



Unlocking the potential of undervalued forest residues: a comprehensive characterization of eucalypt stump in the biorefinery context

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Abstract

Eucalyptus globulus stumps are a by-product from the coppice pulp plantation after three generations. In this study a stump was fractionated in three discs (60 cm between them), and their constituent tissues—heartwood, sapwood and bark—were subjected to further chemical characterization by summative analysis, evaluation of the phytochemical profile and antioxidants activities, plus GC/MS and analytical pyrolysis aiming at their valorization. Wood density was similar between tissues and disc level: values ranging from 0.652 to 0.705 g/cm³ (Disc 1) and 0.605 g/cm³ (Disc 5). Bark had high ash (3.5%), extractives (7.5%) and holocellulose (68.4%) but lower lignin contents (22.0%). Original heartwood contained 0.7% ash, 7.0% extractives, 27.1% lignin, and 67.3% holocellulose. Heartwood showed high extractives (12.1–15.8%), less lignin (23.9–24.5%), and high holocellulose (61.7–64.7%) compared to sapwood which contained 3.9–5.4% extractives, 26.9–27.3% lignin and 68.6–71.5% holocellulose. Water extracts had poor antioxidant activity in contrast to ethanol extracts with high activities in heartwood. All tissues presented GS lignin type with S/G ratios varying from 3.0 to 3.4 (heartwood), 3.2–3.4 (sapwood), bark (3.5) and 3.8 (original heartwood). In wood, fibers and vessels were highly lignified with SG and G-lignin respectively; while rays had low lignin with G-type. Light and fluorescence macroscopic observation of the tissues in Disc 1 revealed a lower proportion and larger vessels in sapwood and high emission fluorescence at 488nm. Overall, these results show that stumps are valuable raw material to be used under the biorefinery context.

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Introduction

Eucalyptus globulus is a crucial species for the pulp and paper industry, prized for its rapid growth and superior wood quality, which make it ideal for producing high-quality bleached pulps. In Portugal, eucalypt is managed in short rotation coppice systems (8 to 14 year rotations) with three cutting cycles. However, other trees must be established at the end of the commercial exploitation. Thus, stumps must be removed from the field before soil preparation or be broken into small pieces and incorporated into the soil for fertilization purposes. Stumps are the basal part of the trees and the principal root that remain in the soil after harvesting. In the case of eucalypts, stumps are uprooted by a machine from the soil, removed from the plantation, left to dry, and used as biofuel to produce heat and energy for the pulp and paper industry (Gominho et al. 2014; Agnes et al. 2021). In fact, a study showed that stumps and roots from different *Eucalyptus* clones had adequate characteristics for bioenergy purposes (Costa et al. 2021). Nevertheless, due to their chemical and anatomical characteristics, stumps have recently been used as feedstock for pulp and paper (Gominho et al. 2014), but more value-added products can be obtained within the concept of a biorefinery (Gominho et al. 2012, 2019; Luís et al. 2014; Pinto et al. 2017). For instance, stump extractive content can reach 15% (Gominho et al. 2012), higher than in eucalypt stemwood, with 3.1% (Morais and Pereira 2012). Luis et al. (2014) reported high concentrations of phenolic compounds (> 200 mg GAE/g extract) and flavonoids (> 10 mg QE/g extract) in stump extracts with antioxidant and antimicrobial activities. Therefore, stump extractives can be collected first, and the bioactive molecules may be used for biomedical purposes. Also, to expand lignin sources not only obtaining it from pulping liquor, a study reported the use of lignin extracted from stumps as a Cr(VI) adsorbent to clean wastewater (Lourenço et al. 2022). In this context, lignin is an important chemical component that requires adequate valorization within the concept of total resource use. Lignin plays a central role in determining the heating value of biomass and offers opportunities for producing a variety of bio-based products such as antioxidants, resins, and polymers (Bajwa et al. 2019). Furthermore, its content and composition significantly impact fractionation and recovery processes (Faustino et al. 2010). Lignin characterization is fundamental groundwork for optimizing its utilization and designing tailored products. To date, the composition of lignin in various fractions of *Eucalyptus globulus* stumps (including sapwood, heartwood, and bark) remains unexplored. This presents a unique opportunity to obtain an initial insight into the extractives and lignin composition within these distinct stump components. Employing a comprehensive multi-analytical approach, encompassing traditional methods like summative analysis, gas chromatography, thioacidolysis, analytical pyrolysis, and FTIR spectroscopy, alongside light and fluorescence macroscopy, we aim to achieve a thorough characterization of stump constituents, particularly lignin. This holistic approach is essential for maximizing the valorization potential of stumps within the framework of biorefineries.

Materials and methods

Collection of raw material

Eucalyptus globulus stump was obtained from a production site of a Portuguese pulp and paper industry (ALTRI SGPS). The stump was cut into discs at five different levels (totalling about 1.50 cm), transported to the laboratory, and left to dry under air conditions. After that, the bottom of each disc was stained with methyl orange to identify the different tissues: heartwood (stained red) and sapwood (yellow-orange, Fig. 1). In Disc 1, methyl orange staining allowed us to distinguish an older part corresponding to the first tree that was formed (called here as original heartwood, since no sapwood is present) and two trees that have grown after cutting the original wood (heartwood 1 and 2, sapwood 1 and 2). Discs 1, 3, and 5 (60 cm between them) were used for chemical analysis, while only Disc 1 was used for the microscopical analysis. The bark was removed by hand using a chisel, in the Disc and collected for both analyses.

Basic wood density was determined gravimetrically after water immersion of original heartwood, heartwood, and sapwood separately. Due to the physical characteristics of the material, it was not possible to measure the basic density of bark samples. Five aliquots were used to calculate the average value and STDEV.

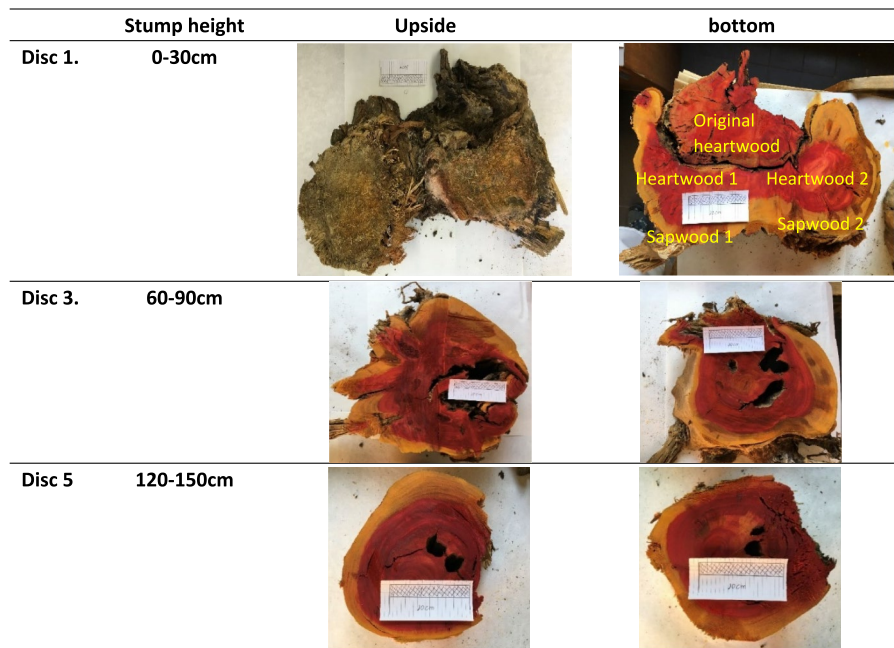


Fig. 1 Discs of *Eucalyptus globulus* stumps stained with methyl orange for identification of sapwood (yellow) and heartwood (red) regions (colour figure online)

Chemical composition

The tissues from Discs 1, 3, and 5 were prepared for chemical analysis. In Disc 1, four tissues were selected: original heartwood, sapwood, heartwood, and bark; in Discs 3 and 5, just sapwood and heartwood. These tissues were milled in a knife mill, first sieved with a mesh of 6mm, then subjected to further grinding with a mesh of 1mm, and then sieved again to collect the 40–60 mesh fraction for chemical analysis. Summative analysis was made according to adaptations of Tappi standards: ashes (Tappi 15 os-58); total extractives were determined in 2 g of sample, in a Soxhlet apparatus using sequential extraction with dichloromethane (6 h), ethanol (16 h), and water (16 h) following Tappi standard 211 om-02; total lignin was determined in extractives-free samples, as the sum of Klason lignin (Tappi T222 om-88) and soluble lignin (Tappi UM 250). Polysaccharides were determined by separating the hydrolyzate obtained from Klason lignin. Neutral monosaccharides and galacturonic and glucuronic acids were quantified by high-pressure ion chromatography (DIONEX ICS3000) as described in Costa et al. (2023). Holocellulose was also determined using the chlorite method according to Browning (1967) and after 240 min reaction time. In the bark sample, the amount of suberin was also determined by methanolysis, according to Pereira (2013). All analyses were made in duplicate.

Extracts composition

The DCM extracts from all three discs and tissues were analyzed by GC–MS analysis. Around 1 mg of the DCM extracts was derivatized with pyridine (Sigma-Aldrich) and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane, Sigma-Aldrich) to form trimethylsilyl ethers/esters (TMS). The mixture was heated at 60 °C for 30 min and then cooled to room temperature. The TMS-extracts were injected into an Agilent 7890 A-5975C MSD GC–MS system equipped with a high-temperature capillary column (Zebtron 5 HT, 30 m×0.25 mm×0.1 µm), using helium as the carrier gas at a constant flow (1 mL/min) in splitless mode. The detailed conditions can be seen in Gominho et al. (2020).

Extracts activities

The ethanol and water extracts obtained from successive Soxhlet extractions from all the tissues were analysed as total phenolics content using the Folin–Ciocalteu method, total flavonoids content via the aluminium chloride colorimetric assay, and condensed tannins using the vanillin-sulfuric acid procedure (Ferreira et al. 2015). Phenolic content was reported as mg gallic acid equivalents, while flavonoids and condensed tannins as (+)-catechin equivalents through a calibration curve obtained with the same methodology. All tests were done in duplicate.

The antioxidant activity of the extracts was determined by the ferric-reducing antioxidant power (FRAP) and free radical scavenging activity (DPPH) as described in Miranda et al. (2016). The FRAP measures the potential of the extracts to reduce

Fe(III) to Fe(II), and results were reported as mmol Fe(II)/g extract. The DPPH was evaluated by the IC₅₀ (concentration of extract necessary to achieve 50% DPPH inhibition) and by the antioxidant activity index AAI, which describes the antioxidant activity of extracts as poor if $AAI < 0.5$, moderate if $0.5 < AAI < 1$, strong if $1 < AAI < 2$ and very strong if $AAI > 2$ (Scherer and Godoy 2009).

Analytical pyrolysis (PY-GC/MS)

The extractives-free samples (O.Heart, heartwood, sapwood and bark) were dried and finely ball-milled in a Retsch MM200 centrifugal ball mill for 10 min. Around 105 µg of each sample was pyrolyzed in a quartz boat at 550 °C for 1 min in a platinum coil Pyroprobe connected to a CDS 5150. The volatiles separation was made in a GC from Agilent (7890B) using a fused-silica capillary column (ZB-1701: 60 m × 0.25 mm i.d. × 0.25 µm film thickness) and applying the chromatography conditions described by Lourenço et al. (2013). For mass spectra analysis (Agilent 5977B MS), electron impact ionization at 70 eV and damping helium gas (1 mL) were used. The total area of the chromatogram was attained by automatic integration (Mass Hunter software), and the lignin and carbohydrates derivatives were identified by comparing their mass spectra with NIST2014, Wiley databases and literature (Faix et al. 1990, 1991; Ralph and Hatfield 1991). The lignin derivatives represented by H, G, and S-lignin units were summed up, and the S/G ratio and the relation H:G:S were determined. Total carbohydrates were determined as the sum of all the carbohydrates derivatives, and the relation carbohydrates-to-lignin ratio was calculated (C/L).

Infrared analysis (FTIR)

O.Heartwood, heartwood, sapwood, and bark-dried samples were ground and analyzed by transmission FTIR carried out using a Nicolet 6700 spectrometer and Omnic software (Thermo Fisher Scientific, USA). KBr pellets were prepared by mixing the sample with potassium bromide. Spectra were obtained through 16 scans from 4000 to 400 cm⁻¹ with 4 cm⁻¹ spectral resolution while subtracting background spectra measured in the air. Spectra were then corrected by baseline subtraction and area normalization to compare samples.

Thioacidolysis analysis

The lignin monomeric composition was also determined by thioacidolysis, which allowed the quantification of the monomers involved in the labile ether linkages (Lapierre et al 1986). Extractives-free samples of sapwood, heartwood original, and bark (40–60 mesh fraction) were incubated at 100 °C under agitation in a solution of ethanethiol/BF₃ etherate/dioxane (2.5/10/87.5 v/v/v) with the addition of 1 mL from a mixture of standards (tetracosane (25.2 mg) and dodecosane (23.8 mg) both dissolved in 100 mL of dioxane). After a 4 h reaction, lignin compounds released by thioacidolysis were extracted using dichloromethane and then silylated before

GC-FID analysis. The guaiacyl (G) and syringyl (S) thioethylated monomers were separated in a fused silica DB-1 column (30 m×250 µm×0.25 µm, J&W Scientific[®]) using He as carrier gas and 160–280 °C temperature gradient at 2°C/min. Three replicates were used for each sample.

Microscopic observations

The anatomical studies were made on the tissues collected from Disc 1: sapwood, heartwood, the original heartwood, and bark. Samples were cut into small blocks (1×1×1.5 cm), then fragments of about 20 mm in length and 3mm in thickness were obtained using blades or saw. The original heartwood could not be cut at once and was conditioned under 85% relative humidity using a solution of KCl. Transverse sections of 30–40 µm thickness were obtained using a microtome equipped with disposable blades (Thermo scientific HM 340E semi-automated microtome). All observations were done using a stereo zoom macroscope (AXIO Zoom.V16, Zeiss) and visible light or fluorescence as described below.

Histochemical analysis (Wiesner and Mäule staining)

The Wiesner and Mäule reactions were used to visualize lignin in each tissue (Day et al. 2005). The Wiesner reaction was performed after staining the sections with phloroglucinol (2% in 96% ethanol) for 5 min and mounting in HCl (2N). For Mäule staining, sections were incubated with KMnO₄ (1% in water) for 5 min and washed with water before adding HCl (2N) until the section was partially decoloured. The section was finally mounted in ammonium hydroxide (28–30%). Samples stained with Wiesner and Maule reagents were imaged using a macroscope and visible light. For each tissue, the images of different sections (5–9 images) acquired at 25× and 63× magnification were analysed by ImageJ software to determine the proportion and size of the vessels in each tissue.

Fluorescence determination

Sections were mounted in deionised water prior to fluorescence observation. Fluorescence images of four sections were acquired at 63× magnification and two excitation/emission wavelengths conditions: (1) 365 nm excitation, 420–470 nm emission (blue fluorescence), and exposure time of 1000 ms, 2000 ms, and 3000 ms; (2) 488 nm excitation, 500–550 nm emission (green fluorescence) and exposure time of 500 ms, 1000 ms, and 1500 ms. The field of view, resolution, and depth of field for fluorescence image acquisition was 3.7 mm, 0.7 µm, and 9 µm. Fluorescence images were analysed using ImageJ software, following an automated procedure as described by Audibert et al. (2023). The procedure was applied to the stack of the four images acquired for each excitation and allowed the quantification of average fluorescence intensity. The stack image was converted to an 8-bit grey level, and an automated threshold called “Huang dark” was applied to detect the cell walls and

extract a selection that is applied in the unmodified image stack to measure the average grey value.

Statistical analysis

A one-way analysis of variance (ANOVA) followed by Holm-Sidak method for pairwise comparison was performed to compare group mean values for each analysis performed at least in triplicate. Statistical tests were conducted using SigmaPlot® software (version 12.0, Systat, USA) a significance level of 5%.

Results and discussion

Basic density and chemical analysis

The emergence of new feedstocks, such as biomass from eucalypt stumps, is a crucial development for biorefineries. Eucalypt stump biomass is gaining attention as a valuable raw material due to its unique physical and chemical properties, as illustrated in Tables 1 and 2. One of the key parameters for assessing wood quality is basic density, which generally increases with tree age and varies across species (Lima 1995). In our findings, the basic density values did not show substantial differences between the tissues and Disc levels. For instance, Disc 1, which includes stumps above soil level, exhibited density values ranging from 0.652 to 0.705 g/cm³. A decrease in the density of heartwood was found significant between the three discs, with the lowest values for the deeper sample, Disc 5, whose sapwood density (0.605 g/cm³) is significantly lower than that of Discs 1 and 3 (Table 1). Notably, these values surpass those reported for stumps and roots from different eucalyptus clones in Brazil, which values ranged from 0.357 to 0.365 g/cm³ (Costa et al. 2021); but also, those reported for *E. globulus* stemwood, 6 and 5.6 years, with 0.478 g/cm³ (Carrillo et al. 2017) and 0.491 g/cm³ (Gominho et al. 2001), respectively. The density of other eucalyptus species, such as *E. nitens*, was recorded at 0.490 g/cm³ at 6-years-old (Carrillo et al. 2017). When examining different tissues, Lourenço (2009) did not observe any variation in basic density between heartwood and sapwood from 18-year-old *E. globulus*, with a consistent density of 0.74 g/cm³. In contrast, *S. mahogany* heartwood presented lower basic density compared to sapwood

Table 1 Basic density values of the heartwood (Heart) and the sapwood (Sap) at different heights and of the bark of the *Eucalyptus globulus* stump. Mean values and standard deviation (STDEV) of five replicates

	Disc 1			Disc 3		Disc 5	
	O.Heart	Heart	Sap	Heart	Sap	Heart	Sap
Density (g/cm ³)	0.678	0.695	0.657	0.620	0.654	0.605	0.605
STDEV	0.028	0.026	0.007	0.032	0.019	0.048	0.012

O.Heartwood = original heartwood, i.e. from the first tree grown.

Table 2 Chemical composition of sapwood and heartwood at different heights and of the bark of *Eucalyptus globulus* stump (mean values of 2 replicates)

Component (% dry matter)	Disc 1			Disc 3		Disc 5		
	O.Heart	Heart	Sap	Heart	Sap	Heart	Sap	Bark
Ash	0.7	0.3	0.7	0.2	0.3	0.2	0.3	3.5
Extractives	7.0	12.1	5.4	15.6	4.5	15.8	3.9	7.5
Dichloromethane	0.5	0.6	0.3	1.0	0.3	1.2	0.2	0.6
Ethanol	2.1	8.4	1.6	11.7	0.8	11.6	0.6	2.8
Water	4.4	3.1	3.5	2.8	3.4	3.0	3.0	4.1
Total Lignin	27.1	24.5	27.3	24.0	26.0	23.9	26.4	22.0
Klason Lignin	23.5	21.4	24.1	21.3	22.9	21.1	23.1	19.3
Soluble Lignin	3.6	3.1	3.2	2.7	3.1	2.8	3.3	2.7
Holocellulose	67.3	64.7	68.6	61.7	71.5	62.5	71.5	68.4
Suberin	–	–	–	–	–	–	–	0.3
Lignin-to-Holocellulose ratio	0.40	0.37	0.39	0.38	0.36	0.38	0.37	0.32
<i>Sugars (% of Total monosaccharides)</i>								
Rhamnose	0.7	0.7	0.5	0.4	0.4	0.4	0.5	0.2
Arabinose	0.6	0.8	0.8	0.4	0.8	0.5	0.5	0.9
Galactose	1.9	2.2	2.6	1.6	1.5	1.4	1.3	1.9
Glucose	63.6	60.8	64.9	66.2	66.1	67.4	66.6	70.1
Xylose	25.6	26.6	22.9	24.6	22.7	23.1	22.8	20.7
Galacturonic acid	1.6	1.6	1.5	1.3	1.2	1.2	1.1	1.2
Acetic acid	6.0	7.5	7.0	5.5	7.2	6.0	7.1	5.0

O.Heart = original heartwood, i.e., from the first tree. Heart = heartwood; Sap = sapwood; In bark, the total lignin value was corrected to extractives and ashes contents

(Arisandi et al. 2023), highlighting the complexity of this topic. The relationship between density, vessel frequency, and diameter adds another layer of complexity. As noted by Pfautsch et al. (2016), the basic density of sapwood tends to increase alongside vessel frequency; however, mean vessel diameter may decrease in trees growing in mesic environments. The implications of vessel diameter differences will be addressed in subsequent sections.

Chemical analysis revealed distinct characteristics among tissues and across the three stump levels (Table 2). Bark had elevated ash content (3.5%) and substantial extractives (7.5%), but a lower lignin content (22.0%) compared to sapwood and heartwood (23.9–27.3%). Stump bark contained 0.3% suberin, typical for *E. globulus* species (0.98%), yet lower than other barks like *Q. faginea* (2.9%) and *B. pendula* (5.9%, Miranda et al. 2013). Holocellulose content in bark (68.4%) fell within the range for eucalypt stem bark (62.6%) but was higher compared to birch (49.8%, Miranda et al. 2013), indicating potential for valorization beyond combustion for power generation. Bark presented the highest concentration of glucose (70.1% of polysaccharides), while displaying the lowest content of xylose (20.7%), as compared to the wood tissues.

Heartwood exhibited a notable abundance of extractives, particularly in Discs 3 and 5 (15.6% and 15.8%), possibly indicating a defensive response or leaching due to rain after tree removal. Interestingly, the original heartwood showed a lower level of extractives (7.0%), likely due to seasoning from previous tree removal (Gutiérrez et al. 1998), allowing the regrowth of the plant. Sapwood consistently had a higher lignin content (26.9–27.3%) compared to heartwood (23.9–24.5%), whatever the Disc analysed. However, original heartwood (27.1%) displayed lignin levels similar to sapwood. Lignin variation between heartwood and sapwood has also been found in a hybrid eucalypt *E. urophylla* X *E. grandis*, but in this case, heartwood presented higher values compared to sapwood, reaching values of 33.8 and 26.0% respectively (Xiao et al. 2019), while other studies have reported higher lignin content in the heartwood of *Michelia macclurei* (Ren et al. 2023). In Disc 1, sapwood exhibited the highest holocellulose content (mean 68.6%), akin to the original heartwood (67.3%), whereas heartwood ranked behind at 64.7%. This trend persisted in Discs 3 and 5, with sapwood exceeding 70% of dry matter. Generally, sapwood across various species displays higher total carbohydrate content than heartwood. For instance, total carbohydrate values of 51.5%, 54.4%, 55.5%, and 61.9% were reported for *E. urophylla*, *E. globulus*, *A. mangium*, and the hybrid *E. urophylla* x *E. grandis* sapwood samples respectively (Çetinkol et al. 2012; Xiao et al. 2020). Overall, xylose and glucose predominate among tissues, consistent with the glucuronoxylan type of hemicelluloses found in hardwoods like *E. globulus* (Rowell et al. 2012). Xylose content prevailed in heartwood polysaccharides (23.1–26.6%), while constant values were reported in sapwood (22.7–22.9%). Glucose content was not clearly consistent between wood tissues (Disc 1: heartwood had the lowest value, but in Disc 3 and 5, the tissues presented similar values). Hemicelluloses isolated from the heartwood, sapwood, and bark of a eucalypt hybrid revealed the highest xylan content mainly in heartwood (73.0%, 58.5%, and 69.9%, respectively), followed by galactose that prevailed in sapwood (3.0%, 14.0% and 3.2%, Xiao et al. 2020). Furthermore, the presence of acetic acid and galacturonic acid, with values exceeding 5% and around 1.5% of total sugars, respectively, suggests partial acetylation of hemicelluloses. Note that the summative composition slightly exceeds 100% since the determination of the different components involves the use of different methods. Also, these are mean values, and we have also to keep in mind that all the chemical components present a range of values, showing the heterogeneity of the material.

Overall, the chemical analysis shows mainly variations between heartwood and sapwood, in particular the contents of extractives, lignin, and holocellulose, but no clear conclusion can be drawn in relation to the distribution of these chemical components along the stump height (data of Disc 3 and 5).

FTIR analysis

FTIR spectra of the samples from Disc 1 are presented in Fig. 2. A broader band appears in all samples at 3416 cm^{-1} followed by the band at 2915 cm^{-1} . In the fingerprint region more, bands were present. The band associated with carbonyl groups from hemicelluloses (1740 cm^{-1}) and absorbed water (1682 cm^{-1} , Popescu et al.

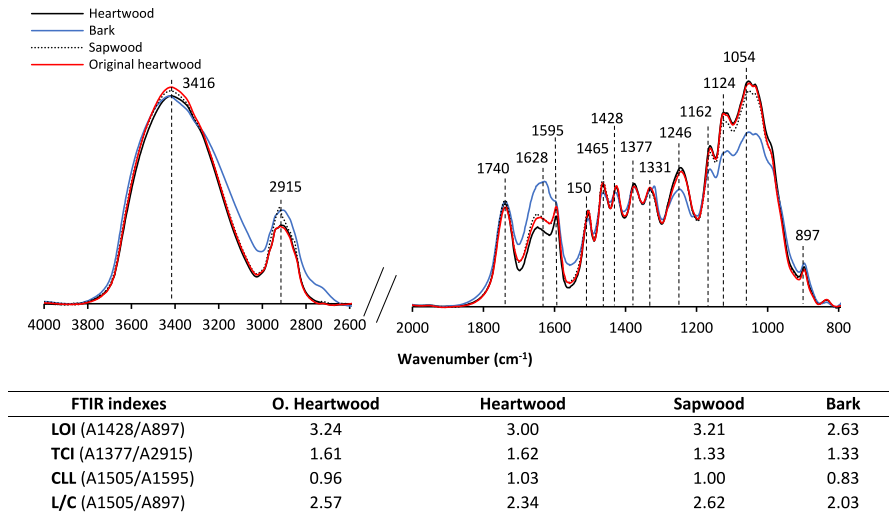


Fig. 2 FTIR spectra of sapwood, heartwood, original heartwood and bark samples from Disc 1. Main bands assignments (cm^{-1}): **3416** O–H stretching and H-bonded; **2915** asymmetric and symmetric stretching vibrations of the methylene groups; **1740** unconjugated C=O stretch in ketons, carbonyl and ester groups in xylans; **1628** adsorbed water and C=O stretching in flavones and calcium oxalate; **1595 and 1505** aromatic skeleton vibration in lignin; **1465 and 1428** C–H deformation in lignin and carbohydrates; **1377** C–H deformation in polysaccharides; **1331** C_{aryl}–O vibration in syringyl derivatives; **1246** C–O stretch in guaiacyl derivatives and O–H in plane in polysaccharides; **1162** C–O–C vibration in polysaccharides; **1124** aromatic skeletal and C–O stretch; **1054** C–O stretch in polysaccharides; **897** C–H deformation in cellulose (amorphous region). FTIR indexes calculated: LOI (lateral order index), TCI (Total crystalline index), CLL (Cross-linked lignin), L/C (Lignin-to-cellulose index)

2009). The cellulose bands were: 1377, 1246, 1054, and 897 cm^{-1} (Popescu et al. 2009; Rodrigues et al. 1998; Gominho et al. 2019; Rubio-Valle et al. 2024). The lignin bands were 1595 and 1505, 1331, 1124 cm^{-1} , while the bands 1737 and 1265 cm^{-1} (Schwanninger et al. 2004; Popescu et al. 2009) were not dominating. Also, all samples have a GS lignin type since the band 1465 is higher than 1505 cm^{-1} (Faix 1991).

Using the FTIR bands intensity, it was possible to calculate different indexes: (i) Lateral order index (LOI): an empirical crystallinity index = A_{1428}/A_{897} , where 1428 cm^{-1} band is associated with the crystalline structure of cellulose, while the band at 897 cm^{-1} corresponds to the amorphous region of cellulose (Nelson and O'Connor, 1964); (ii) Total crystalline index (TCI) = A_{1377}/A_{2915} , the band 1377 cm^{-1} is associated with glycosidic bond β -(1,4) in cellulose and the band 2915 cm^{-1} is related to the crystalline structure of cellulose (Nelson and O'Connor, 1964); (iii) Cross-linked lignin (CLL) indicates the proportion of condensed lignin and cross-linked structures = A_{1505}/A_{1595} ; where both bands 1505 cm^{-1} and 1595 cm^{-1} relate to aromatic skeletal vibration of lignin (Auxenfans et al. 2017); (iv) Lignin-to-cellulose index (L/C) was determined between A_{1505}/A_{897} , where the 1505 cm^{-1} band corresponds to the lignin stretching of C=C, and 897 cm^{-1} is assigned to C–H deformation in cellulose (Gaur et al. 2015). Original heartwood and heartwood attained similar values for LOI and TCI; the LOI values were 3.24 and

3.00, respectively, while their TCI values were 1.61 and 1.62. In bark tissue, the lateral order index (LOI) and total crystalline index (TCI) were the lowest, suggesting that cellulose in bark has a lower degree of crystallinity compared to wood tissues. However, we could not corroborate this with literature for the same tree; *E. globulus* bark relative crystallinity was reported to be 46.4%, determined by X-ray diffraction (Li et al. 2022), while the cellulose extracted from stemwood had a crystallinity of 87% (Magina et al. 2024). The lignin-to-cellulose index (L/C) was also lower in bark (2.03) compared to heartwood and sapwood (2.34–2.62), which recorded the highest value (2.62). This is consistent with the chemical analysis, where the lignin-to-holocellulose ratio was the lowest in bark (0.32, Table 2). The lowest value of the LCC ratio was obtained in the bark, suggesting that lignin is more condensed with crosslinked G-type lignin structures. These infrared data were in the range of values reported in the literature. For instance, industrial chips derived from *E. globulus* stump wood exhibited a high degree of crystallinity, with LOI and TCI values reaching 4.31 and 1.49, respectively. Additionally, the CLL stood at 0.87, and the L/C ratio was 3.29 (Gominho et al. 2019). Other hardwoods, such as poplar, presented an LOI of 1.77 and a CLL of 1.32 (Auxenfans et al. 2017).

Extractives composition

Figure 3 illustrates the distribution of the primary compound families in the dichloromethane (DCM) extracts, and a detailed list of compounds identified is available in Supplementary material (Table S1). On average, approximately 76% of the total chromatogram area was identifiable, with compounds primarily belonging to

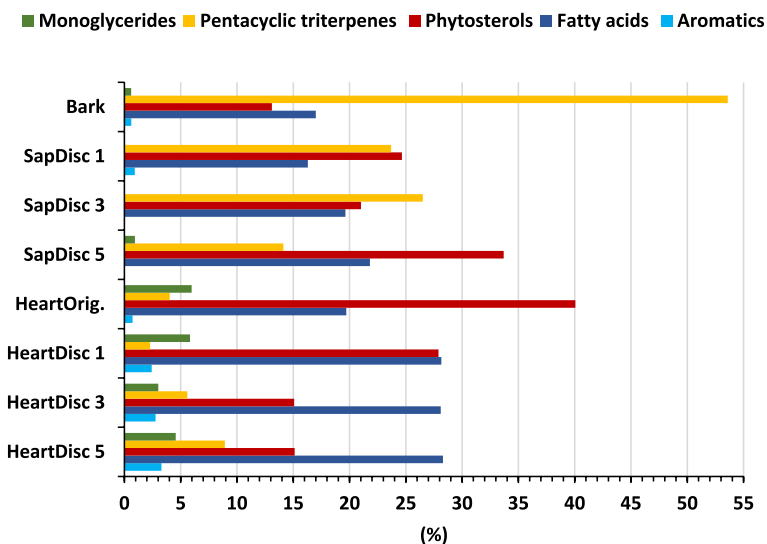


Fig. 3 Distribution of the DCM extract families in the tissues studied and in between discs (% of total chromatographic area)

phytosterols, triterpenes, fatty acids, and aromatics families. Sapwood exhibits a higher concentration of triterpenes, with asiatic and arjunolic acids being the most prominent, followed by phytosterols (predominantly β -sitosterol) and fatty acids (notably hexadecanoic acid and *cis* 9-octadecenoic acid). Conversely, aromatics and monoglycerides are more prevalent in heartwood. This composition pattern of the DCM extract mirrors findings from Gominho et al. (2020) in mature *E. globulus* trees, indicating that heartwood tends to be richer in triterpenes and aromatic compounds. These compounds, which accumulate in the heartwood region, play a crucial role in providing protection against biotic agents. Overall, there appears to be an axial gradient regarding the type of families present: heartwood has more phytosterols and fatty acids (Disc 1), but there is a prevalence of fatty acids in Disc 3 and Disc 5. Sapwood has more phytosterols and triterpenes (Disc 1), while triterpenes prevail in Disc 3 and phytosterols in Disc 5. In turn, the bark is rich in pentacyclic triterpenes (53.6%, with betulinic acid as the main compound), followed by fatty acids (17%) and phytosterols (13%). Our results are comparable with those of other authors for *E. globulus* stemwood as well as for other eucalypt species. Santos et al. (2017) focused on *E. grandis* wood from Portugal, Brazil, and South Africa, which was obtained as the main family sterol, followed by fatty acids, while triterpenic acids were detected only in Brazilian wood. According to Domingues et al. (2010), studying the DCM extracts from eucalypt residues found predominantly triterpenoids and fatty acids type of compounds as mentioned here. Besides, these authors, as well as Gominho et al. (2021), mentioned that this type of biomass could be a good source of bioactive compounds with a wide range of applications from the food to pharmaceutical and biomedical industries.

Extractives activity

Table 3 presents the phytochemical profile of ethanol and water extracts, encompassing total phenolics (TP), total flavonoids (TF), and condensed tannins (CT), whose total values were calculated accounting for the percentage of each extract (Table 2). The antioxidant activities were determined using the FRAP and DPPH methods. Notably, ethanol extracts generally exhibited a higher phytochemical profile compared to water extracts across all samples. Heartwood samples exhibited phenolic levels ranging from 866.7 to 1 066.2 mg GAE/g extract for ethanol extracts and 177.4 to 280.8 mg GAE/g extract for water extracts. Similarly, sapwood ethanol extracts ranged from 758.7 to 860.7 mg GAE/g extract, while water extracts ranged from 149.1 to 231.9 mg GAE/g extract. This variation can be attributed to the higher content of phenolics in ethanol extracts compared to water extracts in heartwood (8.4% vs. 3.1%, as shown in Table 2), whereas the opposite is observed in sapwood (1.6% vs. 3.5%). Interestingly, the original heartwood mimics the behavior of sapwood tissue, exhibiting a TP value of 983.3 and 243.6 mg GAE/g extract, respectively, ethanol and water extracts.

In terms of total flavonoids (TF) and condensed tannins (CT) compositions, both heartwood and sapwood displayed a similar pattern to that of total phenolics (TP). For instance, heartwood TF levels in the ethanol extract ranged from

Table 3 Phytochemical profile and determination of the antioxidant activities of the phenolic extracts from heartwood, sapwood, and bark of eucalypt stumps (mean values of 2 replicates)

	Disc 1			Disc 3		Disc 5		
	O. Heart	Heart	Sap	Heart	Sap	Heart	Sap	Bark
<i>Phytochemical Profile</i>								
Total Phenolics (TP, mg GAE/g extract)								
Ethanol	983.3	1 066.2	758.7	866.7	820.2	884.7	860.7	431.4
Water	243.6	280.8	231.9	177.4	149.1	252.0	174.8	201.0
Flavonoids (TF, mg CE/g extract)								
Ethanol	145.5	143.9	154.9	108.9	221.7	106.8	173.8	135.3
Water	3.0	11.1	2.4	12.5	1.6	11.5	1.3	72.2
Condensed Tannins (CT, mg CE/g extract)								
Ethanol	22.3	21.9	47.5	17.7	45.2	20.3	42.1	149.1
Water	0.5	1.7	0.8	2.0	0.8	2.2	0.3	51.9
<i>Antioxidant Activity</i>								
FRAP (mmol Fe(II)/g extract)								
Ethanol	12.6	12.1	7.6	10.8	7.5	8.8	7.8	4.4
Water	0.3	0.9	0.1	1.2	0.1	1.0	0.1	2.0
DPPH								
IC50 Ethanol (mg/L)	2.6	1.3	2.3	1.9	3.0	2.1	2.5	6.8
AAI Ethanol	9.2	18.3	10.4	12.5	7.9	10.9	9.5	3.5
Water IC50 (mg/L)	12.7	7.3	10.8	17.8	18.8	8.0	13.7	148.4
AAI Water	1.8	3.2	2.6	1.3	1.2	2.9	1.7	0.1

O. Heart—original heartwood; *values reported to the percentage of each extract attained from chemical composition

106.8 mg CE/g extract (disk 5) to 143.9 mg CE/g extract (disk (1). Conversely, bark exhibited intermediate values for TP of the ethanolic extract (431.4 mg GAE/g ethanolic extract) falling between those of heartwood and sapwood, but with generally higher levels for TF and CT (135.3 and 149.1 mg CE/g extract, respectively). Luís et al. (2014) also studied the phenolic compounds of wood, bark, and stumps from *E. globulus*, attaining in the ethanol extracts the values 460.0 (stumps) and 253.9 mg GAE/g extract (bark). These authors measured flavonoids by using a different standard (quercetin), and the values reached 33.6 and 8.3 mg QE/g extract for stump wood and bark, respectively (Luís et al. 2014).

Ethanol extracts from heartwood exhibited superior antioxidant activity compared to water-soluble extracts. FRAP values ranged from 8.8 to 12.6 mmol Fe(II)/g extract in heartwood ethanol extracts and 7.5 to 7.8 mmol Fe(II)/g extract in sapwood ethanol extracts. Conversely, bark showed higher antioxidant activity in water extracts (2.0 Fe(II)/g extract) than in ethanol extracts (4.4 Fe(II)/g extract). Regarding the DPPH method, both heartwood and sapwood ethanol extracts displayed strong antioxidant activity, with AAI values exceeding 2. Heartwood ranged from 9.2 to 18.3, while sapwood ranged from 7.9 to

10.4. Bark ethanol extracts exhibited significant antioxidant activity (AAI of 2.4), whereas water extracts showed poorer activity (AAI of 0.1). Studies by Luís et al. (2014) corroborated these findings, showing higher antioxidant activity in ethanol extracts (AAI 7.39) compared to other solvents, such as n-hexane extracts (AAI 0.25) when analyzing stump wood. Also, Fernández-Agulló et al. (2015), while testing several methods to obtain phenolic compounds from *E. globulus* wood wastes, showed that ethanol was the best solvent compared to water or methanol to yield more compounds with high antioxidant properties. Santos et al. (2017), while studying *E. grandis* wood obtained differences in antioxidant activities of the phenolic extracts regarding the geographic origin: Brazil wood had the highest antioxidant activities, followed by South African and Portugal (IC₅₀ of 5.08, 6.13 and 6.18 µg/mL).

Pyrolysis analysis

Analytical pyrolysis is a robust methodology for characterizing both wood and bark, offering a rapid analysis that enables a relative quantification of carbohydrates and lignin (Lourengo et al. 2019; Rencoret et al. 2011). While versatile for insights into both components, its main interest lies in determining lignin composition. Therefore, we present in more detail the data for lignin characterization and briefly those of carbohydrates. Based on the pyrolysis findings (refer to Table 4, Table S2, and Figure S1), carbohydrate derivatives accounted for 54.6% of the original heartwood composition, comparable to sapwood (ranging from 52.3 to 54%). However, heartwood displayed slightly lower values, ranging from 48.9% (Disc 5) to 51.1% (Disc 1). For a detailed identification of the derived compounds and their relative quantification, please refer to the supplementary information (Table S2). Lignin content remained consistent across all tissues, with heartwood registering 27.8% (Disc 1) and sapwood slightly higher at 29.7% (Disc 1), while bark exhibited a notably lower percentage at 20.1%. Minimal differences were observed among the other two levels. All these values are in the range obtained by chemical analysis (Table 2). In

Table 4 Resume of the results from the pyrolysis analysis (values as % of total chromatogram area)

	Disc 1			Disc 3		Disc 5		
	O. Heart	Heart	Sap	Heart	Sap	Heart	Sap	Bark
Total carbohydrates	54.6	51.1	52.3	49.4	54.0	48.9	52.0	58.9
Total lignin	28.6	27.8	29.7	28.5	28.2	28.2	27.3	20.1
S	22.6	21.3	23.1	21.6	21.7	21.1	20.7	15.4
G	5.9	6.4	6.4	6.9	6.3	7.0	6.5	4.4
H	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.3
Others	0.8	0.7	0.7	0.9	1.0	0.9	0.9	0.9
S/G ratio	3.8	3.4	3.4	3.1	3.4	3.0	3.2	3.5
H:G:S relation	1:20:79	1:22:77	1:21:78	0:24:76	1:22:77	0:25:75	0:24:76	2:21:77

Data from Disc 1: Heartwood values are the mean values of Heart 1 and Heart 2, the same for Sapwood

terms of lignin composition, all tissues showed a prevalence of syringyl (S) over guaiacyl units (G), with a minor presence of *p*-hydroxyphenyl units (H). Consequently, S/G ratios ranged from 3.1 (heartwood) to 3.5 (bark), with slightly lower values recorded in the wood of Disc 5. These findings align with the range of 1.9–5.5 reported for eucalypt stemwood as determined by Py-GC/MS analysis (del Río et al. 2005; Rencoret et al. 2011; Lourenço et al. 2019). Also, when studying *E. globulus* stemwood with 1 month, 18 months, and 9 years, Rencoret et al. (2011) identified more H units like methylphenols and dimethylphenol, particularly in the young wood (9%), decreasing in oldest wood (2%). This is in line with our findings here, where only phenol was identified, maybe because stumps are an older material. During wood maturation, there is an increase of G and S-units being deposited in the cells (Terashima et al. 1986).

Thioacidolysis analysis

Even though pyrolysis is an interesting technique for attaining the lignin monomeric composition of the whole polymer, thioacidolysis can allow the detection of the monolignols that are linked only through labile aryl ether linkages, thereby providing an estimation of the proportion of non-condensed lignin structure (Lapierre 1993). In agreement with pyrolysis data, the β -O-4' linked lignin structures in all stump tissues contained both G and S units with S/G molar ratio ranging from 3.3 to 4.0 (Table 5), whereas H units were at trace levels (data not shown). This result agrees with a previous study reporting an S/G value close to 3.3 in mature wood of *Eucalyptus*. Xiao et al. (2019) found that the lignin of sapwood of *Eucalyptus* displayed increasing S/G ratios of lignin from heartwood to sapwood. Albeit significant, the weak S/G variation shown for the β -O-4' linked lignin structures in stump tissues indicates that sapwood and heartwood reached quite similar maturity in terms of lignin as the S units increased during lignification (Lourenço et al. 2016).

Based on the total recovery yields from thioacidolysis and an average monolignol molecular weight of around 200, the fraction of non-condensed lignin structure was estimated and accounted for 39 to 53% of Klason lignin with significant variation between wood tissues (Lapierre et al. 1993). The lowest frequency was found in the bark tissue, indicating that lignin is more condensed than other tissues. This result thus confirmed that the FTIR analysis showed the lowest CLL ratio in the bark. However, a higher proportion of β -O-4' linkages have been reported in lignin isolated from the wood bark of *Eucalyptus urophylla* $\times$ *E. grandis* (Xiao et al. 2019). A

Table 5 Monolignols quantification after thioacidolysis (as $\mu\text{mol/g}$ Klason lignin $\pm$ STDEV)

Sample	G	S	G + S	S/G
Original heartwood	455.6 $\pm$ 29.9	1 807.3 $\pm$ 107.1	2 262.8 $\pm$ 135.3	4.0 $\pm$ 0.1
Heartwood	557.1 $\pm$ 4.2	2 082.9 $\pm$ 21.2	2 640.1 $\pm$ 25.2	3.7 $\pm$ 0.0
Sapwood	554.6 $\pm$ 17.9	1 832.4 $\pm$ 7.0	2 387.1 $\pm$ 22.1	3.3 $\pm$ 0.1
Bark	432.8 $\pm$ 31.9	1 518.4 $\pm$ 198.4	1 951.2 $\pm$ 230.3	3.5 $\pm$ 0.2

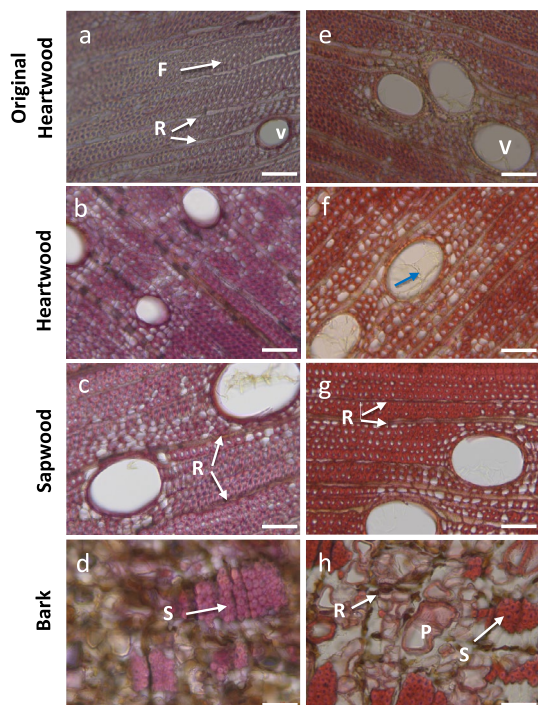
deeper investigation using spectroscopic analysis could allow a more detailed characterization of the bonding pattern of lignin in the different tissues, as shown by 2D NMR analysis of *Eucalyptus* wood and the constitutive tissues (Rencoret et al. 2011; Xiao et al. 2019).

Histochemical analysis

Lignin was visualized in the stump tissues using Mäule and Wiesner staining techniques (Fig. 5). Wiesner staining imparted a red or dark rose in the cell walls rich in lignin cinnamaldehyde groups (Clifford 1974). This staining method unveiled extensive lignification in both heartwood and sapwood, notably in fibers (F) and vessels (V), whereas rays (R) exhibited a darker brown coloration, indicating lower lignification levels (Fig. 5.a–c). Conversely, sclerenchyma (S) showed a deep rose coloration in bark, indicative of lignification, particularly notable in the middle lamella and cell corners (Fig. 5d). However, other cell types, such as parenchyma, showed less pronounced lignification. Obtaining high-quality microscope sections from original heartwood and bark posed challenges due to their stricter texture, necessitating additional effort during sample preparation.

Following Mäule staining (Fig. 5e–h) that gives a purple-red coloration with syringyl unit (Meshitsuka and Nakano 1979), the prevalence of syringyl and guaiacyl lignin types in the cell walls of fibers (F) was evident in original heartwood,

Fig. 5 Lignin visualization in stump tissues using Wiesner (a–d) and Mäule (e–h) staining (magnification 258x). Legend: V vessel; F fibers; FT fibrotracheides; R ray cell; S sclerenchyma; P parenchyma cells; Tylose in blue arrow. Scale bars are 100 µm



heartwood, and sapwood, while vessels (V) and ray cells (R) displayed a G-type lignin, appearing orange to brown as already reported in hardwood vessels (Fergus and Goring 1970; Donaldson 2001). In the bark, sclerenchyma (S) stained red and exhibited lignin of the SG type. Parenchyma cells (P), appearing pink-rose, were at a distinct stage of lignification, while the rays, displaying a brown coloration, were primarily composed of G-lignin. The tyloses observed in vessels of both original and heartwoods (blue arrow) displayed pale staining with Maule reagent. Using an improved Mäule reaction for fluorescence observation, Yamashita et al. (2016) suggested that tyloses differ from vessels in their syringyl proportion.

Image analysis revealed differences between different wood regions in Disc 1 (7 replicates per tissue). Both original heartwood and heartwood had a significantly higher proportion of vessels (7.8 ± 0.3 and 7.2 ± 3.5 vessels/mm² respectively) than sapwood (3.3 ± 0.8 vessels/mm²). Vessels in sapwood were larger ($9.1 \times 10^3 \pm 1.4 \times 10^3 \mu\text{m}^2$) than in original heartwood $5.8 \times 10^3 \pm 0.6 \times 10^3 \mu\text{m}^2$) and in heartwood ($4.8 \times 10^3 \pm 1.4 \times 10^3 \mu\text{m}^2$). This result suggests that stump anatomy is very similar to stemwood, for which different studies have reported that vessel diameter increased from heartwood to sapwood (Longui et al. 2014).

Stump tissues were also observed using fluorescence macroscopy at two different excitation wavelength, 353 nm which can be attributed mainly to lignin fluorescence and 488 nm that may correspond to the presence of phenolic extractives such as flavonoids (Donaldson 2020). All the cell walls showing positive reaction with Wiesner and Maule reagents displayed intense blue and green fluorescence when

Fig. 6 Fluorescence macroscope imaging of stumps tissues at 353 nm (a–d) and 488 nm (e–h). All images were acquired at 1000 ms of exposition, magnification 63x. Scale bars are 500 μm . Intense green fluorescence in rays (arrow)

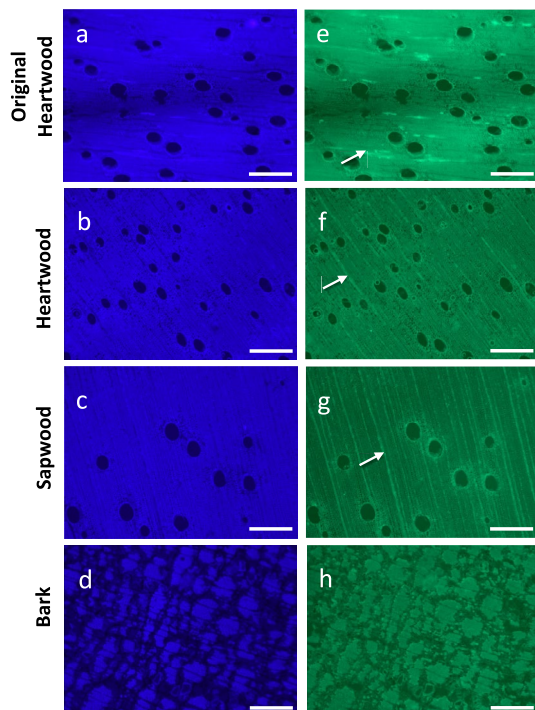


Table 6 Values of the fluorescence emission were determined using the images acquired at 63× magnification. Mean ± STDEV of three to four images. (mean grey value ± STDEV)

63× magnification; 353 nm excitation				
Exposition time (ms)	O. Heartwood	Heartwood	Sapwood	Bark
1000	15.4 ± 0.6	14.2 ± 1.4	12.8 ± 0.4	7.8 ± 0.9
2000	31.4 ± 1.2	28.9 ± 2.9	26.3 ± 0.8	16.0 ± 1.8
3000	43.7 ± 2.1	41.3 ± 3.2	39.4 ± 1.3	24.2 ± 2.8
63× magnification; 488 nm excitation				
500	19.5 ± 4.4	8.6 ± 1.9	10.4 ± 0.6	12.4 ± 2.1
1000	39.7 ± 8.8	17.8 ± 3.6	21.5 ± 1.3	25.1 ± 3.8
1500	56.7 ± 9.5	27.2 ± 5.4	32.9 ± 2.0	37.6 ± 5.2

illuminated with 353 and 488 nm respectively (Fig. 6). In contrast to the blue fluorescence, a higher green fluorescence could be observed in ray cells and vessels cell walls as compared to fibers. This observation is consistent with studies reporting that extractives accumulate in rays and impregnate cell walls (Watanabe et al 2004; Koch and Kleist 2001; Mishra et al 2018). Image analysis allowed quantification of the emission fluorescence, thus enabling us to compare samples (Table 6).

Fluorescence quantification in stump samples was performed at a standard exposure time and similar magnification (three replicates per tissue). The results indicate significantly stronger fluorescence at 488 nm, except for heartwood. For both excitation wavelengths, the highest fluorescence intensity was obtained for the original heartwood, which contains more lignin than heartwood in Disc 1 and almost similar content of phenolic extractives. Lignin would mainly contribute to the blue fluorescence with maximum excitation at UV wavelength. However, it can still have weak fluorescence at 488 nm excitation, and some phenolic extractives also display autofluorescence under 355 nm excitation (Donaldson, 2020). Thus, stump wood autofluorescence can include lignin and extractives (Donaldson, 2019). Nevertheless, the lowest autofluorescence of bark at 355 nm excitation is consistent with the lowest content in lignin and phenolic extractives. Further investigation combining confocal spectral characterization and chemical extraction of extractives would give more detailed information on the distribution of lignin and extractives in stump tissues as reported for *Eucalyptus* stemwood (Speranza et al. 2009). Overall, this data, along with those from histochemical, could provide us a first glance at the presence of lignin and extractives on our samples, even before the chemical analysis.

Conclusion

Eucalypt stumps, a by-product of eucalypt wood exploitation valued primarily for pulp production, represent a significant opportunity for biomass valorization encompassing the waste reduction during the management of this species. In this study, we comprehensively characterized the stump tissues, including original heartwood (from the first tree), heartwood, sapwood, and bark, to uncover their

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Data Availability No datasets were generated or analysed during the current study.

Competing Interest The authors declare no competing interests.

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