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Article

Studies on Polyphenols and Lignin of *Astronium urundeuva* Wood

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Uma amostra da madeira de aroeira preta (*Astronium urundeuva*) moída em moinho de bolas foi extraída, sucessivamente, com benzeno, benzeno:etanol (2:1, vv^{-1}), clorofórmio e água num soxhlet. A madeira extraída foi secada ao ar livre resultando o resíduo **A** que, em seguida, foi extraído com acetona:água (9:1, vv^{-1}), também num soxhlet. Do extrato resultante foi obtida a lignina de madeira moída (MWL). Não considerando a MWL, a soma de todos os extratos alcançou 18,19% da madeira seca. O conteúdo em lignina de Klason de **A** e o conteúdo de grupos metoxila da MWL foram 23,84% e 19,00%, respectivamente. Outra amostra de **A** apresentou conteúdos em polissacarídeos de difícil e fácil hidrólise de 34,86% e 18,81%, respectivamente. Uma Segunda amostra de madeira moída foi submetida a uma extração única com metanol, à temperatura ambiente, fornecendo o extrato metanólico **B** (23,85% da madeira seca). Espectros de RMN do ^{13}C nas fases líquida e sólida indicaram que **B** está constituído fundamentalmente por proantocianidinas dos tipos profisetinidina e prorobinetinidina.

An *Astronium urundeuva* ball-milled wood sample was successively extracted with benzene, benzene-ethanol (2:1, v/v^{-1}), chloroform, and water in a soxhlet apparatus. The extracted wood was air-dried giving the residue **A** that was then treated with ketone-water (9:1), in a soxhlet apparatus. From the ketone-water extract was precipitated the milled wood lignin (MWL). With exception of MWL, the extract contents summed up 18.19% (o.d.w.). The Klason lignin content of **A** and the methoxyl content of the MWL were 23.84% and 19.00%, respectively. Another sample of **A** gave hardly and easily hydrolysable polysaccharide contents of 34.86% and 18.81%, respectively. A second sample of ball-milled wood was submitted to a single extraction with methanol, at room temperature, affording the methanol extract **B** (23.85% o.d.w.). Liquid and solid state ^{13}C-NMR spectroscopies indicated that **B** is mainly constituted by proanthocyanidins of profisetinidin and prorobinetidin types.

Keywords: *lignin, polyphenols,* Astronium urundeuva, *cerrado vegetation*

Introduction

Astronium urundeuva wood is well known due to its very good mechanical, biological and chemical resistance and therefore it is widely used in Brazil[1]. In spite of this, we could not find any systematic study on the chemical composition of this wood in the literature. In order to investigate whether there is a relationship between resistance and chemical constitution, we have initiated studies to characterize the polyphenols and the lignin structure of this wood.

To obtain a lignin mostly free of polyphenol contamination, we employed the Björkmann procedure adapted for *Eucalyptus grandis* wood cultivated in the central region of Brazil. This wood also contains a large amount of polyphenols[2].

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Polyphenol studies were carried out using classical chemical techniques[3-7] associated with modern high performance liquid chromatography (HPLC)[8-13] and high resolution liquid and solid state ^{13}C-NMR[11,14,15] and FTIR spectroscopies.

This work presents the results of our studies on the *Astronium urundeuva* wood composition.

Materials and Methods

Astronium urundeuva *wood*

A stem of a thirty year old tree from Uberlândia (Triângulo Mineiro) was disk cut and air dried for 12 months.

Extracts

A ball-milled wood sample (100 g) was sequentially extracted in a soxhlet apparatus with benzene, benzene: ethanol (2:1), chloroform, and water. The isolated extracts were dried in an oven at 70 °C for 12 h and the residual air-dried wood powder (**A**) was used for MWL preparation. Another sample of ball-milled wood (50 g) was stirred with 300 mL of methanol in a beaker at room temperature for 24 h and, then, the mixture was filtered and the methanol evaporated under reduced pressure, at 40 °C, yielding the extract **B**.

A third sample of ball-milled wood (50 g) was submitted to an extraction with methanol: water (80:20) in a beaker at room temperature with stirring for 24 h. Then the mixture was filtered and the methanol was evaporated under reduced pressure, at 40 °C. The remaining aqueous extract (**C**) was divided in four fractions and freeze-dried for its conservation. One fraction of **C** was used for total phenols analysis and another for proanthocyanidins determination; a third was extracted with ethyl ether, and the fourth fraction was used in the HPLC analysis as well as the ethereal extract.

High performance liquid chromatography

HPLC analysis was carried out in a chromatograph equipped with diode array detector and Hypersil ODS (200 x 4 mm i. d.) column protected by a precolumn of the same material. Two solvent mixtures were employed for elution: X: MeOH:H_3PO_4 (999:1) and Y: H_2O:H_3PO_4 (999:1) and the gradient profile was: 0 min - 40 min, 20% - 100% X; 40 min - 45 min, 100% X. The flow rate was 1 mL/min and column oven temperature 30 °C. Detection was carried out at 270 ± 15 nm, 325 ± 75 nm and 365 ± 2 nm. The compound identification was based on a private library of UV spectra of phenolics and flavanoids and on retention times.

Total phenol contents

Total phenol content was determined by the Folin-Ciocalteu method[2]. 2,5 mL of Folin-Ciocalteu reagent (Fluka, diluted 10 times by water) and 2 mL of aqueous solution of sodium carbonate (75 g L^{-1}) were added to 0.5 mL of the aqueous extract (**C**), and the mixture was kept 5 min at 50 °C. After cooling, absorbance was measured at 760 nm. Aqueous solution of gallic acid (2 μg – 40 μg) was used as standard. The molar absorptivity obtained in our conditions was 22.3 x 10^3.

Proanthocyanidin contents

Proanthocyanidin determination was carried out by the vanillin method[4]. 1 mL of the aqueous extract was mixed with 2 mL of a freshly prepared vanillin solution (1 g/ 100 mL of 70% H_2SO_4) and maintained 15 min at 20 °C. The absorption was measured at 500 nm. Calibration was performed with catechin aqueous solutions (2 μg - 40 μg mL^{-1}) and a molar absorbance of 34.3 x 10^3 was attained in our conditions.

Anthocyanidin contents[3-4]

To a reaction vial were added 6 mL of concentrated HCl:n-BuOH (2:3, v v^{-1}), 1 mL of aqueous extract and 0.9 mg of $FeSO_4.7H_2O$. The vial was closed and left in the oven for 40 min at 90 °C. After cooling, 0.1 mL of reaction mixture was dissolved in 1 mL of MeOH:H_2O (1:1) and injected in the HPLC chromatograph.

Milled wood lignin

MWL was prepared from **A** according to Björkmann technique[16] adapted by us to *Eucalyptus grandis* wood: the dried wood powder (100 g) was extracted in a soxhlet apparatus with ketone-water (9:1) and the organic solvent was evaporated under reduced pressure at 70 °C. After that, the aqueous mixture was acidified with diluted HCl until pH 2 and the precipitated lignin was filtered and washed with a small amount of water. The lignin was dried at 70 °C for 12 h.

Easily and hardly hydrolysable polysaccharides

The polysaccharides were determined according to the Kiesel-Semiganovski method[17]: 4.0 g of **A** were refluxed with 2% HCl for 3 h and filtered. The residue obtained was washed and dried at 105 °C. The weight difference corresponds to the easily hydrolysable polysaccharides (EHP). Then, the residue was treated with 80% H_2SO_4 for 2 h at room temperature and, thereafter, it was diluted up to 1 L and refluxed for 3 h. The mixture was filtered and the residue was washed and dried at 105 °C. The weight loss was due to the hardly hydrolysable polysaccharides (HHP).

Holocellulose

Holocellulose content was determined applying Browning procedure[18] to non-extracted ball-milled wood. The obtained holocellulose still showed a pale brown color.

NMR measurements

Solid state ^{13}C-NMR spectra were recorded on a BRUKER DRX spectrometer operating at a frequency of 100.6 MHz using the pulse sequence VACP, pulse delay 0.5 s, acquisition time 25 ms, rotor speed 4000 rpm, and 1520 scans were collected. Liquid ^{13}C-NMR spectra were recorded at the same frequency using DMSO-d_6 as solvent and pulse sequence ZGPG30, power gated and angle of 30° at the principal pulse; pulse delay was 1.0 s and 10240 scans were collected.

FTIR spectra

They were recorded on a PERKIN-ELMER SPECTRUM 1000 spectrometer in KBr pellets.

Results and Discussion

Table 1 presents the composition of *Astronium urundeuva* wood. The total of substances extracted by benzene, benzene: ethanol (2:1, v v^{-1}), water and chloroform was very large 18.19%. However, when only methanol was used as extraction solvent, at room temperature, the extract yield was still greater: 23.85%. The sum of extracts, Klason lignin, EHP, plus HHP was 95.70%. Holocellulose plus Klason lignin and extracts reached, however, 105.03%. These discrepancies are considered normal[18] and reflect the difficulties in analyzing woody materials.

In spite of *Astronium urundeuva* wood being very hard, its content of structural polymer cellulose is low as can be verified from the low content of hardly hydrolysable polysaccharides. However, the relatively high content of lignin and extractives is not sufficient to impart resistance to wood species against chemical and biological attacks. For instance, *Eucalyptus grandis* wood cultivated in the same region also contains approximately 40% of lignin plus extracts and, nevertheless, it is well known that this wood easily degrades under a tropical climate.

Table 1. Composition of *Astronium urundeuva* wood.

Extracts and other wood components	Percentage of dried wood
Benzene	1.09
Ethanol-benzene	13.68
Chloroform	0.29
Aqueous	3.13
Methanol[#]	23.85
Klason lignin from extracted wood *	23.84
Hardly hydrol. carboh. (HHC) *	34.86
Easily hydrol. carboh. (EHC) *	18.81
Holocellulose	63.00

* Corrected for non-extracted wood; [#] single extraction.

The structure of lignin or more probably the composition of phenolics could account for the great resistance of *Astronium urundeuva*.

Figure 1 presents the IR spectrum of the MWL in KBr pellet. The band profile in the finger print region is typical of lignins[19-20].

Figure 2 shows the IR spectrum of the methanolic extract **B**. The band distribution is characteristic of tannins[21]. In addition, the great intensity of the band at 1513 cm^{-1} suggests that **B** is contaminated by lignin, as was the case for tannins from *Eucalyptus* wood[22].

Due to great insolubility of the *Astronium urundeuva* MWL in the common NMR solvents and to the low yield of the MWL acetylation, solid state ^{13}C-NMR spectroscopy was employed. Figure 3 presents the CP/MAS ^{13}C-NMR spectrum of the MWL. As can be seen, the characteristic signals of guaiacyl-siringyl (G-S) lignins are present[18,23-24]. However, in spite of the exhaustive extraction using different solvents, MWL still contains contaminants, as can be suggested by the intense signal at δ 143.16 and by the

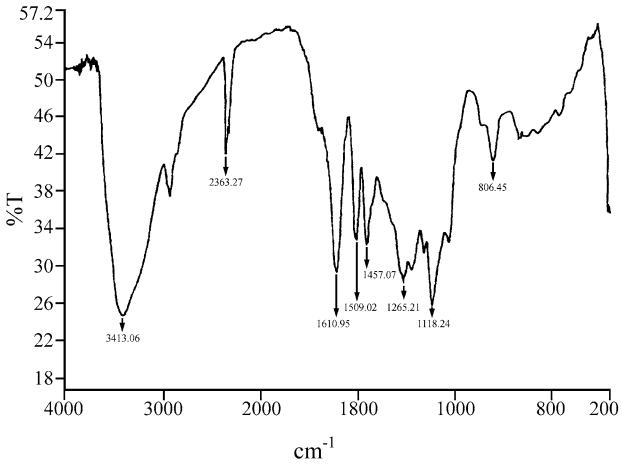


Figure 1. FTIR spectrum of the *Astronium urundeuva* MWL in KBr pellet.

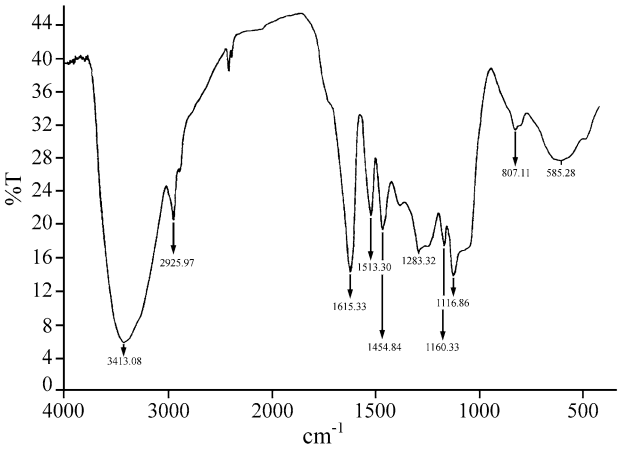


Figure 2. FTIR spectrum of the methanol extracts (**B**) in KBr pellet.

relatively large signals in the range δ 170-182 and at δ 191.99. The signal at δ 181.81 suggests the presence of COOH (from cinnamic acid) in the lignin structure.

Figure 4 presents the CP/MAS ^{13}C-NMR spectrum of the methanolic extract (**B**). With the exception of the signal at δ 56.47 (due to -OCH_3), the chemical shifts of the signals in the range δ 30-160 are typical of proanthocyanidins[4,11]. In addition, Fig. 4 contains a signal at δ 175.97 due to C=OOR and C=OOH and another two at δ 189.40 and δ 199.88 that may be due to C=O from flavanons, dihydroflavanols, ketones and aldehydes[2,25].

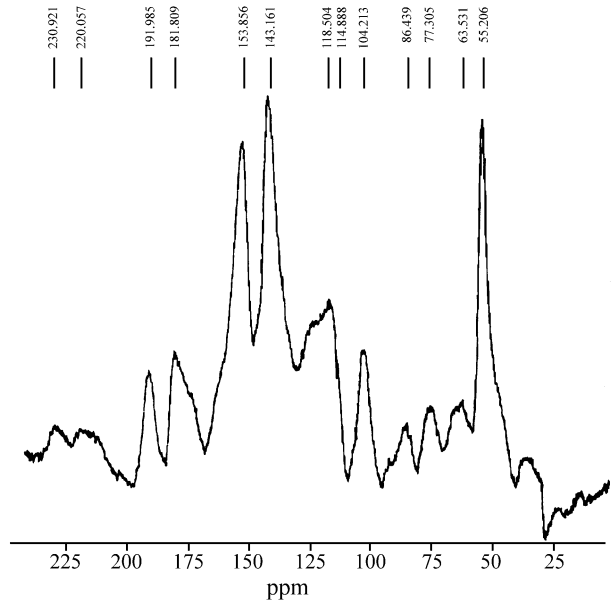


Figure 3. CP/MAS ^{13}C-NMR spectrum of the *Astronium urundeuva* MWL.

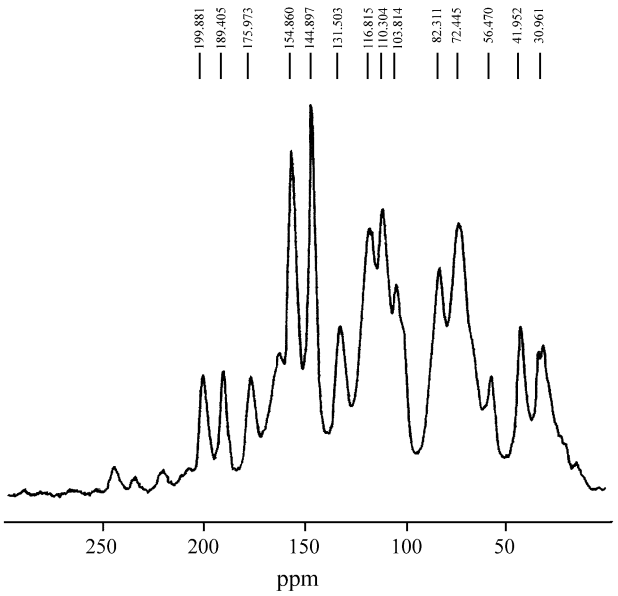


Figure 4. CP/MAS ^{13}C-NMR spectrum of the methanolic extracts (**B**).

By comparing the spectra of Figs. 3 and 4, we can conclude that the contamination of lignin arises from tannins. On the other hand, the presence of the signal at δ 56.47 in Figure 4 suggests that the tannins contain some lignin contaminant.

As lignin is slightly soluble in DMSO, we dissolved the methanolic extract in $(CD_3)_2CO$, filtered the mixture and recorded the ^{13}C-NMR spectrum of the resulting solution (Fig. 5). This spectrum is then more representative of **B**.

Considering that condensed tannins (proanthocyanidins) are polymers constituted by the flavan-3-ol monomeric units shown in Fig. 6, the most outstanding characteristic of Fig. 5 is the chemical shift assigned to carbon-8 of the A ring at δ 102.82[4]. This chemical shift indicates that the hydroxylation pattern in the A ring is of resorcinol type, *i.e.*, only carbon-7 is hydroxylated. This finding is very important because it eliminates other monomeric units with the exception of fisetinidol and robinetinidol (Fig. 6). In order to characterize whether fisetinidol or robinetinidol or both monomeric units are present in the extract **B**, the reported characteristic ^{13}C-NMR chemical shifts for B-ring oxidation patterns[4,15] were used to further analyze Fig. 5. Table 2 presents the reported ^{13}C-NMR chemical shifts and those obtained from Fig. 5. The found and reported data are in good agreement and so Table 2 suggests that fisetidinol and robinetinidol polymers may be the main components of the studied condensed tannins.

Figure 7 presents the HPLC chromatogram of the ethereal extract obtained from the aqueous extract. Fisetin is the main component; gallic and ellagic acids were also detected; minor concentrations of compounds showing UV

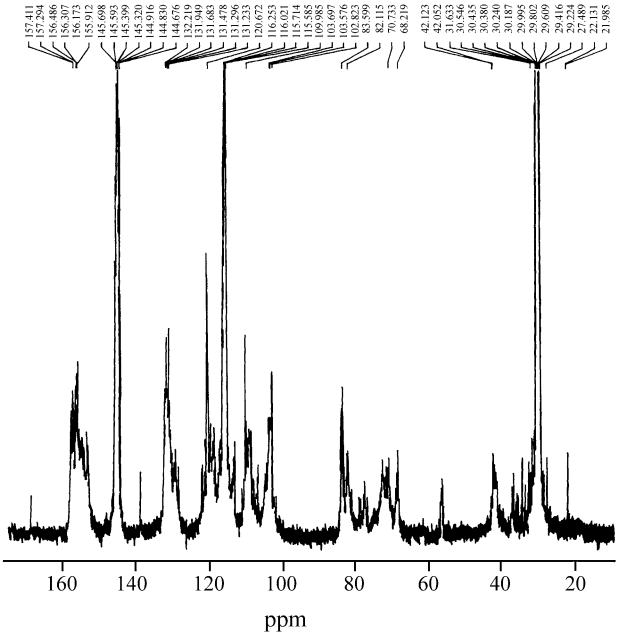
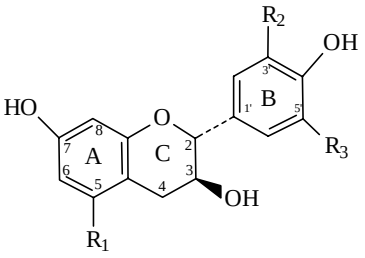


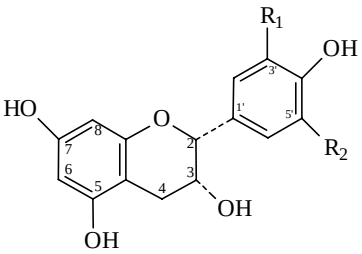
Figure 5. ^{13}C-NMR spectrum of the methanol extract (**B**) in $(CD_3)_2CO$.

Table 2. ^{13}C-NMR chemical shifts for B-ring hydroxylation patterns.

	Carbon atom											
B-ring	1'		2'		3'		4'		5'		6'	
3',4'-dihy-droxy	131.5[b]	(132)	115.7[b]	(115)	144.8[b]	(145)	144.8[b]	(145)	116.0[b]	(116)	120.7	(120)
3',4',5'-tri-hydroxy	131.5[b]	(132)	108.0[b]	(108)	145.5[b]	(146)	132.2	(133)	145.6[b]	(146)	108.0[b]	(108)n

[a] In $(CD_3)_2CO$; reported ^{13}C-NMR chemical shifts[4,15] are given in parentheses.
[b] Average of various values obtained from Fig. 5.





$R_1 = R_2 = OH$, $R_3 = H$; Catechin
$R_1 = R_2 = R_3 = OH$; Gallocatechin
$R_1 = OH$, $R_2 = R_3 = H$; Afzelechin
$R_1 = R_3 = H$, $R_2 = OH$; Fisetinidol
$R_1 = H$, $R_2 = R_3 = OH$; Robinetinidol

$R_1 = OH$, $R_2 = H$; Epicatechin
$R_1 = R_2 = H$; Epiafzelechin
$R_1 = R_2 = OH$; Epigallocatechin

Figure 6. Proanthocyanidin precursores.

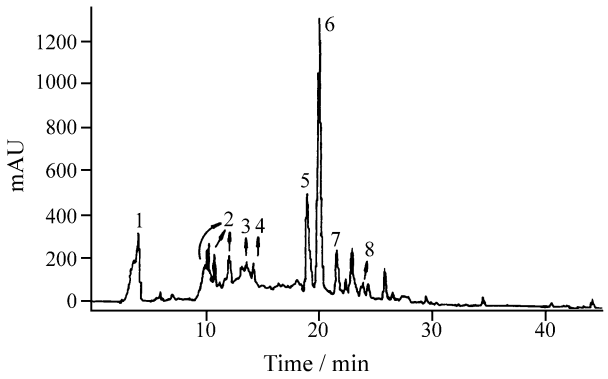


Figure 7. HPLC chromatogram of the ethereal extract: gallic acid (1), flavanons (2), hydroxyacid (3), ellagic acid (4), fisetin (5), ellagitannins (6), and flavanol (7).

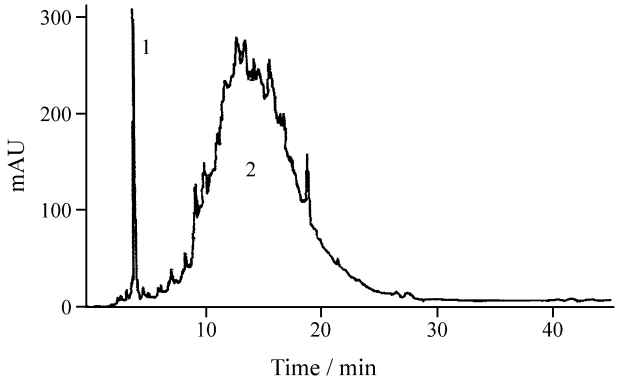


Figure 8. HPLC chromatogram of the aqueous extract: gallic acid (1), and proanthocyanidins (2).

spectral characteristics of flavanons, flavanols and ellagitannins were observed. The presence of fisetin constitutes further evidence of the occurrence of profisetinidins in *Astronium urundeuva* wood.

Figure 8 presents the HPLC chromatogram of the aqueous extract after ether extraction. Gallic acid and a broad band composed of many proanthocyanidins could be detected.

In order to detect other monomeric compounds that constitute the polymeric structure of the proanthocyanidins, we then tried cleaving the polymer bonds with HCl/n-butanol. In spite of strong conditions, the proanthocyanidins of the aqueous extract were not degraded, as can be shown by the HPLC chromatogram of the reaction extract (Fig. 9). Only gallic acid, n-butylgallate (resulting from gallic acid and n-butanol) and proanthocyanidins were detected.

At this point, it is interesting to compare the elemental analysis of the methanolic extract and lignin: C: 56.84%; H: 5.25%; N: 0.66%; O: 37.25% and C: 59.50%; H: 4.38%; N: 0.77%; O: 35.35%, respectively. The high oxygen content in the methanol extract may indicate that this material suffered oxidation during the long exposure to air in the drying process.

Using the methoxyl content found for lignin (19.0%), the calculated C_9-unit formula is $C_9H_{5.21}O_{3.31}(OCH_3)_{1.25}$ which also indicates a relatively high oxygen content[26] probably due to contamination by tannins.

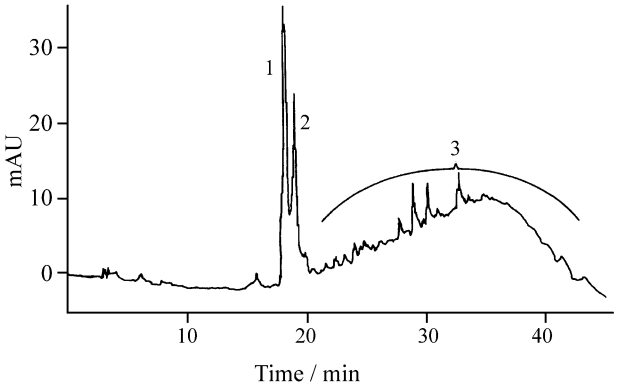


Figure 9. HPLC chromatogram of the mixture resulting from the reaction of aqueous extract with HCl/n-butanol: ellagic acid (1), n-butylgallate (2), and proanthocyanidins (3).

Finally, the measured phenolic OH-content in MWL (3.2%) is too high for lignins and this result is also in good agreement with the supposed contamination by the oxidized tannins.

Regarding the classical chemical assays, the aqueous extract presented 100.7 mg of gallic acid equivalents and 12.9 mg of catechin equivalents for 1 g of extracted milled wood (o.d.w.). These results are much higher than those reported in the literature for *Eucalyptus* species[2,27] and they confirm once more that *Astronium urundeuva* wood contains a large amount of tannins.

Conclusions

Astronium urundeuva wood contains a large amount of extracts, which are constituted mainly by proanthocyanidins, probably of profisetinidin and prorobinetinidin types.

The milled wood lignin could not be isolated in pure form even when extracting the milled wood sequentially with benzene, benzene:ethanol, chloroform, and water before the final extraction with ketone:water (9:1). Some contamination by tannins still remains. This suggests a strong association between lignin and tannins.

The association of lignin and tannins and/or the presence of profisetinidins and prorobinetinidins could account for the great resistance of *Astronium urundeuva* wood regarding chemical and biological degradation.

Acknowledgments

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