

Tannin Extraction and Characterization of Polar Extracts from the Barks of Two *Eucalyptus urophylla* Hybrids

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Barks from two commercial clones of *Eucalyptus urophylla* hybrids used by the charcoal industry in Brazil were analyzed, with their polar extracts characterized. Also, tannins were extracted with water and sodium sulfite. The mean bark chemical composition showed 2.6% ash, 16% extractives (89% of which were polar extractives), 1.2% suberin, 18.9% lignin, and 61.4% polysaccharides composed mainly of glucans (glucose 48.3% of bark) and xylans (xylose plus arabinose 10.1% of bark). The polar extracts included high mean contents of total phenolics (380.9 mg GAE / g extract), flavonoids (178.4 mg CE / g extract), and tannins (67.7 mg CE / g extract). The mean antioxidant activity of the extracts was low (53.6 mg Trolox / g of bark extract). The extracts had Stiasny indices of 20% with water and 45% with sodium sulfite solution. The FTIR spectra showed condensed tannin bands characteristic of C=C, C-C, and O-H bonds. The barks of *E. urophylla* hybrids are a potential source of polar extractives that may represent an important valorization.

Keywords: *Eucalyptus urophylla* hybrids; Bark; Tannin; Chemical composition

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INTRODUCTION

Plantations of fast-growing tree species have increased due to both industrial and community demands. In this context, Brazil is an important example, with extensive *Eucalyptus* plantations to supply the pulp and charcoal industries, totaling 5.7 million hectares in 2016 (IBÁ 2017). The species *Eucalyptus urophylla* Blake has been widely used, mainly as hybrids with *E. grandis* Hill (namely, the well-known *E. urograndis* hybrid, used for pulping) and *E. camaldulensis* Dehnh. The plantations are usually clonal, and a continual breeding effort is maintained to develop improved clones.

Large quantities of forest biomass residues are generated during collection and processing; for instance, 10 to 12% of the volume corresponds to bark that is burned for power generation for the industry itself, though a large underused surplus remains (Foelkel 2010). Barks, being widely available and chemically interesting, have been considered as biorefinery resources (Miranda *et al.* 2013; Sartori *et al.* 2016). Bark extractives, namely, their phenolic components, have been studied for some eucalypt species, including *E. camaldulensis* (Conde *et al.* 1996; Cadahía *et al.* 1997), *E. sideroxylon* (Miranda *et al.* 2016), *E. globulus* (Conde *et al.* 1995; Vázquez *et al.* 2008; Mota *et al.* 2012; Santos *et al.* 2012a, 2012b), and other eucalyptus species (Luís *et al.* 2014; Lima *et al.* 2017).

The soluble phenolic compounds that result from the secondary metabolism of plants and are enhanced by stress conditions act as protection against infection, injury, and ultraviolet radiation; therefore, they are found in higher concentrations in the bark (Naczka and Shahidi 2004). Among bark phenolics, the tannins have been scrutinized due to the diversity of their potential applications. Tannins have been used since antiquity in leather tanning due to their ability to form complexes with proteins, in treating drinking water because they act as coagulants and flocculants, in pharmaceuticals and cosmetics due to their antioxidant properties, and in the beverage industry to give an astringent taste (Pizzi 1994). Tannins may also be used as natural adhesives due to their ability to react with formaldehyde, and many studies have been developed to partially or totally replace conventional commercial adhesives, such as wood adhesives (Carneiro *et al.* 2010). Extensive research has been done in the use of bark tannins from different species as substitutes for phenol in wood adhesive formulations (Feng *et al.* 2013), *e.g.*, from *Acacia mangium* (Hoong *et al.* 2011); *P. caribaea*, *P. elliottii*, *P. pinaster*, and *P. sylvestris* (Yazaki and Collins 1994); tropical hardwoods *Alstonia boonei*, *Funtumia elastica*, and *Tetrapleura tetraptera* (Antwi-Boasiako and Animapauh 2012); spruce (Roffael *et al.* 2000); and *Acacia mearnsii* (Yazaki *et al.* 2009).

This study reports the extraction of tannins from the barks of two commercial clones of *E. urophylla* hybrids used by the charcoal industry in Brazil and their characterization by Fourier transform infrared (FTIR) spectroscopy, which has been applied to tannins of various origins, including barks of *P. pinaster* (Chupin *et al.* 2013) and *P. halepensis* (Saad *et al.* 2014), grape pomace residues (Ping *et al.* 2012), the fruit of *Bixa orellana* (Lima *et al.* 2006), wine tannins (Fernandez and Agostin 2007), and proanthocyanidins (Foo 1981). The polar extractives of the two *E. urophylla* hybrids were also characterized by total contents of phenolics, tannins, and flavonoids, and their antioxidant capacities were analyzed. Further, the overall chemical composition of these eucalypt barks was also analyzed, namely their content in structural components. It is the aim to contribute to a full valorization of the potentially available barks of *E. urophylla* hybrids as industrial residual streams by using them as a biorefinery biomass source thereby providing a complete use of the resource.

EXPERIMENTAL

Material and Sample Preparation

Barks from two commercial clones of *E. urophylla* were studied: a hybrid of *E. urophylla* × *E. camaldulensis* (here named clone 1) and an undisclosed hybrid of *E. urophylla* (here named clone 2). The barks were obtained from a commercial plantation belonging to a charcoal company associated with the Brazilian Association of Forest Plantation Producers (ABRAF). The trees were harvested at 7 years of age at average breast height diameters of 17.5 cm (clone 1) and 17.3 cm (clone 2). The bark samples were taken at several locations from the bark residue pile established in the field for each clone and were afterwards homogenized to obtain a representative sample of the residual bark from these commercial plantations.

The barks were air dried, ground in a cutting mill (Retsch SM 2000, Retsch GmbH, Haan, Germany) with a sieve opening of 1 mm², and sieved in a vibratory apparatus to separate the 40 to 60 mesh fraction that was used for chemical analysis. The samples were stored in the dark.

Summative Chemical Characterization

The summative chemical analysis included determinations of ash, extractives soluble in dichloromethane, ethanol, and water, suberin, Klason and acid-soluble lignin, and the monomeric composition of polysaccharides.

The ash content was determined according to TAPPI 211-om-93 (1993). Successive Soxhlet extractions were made with dichloromethane, ethanol, and water, for 6 h, 16 h, and 16 h, respectively, and the extractives solubilized by each solvent were determined gravimetrically. The suberin content was determined on the extractive-free material with depolymerization by methanolysis (Pereira 1988). Lignin content was determined with the previously extracted and desuberinised material as Klason lignin (TAPPI 222 om-02 (2002)) and soluble lignin by UV absorbance at 206 nm (TAPPI UM 250 (1991)).

The polysaccharides were calculated based on the amount of neutral sugar monomers released in the hydrolysate obtained for lignin determination. The neutral monosaccharides were quantified by high performance ion chromatography with pulsed amperometric detection (HPIC-PAD) using a Dionex ICS-3000 system. The separation was performed with Aminotrap plus CarboPac SA10 anion-exchange columns (Thermo Scientific, Waltham, MA, USA), using as mobile phase an aqueous NaOH and CH₃COONa solution at a flow rate of 1.0 mL/min (0±20 min 18 mM NaOH; 20±25 min 50 mM NaOH+170 mM CH₃COONa; 25±40 min 50 mM NaOH+170 mM CH₃COONa) at 30 °C.

Composition of Bark Extracts

Approximately 1 g of ground bark was extracted with ethanol/water (50/50, v/v) with a solid:liquid ratio of 1:10 (m/v) for 60 min at 50 °C using an ultrasonic bath (Branson 2200, Scientific Support, Inc., Hayward, CA, USA).

Total phenolic content was determined by the Folin-Ciocalteu method, and expressed as mg of gallic acid equivalents (GAE) / g of the dry bark extract (Singleton and Rossi 1965; Miranda *et al.* 2016). Flavonoid content was determined with an aluminum chloride colorimetric assay, and expressed as mg of (+)-catechin equivalents (CE) / g of the dry bark extract (Jia *et al.* 1999; Miranda *et al.* 2016). Tannin content was determined by the vanillin-H₂SO₄ method, and expressed as mg of (+)-catechin equivalents (CE) / g of the dry bark extract (Abdalla *et al.* 2014; Miranda *et al.* 2016).

Antioxidant Activity of Bark Extract

The antioxidant activity of the bark extracts was determined using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (Sharma and Bhat 2009; Miranda *et al.* 2016) and expressed in terms of the amount of extract required to reduce the DPPH concentration by 50% (IC₅₀) and Trolox equivalents (TEAC) on a dry extract base (mg Trolox / mg dry extract).

Extraction of Tannins

Two extractions of bark tannins were made using pure water and 5% sodium sulfite solution based on dry weight of the barks. For each extraction, an aliquot of 100 g of bark was taken with a solid:liquid ratio of 1:15 (m/v), and the extraction was made in a water bath at 70 °C for 3 h under periodic agitation. After extraction, the material was filtered through a 1.0 mm² flannel, and then vacuum filtered through a No. 1 porosity sintered glass funnel. The extracts were concentrated by rotary evaporation at 60 °C to

approximately 150 mL. The solids were determined by total evaporation of two 10 g samples and oven drying at 100 °C. The result was reported as a percentage of initial dry mass of the bark.

The extracts' reactivity to formaldehyde was determined by measuring the Stiasny index (Sartori *et al.* 2014). After each extraction, an aliquot of 20 g of concentrated extract was supplemented with 4 mL of formaldehyde (37%, m/m) and 2 mL of 10 M HCl, heated under reflux for 35 min, and filtered. The precipitate was washed and dried. The Stiasny index (SI) is the yield of formaldehyde-condensable tannins (precipitate) expressed as a percentage of the extract. The tannin yield (% of tannin in the initial bark) was calculated by multiplying the solid extract yield (% of extract in the initial bark) by SI. The non-tannin yield was calculated by difference.

FTIR Analysis

Fourier transform infrared spectroscopy (FTIR) was performed using KBr pellets. Each ultra-milled sample was oven dried at 60 °C overnight, and a 1.5 mg aliquot was mixed with 200 mg KBr and milled in a mortar. The KBr pellets were prepared with a compressor (Carver C40000-544, Wabash, USA) and analyzed on a Bruker FTIR spectrometer (Alpha 100382, Bruker OptiK GmbH, Ettlingen, Germany) equipped with a KBr pellet detector accessory (Alpha-T, Bruker OptiK GmbH, Ettlingen, Germany), collecting 24 scans per sample at a resolution of 4 cm⁻¹ and wavenumber range of 375 to 4000 cm⁻¹. Data analysis was performed using OPUS 6.5 software from Bruker (Ettlingen, Germany).

RESULTS AND DISCUSSION

Bark Chemical Composition

The chemical composition results for the bark samples from the two commercial clones of *E. urophylla* hybrids are shown in Table 1. The barks of both hybrids had similar chemical composition: low ash content (2.9 and 2.2%), high extractives content (17.1 and 14.9%), little suberin (1.3 and 1.1%), moderate lignin content (19.4 and 18.3%), and high polysaccharide content (59.3 and 63.5%), composed mainly of glucans (glucose content being 46.7 and 49.8% of the dry bark) and xylans (xylose and arabinose content being 9.4 and 10.8% of the dry bark).

The bark chemical composition of the studied *E. urophylla* hybrids was similar to what was previously reported by Sartori *et al.* (2016) for the bark of other *E. urophylla* hybrids: extractives 14.3 to 17.6%, suberin 0.8 to 1.5%, lignin 18.1 to 21.2%, and polysaccharides 59.3 to 61.2%. Compared to the bark of *E. grandis* × *E. urophylla* (Lima *et al.* 2014), they contained less extractives (28.1%) but similar amounts of polysaccharides (56.8%) and lignin (22.2%). The monomeric composition of polysaccharides showed the predominance of cellulose (on average 48.3%, estimated from the glucose content), while the hemicelluloses were constituted mainly by xylans (10.1% estimated by the xylose and arabinose content). Similar values were obtained for other *urophylla* eucalypt bark (Sartori *et al.* 2016). Bragatto (2010) reported the carbohydrate composition of *E. grandis* × *E. urophylla* bark (in % of initial bark) as glucose 46.2% and xylose 14.1%. Vázquez *et al.* (2008) and Miranda *et al.* (2013) reported similar monomeric composition of polysaccharides for *E. globulus* bark: a

predominance of glucose (70.4% and 68.4% of the neutral monosaccharides) and a notable content of xylose (20.8% and 23.2%).

Suberin content was very low due to the small proportion of phellem in the periderm of these young eucalyptus trees; suberin is a structural cell wall component only present in phellemic cork cells (Leite and Pereira 2017). These anatomical characteristics have been shown for *E. globulus* bark (Quilhó *et al.* 1999), and other eucalypt species also show similarly low suberin contents (Miranda *et al.* 2013; Lima *et al.* 2017).

Table 1. Summative Chemical Composition (% of Total Dry Initial Bark) of the Barks of the *Eucalyptus urophylla* Hybrid

Component	Clone 1	Clone 2
Ash	2.9	2.2
Extractives	17.1	14.9
Dichloromethane	1.8	1.7
Ethanol	9.8	8.7
Water	5.5	4.5
Suberin	1.3	1.1
Lignin	19.4	18.3
Acid-insoluble lignin (Klason)	16.4	16.1
Acid-soluble lignin	3.0	2.2
Cellulose (as glucan)	46.7	49.8
Hemicelluloses	12.6	13.7
Xylan (xylose + arabinose)	9.4	10.8
Galactomannan (mannose + galactose + rhamnose)	3.2	2.9

Table 2. Extraction Yield, Total Phenolic Content, Tannins, Flavonoid Content, and Antioxidant Activity of Bark Extracts of *Eucalyptus urophylla* Hybrids

	Clone 1	Clone 2
Extraction yield (% of initial bark)	11.75	7.15
Total phenolic content (mg GAE / g of extract)	360.5	401.2
Flavonoids (mg catechin / g of extract)	204.0	152.8
Tannins (mg catechin / g of extract)	74.3	61.1
Antioxidant capacity TEAC (mg Trolox / g of extract)	511.1	659.9
Antioxidant capacity TEAC (mg Trolox / g of bark)	59.9	47.2
IC ₅₀ values (µg extract/mL)	5.9	4.9
IC ₅₀ Trolox in ethanol-water = 2.6 µg Trolox/mL		

The non-structural materials obtained by extraction with solvents were mostly polar extractives (soluble in ethanol and water), which represented 89% of the total extractives. Approximately half of the polar extractives (36 and 40%) were of phenolic character (as given by the Folin-Ciocalteu method), including flavonoids and tannins, which amounted to 77 and 53% of the total phenolics (Table 2). In comparison with barks of other *Eucalyptus* species, the polar extractives contents were similar to the reported values; for example, in *E. urophylla* hybrids' barks the ethanol and water soluble compounds represented about 89.6% of the total extractives (Sartori *et al.* 2016), and in *E. globulus* bark 79.9 to 83.0% of the total extractives (Miranda *et al.* 2013; Neiva *et al.* 2014).

Characterization of Bark Polar Extracts

The ethanol-water extraction yields of 11.8 and 7.2% (Table 2) confirmed the high content of soluble materials in these barks. The phenolic and polyphenolic nature of the extracts was shown by the high mean contents of total phenolics (380.9 mg GAE / g extract), flavonoids (178.4 mg CE / g extract), and tannins (67.7 mg CE / g extract).

These values are similar to those found in reports for extracts of *Eucalyptus* barks: 210.9 to 550.9 mg GAE / g of ethanol extract of *E. urophylla* hybrids (Sartori *et al.* 2016), 223 to 253 mg GAE / g of ethanol-water extract and 201 to 413.8 mg GAE / g of methanol-water extract of *E. globulus* (Vázquez *et al.* 2008; Santos *et al.* 2011; Luís *et al.* 2014), and 203.9 and 385.6 mg GAE / g of extracts of *E. grandis*, *E. urograndis*, and *E. maidenii* (Santos *et al.* 2012a).

The flavonoid concentrations were similar to those found by Sartori *et al.* (2016) for *E. urophylla* hybrids (92.6 to 234.5 mg CE / g of extract) and higher than the 8.5 mg quercetin equivalent / g ethanol-water extract of *E. globulus* stump bark (Luís *et al.* 2014).

The tannin content was lower than the 76.5 to 183.8 mg CE / g extract of *E. urophylla* hybrids (Sartori *et al.* 2016) but higher than in other eucalypt barks: 29.0 mg GAE / g ethanol-water extract in *E. globulus* stump bark (Luís *et al.* 2014) and 39.2, 7.4, and 0.5 mg CE / g methanol-water extract of *E. camaldulensis*, *E. globulus*, and *E. rudis* barks (Cadahía *et al.* 1997).

Antioxidant Activity

Table 2 shows that these extracts had weak free radical scavenging activity, with mean IC₅₀ values of 5.87 and 4.89 µg/mL, as compared to Trolox (IC₅₀ of 2.63 µg/mL), which is used as an antioxidant standard. This antioxidant activity was similar to that reported for hydroalcoholic extracts of six *E. urophylla* hybrid clone barks for which the IC₅₀ values were between 7.1 and 11.2 µg of extract/mL (compared with 2.90 µg/mL for Trolox) (Sartori *et al.* 2016). This antioxidant activity was also similar to that reported for methanol-water extracts of *E. grandis*, *E. urograndis*, and *E. maidenii* barks, for which the IC₅₀ values were, respectively, 6.3 µg/mL, 6.1 µg/mL, and 8.2 µg/mL, compared with 2.2 µg/mL for ascorbic acid (Santos *et al.* 2012a).

The antioxidant activity expressed as mg of Trolox / g of bark (511.1 and 659.9 mg Trolox / g extract, corresponding to 59.9 and 47.2 mg Trolox / g of bark) showed values significantly higher than those reported for *E. grandis*, *E. urograndis*, and *E. maidenii* bark extracts expressed in terms of ascorbic acid equivalents (AAE) on a bark basis: respectively, 36.7, 53.2, and 34.5 mg AAE / g bark (Santos *et al.* 2012a).

Extraction of Tannins and Stiasny Number

The extraction of tannins from clones of *E. urophylla* hybrid barks was carried out with water and with 5% sodium sulfite solution at 70 °C. The yields obtained, the polyflavanoid contents, and the reactivities of the extracts with formaldehyde given by the Stiasny index are summarized in Table 3.

Table 3. Stiasny Index of the Solids (SI, % of Solids in the Extract) and Yields of Total Solids (TS, % of Initial Bark), of Condensed Tannins (CT, % of Initial Bark), and of Non-Tannin Solids (NT, % of Initial Bark)

Extraction	SI (%)	TS (%)	CT (%)	NT (%)
Clone 1, water	43.41	4.97	2.16	2.81
Clone 1, 5% sodium sulfite	44.79	7.61	3.41	4.20
Clone 2, water	20.13	3.94	0.79	3.15
Clone 2, 5% sodium sulfite	27.47	4.99	1.37	3.62

The yields with water (4.97 and 3.94% of bark) and with 5% sodium sulfite solution (7.61 and 4.99%) were near to those described for *Eucalyptus globulus* bark (Vázquez *et al.* 2009): 6.8 % (with water), 6.8 and 8.6% (with 2.5% sodium sulfite solution at 70 and 90 °C, respectively), and 6.8 and 10.2% (with 10% sodium sulfite solution at 70 and 90 °C, respectively). However, they were less than those obtained by Mori *et al.* (2001) with the barks of *Eucalyptus urophylla*, *Eucalyptus citriodora*, and *Eucalyptus grandis* with 5% sodium sulfite (at 70 °C): extraction yields of 13.23, 17.99, and 11.35%, respectively, and tannins of 6.28, 5.27, and 10.42%, respectively.

The addition of sodium sulfite increased the extraction yields (increase of 53% in clone 1 and 27% in clone 2) and the condensed tannin contents (increase of 59% in clone 1 and 73% in clone 2). Mori *et al.* (2001) found that the addition of 5% sodium sulfite in the extractions of the barks of *Eucalyptus urophylla*, *Eucalyptus citriodora*, and *Eucalyptus grandis* resulted in increases in extraction yields of 147, 97, and 160%, and in tannin yields of 124, 109, and 91%, respectively. Vital *et al.* (2004) also found for barks of *E. grandis* and *E. pellita* that the addition of 1.5, 3, and 4.5% sodium sulfite increased the extract yields by 29, 43, and 52% and the tannin yields by 10, 21, and 31%.

The Stiasny index gives information about whether the extracts can be used as adhesives (Chupin *et al.* 2013), and a value of at least 65% is needed for a high-quality adhesive (Yazaki and Collins 1994). However, Ping *et al.* (2011) produced good-quality adhesives with a Stiasny index of 46%. The Stiasny indices obtained in this study were 20% and 43% with water and 28% and 45% with sodium sulfite solution. The extractions with sodium sulfite showed increased reactivity of the extracts to formaldehyde. This is contrary to other authors who found that the addition of sodium sulfite decreased the Stiasny index of the extract (Mori *et al.* 1999; Vital *et al.* 2004; Vázquez *et al.* 2009).

FTIR Analysis of Tannins

The FTIR spectra of water and 5% Na₂SO₃ bark extracts are compared in Fig. 1. They show the same absorbances attributed to phenolic compounds.

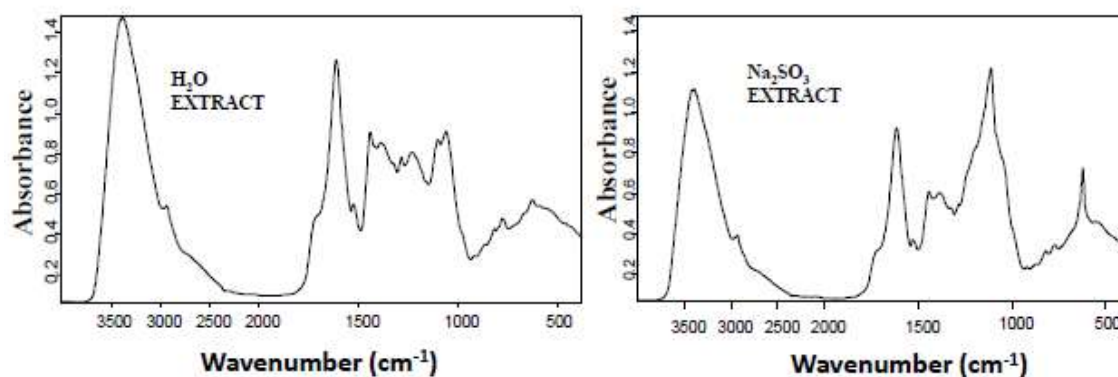


Fig. 1. Infrared spectroscopy of water and 5% Na₂SO₃ bark extracts

The band in the region of 3500 to 3000 cm⁻¹ corresponds to O-H stretching vibration in phenolic and aliphatic structures. For tannin extracts characterized by a high degree of polymerization and a low percentage of monomeric units, the maximum can be located around 3400 cm⁻¹. The peaks near 2930 cm⁻¹ are due to -CH and -CH₂ vibration of aliphatic hydrocarbons (Lee and Lan 2006; Zhang *et al.* 2017). This band originates from -CH stretch vibrations in the aromatic methoxyl groups and in methyl and methylene groups of side chains (Soto *et al.* 2005; Chupin *et al.* 2013; Zhang *et al.* 2017). The absorption band at 1715 cm⁻¹ is attributed to carbonyl groups; in tannins this band was attributed to the carboxylic acid -C=O of the galloyl unit on the epicatechin gallate (Falcão and Araújo 2013; Zhang *et al.* 2017). The bands at 1610, 1520, and 1444 cm⁻¹ are assigned to aromatic ring stretching vibrations (C=C). The peaks at 1103 and 1286 cm⁻¹ are assigned to C-O asymmetric stretching vibrations arising from the pyran-derived ring structure of condensed tannins and therefore show the presence of condensed tannins (Ricci *et al.* 2015). The intense band at 1138 cm⁻¹ detected in 5% Na₂SO₃ bark extract is characteristic of S=O stretching and therefore was not observed in the water extract.

CONCLUSIONS

1. The barks of commercial clones of *Eucalyptus urophylla* hybrids were chemically characterized by notable content of extractives, including mainly polar compounds.
2. The polar extracts included high contents of total phenolics (380.9 mg GAE / g extract) and tannins (67.7 mg CE / g extract) and revealed moderate antioxidant activity.
3. Extraction with a 5% Na₂SO₃ aqueous solution increased extraction yield notably compared to extraction with water, and also increased the Stiasny index of the extracts.

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