JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification and Quantification, by HPLC-DAD-MS/MS, of Carotenoids and Phenolic Compounds from the Amazonian Fruit *Caryocar villosum*

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Supporting Information

ABSTRACT: The Amazonian region from Brazil has a wide variety of native and wild noncommercially cultivated fruits. This article reports for the first time the composition of carotenoids and phenolic compounds from *Caryocar villosum* fruit pulp, and, in addition, its proximate composition and antioxidant capacity (ORAC assay) were determined. According to the nutritional composition, water (52%) and lipids (25%) were the major components found in the pulp, and the total energetic value was 291 kcal/100 g. The major phenolic compounds identified by HPLC-DAD-ESI-MS/MS were gallic acid (182.4 μg/g pulp), followed by ellagic acid rhamnoside (107 μg/g pulp) and ellagic acid (104 μg/g pulp). The main carotenoids identified by HPLC-DAD-APCI-MS/MS were all-*trans*-antheraxanthin (3.4 μg/g pulp), all-*trans*-zeaxanthin (2.9 μg/g pulp), and a lutein-like carotenoid (2.8 μg/g pulp). The antioxidant capacity of the pulp (3.7 mMol Trolox/100 g pulp) indicates that it can be considered a good peroxyl radical scavenger.

KEYWORDS: *piquiá, Amazonian fruit, LC−MS, phenolic acids, xanthophylls, ORAC*

INTRODUCTION

Caryocar villosum (Aubl.) Pers. (Brazilian name: piquiá), from the Caryocaraceae family, is a very large tree (up to 40−50 m high) native from the Amazonian forest, especially abundant in Pará State, which is located in the North region of Brazil.[1,2] The main commercial use of the trees of *Caryocar* genus is the wood, due to the high quality of its timber, frequently used for the construction of houses and boats. However, the pulp of its fruits is consumed by the local people for cooking with rice, or its edible oil can be used to prepare regional dishes as a substitute for butter, as well as for soaps or cosmetic applications.[1,3] The endocarp normally surrounds one seed, whereas sometimes there can be up to four seeds. In the Amazonian region, the flowering occurs in the dry season (from July to November) with fruiting in the rainy season (from March to May).[1] Furthermore, the consumption of *C. villosum* pulp is similar to the pulp of *Caryocar brasiliense* Camb. (Brazilian name: pequi), another fruit from the same Caryocaraceae family, which is widely known in Brazil with spontaneously occurrence in the Northeast and Midwest regions.

The fruits of *Caryocar villosum* can be considered an interesting source of bioactive compounds, because they showed the highest contents of total phenolic compounds, flavonoids, and antioxidant activity as compared to the 18 other tropical fruits (nine of them from the Amazonian region) in a previous screening carried out by our research group.[4] The main components of *Caryocar villosum* pulp, such as carbohydrates (starch, glucose, sucrose, fructose), free amino acids (asparagine, γ-aminobutyric acid, alanine, leucine, valine), minerals and trace elements (calcium, magnesium, phosphorus), unsaponifiable matter (sterols), biogenic amines (*O*-phosphoethanolamine, taurine) and volatile compounds (*trans*-nerolidol, 2-heptanone, β-bisabolene), were already studied.[2] Magid et al.[5] identified seven new phenolic glycosides, most of them galloyl and ellagic acid derivatives from the stem bark of *Caryocar villosum* and *C. glabrum*, along with 15 other known compounds. Regarding the other phytochemicals, the triterpenoid saponins (compounds with detergent and surfactant properties) were isolated from the methanol extract of dry peel, pulp, and stem bark of *C. villosum*, and their toxicity, antimicrobial, cytotoxic, lipolytic, and tyrosinase inhibitory activities were also reported.[6,7]

Bioactive compounds, such as carotenoids and phenolic compounds, have been associated with the reduction of the risks of various chronic degenerative disorders, such as cancer, inflammations, cardiovascular diseases, cataracts, and macular degeneration.[8,9] Although *Caryocar villosum* pulps are reported to contain phenolic compounds and carotenoids,[4] no information about the composition of phenolics and carotenoids was found yet. However, the composition of carotenoids and phenolic compounds of the pulp of *Caryocar brasiliense* was reported in the literature.[10,11]

Considering that the essential role of biodiversity for its sustainable use in food security and nutrition is worldwide recognized,[12] and that Brazil has a wide variety of native, wild, and noncommercially cultivated fruits and little information about their constituents is available, this study was designed to identify and quantify the phenolic compounds and carotenoids in the pulp of *C. villosum* by high performance liquid chromatography coupled to diode array and mass spectrometric

Received: February 23, 2012
Revised: May 18, 2012
Accepted: May 22, 2012
Published: May 22, 2012

detectors (HPLC-DAD-MS/MS). Additionally, the proximate composition, as well as the peroxyl radical scavenging (ROO•) capacity of *Caryocar villosum* pulp, was determined in the present study.

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), catechin, epicatechin, quinic acid, rutin, taxifolin, naringin, hesperidin, neohesperidin, luteolin, quercetin, naringenin, apigenin, rhamnetin, fluorescein, AAPH (α,α′-azodiisobutyramidine dihydrochloride), and all-*trans*-β-carotene were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, methyl tert-butyl ether (MTBE), and acetonitrile, all of chromatographic grade, were obtained from J. T. Baker (Phillipsburg, NJ), and ultrapure water was obtained from the Millipore system (Billerica, MA). The gallic acid, ellagic acid, hydroxybenzoic acid, caffeic acid, 4-coumaric acid, ferulic acid, myricetin, and kaempferol standards were purchased from Extrasynthèse (Lyon Nord, France). Standards of all-*trans*-lutein and all-*trans*-zeaxanthin were provided by DSM Nutritional Products (Basel, Switzerland). The carotenoid standards of 9-*cis*-neoxanthin, all-*trans*-violaxanthin, and all-*trans*-antheraxanthin were purchased from CaroteNature (Lupsingen, Switzerland). All standards showed at least 95% of purity, determined by HPLC-DAD. All other chemical salts and solvents of analytical grade were purchased from Synth (São Paulo, Brazil) or Merck (Darmstadt, Germany). For chromatographic analysis, samples and solvents were filtered using, respectively, membranes of 0.22 and 0.45 μm, both from Millipore.

Materials. The *Caryocar villosum* fruits (piquiá) were acquired at the "Ver-O-Peso" market located in the city of Belém, Pará State, Brazil (latitude 01°27′21″ S and longitude 48°30′16″ W) in March 2010. All ripe fruits (9 kg) were cut in half, and the shells were manually removed from the pulp and seeds. The yellow colored pulp was separated from the seeds, weighed, grinded, and divided in two parts: one was used for the proximate composition (wet basis), and the other one was immediately lyophilized (dry basis) in a freeze-drier (Liobras equipment, São Paulo, Brazil) for all other analysis. Both fresh and lyophilized materials were thoroughly mixed, vacuum packed, and stored under light-free conditions at −36 °C until analysis.

Proximate Composition Analysis. The recommended methods of the Association of Official Analytical Chemists[13] were used to determine the moisture, ash, total lipids, and total protein (conversion factor of 6.25 from total nitrogen to total protein) in *Caryocar villosum* fresh pulp. The content of total carbohydrates was obtained by the difference of the sum of the percentage contents of moisture, ashes, total lipids, and proteins.[14] All of the experiments of proximate composition were performed in triplicate and expressed in g/100 g of fresh pulp (%). The total energetic value was calculated according to the Atwater specific energy conversion factors for fruits,[14] as follows: total energetic value (kcal/100 g) = (protein percentage × 3.36 kcal/g) + (lipid percentage × 8.37 kcal/g) + (total carbohydrate percentage × 3.60 kcal/g).

ROO• Scavenging Assay. Five grams of the freeze-dried pulp was used to obtain a methanol/water extract to estimate the scavenging capacity of *C. villosum* pulp against ROO•. The extraction procedure was performed with methanol/water (8:2, v/v) five times, followed by concentration in a rotary evaporator (T < 38 °C), and filled to the final volume (50 mL) with the same solvent used in the extraction. The ROO• scavenging assay was carried out by monitoring the effect of different dilutions of the extract on the fluorescence decay resulting from ROO•-induced oxidation of fluorescein and expressed as the "oxygen radical absorbance capacity" (ORAC).[15] Trolox was used as the standard control, and the concentration in the eight-point analytical curve varied from 8 to 96 μM. The results were expressed as mMol Trolox equivalent per 100 g of freeze-dried pulp. All measurements were performed in triplicate in three different microplates using a microplate reader (Synergy MX, Biotek, Winooski, VT).

HPLC-DAD-MS/MS Analysis of Carotenoids and Phenolic Compounds. HPLC-DAD analysis of carotenoids and phenolic compounds was performed in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with a 20 μL loop, and a diode array detector (DAD) (SPD-M20A). The equipment was also connected in series to a mass spectrometer (MS/MS) from Bruker Daltonics (Esquire 4000 model, Bremen, Germany) with APCI (atmospheric pressure chemical ionization) and ESI (electrospray ionization) sources, and an ion-trap analyzer.

The extraction and HPLC-DAD-APCI-MS/MS analysis of carotenoids from the freeze-dried pulp of *Caryocar villosum* (5 g) was performed according to the procedure previously described by De Rosso and Mercadante[16] for carotenoids from Amazonian fruits. Briefly, after exhaustive extraction, an aliquot of the carotenoid extract was evaporated under N_2 flow, dissolved in methanol/MTBE (70:30, v/v), and injected into the chromatographic system. The carotenoids were separated on a C_{30} YMC column (5 μm, 250 mm × 4.6 mm) using as mobile phase a linear gradient of methanol/MTBE from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min.[16] The flow rate was 0.9 mL/min, and the column temperature was set at 29 °C. The MS parameters were set as follows: positive mode; current corona, 4000 nA; source temperature, 450 °C; dry gas, N_2; temperature, 350 °C; flow, 60 L/h; nebulizer, 5 psi; MS/MS fragmentation energy, 1.4 V. The mass spectra were acquired with scan range of m/z from 100 to 700.[16] The carotenoids were tentatively identified according to the following combined information: elution order on C_{30} column, cochromatography with authentic standards, UV−visible spectrum (λ_{max}, spectral fine structure (%III/II), peak *cis* intensity (%A_B/A_{II})), and mass spectrum compared to data available in the literature.[16] Because the *cis*-isomers of carotenoids cannot be distinguished from the corresponding all-*trans* by MS analysis, its final characterization was based on the observation of the protonated molecule by MS, decreased %III/II, and by the %A_B/A_{II} values (∼10% = 9-*cis*; ∼45% = 13-*cis*, and ∼56% = 15-*cis* carotenoid).[17] The carotenoids were quantified by HPLC-DAD, using external seven-point analytical curves (in duplicate) for 9-*cis*-neoxanthin (0.9−17.1 μg/mL), all-*trans*-violaxanthin (0.7−13.6 μg/mL), all-*trans*-antheraxanthin (0.8−15.9 μg/mL), all-*trans*-lutein (1.0−59.5 μg/mL), all-*trans*-zeaxanthin (1.3−59.7 μg/mL), and all-*trans*-β-carotene (1.1−30.2 μg/mL). All other carotenoids were estimated using the curve of zeaxanthin and the *cis*-isomers using the corresponding curve of its all-*trans*-carotenoid. For all analytical curves of carotenoids, the r^2 = 0.99, the limit of detection (LOD) was 0.1 μg/mL, and the limit of quantification (LOQ) was 0.5 μg/mL, calculated using the parameters of the analytical curves (standard deviation and the slope).[18] The NAS-IOM[19] conversion factor was used to calculate the vitamin A value, with 12 μg of dietary all-*trans*-β-carotene corresponding to 1 μg of retinol activity equivalent (RAE), and the activity used was 100% for all-*trans*-β-carotene.

The phenolic compounds were analyzed by HPLC-DAD-ESI-MS/MS using an aliquot of the same extract obtained for the ROO• scavenging assay. To provide additional information, the extract was also subjected to acid hydrolysis[20] and analyzed by HPLC-DAD-ESI-MS/MS. The phenolic compounds were separated on a C_{18} Synergi Hydro column (4 μm, 250 × 4.6 mm, Phenomenex) at 0.9 mL/min, column temperature at 29 °C, with a mobile phase consisting of water/formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile/formic acid (99.5:0.5, v/v) (solvent B) in linear gradient from A:B 99:1 to 50:50 in 50 min, following from 50:50 to 1:99 in 5 min. This latter ratio (1:99) was maintained for an additional 5 min. The column eluate was split to allow only 0.15 mL/min to enter the ESI interface. The spectra were obtained between 200 and 600 nm, and the chromatograms were processed at 271 and 367 nm. The mass spectra were acquired with a scan range from m/z 100 to 800; the MS parameters were set as follows: ESI source in positive and negative ion modes; capillary voltage, 2000 V; end plate offset, −500 V; capillary exit, −110 V; skimmer 1, 10 V; skimmer 2, 5 V; dry gas (N_2) temperature, 310 °C; flow rate, 5 L/min; nebulizer, 30 psi; MS/MS fragmentation energy, 1.4 V; and MS^3 fragmentation energy, 1.8 V. The phenolic compounds were tentatively identified on the basis of the following information: elution order and retention time of the peaks in relation to standards, UV−visible and mass spectra features as

compared to standards analyzed under the same conditions, and data available in the literature.[5,21−25] Phenolic compounds were quantified by comparison to external standards using seven-point analytical curves (in duplicate) for gallic acid (0.5−51.5 μg/mL), 4-coumaric acid (0.5−49.5 μg/mL), ellagic acid (0.5−52 μg/mL), and methyl quercetin (0.2−19.2 μg/mL). For all of these compounds, r^2 = 0.99, limit of detection was 0.1 μg/mL, and limit of quantification was 0.4 μg/mL, calculated using the parameters of the analytical curves (standard deviation and the slope).[18]

The contents of carotenoids and phenolic compounds determined by HPLC-DAD were expressed as μg/g of freeze-dried pulp (dry basis), considering three independent extraction procedures (n = 3).

Statistical Analysis. The mean and standard deviation (SD) results related to the proximate composition, antioxidant capacity, and HPLC-DAD analysis of *Caryocar villosum* pulp were calculated with the Statistica 6.0 software.

RESULTS AND DISCUSSION

Proximate Composition and Antioxidant Capacity of *Caryocar villosum* Pulp. The fruits were irregularly oblong-globose, approximately 7−9 cm in diameter, and the yellowish pulp represented approximately 12% of total weight from the nonprocessed fruit (24% of seed and 64% of shell), in accordance with the values previously reported.[1,2] The analysis of the main chemical constituents was performed to obtain an overview of the chemical composition and its nutritive value.

According to the nutritional composition (Figure 1), water and lipids are the major components in the pulp. The

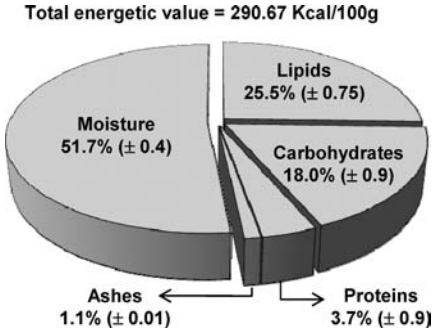

Figure 1. Proximate composition (wet basis) of *Caryocar villosum* pulp.

nutritional composition of *Caryocar villosum* is similar to that from other Amazonian fruits with high lipid contents (12.8−47.2%), such as *Jessenia pataua* (patauá), *Endopleura uxi* (uxi), *Bactris gasipaes* (pupunha), *Poraqueiba sericea* (mari), and *Astrocaryum vulgare* (tucumã), which also all presented low water contents (35.6−55.6%) and total energetic values ranging from 252.4 to 474.0 kcal/100 g.[26] Because of its high lipid content, the nutritive value of *C. villosum* pulp seems to be substantially determined by the composition of the lipid fraction (mainly palmitic and oleic acid).[2] Furthermore, *C. villosum* pulp showed higher contents of water, proteins, ashes, and carbohydrates than did pequi (*Caryocar brasiliense*).[27]

The antioxidant capacity of *C. villosum* pulp, as ORAC, was 3.74 ± 1.09 mmol Trolox/100 g, a high value as compared to the average reported value for 41 different fruits (2.7 mmol Trolox/100 g),[28] and higher than other Amazonian fruits, such as *B. crassifolia* (2.65 mmol Trolox/100 g) and *I. edulis* (2.34 mmol Trolox/100 g),[29] but much lower than freeze-dried acai (*Euterpe oleraceae* Mart) (99.7 mmol Trolox/100 g).[30]

Composition of Phenolic Compounds. The HPLC-DAD-ESI-MS/MS method allowed the separation and tentative identification of 17 phenolic compounds in the pulp (Figure 2a,b). The retention time, UV−vis, and mass spectra data of 20 standards of phenolic compounds were used to confirm their presence or absence (data not shown). Moreover, the MS/MS fragmentation pattern of the aglycone standard was compared to the corresponding acylated or glycosylated phenolic compound observed in the pulp. The HPLC coupled to tandem mass spectrometry, using electrospray ionization (ESI), proved to be extremely useful for peak assignment and further characterization of individual compounds described in Table 1, because only gallic and ellagic acids were commercially available as reference compounds, among all phenolic compounds identified in *Caryocar villosum* pulp. The ESI in the negative ion mode provided a very sensitive, selective method and produced by far the most characteristic data for the identification of phenolic compounds in this pulp extract. In our study, no ionization in the MS was obtained in positive ion mode, according to previous studies with similar phenolic compounds.[21,31]

Phenolic compounds in nature generally occur as conjugates of sugars, usually *O*-glycosides. Glucose, fructose, and sucrose were the sugar molecules already reported in the pulp of *Caryocar villosum*.[2] Additionally, rhamnose and glucose conjugated with phenolic acids were identified in its stem bark,[5] and arabinose was found in the peel extract of this fruit.[22] In the present study, the identity of the sugar moiety could not be determined by the applied methodology; however, MS/MS analysis indicated that cleavage of the glycosidic linkage with concomitant H rearrangement leads to elimination of the sugar residue, that is, 162 u (an hexose), 146 u (a deoxyhexose), and 132 u (a pentose).

Peak 1 was assigned as a monogalloyl hexoside, showing deprotonated molecule at m/z 331 and fragment at m/z 169 (loss of hexoside), which corresponds to the gallic acid moiety, and the fragment at m/z 125, which corresponds to the consecutive losses of 162 u (hexoside) and 44 u (CO_2), as reported for phenolic compounds in seed, skin, and pulp of muscadine grapes.[23] Peak 2 was positively identified as gallic acid on the basis of coelution and comparison of UV and mass spectra with authentic standard, and the MS spectrum showed $[M - H]^-$ at m/z 169 with MS/MS fragment at m/z 125, corresponding to the neutral loss of 44 u (CO_2). Moreover, the gallic acid was the major compound identified in the *Caryocar villosum* extract after acid hydrolysis (Figure 2c). Gallic acid was also identified as one of the major phenolic compounds in ethanolic extract from the peel of *Caryocar brasiliense* fruit.[22] Peak 3, tentatively identified as a hexahydroxydiphenoyl (HHDP) hexoside, showed $[M - H]^-$ at m/z 481 and MS/MS fragments at m/z 463 (loss of H_2O), m/z 319 (loss of hexose), which corresponds to the HHDP moiety, and the fragment at m/z 301 corresponding to the consecutive neutral losses of hexoside and water molecules.

Peak 4 was tentatively identified as a coumaroyl-galloyl hexoside, because the deprotonated molecule showed $[M - H]^-$ at m/z 477 and MS/MS fragments at m/z 307 (loss of a gallic acid moiety), m/z 163 (loss of a galloyl hexose), which corresponds to the coumaric acid moiety, and m/z 145 (loss of H_2O from m/z 163), as previously reported for phenolic compounds of strawberry.[31] Peak 5 was tentatively identified as a coumaroyl quinic acid, based on the similar characteristics found for the same compound in *Erigeron breviscapus*,[32] that is,

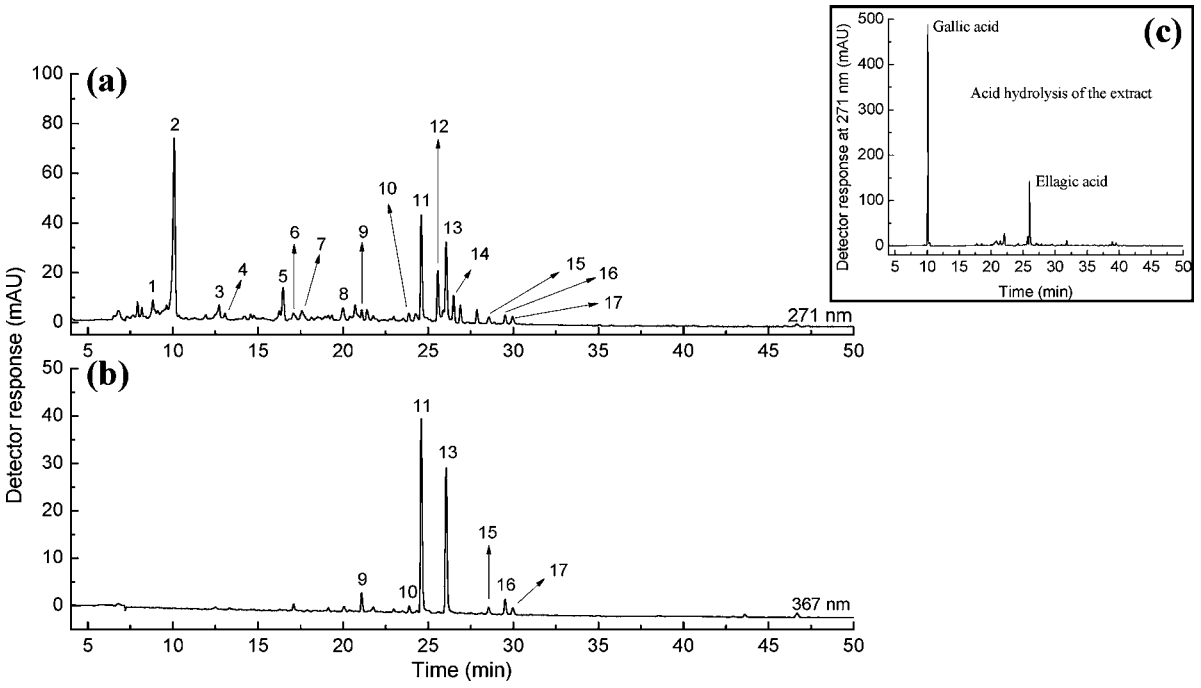

Figure 2. Chromatograms obtained by HPLC–DAD of phenolic compounds from *Caryocar villosum* pulp, (a) processed at 271 nm, (b) processed at 367 nm, and (c) acid hydrolysis of the extract processed at 271 nm. Chromatographic conditions: see text. Peak characterization is given in Table 1.

Table 1. Chromatographic, Spectroscopic Characteristics, and Content of Phenolic Compounds from *Caryocar villosum* Pulp

peak	phenolic compound[a]	concentration (μg/g pulp)[b]	t_R range (min)[c]	λ_{max} (nm)[d]	$[M-H]^-$ (m/z)	MS/MS (−) (m/z)[e]	MS^3 (m/z)[j]	identity confirmation
1	monogalloyl hexoside[f]	21.1 ± 1.4	8.6–8.8	274	331	**169**, 125	**125**	23
2	gallic acid[f]	182.4 ± 17.0	9.9–10.1	271	169	**125**		standard
3	HHDP hexoside[f]	20.4 ± 2.4	12.6–12.8	269	481	463, **319**, 301	**147**	23, 24
4	coumaroyl-galloyl hexoside[g]	4.5 ± 0.6	12.9–13.1	276, 303	477	**307**, 163, 145	**163**, 145	25
5	coumaroyl quinic acid[g]	2.7 ± 0.6	16.3–16.5	278	337	191, 173, **163**, 119	148, **119**	25
6	HHDP-dihexoside[f]	17.4 ± 2.4	17.0–17.2	278	625	539, 523, 481, **463**, 319, 301	nd[k]	23
7	digalloyl hexoside[f]	18.4 ± 5.7	17.6–17.8	273	483	**331**, 313, 271, 193, 169	271, **169**	23
8	galloyl-HHDP hexoside[f]	20.6 ± 4.8	19.9–20.0	279	633	615, 481, 463, **301**, 275, 247, 231	285, **257**, 229	23, 31
9	ellagic acid hexoside[h]	16.9 ± 2.0	21.3–21.5	360	463	347, **301**	257, 229, **185**	31
10	ellagic acid pentoside[h]	9.3 ± 1.8	23.8–23.9	361	433	405, **301**	**257**, 229, 185	23, 31
11	ellagic acid deoxyhexoside[h]	107.0 ± 9.4	24.5–24.7	253, 300(sh), 361	447	373, **301**	273, **257**, 229, 185	23, 31
12	not identified[f]	29.3 ± 3.6	25.4–25.6	285	495	467, 449, **427**, 383, 359	**427**	
13	ellagic acid[h]	104.0 ± 10.5	25.9–26.1	253, 300(sh), 367	301	**257**, 229, 185		standard
14	methyl ellagic acid pentoside[h]	8.2 ± 2.2	26.4–26.6	279, 366	447	**315**, 300	**300**, 257, 229, 185	5, 31
15	methyl ellagic acid deoxyhexoside[h]	10.5 ± 2.1	28.4–28.6	300(sh), 366	461	417, **315**, 300	**300**, 257, 185	5, 31
16	methyl quercetin dihexoside[i]	6.3 ± 1.1	29.4–29.6	365	639	595, 459, **315**, 300, 165	**300**, 273, 179, 151	25
17	dimethyl ellagic acid pentoside[h]	10.3 ± 1.6	29.9–30.1	360	461	417, **329**, 315, 300	**300**, 257, 229	5, 31
total phenolic (μg/g pulp)		589.4 ± 41.5						

[a]Tentative identification based on UV–vis and mass spectra as well as relative HPLC retention times and published data. [b]n = 3 (dry basis). [c]Retention time on the C_{18} Synergi Hydro (4 μm) column. [d]Solvent: gradient of 0.5% formic acid in water and acetonitrile with 0.5% formic acid. [e]In the MS/MS from $[M-H]^-$, the most abundant ion is shown in boldface. HHDP = hexahydroxydiphenoyl. [f]Peaks were quantified as equivalent to gallic acid. [g]Peaks were quantified as equivalent to coumaric acid. [h]Peaks were quantified as equivalent to ellagic acid. [i]Peaks were quantified as equivalent to methyl quercetin. [j]The MS^3 fragments were obtained from the most abundant ion in the MS/MS experiment. [k]nd = not detected.

the presence of $[M-H]^-$ at m/z 337 and MS/MS fragments at m/z 191 (loss of a coumaroyl group), which corresponds to the presence of quinic acid, m/z 173 (loss of H_2O from quinic acid), m/z 163 originated from coumaric acid moiety, and m/z

dx.doi.org/10.1021/jf301904f | J. Agric. Food Chem. 2012, 60, 5884–5892

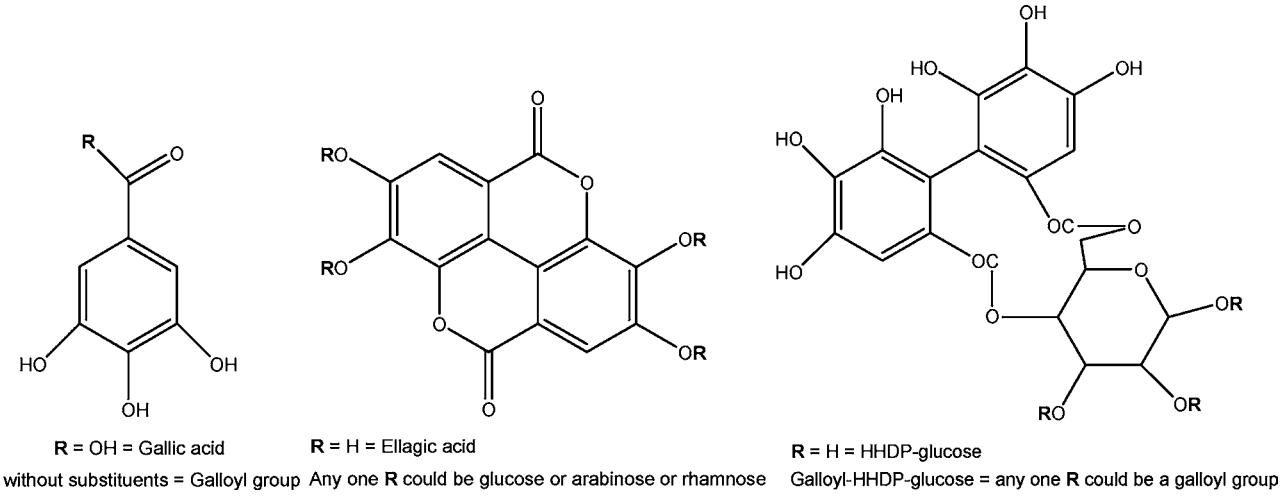

Figure 3. Chemical structures of phenolic compounds proposed for *Caryocar villosum* pulp.

119 (loss of CO_2 from coumaric acid). Peak 6 had a deprotonated molecule at m/z 625 $[M - H]^-$ that dissociated to give an intense MS/MS at m/z 463 $[M - H - 162]^-$, indicating the loss of a hexose unit, and the presence of the ion at m/z 301, suggesting the loss of another hexose unit $[M - H - 162 - 162]^-$. On the basis of fragmentation data, peak 6 was tentatively identified as HHDP dihexoside. Peak 7 was assigned as a digalloyl hexoside due to the presence of the deprotonated molecule at m/z 483 $[M - H]^-$ and MS/MS fragments at m/z 331 $[M - H - 152]^-$ and at m/z 169 $[M - H - 152 - 162]^-$, after sequential removal of the galloyl group (152 u) and the hexose moiety (162 u), respectively. Peak 8, tentatively identified as a galloyl-HHDP hexoside, had an ion at m/z 633 $[M - H]^-$ and MS/MS fragments at m/z 481, indicating the presence of HHDP-hexoside via the loss of a galloyl unit $[M - H - 152]^-$, and at m/z 301, suggesting the loss of a hexose unit. The loss of a galloyl unit suggested that this unit was attached directly to the hexose, because a galloyl bond via a *m*-depside bond is more prone to cleavage, resulting in loss of a galloyl unit (152 u).[23,31]

Peaks 9, 10, and 11 were tentatively identified as ellagic acid hexoside ($[M - H]^-$ at m/z 463), ellagic acid pentoside ($[M - H]^-$ at m/z 433), and ellagic acid deoxyhexoside ($[M - H]^-$ at m/z 447), respectively. All three compounds showed MS/MS fragment at m/z 301 as the most abundant ion, suggesting neutral losses of the sugar moiety, hexose (loss of 162 u from $[M - H]^-$ of peak 9), pentose (loss of 132 u from $[M - H]^-$ of peak 10), and deoxyhexose (loss of 146 u from $[M - H]^-$ of peak 11), as previously reported.[23,31] The MS^3 spectra from the ion at m/z 301 of peaks 9, 10, and 11 presented the same fragmentation pattern described below for ellagic acid (peak 13) (available in Figure 1 of the Supporting Information). Peak 12 was not identified, whereas the absorption maximum was at 285 nm, $[M - H]^-$ was at m/z 495, and the most abundant ion in MS/MS was at m/z 427 (loss of 68 u), which did not show any fragments during the MS^3 experiment.

Peak 13 was positively identified as ellagic acid based on coelution and comparison of UV and mass spectra with authentic standard. This peak presented $[M - H]^-$ at m/z 301 and typical MS/MS fragmentation ions of ellagic acid at m/z 257 (loss of CO_2), 229 (loss of CO_2 and CO), and 185 (loss of two CO_2 and one CO).[24,25] Furthermore, the ellagic acid was the second major compound identified in the *Caryocar villosum* extract after acid hydrolysis (Figure 2c). Peak 14, tentatively identified as a methyl ellagic acid pentoside, showed $[M - H]^-$ at m/z 447 and presented MS/MS fragments at m/z 315 (loss of an pentose moiety), and at m/z 300, corresponding to ellagic acid after neutral loss of one methyl group from the fragment at m/z 315. Peaks 15 and 17 were tentatively identified as methyl ellagic acid deoxyhexoside and dimethyl ellagic acid pentoside, respectively, both with deprotonated molecule at m/z 461 ($[M - H]^-$) and the first MS/MS fragment at m/z 417 ($[M - H - CO_2]^-$). Moreover, in peak 15, the neutral loss of deoxyhexose was detected by the MS/MS fragment at m/z 315 ($[M - H - 146]^-$), and in peak 17 the fragment at m/z 329 ($[M - H - 132]^-$) was attributed to the neutral loss of a pentose. Additionally, peaks 15 and 17 showed the same MS/MS fragment at m/z 300, probably due to the neutral loss of one (peak 15) or two methyl groups (peak 17) after losing their sugar moieties. The structure of 3-*O*-methyl-4′-(3″-*O*-acetyl)-α-L-rhamnopyranosyl ellagic acid, confirmed by NMR, was previously reported in the stem bark of *Caryocar villosum* and *Caryocar glabrum*,[5] confirming the assignment of peaks 11 (ellagic acid deoxyhexoside) and 15 (methyl ellagic acid deoxyhexoside). In addition, the MS^3 experiment from the most abundant ion at m/z 315 (peaks 14, 15, and 17) showed the same fragments found after the fragmentation of ellagic acid (peak 13). Finally, peak 16, with $[M - H]^-$ at m/z 639, was tentatively identified as methyl quercetin dihexoside after considering the MS/MS fragments at m/z 595 (loss of CO_2), at m/z 315 (loss of dihexose moiety, 324 u), and at m/z 300 (loss of methyl group after losing the dihexose moiety), also observed in the same compound found in Sicilian wines.[25] The fragment at m/z 300 found in peak 16 was considered as quercetin and not as ellagic acid, because a methyl ellagic acid dihexoside should elute before peak 15 and the MS^3 experiment from ion at m/z 315 showed some characteristics fragments attributed to quercetin.[23,25,31]

The phenolic profile here reported for *Caryocar villosum* pulp was very similar to those of other fruits, which were found to contain appreciable concentrations of ellagitannins (including HHDP derivatives) and ellagic acid glycosides in raspberry, blackberry, strawberry, cloudberry, boysenberry, pomegranate, persimmon, and muscadine grape.[33] As a result, this is the first report related to the identification of phenolic compounds in *Caryocar villosum* pulp, and the major phenolic compounds

identified in the pulp (dry basis) were gallic acid (182.4 μg/g pulp), followed by ellagic acid rhamnoside (107.0 μg/g pulp) and ellagic acid (104.0 μg/g pulp). The basic chemical structures of the phenolic compounds proposed for *Caryocar villosum* pulp are shown in Figure 3.

Composition of Carotenoids. The C_{30} column allowed the separation of 12 carotenoids, of which only one could not be identified by HPLC-DAD-APCI-MS/MS (Figure 4). The

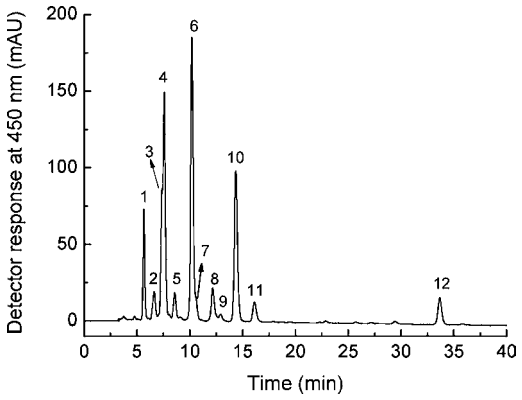

Figure 4. Chromatogram obtained by HPLC-DAD of carotenoids from *Caryocar villosum*. Chromatographic conditions: see text. Peak characterization is given in Table 2.

MS/MS experiments confirmed the assignment of the protonated molecule ($[M + H]^+$) of all identified peaks through the fragments expected for the carotenoid polyene chain and functional groups, along with the UV−visible spectra features (Table 2). Although in-source fragmentation occurred in the APCI interface, the MS/MS unambiguously allowed the assignments of some functional groups due to the specific losses from the protonated molecule. Considering that a detailed description of carotenoid identification using the above information was already reported,[16,17,34] only some of the most important aspects were discussed below.

All identified carotenoids presented two hydroxyl groups, with the exception of β-carotene (peak 12), and as expected for reversed-phase columns, all nonesterified xanthophylls eluted before this carotene. The carotenoids with 5,6-epoxide and/or 5,8-furanoid groups located at the 3- or 3′-hydroxy β-rings, all-*trans*-neoxanthin (peak 1), 9-*cis* or 9′-*cis* isomers of neoxanthin (peaks 2 and 5), all-*trans*-violaxanthin (peak 3), 9-*cis*-violaxanthin (peak 7), all-*trans*-antheraxanthin (peak 6), 9-*cis*-mutatoxanthin (peak 9), and 9-*cis*-antheraxanthin (peak 11), showed UV−vis and mass spectra features similar to those reported in the literature.[16,17,34] Peak 5 was assigned as 9′-*cis*-neoxanthin after comparing the UV−vis and MS features of 9′-*cis*-neoxanthin isolated from spinach and previously identified by NMR.[35] The identification of 9-*cis*-neoxanthin (peak 2), all-*trans*-violaxanthin (peak 7), all-*trans*-antheraxanthin (peak 6),

Table 2. Chromatographic, UV−Vis, Mass Spectroscopy Characteristics, and Content of Carotenoids from *Caryocar villosum*, Obtained by HPLC-DAD-APCI-MS/MS

peak[b]	carotenoid[a]	concentration (μg/g pulp)[c]	t_R range (min)[d]	λ_{max} (nm)[e]	%III/II	$\%A_B/A_{II}$	$[M + H]^+$ (m/z)	MS/MS (+) (m/z)[f]
1	all-*trans*-neoxanthin[h]	2.3 ± 0.6	5.6−5.7	416, 440, 469	90	0	601	**583** $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 509 $[M + H - 92]^+$, 393, 221
2	9-*cis*-neoxanthin[h]	0.8 ± 0.2	6.5−6.6	327, 416, 440, 469	83	13	601	**583** $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 509 $[M + H - 92]^+$, 393, 221
3	all-*trans*-violaxanthin[i]	1.1 ± 0.2	7.2−7.4	415, 439, 468	92	0	601	**583** $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$, 491 $[M + H - 92 - 18]^+$, 221
4	lutein-like[l]	2.8 ± 0.4	7.5−7.6	420, 445, 473	55	0	nd[n]	**583** $[M + H - 18]^+$,[g] 565 $[M + H - 18 - 18]^+$, 547 $[M + H - 18 - 18 - 18]^+$, 491 $[M + H - 92 - 18]^+$, 211, 166
5	9′-*cis*-neoxanthin[h]	0.9 ± 0.2	8.5−8.6	325, 416, 439, 467	64	6	601	**583** $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$, 491 $[M + H - 92 - 18]^+$, 221
6	all-*trans*-antheraxanthin[j]	3.4 ± 0.8	10.1−10.2	420, 444, 472	61	0	585	**567** $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 529 $[M + H - 56]^+$, 221
7	9-*cis*-violaxanthin[i]	0.4 ± 0.1	10.6−10.7	325, 413, 435, 464	73	8	601	**583** $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$, 221
8	all-*trans*-lutein[l]	0.9 ± 0.2	12.1−12.2	420, 444, 471	55	0	nd	**551** $[M + H - 18]^+$,[f] 533 $[M + H - 18 - 18]^+$, 495 $[M + H - 18 - 56]^+$, 477 $[M + H - 92]^+$
9	9-*cis*-mutatoxanthin[k]	0.6 ± 0.5	12.8−12.9	310, 405, 427, 452	78	11	585	**567** $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 493 $[M + H - 92]$, 221
10	all-*trans*-zeaxanthin[k]	2.9 ± 0.3	14.3−14.4	425, 450, 476	33	0	569	**551** $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
11	9-*cis*-antheraxanthin[j]	0.6 ± 0.2	16.1−16.2	325, 417, 440, 468	63	7	585	**567** $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 221
12	all-*trans*-β-carotene[m]	0.7 ± 0.04	33.6−33.7	425, 451, 477	40	0	537	**444** $[M + H - 92]^+$
total carotenoids (μg/g pulp)		17.3 ± 2.4						
vitamin A value (μg RAE/g pulp)		0.06 ± 0.003						

[a]Tentative identification based on UV−vis and mass spectra as well as relative HPLC retention times and published data. [b]Numbered according to the chromatogram shown in Figure 4. [c]$n = 3$ (dry basis). [d]Retention time on the C_{30} column. [e]Linear gradient of methanol/MTBE. [f]In the MS^2, the most abundant ion is shown in boldface. [g]In-source detected fragment. [h]The peaks were quantified as equivalent to 9-*cis*-neoxanthin. [i]The peaks were quantified as equivalent to violaxanthin. [j]The peaks were quantified as equivalent to antheraxanthin. [k]The peaks were quantified as equivalent to zeaxanthin. [l]The peaks were quantified as equivalent to lutein. [m]The peaks were quantified as equivalent to β-caroten. [n]nd = not detected. RAE = retinol activity equivalent.

dx.doi.org/10.1021/jf301904f | J. Agric. Food Chem. 2012, 60, 5884−5892

and all-*trans*-β-carotene (peak 12) was confirmed through coelution with authentic standards, and UV−vis and mass spectra features in comparison with standards and with the literature.[16,17,34] Because the major carotenoids identified presented epoxide groups, all analyses were performed again in both fresh and freeze-dried pulps, with and without addition of $NaHCO_3$ during the extraction step, and the carotenoid profiles were not different.

Despite all-*trans*-lutein (peak 8) and all-*trans*-zeaxanthin (peak 10) having the same chemical formula ($C_{40}H_{56}O_2$) and, therefore, identical protonated molecule (m/z 569), zeaxanthin possesses two β-rings, while lutein has one β-ring and one ε-ring. Thus, one of the hydroxyl groups of lutein is allylic to the double bond in the ε-ring and not conjugated with the polyene chain, resulting in 10 conjugated double bonds (cdb). On the other hand, zeaxanthin has both double bonds in β-ring conjugated to the polyene chain, and consequently its cromophore shows 11 cdb. Therefore, it is possible to distinguish them by UV−visible and mass spectra fragmentations. As expected, zeaxanthin showed λ_{max} values (425, 450, 476 nm) higher than those of lutein (420, 444, 471 nm). The mass spectrum of zeaxanthin showed a more intense protonated molecule peak (m/z 569) in comparison with the fragment at m/z 551 $[M + H - 18]^+$, whereas the contrary was observed for lutein, as previously reported by De Rosso and Mercadante.[16,34] Moreover, the identities of the peaks of lutein and zeaxanthin were confirmed by coelution with authentic standards.

Peak 4 was assigned as lutein-like carotenoid in *Caryocar villosum* pulp (2.84 μg/g) because it presented similar UV−vis to that of lutein (peak 8), such as the λ_{max} at 445 nm in methanol/MTBE (%III/II = 55 and no *cis* peak), indicating the presence of 10 cdb. The lutein-like carotenoid presented an in-source fragment at m/z 583 ($[M + H - 18]^+$) in the MS spectrum and a possible molecular weight of 600. However, the protonated molecule ($[M + H]^+$) at m/z 601 was not detected. This carotenoid did not coeluted neither with the available standards (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, and β-carotene) nor with the carotenoids from saponified extracts of kale, mango, and flowers of chrysanthemum, with and without HCl addition for 5,8 epoxide formation, or heat treatment for *cis* isomerization. Thus, the identification of this compound by NMR must be performed to establish the correct structure of this possible new carotenoid.

This is the first report about the carotenoid composition from *Caryocar villosum* (piquiá) pulp, and the major identified carotenoids were all-*trans*-antheraxanthin (3.4 μg/g pulp), followed by all-*trans*-zeaxanthin (2.9 μg/g pulp) and the lutein-like carotenoid (2.8 μg/g pulp). Antheraxanthin was also one of the major carotenoids found in persimmons (*Diospyros kaki*) and papayas (*Carica papaya*),[36] and a *cis* isomer of antheraxanthin, probably the 9- or 9′-*cis* isomer, was the major compound identified in orange juice.[37] Azevedo-Meleiro and Rodriguez-Amaya[10] reported the carotenoid composition of *Caryocar brasiliense* (pequi), and the main carotenoids were violaxanthin, lutein, and zeaxanthin, with smaller amounts of β-cryptoxanthin, β-carotene, and neoxanthin. Although having similar popular names, these fruits are from different species; therefore, the differences in composition are not surprising.

For vitamin A activity, a carotenoid must have at least one unsubstituted β-ionone ring with an attached polyene side chain of at least eleven carbons. According to these chemical structural requirements, among the identified carotenoids of *C. villosum* pulp, only β-carotene possess vitamin A activity (0.06 μg RAE/g pulp), but much lower than those reported for other Amazonian fruits (3.44−36.4 μg RAE/g pulp).[16] Thus, the pulp of *Caryocar villosum* should not be considered as a good pro-vitamin A source. On the other hand, considering that high consumption of fruits and vegetables, specifically rich in lutein and zeaxanthin, was correlated with a lower risk for age-related macular degeneration,[38] the contents of lutein (0.9 μg/g pulp) and zeaxanthin (2.9 μg/g pulp) of *Caryocar villosum* pulp were in the same range and superior, respectively, as compared to other Amazonian fruits (0.03−1.44 μg of lutein/g pulp and 0.16−0.61 μg zeaxanthin/g pulp),[16] while both lutein and zeaxanthin contents were lower as compared to some wild fruits from Panama (2.3−36.8 μg of lutein/g pulp and 2.8−84.7 μg zeaxanthin/g pulp).[39] However, according to the classification of good sources of carotenoids by content range, indicated by Britton and Khachik[40] (low, 0−100 μg/100 g; moderate, 100−500 μg/100 g; high, 500−2000 μg/100 g; very high, >2000 μg/100 g), *Caryocar villosum* pulp can not be considered as a good source of lutein (90 μg/100 g pulp, low) or zeaxanthin (290 μg/100 g pulp, moderate).

This is the first time that the composition of phenolic compounds and carotenoids of *Caryocar villosum* (piquiá) pulp was reported. Furthermore, not only the major phenolic compounds and carotenoids were determined by HPLC-MS/MS, but also 13 minor phenolic compounds and 7 carotenoids were identified. The daily intake of bioactive compounds, such as phenolic compounds and carotenoids, seems to be associated with the reduction of the risks of various chronic degenerative disorders;[8,9] however, these potential benefits depend on the bioavailability of these compounds, specially related to the intestinal absorption and concentration in the human plasma. Gallic acid, ellagic acid, and its glycoside derivatives, which are the major phenolic compounds found in *Caryocar villosum* pulp, as well as the ellagitannins, are absorbed (as aglycone) and rapidly methyl conjugated or glucorinated by the intestinal cells.[41] Additionally, the daily exposure to grape seed polyphenolic extract, containing gallic acid, demonstrated a significant increase in the bioavailability of gallic acid in brain tissues in a mouse model of Alzheimer's disease.[42] On the other hand, epoxy-xanthophylls seems to be not bioavailable in human plasma according to the results obtained by Barua and Olsen,[43] where violaxanthin and 5,6-epoxy-lutein were not detected in human plasma after oral administration of these carotenoids. A more recent study demonstrated that even after 1 week of spinach intake (3.0 mg neoxanthin/day), the concentrations of neoxanthin and its metabolites (neochrome stereoisomers) in plasma remained very low (about 1 nmol/L), whereas those of β-carotene and lutein markedly increased.[44]

In conclusion, the pulp of *Caryocar villosum* presents a promising chemical composition for the research of bioactive compounds with antioxidant properties. Additionally, due to its nutritional composition and high energetic value, the consumption of *C. villosum* pulp can be important to the Amazonian population who lives around the occurrence area of this species.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures of MS, MS/MS, and MS^3 spectra of some phenolic compounds identified in *Caryocar villosum* pulp. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We thank the Brazilian foundation FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial support.

Notes

The authors declare no competing financial interest.

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