtures were incubated in a water bath at 37 °C for 30 min. The absorbance of the reaction mixture was read at 593 nm in a Lambda 35 UV-Vis spectrophotometer (PerkinElmer) using FRAP solution as blank. The calibration curve was constructed using different concentrations of $FeSO_4.7H_2O$ (0–2000 μM) ($r^2 = 0.9987$) and the results are expressed as mmol Fe^{2+}/g sample. Standards of Trolox and catechin were treated under the same conditions. All analyses were conducted in triplicate.

Vanillin-HCl assay

Proanthocyanidins were determined by the vanillin-HCl assay as described by Sun et al. [25] with modifications. HEB and fractions were diluted in methanol to a concentration of 200 μg/mL. In a test tube, were added 1 mL of sample or standard, 2.5 mL of reagent A (vanillin solution 1% w/v in methanol) and 2.5 mL of reagent B (HCl solution 9M in methanol). The reaction mixture was incubated for 20 min at 30°C and the absorbance at 500 nm was measured in a Lambda 35 UV-Vis spectrophotometer (PerkinElmer). Concentration of proanthocyanidins (CPAs) was calculated from the calibration curve constructed with standard solutions of catechin (1.0-25.0 μg/mL) ($r^2 = 0.998$) and is expressed as milligrams of catechin equivalents per gram of babassu mesocarp (mg CE/g). All analyses were conducted in triplicate.

Tyrosinase inhibitory activity assay

Tyrosinase inhibitory activity was determined as described by Momtaz et al. [26] with modifications. HEB and fractions were diluted in 50 mM potassium phosphate buffer (pH 6.5) in different concentrations (50.0-200.0 μg/mL) were combined with 30 μL mushroom tyrosinase solution (313 Units/mL in phosphate buffer, pH 6.5) in triplicate in a 96-well plates. L-tyrosine was selected as substrate for the monophenolase reaction assay and 3,4-dihydroxyphenylalanine (L-DOPA) was selected as substrate for the diphenolase reaction assay. After incubation at room temperature for 5 min, 110 μL of substrate (2 mM L-tyrosine or 12 mM L-DOPA) were added to each well. Plates were incubated for 30 min at room temperature and the absorbance were determined at 492 nm with the Multiskan EX spectrophotometer (Labsystems). Mixtures without tyrosinase and without the sample solution were used as blank and control, respectively. Tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = 100 - [(A_{sample} - A_{blank}) \times 100/A_{control}],$$

Where A_{sample} = absorbance of the sample after 30 min of reaction, A_{blank} = absorbance of the blank, and $A_{control}$ = absorbance of the control. The results were expressed as IC_{50} values (μg/mL) corresponding to the sample concentration required to inhibit 50% of enzyme activity.

HPLC-UV-Vis analysis

HPLC analysis was carried out in a Thermo Finnigan Surveyor Autosampler liquid chromatograph (San Jose, CA, USA) equipped with an injector with a 25-µL loop and a UV detector. A Hypersil BDS C-18 column (250 x 4.6 mm, 5 µm; Thermo Electron Corporation, Waltham, MA, USA), protected by a C-18 pre-column (4 x 3 mm, 5 µm; Gemini, Phenomenex), was used. The compounds of the EAF were separated at room temperature using a gradient elution program at a flow rate of 1.0 mL/min. The mobile phase consisted of Milli-Q water containing 0.1% formic acid (A) and methanol (B). The following linear gradient was applied: 0-1 min, 5% B; 1-40 min, 5-30% B; 40-90 min, 30-100% B. The column was re-equilibrated for 10 min before the next run. The injection volume into the HPLC system was 25 µL and UV-Vis detection was performed at 280 nm. Before injection into the HPLC system, EAF was dissolved in the same solvent used as mobile phase (HPLC grade) to obtain a final concentration of 1 mg/mL and then filtered through a 0.22-µm Nylon syringe filter obtained from Allcrom (São Paulo, SP, Brazil).

Mass spectrometry analysis

The EAF was analyzed in HPLC system (LC-10AD, Shimadzu) equipped with a photodiode array detector, which was coupled to an Esquire 3000 Plus ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) using electrospray ionization (ESI). The conditions for dilution of the samples and the mobile phase composition were the same as described above. The ionization conditions were adjusted as follows: electrospray voltage of the ion source of 40 V, capillary voltage of 4.0 kV, and capillary temperature of 300 °C. Ultrahigh-purity helium (He) was used as the collision gas and high-purity nitrogen (N_2) as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 12 psi. Desolvation was facilitated using a counter current nitrogen flow set at a rate of 5.0 L/ min. Analyses were carried out using full-scan mass spectra in the negative ionization mode and data-dependent MS^2 scans from *m/z* 100 to 3000 Da. The compounds were identified on the basis of the molecular ion mass, fragmentation and UV-visible spectra.

Statistical analysis

All analyses were performed in triplicate. The results are expressed as the mean ± standard deviation and were analyzed using the GraphPad Prism 5.0 program. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were used to determine significant differences between means. A level of significance of $p < 0.05$ was adopted. Pearson's correlation test was used to evaluate the correlation between TPC (mg GAE/g), CPAs (mg CE/g), antioxidant and tyrosinase inhibitory activities (µg/mL).

RESULTS AND DISCUSSION

Extraction yields and total phenolic content

The HEB yield was 2.15% and fraction yield was 3.51% for HF, 2.43% for CF, 25.19% for EAF, and 61.78% for HAF. The TPC of HEB and fractions is shown in Table 1. EAF contained the highest concentration of total phenols (646.5 mg GAE/g). There are no data in the literature regarding the TPC of the mesocarp of *A. speciosa*. The TPC of babassu mesocarp is higher than that reported for the fruits of other native palm trees of the family Arecaceae, such as *Oenocarpus bacaba* Mart. [27], *Euterpe oleracea* Mart., *Euterpe precatoria* Mart. [28], and *Astrocaryum aculeatum* G. Mey. [29]. This result shows that the *A. speciosa* mesocarp is a rich source of phenolic compounds.

Table 1: Total Phenolic Content and Antioxidant Activity of the Extract and Fractions of the Mesocarp of *Attalea speciosa* Mart. ex Spreng. Fruits[a]

Sample	TPC (mg GAE/g)	$DPPH^{\bullet}$ $IC_{50\ (\mu g/mL)}$	$ABTS^{\bullet+}$ $IC_{50\ (\mu g/mL)}$	FRAP (mmol Fe2+/g)
HEB	414.40 ± 28.80a	4.00 ± 0.03ab	2.74 ± 0.01a	11.46 ± 0.56ab
HF	12.29 ± 0.27b	41.19 ± 2.80c	nd	nd
CF	176.60 ± 8.58c	25.89 ± 0.42d	2.97 ± 0.07b	9.02 ± 0.47ab
EAF	646.50 ± 2.93d	3.38 ± 0.05ab	2.04 ± 0.03c	15.41 ± 0.18a
HAF	508.90 ± 2.40e	3.82 ± 0.08ab	2.98 ± 0.13b	4.89 ± 0.53b
Trolox	-	5.15 ± 0.01b	2.36 ± 0.04d	8.91 ± 0.17ab
Catechin	-	1.76 ± 0.06a	0.75 ± 0.01e	54.89 ± 6.75c

[a]Values represent the mean of triplicate measurements ± standard deviation. Different letters in the same column indicate a significant difference (Tukey test, $p < 0.05$). TPC, total phenolic content; HEB, hydroalcoholic extract of babassu; HF, hexane fraction; CF, chloroform fraction; EAF, ethyl acetate fraction; HAF, hydroalcoholic fraction; nd, not detected

Antioxidant activity

The antioxidant activity of the extract and fractions was evaluated using three common chemical model systems, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP assays (Table 1). In the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, a lower IC_{50} value indicates the greatest antioxidant activity. EAF exhibited the highest activity among the samples analyzed by the $DPPH^{\bullet}$ radical-scavenging assay, with an IC_{50} value of 3.38 µg/mL which was statistically similar to obtained for Trolox (IC_{50} = 5.15 µg/mL) and catechin (IC_{50} = 1.76 µg/mL) standards. Silva et al. [30] reported an IC_{50} value of 27.0 µg/mL for mesocarp and epicarp ethanol extracts of fruits of this same species. By the $ABTS^{\bullet+}$ assay, EAF exhibited the lowest IC_{50} (2.04 µg/mL), indicating an antioxidant activity higher than that of Trolox (IC_{50} = 2.36 µg/mL). By the FRAP assay, EAF showed the best ferric-reducing ability (15.41 mmol Fe^{2+}/g), which was statistically similar to Trolox (8.91 mmol Fe^{2+}/g).

The highest antioxidant activity evaluated by the DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP assays was obtained to EAF, the fraction that also contained the highest concentration of phenolic compounds (646.5 mg GAE/g).

Concentration of proanthocyanidins (CPAs)

Considering the largest TPC among the samples analyzed, HEB, HAF and EAF were evaluated for concentration of proanthocyanidins (Table 2). EAF showed higher CPAs (453.70 mg CE / g) which can be related to its higher antioxidant capacity. Proanthocyanidins have been identified in other edible parts of palm species, such as in the fruits of *Cocos nucifera* L. [31] and *Areca catechu* L. [32] which are rich in catechin and its derivatives. CPAs in babassu mesocarp are significantly higher than those found in fruits of mesocarp *Euterpe oleracea* Mart. (12,89 mg/g) [33], *Astrocaryum aculeatum* Meyer (8,24 mg EC/g) [34] e *Syagrus coronata* (Mart.) Becc. (146 mg EC/g) [35].

Table 2: Procyanidin Concentration and Tyrosinase Inhibitory Activity of *Attalea speciosa* HEB and Fractions by Monophenolase and Diphenolase[a]

Sample	CPAs (mg CE/g)	Monophenolase IC50 (µg/mL)	Diphenolase IC50 (µg/mL)
HEB	421.70 ± 7.42a	50.07 ± 14.16a	271.55 ± 8.24a
HAF	335.60 ± 12.81b	53.98 ± 6.06b	213.27 ± 21.35b
EAF	453.70 ± 12.33c	48.43 ± 29.51c	132.63 ± 5.71c

[a]Values represent the mean of triplicate measurements ± standard deviation. Different letters in the same column indicate a significant difference (Tukey test, $p < 0.05$). CPAs, concentration of proanthocyanidins; HEB, hydroalcoholic extract of babassu; EAF, ethyl acetate fraction; HAF, hydroalcoholic fraction

Proanthocyanidins possess multiple biological activities attributed to their antioxidant properties [36, 37], since phenolic derivatives play an important role in the elimination of free radicals such as hydroxyl, peroxyl and superoxide radicals and nitric oxide in living systems [38].

Inhibitory effects of babassu mesocarp on mushroom tyrosinase

The effect of the HEB, HAF e EAF on monophenolase and diphenolase activity of mushroom tyrosinase was shown in Table 2. Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [39]. The monophenolase and diphenolase activities may be both negatively affected by adding of babassu mesocarp. EAF showed greater inhibitory activity of tyrosinase by monophenolase (IC_{50} = 48.43 µg/mL) and diphenolase actions (IC_{50} = 132.63 µg/mL), results can be explained by the higher concentration of proanthocyanidins (453.7 mg CE/g) while HAF presented lower PAs content and therefore lower inhibitory activity of the enzyme. In ethnobotanical studies, the fruit extracts of *A. speciosa* have been reported to be useful for the treatment of skin diseases. Baurin et al. [40] reported 58% inhibition of mushroom tyrosinase for the species. Our data are similar to those reported for the fruits of *Actinidia deliciosa*, also rich in proanthocyanidins, which has showed an inhibitory effect of tyrosinase with IC_{50} values of 48.9 e 64.9 µg/mL for L-tyrosine and L-DOPA, respectively [41].

Correlation between total phenolic content, concentration of proanthocyanidins, antioxidant and tyrosinase inhibitory activities

Figure 2 shows the correlations between the results of the DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP methods and TPC. A significant negative correlation was observed between DPPH$^{\bullet}$ and phenolic compounds (-0.947) and between ABTS$^{\bullet+}$ and phenolic compounds (-0.722), with high levels of phenolic compounds being correlated with a low IC_{50} value in the two methods. An inverse correlation was found between ABTS$^{\bullet+}$ and FRAP (-0.948), with low IC_{50} values in the ABTS$^{\bullet+}$ methods being associated with a higher reducing ability in the FRAP method. Similar to the present results, a strong correlation between TPC and antioxidant activity has been reported for tropical fruits [29, 42].

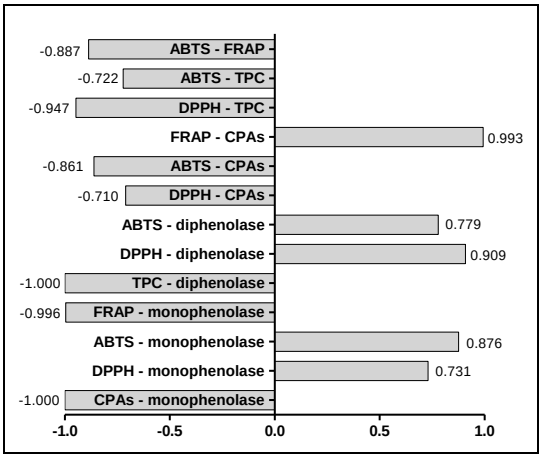

Figure 2: Pearson correlation coefficient (r) between the DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP methods and total phenolic content of the mesocarp of *Attalea speciosa* Mart. ex Spreng

A strong negative correlation (-1.000) was observed between CPAs and inhibitory activity by monofenolase action with high proanthocyanidins content associated with greater inhibition of tyrosinase using L-tyrosine as a substrate.
CPAs showed strong correlation with all the antioxidants tested methods, DPPH (-0.710), ABTS (-0.861) and FRAP (0.993), showing that higher levels of proanthocyanidins are related to higher antioxidant capacity. It was also observed correlation between antioxidant activity and inhibitory activity of tyrosinase, proving that the two activities are directly related.

Identification of phenolic compounds

Since EAF exhibited the highest TPC, CPAs, scavenging activity of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical and greatest ferric-reducing ability in the FRAP assay and tyrosinase inhibitory activity, the chemical composition of this fraction was analyzed by HPLC-MS/MS.
Table 3 shows the identification of phenolic compounds based on their retention time, maximum UV-Vis absorbance (λ_{max}), molecular weight, and molecular ion $[M - H]^-$ and main product ions obtained by HPLC-MS/MS for the nine compounds detected in EAF. Compounds 1-9 were identified as procyanidins by comparing their fragmentation profile with literature data. The results indicated the presence of molecular ions corresponding to monomers, dimers, trimers, and tetramers (Figure 3).

Table 3: Identification of Phenolic Compounds in the Ethyl Acetate Fraction of the Mesocarp of *Attalea speciosa* Mart. ex Spreng. Fruits by HPLC-MS/MS[a]

Compound	Proposed identifications	RT (min)	$\lambda_{max\ (nm)}$	MW	Parent íon $[M-H]^-$ (*m/z*)	MS/MS fragments (m/z)
1	(epi)catechin[b]	18.4	280	290	289	179, 205, 245
2	(epi)catechin-(epi)catechin[b]	15.2	280	578	577	289, 407, 425, 451, 559
3	(epi)catechin-(epi)catechin[b]	17.1	280	578	577	4,25,559
4	(epi)catechin-(epi)catechin[b]	17.5	280	578	577	289, 407, 425, 451, 559
5	(epi)catechin-(epi)catechin[b]	17.7	280	578	577	289, 407, 425, 451, 559
6	(epi)catechin-(epi)catechin-(epi)catechin[b]	18.4	280	866	865	575, 577,695, 713,739
7	(epi)catechin-(epi)catechin-(epi)catechin[b]	19.2	280	866	865	575, 577,695, 713,739
8	(epi)catechin-(epi)catechin-(epi)catechin[b]	23.8	280	866	865	575, 577,695, 713,739
9	(epi)catechin-(epi)catechin-(epi)catechin-(epi)catechin[b]	18	-	1154	1153	n.a.

[a]RT, retention time; λ_{max}, UV-vis absorption maxima; MW, molecular weight; $[M - H]^-$ molecular ion; n.a., not analyzed; [b]Compounds identified for the first time in *Attalea speciosa* Mart. ex Spreng.

Since mass spectrometry does not permit differentiation of the stereochemistry of the C2 and C3 carbons of monomeric and oligomeric catechins, the *m/z* 289 fragments were attributed to (epi)catechin, where 'epi' refers to the epimer and the parentheses indicate any epimer. The presence of precursor ions of a monomer (*m/z* 289), dimer (*m/z* 577), trimer (*m/z* 865) and tetramer (*m/z* 1153) of (epi)catechin indicates the presence of oligomeric procyanidins separated by 288 Da (Figure 3). The main fragmentation pathways of proanthocyanidins include quinone methide (QM) formation, retro-Diels-Alder (RDA) reaction, and heterocyclic ring fission (HRF) [43]. Compound 1 was identified as (epi)catechin. Fragmentation of the molecular ion at *m/z* 289 $[M - H]^-$ produced fragment ions at *m/z* 245, 205 and 179 resulting from three rearrangements that involved the loss of small molecules [43, 44]. Compounds 2-5 yielded a molecular ion at *m/z* 577 $[M - H]^-$, followed by fragment ions at *m/z* 425 $[M - H - 152]^-$ due to RDA, *m/z* 407 $[M - H - 152 - 18]^-$ due to the loss of a water molecule, *m/z* 289 $[M - H - 288]^-$ due to QM, *m/z* 451 $[M - H - 126]^-$ due to HRF, and *m/z* 559 $[M - H - 18]^-$ due to the loss of a water molecule. These compounds were tentatively identified as (epi)catechin-(epi)catechin [44]. Compounds 6-8 produced a molecular ion at *m/z* 865 $[M - H]^-$ and fragment ions at *m/z* 713 $[M - H - 152]^-$ due to RDA, *m/z* 695 due to the subsequent elimination of a water molecule $[M - H - 152 - 18]^-$, *m/z* 577 $[M - H - 288]^-$ due to QM cleavage of the interflavan bond, and *m/z* 739 $[M - H - 126]^-$ due to HRF. These compounds were tentatively identified as (epi)catechin-(epi)catechin-(epi)catechin [45]. The analyses indicate the presence of nine identified compounds in EAF, including 1 monomer, 4 dimers, 3 trimers and 1 tetramer of procyanidin. The chemical structures of these compounds are shown in Figure 4. All of these compounds were detected here for the first time in the mesocarp of *A. speciosa* and only contained (epi)catechin units, characterizing procyanidins, in contrast to other plant species in which the presence of prodelphinidins, formed by subunits of (epi)gallocatechin, has been reported [46, 47]. This is an interesting finding considering the possible role of catechins and their polymer derivatives in the biological activities related to the traditional use of the mesocarp. The EAF has exhibited the highest antioxidant and tyrosinase inhibitory activities probably because of its high content of phenolic compounds identified as monomers, dimers, trimers and tetramers of procyanidins.
This study reports for the first time the presence of procyanidins with antioxidant and tyrosinase inhibitory activities in the mesocarp of fruit this species. This is an important finding considering the possible relationship of these compounds with the biological activities and traditional use of the mesocarp. HPLC-MS/MS allowed the identification of nine (epi)catechin oligomers.

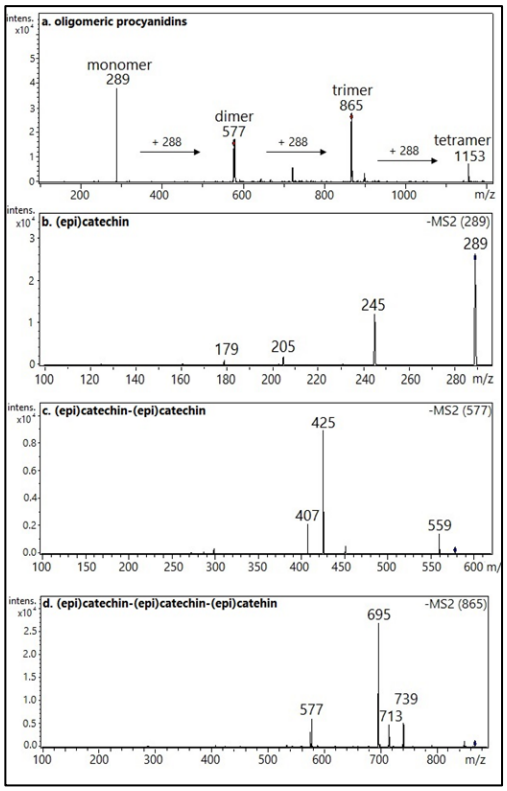

Figure 3: Mass spectra obtained in the negative ion mode for the ethyl acetate fraction (EAF) of the mesocarp of *Attalea speciosa* Mart. ex Spreng. a. oligomeric procyanidins; b. monomer (*m/z* 289); c. dimer (*m/z* 577); d. trimer (*m/z* 865)

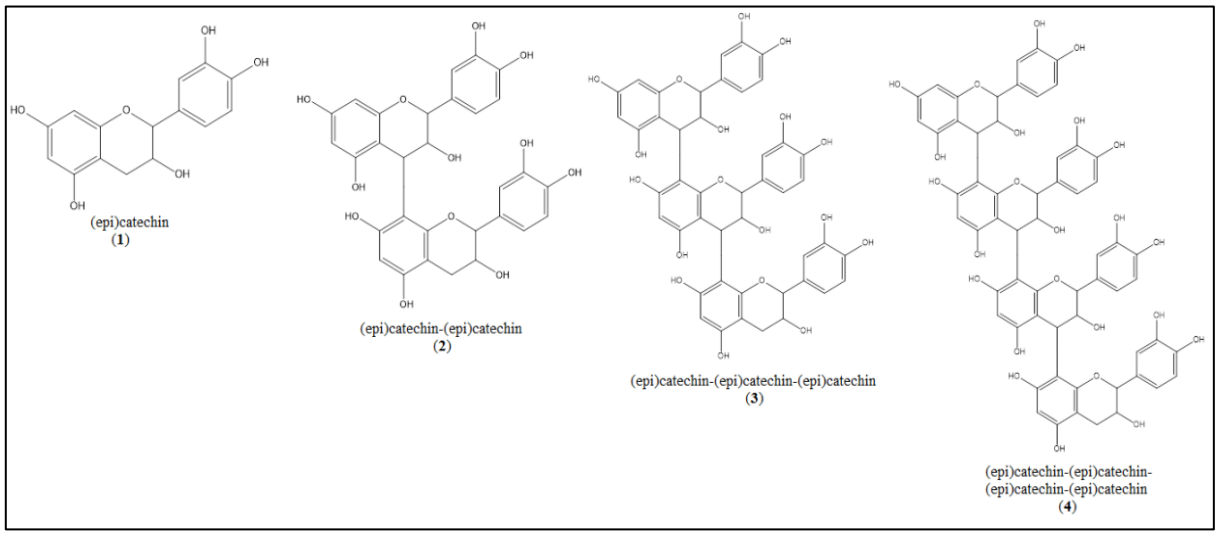

Figure 4: Chemical structure of the procyanidins detected in the mesocarp of *Attalea speciosa* Mart. ex Spreng.

CONCLUSION

In this study, our results revealed that proanthocyanidins from *Attalea speciosa* Mart. ex Spreng. mesocarp fruit can be an industrial resource for further production of antioxidant and anti-tyrosinase agents.

ACKNOWLEDGEMENTS

To CAPES and CNPq for the fellowships, and to FAPEMA for financial support.
Notes: The authors declare no competing financial interest.

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