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Article

Integrative Analysis Based on HPLC-DAD-MS/MS and NMR of *Bertholletia excelsa* Bark Biomass Residues: Determination of Ellagic Acid Derivatives

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Bertholletia excelsa Bonpl. (Lecythidaceae) is a South American tree worldwide known for providing the Brazil nuts. In the Amazon Region, *B. excelsa* is found in monocultures, integrating agroforestries and providing raw materials for food and timber industries. Through the application of an integrative analysis based on high-performance liquid chromatography coupled with diode array detector and tandem mass spectrometry (HPLC-DAD-MS/MS) and nuclear magnetic resonance (NMR) techniques, the present study showed that *B. excelsa* bark biomass residues contain large quantities of ellagic acid (EA) and its derivatives. Qualitatively, five compounds were characterized for the first time in this species. Quantitations were carried out to determine the total amount of these compounds in outer and inner bark tissues. A total of 4.96 and 44.09 g of EA derivatives *per* kg of dry residues was determined for the outer and inner barks, respectively. Among the EA derivatives, eschweilenol C, ellagic acid and valoneic acid dilactone were the main compounds. These results pointed *B. excelsa* barks as a valuable biomass residue with potential to be source of health-promoting compounds. Therefore, a potential raw material as source of valuable bioactive phenolic compounds is described herein.

Keywords: *Bertholletia excelsa*, Brazil nut, ellagic acid, eschweilenol C, timber biomass residues

Introduction

The Brazil nut (*Bertholletia excelsa*, Bonpl.) is a South American tree and is also the name of its commercially harvested edible seeds.[1,2] Especially in the Amazon region, the Brazil nut tree is a frequent component of agroforestry systems because of its adaptation to nutrient-poor upland soils and multiple uses.[3-5] The economic interest for exportation relies basically on the edible seeds that are largely applied in food and cosmetic industries, generating $ 30 million annually in Brazil.[6] This species also produces high-quality timber, whose extraction is allowed only for planted trees by the Brazilian law.[5,7]

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Every year, farms and furniture industries in the Amazonas state of Brazil generate tons of cake and woods residues. So far, only waste material from pressed nuts were proposed as addictive ingredients for the enrichment of animal food and nutritional bars due to its polyphenol content.[8] This material has been proved to be an interesting source of hydrophilic bioactive compounds such as phenolic acids and flavonoids.[8] About the timber industry, the destination of trunk barks and sawdust are simply their discharge.[7,9]

In the last years, search of inexpensive and renewable sources of valuable polyphenols has been attracting interest.[10-13] For that reason, the number of investigations concerning the extraction of these compounds from biomass wastes increased.[8] Furthermore, the application of modern analytical techniques, such as high-performance

liquid chromatography coupled with diode array detector and tandem mass spectrometry (HPLC-DAD-MS/MS) has been demonstrated to be useful to confident identification of polyphenol in complex matrices, in special hydrophilic bioactive compounds.[14-16] Although less sensitive than mass spectrometry (MS), nuclear magnetic resonance (NMR) likewise provides a powerful complementary technique for the confirmation and quantification of metabolites directly in plant extracts.[17,18] In an integrative way, these techniques enable numberless analytical approaches, whether in the prior recognition of complex matrices, or seeking pharmacologically active substances.[19,20]

Thus, the aim of the present work was the qualitative and quantitative characterization of phenolic compounds in the barks waste of *B. excelsa* from a regularized Brazil nut crop. In order to achieve this goal, an integrative strategy consisting of HPLC-DAD-MS/MS and NMR was applied. Moreover, a simplified protocol for selective extraction and quantification of phenolic compounds was proposed.

Experimental

Chemicals

Deuterated solvent dimethyl sulfoxide (DMSO-d_6) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC grade DMSO, methanol and formic acid were from Tedia (Fairfield, OH, USA). Ellagic acid (EA, analytical standard) was bought from Sigma-Aldrich (St. Louis, MO, USA). Distilled and deionized water was obtained from a Millipore Milli-Q apparatus (Bedford, MA, USA).

Biomass residue sample

A piece of log wood (ca. 10 kg) from *B. excelsa* was provided by the agroindustry Agropecuária Aruanã S. A. during September of 2016 (Itacoatiara, Amazonas State, Brazil, 58°49'48.0"W 03°00'17.0"S which constituted the largest monoculture of *B. excelsa* of the state. The sample was obtained directly from a living tree. The access to genetic heritage was registered at Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen) under the code No. A442001.

Biomass residue extraction and fractionation

The plant material was cleaned and dried at ambient temperature (ca. 20 °C) for 20 days. Then, the barks were manually separated from the wood, and split off in outer and inner barks. An aliquot of each material (ca. 15 g) was powdered using an analytical knife micro-mill (Q298A21, Quimis, Diadema, SP, Brazil). An amount of 1 g of pulverized material was extracted with distilled water (50, 100 or 150 mL) under agitation for 24 h at 20 °C. All extracts were filtered through Whatman 43 filter paper (Sigma-Aldrich, St. Louis, MO, USA) and then freeze-dried in a ModulyoD-230 apparatus (Thermo Fisher, New York, NY, USA). In order to evaluate the clean-up efficiency of the solid phase extraction (SPE), the inner bark aqueous extract prepared by the 100 mL-protocol was passed through a cartridge containing 10 g of KP-C18-HS phase (Biotage, VA, USA) previously activated with methanol HPLC (20 mL) and conditioned with water (60 mL). The column was washed with water (60 mL) and further eluted with methanol HPLC (60 mL). The methanol fraction was dried under a nitrogen gas stream, while the aqueous fraction was freeze-dried. All the experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD).

Qualitative analysis of the phenolic compounds

Aqueous extracts and SPE fractions were solubilized in methanol at 1 mg mL^{-1} and injected (2 µL) into a Phenomenex Luna C18 column (5 µm, 150 × 4.6 mm i.d.) (Torrance, CA, USA). Separations were performed by an Accela HPLC system (Thermo Scientific, Waltham, MA, USA). The mobile phase consisted of 1% formic acid aqueous solution (A) and methanol (B) at a flow rate of 1 mL min^{-1}. Elution was performed in gradient mode consisting of 20% B to 100% B over 20 min, followed by 10 min at isocratic mode with 100% B. Absorptions were registered from 240 to 400 nm. The outlet from the diode array detector (DAD) was connected through a split valve (flow rate of 0.3 mL min^{-1}) to a triple quadrupole mass spectrometer model TSQ Quantum Access (Thermo Scientific, Waltham, MA, USA), equipped with an electrospray ionization (ESI) interface and running in the negative ion mode. The ionization settings were as follows: spray voltage, 3000 V; sheath gas pressure, 35 (arb); ion sweep gas pressure, 0.0 (arb); aux gas pressure, 15 (arb); capillary temperature, 270 °C; capillary offset, −35 V; skimmer offset, 0 V; mass range, *m/z* 150 to 950; collision energy, 30 eV.

NMR analysis

The methanol fraction obtained from the SPE fractionation (30 mg) was solubilized in 600 µL of DMSO-d_6 and subjected to one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy on the AVANCE III HD spectrometer (Bruker, Karlsruhe, Germany) operating at 11.75 T, observing ^{1}H and ^{13}C at

500.13 and 125.76 MHz, respectively. Chemical shifts (δ) were presented in ppm relative to the tetramethylsilane signal at 0.00 ppm as internal reference and the coupling constants (J) were given in hertz.

Quantitative analysis

The quantification experiments proceeded by HPLC-DAD using the same chromatographic system and elution conditions of qualitative analysis. The content of EA and its derivatives was established with reference to a calibration curve built with EA at 254 nm.[21,22] A stock solution of EA (1 mg mL^{-1}) was prepared in DMSO and dilutions were done at 5 different levels for calibration curve (0.3, 5, 15, 25 and 50 μg mL^{-1}) ($y = 1 \times 10^{-5x} - 0.204$, correlation coefficient, $R^2 = 0.999$). This solvent was chosen to overcome the solubility issues previously reported to EA.[23] Outer and inner bark aqueous extracts, and methanol SPE fraction were diluted in DMSO at 1 mg mL^{-1}. All injections were performed in triplicate and the results were expressed as mean ± standard deviation (SD).

Statistical analysis

Results were expressed by means of values ± standard error of three separate determinations. Comparison of means was performed by one-way analysis of variance (ANOVA) ($p < 0.05$) followed by *post hoc* Tukey honestly significant difference (HSD) test ($p < 0.01$).

Results and Discussion

Biomass residue extraction

Since chemical differences between inner and outer barks were previously established[24] and water has been preferentially used to prepare folk medicines *B. excelsa*,[25] we choose to study the phenolic composition of aqueous extracts from the biomass waste of Brazil nut timber industry.

The extraction with water followed by lyophilization yielded a powder material. This process was tested with different amounts of distilled water for each 1 g of biomass powdered. A difference of yield (%mg of extract *per* g of dry plant material) was observed among the use of 50 and 100 mL of distilled water, increasing from 94.6 ± 2.1 (9.5%, m/m) to 103.4 ± 2.8 mg (10.3%, m/m) for the outer bark, and from 443.6 ± 4.2 (44.4%, m/m) to 473.7 ± 5.5 mg (47.3%, m/m) for the inner bark. No significant differences were observed between the use of an amount of 100 and 150 mL of water for extractions (Table 1). Furthermore, *post hoc* Tukey HSD test confirmed the raw observation of the extract yields, in which the lack of significance ($p > 0.01$) among 100 and 150 mL volumes highlighted the limit of extract recovery to a given solvent amount. These observations enabled the choice of 100 mL as solvent volume and the inner bark extract as the raw material to be investigated concerning to the clean-up efficiency by the SPE. This procedure yielded 320.7 ± 6.8 mg (32.1%, m/m) of methanol fraction and 151.4 ± 4.4 mg (15.1%, m/m) of aqueous fraction, providing a yield for total fractions of 47.2%.

Characterization of phenolic compounds

The analysis of the crude extracts by HPLC-DAD-MS/MS displayed that both parts of the timber waste, outer and inner barks, had similar chemical profiles (Figures 1a and 1b). A discrete difference regarding the relative abundance of individual compounds was noticed. The main peaks marked as compounds **1** to **6** displayed UV spectrum profiles with absorbances between 200-400 nm. Two characteristic absorption bands at 254-255 and 361-368 nm were compatible with phenolic compounds (Table 2).[21,22]

Through the MS spectra, the phenolic compounds were identified based on their fragmentation under collision-induced dissociation (CID). Compound **1** (Rt 7.0 min) displayed a deprotonated peak at *m/z* 469, and three main fragments at *m/z* 299, 300 and 301, consistent with the structure of valoneic acid dilactone

Table 1. Yields of extracts and fractions of *Bertholletia excelsa* biomass residues

Volume of solvent / mL	Inner bark[a] / (mg *per* g of dry plant material)	Outer bark[a] / (mg *per* g of dry plant material)	$SPE_{meth.}$[b,c] / (mg *per* g of dry plant material)	$SPE_{aqu.}$[c,d] / (mg *per* g of dry plant material)
50	443.6 ± 4.2[e,f]	94.6 ± 2.1[e,f]	–	–
100	473.7 ± 5.5	103.4 ± 2.8	320.7 ± 6.8[e]	151.4 ± 4.4[e]
150	475.4 ± 7.2	104.8 ± 5.3	–	–

[a]Aqueous extract; [b]SPE: solid phase extraction, methanol fraction; [c]extraction performed with inner bark; [d]SPE: solid phase extraction, aqueous fraction; [e]mean ± standard deviations (SD); [f]distribution analysis by one-way analysis of variance (ANOVA) ($p < 0.05$) and significance analysis by *post hoc* Tukey HSD test ($p < 0.01$).

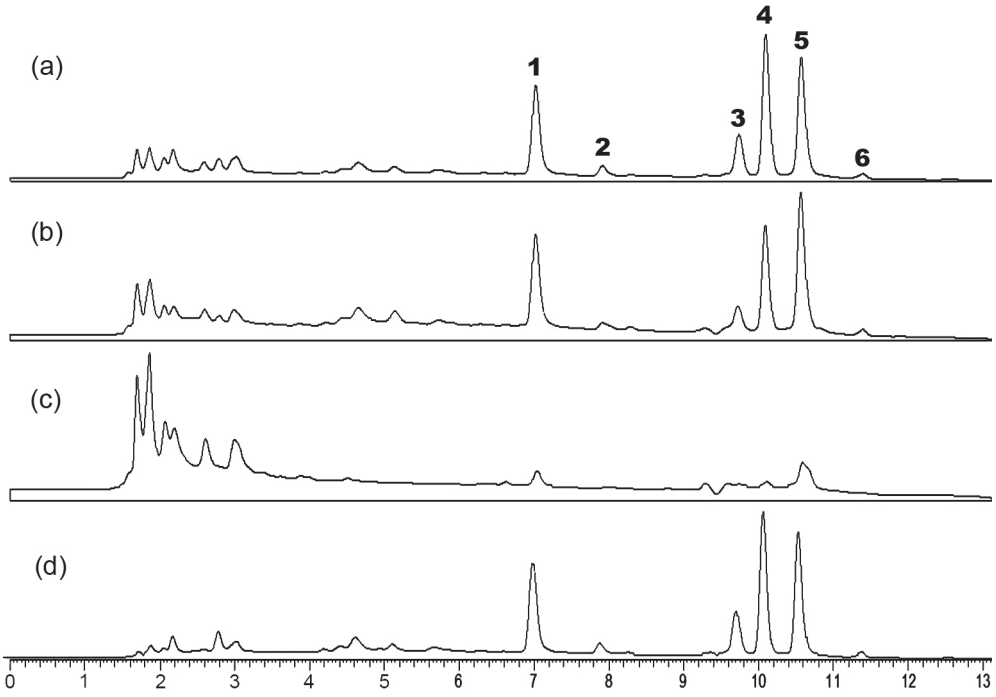

Figure 1. HPLC chromatograms of the inner (a) and outer bark (b) aqueous extracts; and aqueous (c) and methanol SPE fraction (d) from inner bark at 254 nm. The peaks labeled 1 to 6 correspond to the compounds listed in Table 2.

(Figure 2).[26] Compounds **2** (Rt 7.9 min), **3** (Rt 9.7 min) and **4** (Rt 10.1 min) displayed the deprotonated *m/z* at 463, 433 and 447, respectively. After fragmentation, all compounds displayed a single fragment at *m/z* 301, indicative of EA glycosides. Comparison with compounds previously isolated in Brazil nut pointed to ellagic acid hexoside (**2**), ellagic acid pentoside (**3**) and ellagic acid desoxyhexoside (**4**).[21,22] Compound **5** (Rt 10.6 min) was identified as EA by means of its deprotonated mass (*m/z* 301) and fragments (*m/z* 185 and 229) in comparison with an original standard (Figure 3). This reinforced our structural proposals about the phenolic content in wasted barks of Brazil nut timber. The presence of EA (**5**) in the bark of *B. excelsa* was early cogitated,[27] however, its confirmation through analytical instrumentation was only performed in the nuts.[28] According to this previous report, a similar compound related to **4** (*m/z* 447 → 301) was detected in Brazil nut brown skin, but its content was not determined. In addition, compound **6** (Rt 11.4 min) was also an EA derivative. Its deprotonated ion at *m/z* 461 displayed as main fragments the *m/z* 300 and 315. Thus, this phenolic was identified as methyl ellagic acid deoxyhexoside (**6**).[21,22]

To obtain more structural informations regarding to the main compound **4**, the SPE methanol EA derivatives-enriched fraction (Figure 1d) was directly subject to one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy. Several diagnostic signals were observed in the ^{1}H NMR spectrum, among them, aromatic protons at δ_H 7.74-7.46, an anomeric hydrogen at δ_H 5.46 (1H, d, 1.5 Hz) and a methyl group at δ_H 1.14 (3H, d, 6.4 Hz) might be highlighted. The heteronuclear multiple bond correlation (HMBC) experiment evidenced correlations

Table 2. Ellagic acid and derivatives identified in *Bertholletia excelsa* biomass residues using HPLC-DAD-MS/MS analyses

Peak	Compound	Rt / min	$[M - H]^-$	MS fragments[a]	λ_{max} / nm
1	valoneic acid dilactone	7.0	469	300, 301	256, 365
2	ellagic acid hexoside	7.9	463	301	255, 361
3	ellagic acid pentoside	9.7	433	301	255, 361
4	eschweilenol c	10.1	447	301	255, 361
5	ellagic acid	10.6	301	185, 229	254, 368
6	methyl ellagic acid deoxyhexoside	11.4	461	300, 315	257, 365

[a]Main observed fragments.

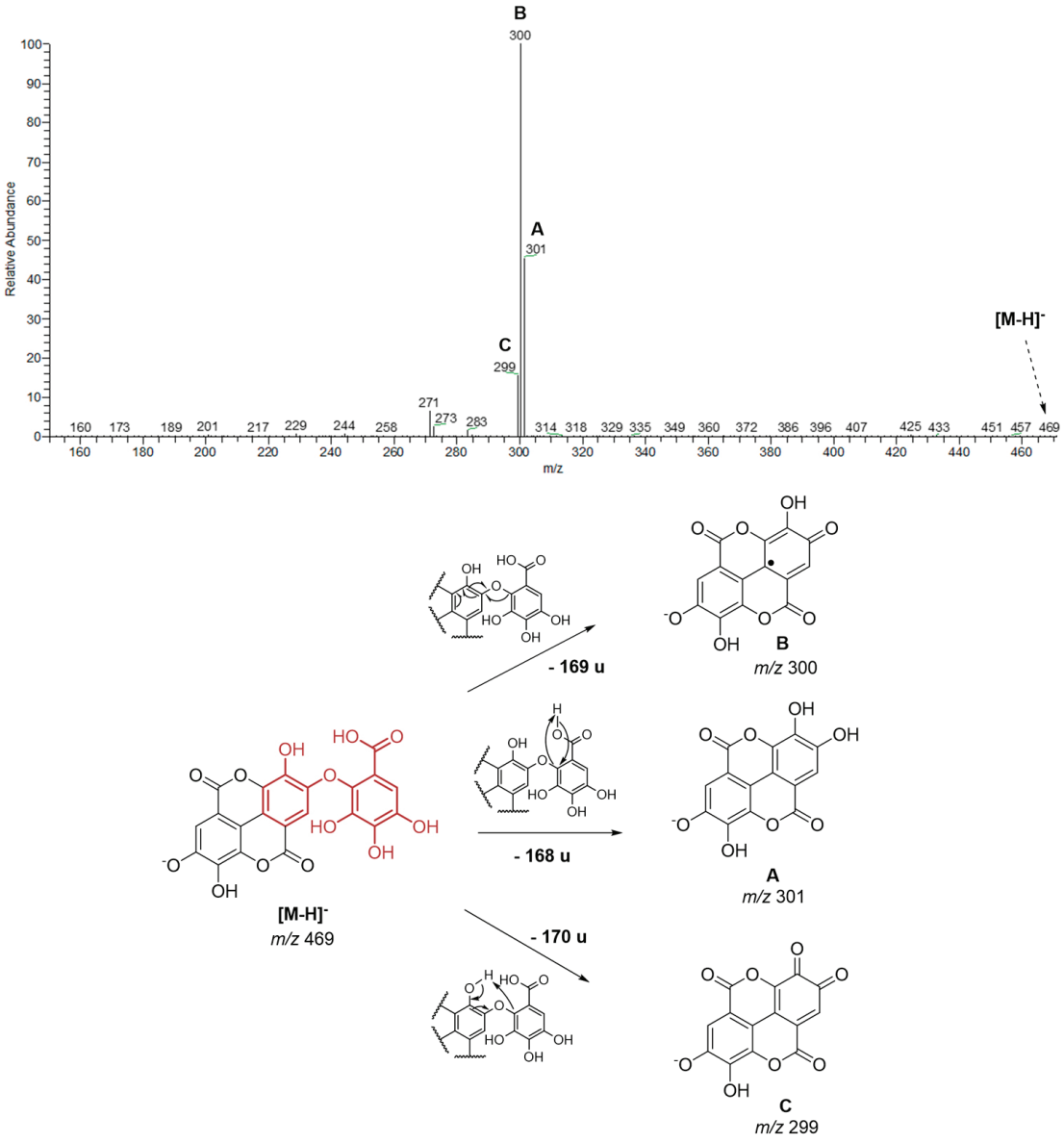

Figure 2. Product ion spectrum of the deprotonated molecule at *m/z* 469 arising from peak 1 and fragmentation proposals for the product ions *m/z* 301 (−168 u), 300 (−169 u) and 299 (−170 u).

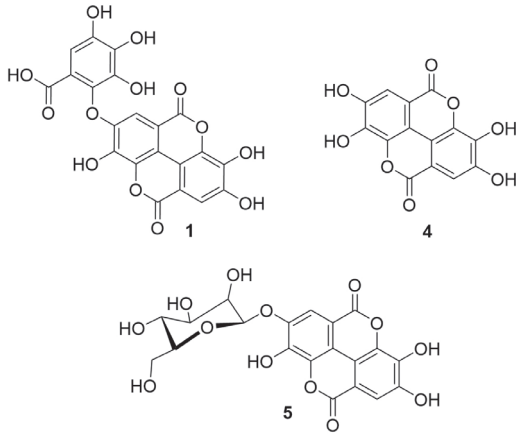

Figure 3. Chemical structures of the phenolic compounds identified in *Bertholletia excelsa* residues.

for deshielded proton at δ_H 7.74 with carbons at δ_C 107.3, 114.5, 141.3, 146.2 and 158.9 (Figure 4a), consistent for an EA unit.[29] Additionally, the anomeric hydrogen displayed HMBC correlations with the carbon atoms at δ_C 69.9 and 146.2 (Figure 4b), which is characteristic of a rhamnose moiety. This structural evidence was confirmed upon the correlations between the methyl group and the carbon at δ_C 69.9 (Figure 4c).

Through the observation of a nuclear Overhauser effect (NOE) between H-5 (δ_H 7.74) and H-1" (δ_H 5.46) in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum (Figure 4d), the rhamnose moiety was confirmed to be linked to C-4. The anomeric configuration was attributed as α through the characteristic chemical shift and

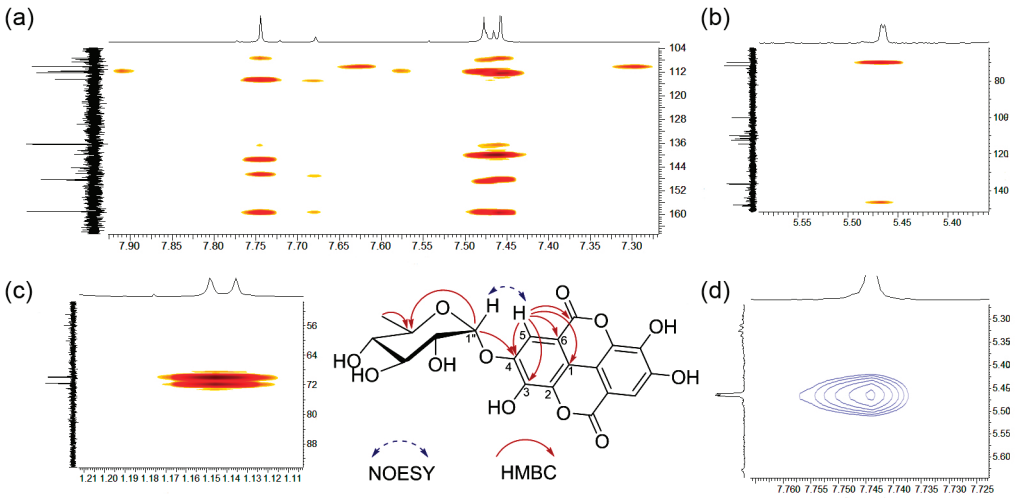

Figure 4. Main correlations observed through HMBC (a, b and c) and NOESY (d) experiments for methanol fraction (DMSO-d_6) and eschweilenol C tentatively identified.

small coupling constant (*J* 1.5 Hz) expected for α anomeric hydrogens.[30] Therefore, compound **4** was identified as 4-(α-rhamnopyranosyl) ellagic acid (eschweilenol C). This compound has been previously reported in the bark of *Eschweilera coriacea* (Lecythidaceae).[31]

According to previous reports, EA derivatives have important influences on human nutrition and possess several biological properties, such as antioxidant, anti-inflammatory, anticancer and antibacterial activities.[21-23,32] As important natural polyphenols, EA compounds are widely contained in various mushy fruits. Due to their strong antioxidant activities, some compounds have been used to scavenge cancer-causing toxins from the human body and improve immunity.[32]

Quantification of phenolic compounds

The total amounts of EA and its derivatives in outer and inner bark residues found by HPLC analyses tissues were 4.96 and 44.09 g of EA derivatives *per* kg of dry residues, respectively (Table 3). Individually, the main compounds in the outer and inner bark wastes were eschweilenol C (1.36 ± 0.09 and 14.97 ± 0.57 g kg^{-1}), ellagic acid (1.85 ± 0.19 and 13.25 ± 0.62 g kg^{-1}), valoneic acid dilactone (1.27 ± 0.11 and 10.10 ± 0.48 g kg^{-1}) and ellagic acid pentoside (0.26 ± 0.02 and 4.45 ± 0.37 g kg^{-1}). Alternatively, the SPE methanol fraction for the inner bark presented an amount of 43.56 g of EA derivatives *per* kg of dry residues, providing to be a simple protocol for selective extraction of EA derivatives. This high concentration of phenolic compounds was close to that reported to Longan seeds (*Dimocarpus longan*).[33] Nowadays, the extracts or products of EA have been put into use in food industry, disease prevention and treatment, and cosmetic production in several countries.[32,33] Therefore, the barks waste from the timber obtaining of Brazil nut are an abundant source of EA derivatives.

Conclusions

The integrative approach proposed to directly analyze the crude extracts of *Bertholletia excelsa* bark residues from

Table 3. Concentration of ellagic acid and derivatives in *Bertholletia excelsa* biomass residues according to HPLC-UV at 254 nm

Peak	Compound	Inner bark[a] / (g *per* kg of dry plant material)	Outer bark[a] / (g *per* kg of dry plant material)	$SPE_{meth.}$[b,c] / (g *per* kg of dry plant material)
1	valoneic acid dilactone	10.10 ± 0.48[d]	1.27 ± 0.11[d]	10.15 ± 0.34[d]
2	ellagic acid hexoside	0.83 ± 0.07	0.12 ± 0.01	0.81 ± 0.02
3	ellagic acid pentoside	4.45 ± 0.37	0.26 ± 0.02	4.38 ± 0.32
4	eschweilenol c	14.97 ± 0.57	1.36 ± 0.09	14.86 ± 0.25
5	ellagic acid	13.25 ± 0.62	1.85 ± 0.19	12.92 ± 0.74
6	methyl ellagic acid deoxyhexoside	0.49 ± 0.03	0.10 ± 0.01	0.44 ± 0.04

[a]Aqueous extract prepared by the 100 mL-protocol; [b]SPE: solid phase extraction, methanol fraction; [c]extraction performed with inner bark; [d]mean ± standard deviations (SD).

the Amazon timber industry showed to be a useful strategy for characterization and quantification of ellagic acid (EA) and its derivatives. Besides, the SPE protocol proved to be a simple way to selectively extract of phenolic compounds in this matrix. The results of this study highlighted for the first time the *B. excelsa* bark residues as a promising source of health-promoting compounds, therefore a potential raw material for food, pharmaceutical and chemical industries. The findings of this work may guide future uses of rich biomass that actually is simply dumped into the environment.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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