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Characterization of Phenolic Components in Polar Extracts of *Eucalyptus globulus* Labill. Bark by High-Performance Liquid Chromatography−Mass Spectrometry

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ABSTRACT: High-performance liquid chromatography−electrospray ionization mass spectrometry (HPLC-ESI-MS) and tandem mass spectrometry (MS^n) were used to investigate the phenolic constituents in methanol, water, and methanol/water extracts of *Eucalyptus globulus* Labill. bark. Twenty-nine phenolic compounds were identified, 16 of them referenced for the first time as constituents of *E. globulus* bark, namely, quinic, dihydroxyphenylacetic, and caffeic acids, bis(hexahydroxydiphenoyl (HHDP))-glucose, galloyl-bis(HHDP)-glucose, galloyl-HHDP-glucose, isorhamentin-hexoside, quercetin-hexoside, methylellagic acid (EA)−pentose conjugate, myricetin-rhamnoside, isorhamnetin-rhamnoside, mearnsetin, phloridzin, mearnsetin-hexoside, luteolin, and a proanthocyanidin B-type dimer. Digalloylglucose was identified as the major compound in the methanol and methanol/water extracts, followed by isorhamnetin-rhamnoside in the methanol extract and by catechin in the methanol/water extract, whereas in the water extract catechin and galloyl- HHDP-glucose were identified as the predominant components. The methanol/water extract was shown be the most efficient to isolate phenolic compounds identified in *E. globulus* bark.

KEYWORDS: *Eucalyptus globulus* Labill., bark, phenolic compounds, flavonoids, HPLC-ESI-MS, tandem mass spectrometry

INTRODUCTION

Eucalyptus globulus Labill. is one of the main wood species produced in Portugal; it ranks third in terms of Portuguese forest area (about 672,000 ha), representing about 31% of the world production of *E. globulus*, and it is the main raw material for pulp and paper production in Portugal and Spain.[1] The pulp industries generate substantial amounts of biomass residues, among which bark is the most abundant and is currently simply burned to produce energy. In the case of *E. globulus*, bark represents about 11% of the stem dry weight.[2] Thus, a pulp mill with a production capacity of 5.0×10^5 tons/year of bleached kraft pulp can generate around 1.0×10^5 tons/year of bark, showing the enormous potential for the upgrading of this biomass residue. Therefore, detailed study of its chemical composition is a key step toward the implementation of strategies for the recovery of valuable components from this biomass residue. Moreover, this strategy is perfectly in tune with the emerging biorefinery concept,[3] which has been attracting increasing interest in recent years, from the perspective of promoting the integrated exploitation of agro-forest biomass resources in the search for new alternatives to petrochemical-derived products.

In recent years, we have demonstrated[4,5] that the lipophilic fraction of *E. globulus* bark (and particularly its outer fraction) is quite rich in high-value triterpenic acids such as ursolic and oleanolic acids (up to 25 g/kg). However, bark is also known to be a promising source of phenolic compounds. In fact, several studies have already addressed the phenolic composition of wood and barks from several *Eucalyptus* species. Methyl and glycosyl derivatives of ellagic acid and free ellagic and gallic acids have already been reported in methanolic extracts from the bark of *Eucalyptus regnans* and *E. globulus*.[6] Fechtal and Riedl[7] have also reported the presence of gallic and ellagic acid derivatives and catechin in extracts obtained after acid hydrolysis of bark from four *Eucalyptus* species. Conde[8] and Cadahía[9] detected gallic and ellagic acids, vanillin, syringaldehyde, sinapaldehyde, and quercetin in methanolic extracts from *E. globulus* wood and gallic, protocatechuic, vanillic, and ellagic acids, protocatechuic aldehyde, taxifolin, eriodictyol, quercetin, and naringenin in the corresponding bark. More recently, Vázquez[10] identified some phenolic compounds from an aqueous extract of *E. globulus* bark, including ellagic acid, galloylglucose derivatives, and flavonoids and reported also their potential as natural antioxidants. Besides this well-known property of phenolic compounds, their interest is also based in a wide variety of other valuable properties, namely, anti-inflammatory, antithrombotic, antimicrobial, and antibacterial capacities,[11] among others.

The identification of phenolic compounds in vegetal matrices is a relatively complex task due to the wide variety of structures that can be found. Flavonoid glycosides are predominant forms of secondary metabolites in plants, in which the flavonoid moiety can be bound to up to five different sugar moieties, either through phenolic −OH groups in the case of *O*-glycosides or directly to carbon atoms in ring A of the flavonoid moiety in *C*-glycosides.[12] Other groups of well-known plant phenolics are hydrolyzable tannins, containing both galloylglucose derivatives and ellagitannins.[13] Analysis of these compounds is usually carried out by using high-performance liquid chromatography, although gas chromatography and capillary electrophoresis have also been employed.[13] All of these techniques are coupled to different detection systems, but mostly with mass spectrometry, which provides valuable

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Table 1. Calibration Data Used for the HPLC-UV Semiquantification of Phenolic Components of *E. globulus* Bark Extracts

compound name	λ (nm)	concn range (mg mL^{-1})	calibration curve[a]	r^2	LOD[b] (mg mL^{-1})	LOQ[c] (mg mL^{-1})
gallic acid	280	0.01−0.60	$y = 535985x + 1092$	0.999	0.021	0.069
protocatechuic acid	280	0.05−1.20	$y = 527991x - 5289$	1.000	0.029	0.096
catechin	280	0.01−1.20	$y = 100865x + 2057$	1.000	0.028	0.092
chlorogenic acid	280	0.01−0.80	$y = 229162x - 1450$	0.999	0.029	0.097
caffeic acid	280	0.01−0.33	$y = 992484x - 1804$	0.997	0.023	0.075
ellagic acid	340	0.01−0.44	$y = 300168x + 3045$	0.998	0.023	0.076
quercetin	340	0.01−0.33	$y = 619494x + 2454$	0.999	0.014	0.046
naringenin	280	0.01−0.28	$y = 722267x + 1939$	0.998	0.016	0.052

[a] y = peak area, x = concentration in mg mL^{-1}. [b] LOD, limit of detection. [c] LOQ, limit of quantification.

structural information about the eluted compounds, especially when tandem mass spectrometry techniques are available and even when coelution might occur.

Due to the importance of phenolic compounds, as well as the interest in their identification and quantification in *E. globulus* bark, the present study reports the detailed characterization of the phenolic fraction of *E. globulus* methanol, methanol/water, and water bark extracts, taking advantage of the use of MS/MS (obtained in a triple quadrupole) and MS^n (acquired in an ion trap mass spectrometer).

MATERIALS AND METHODS

Chemicals. Dichloromethane (99% purity), gallic acid (purity > 97.5%), quercetin (purity > 98%), and luteolin (purity > 98%) were supplied by Sigma Chemical Co (Madrid, Spain). Protocatechuic acid (purity > 97%), chlorogenic acid (purity > 95%), caffeic acid (purity > 95%), and naringenin (98% purity) were obtained from Aldrich Chemical Co. (Madrid, Spain). HPLC-grade methanol, water, and acetonitrile were supplied from Fisher Scientific Chemicals (Loures, Portugal). Formic acid (purity > 98%), methanol (purity > 99.8%), catechin (purity > 96%), and ellagic acid (96% purity) were purchased from Fluka Chemie (Madrid, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA).

Materials. *E. globulus* bark samples were taken from 16-year-old *E. globulus* trees randomly harvested from a clone plantation cultivated by RAIZ − Forest and paper Research Institute in the Eixo (40° 37′ 13.56″ N, 8° 34′ 08.43″ W) region of Aveiro, Portugal.

Sample Preparation. *E. globulus* bark samples were air-dried until a constant weight was achieved and ground to granulometry lower than 2 mm prior to extraction. About 45 g of dried bark was submitted to a Soxhlet extraction with dichloromethane for 6 h to remove the lipophilic components.[4,5] Then, the solid bark residue was divided in two fractions (I and II), which followed two distinct extraction pathways. Fraction I was submitted to methanol (MeOH) extraction (m/v 1:100) for 24 h under constant stirring, followed by an extraction with water (m/v 1:100) for 24 h both at room temperature. Methanol was then removed from the liquid extracts by low-pressure evaporation, and the residues/aqueous solutions were freeze-dried. Fraction II was suspended (m/v 1:100) in a methanol/water ($MeOH/H_2O$) mixture, 50:50 (v/v) at room temperature for 24 h under constant stirring. The suspension was then filtered, MeOH removed by low-pressure evaporation, and the extract freeze-dried.

Total Phenolic Content. The total phenolic content (TPC) of the extracts was determined by the Folin−Ciocalteu method.[14,15] Two and a half milliliters of Folin−Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 mL of aqueous sodium carbonate (75 g/L) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of water for the H_2O extract and in methanol for the others, corresponding to concentration ranges between 35 and 500 μg of extract/mL. Each mixture was kept for 5 min at 50 °C and, after cooling, the absorbance was measured at 760 nm, using a UV−vis V-530 spectrophotometer (Jasco, Tokyo, Japan). The TPC was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (1.5 −60.0 μg/mL) and expressed as mg of gallic acid equivalent (GAE)/g of dry extract. The analyses were carried using three aliquots of each extract, measured in triplicate, and the average value was calculated in each case.

HPLC-UV analyses were carried in a Hewlett-Packard (HP) 1050 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Rheodyne injector with a 10 μL loop, a quaternary pumping system, and a UV detector. The column used was a Discovery C-18 (15 cm × 2.1 mm × 5 μm) supplied by Supelco (Agilent Technologies). The separation of the compounds from *E. globulus* bark extracts was carried out at room temperature with a gradient elution program at a flow rate of 0.2 mL min^{-1}. The mobile phases consisted of water/acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0−3 min, 0% B; 3−10 min, 0−10% B; 10−30 min, 10−20% B; 30−35 min, 20−25% B; 35−50 min, 25−50% B; 50−60 min, 50−0% B; followed by re-equilibration of the column for 10 min before the next run. The injection volume in the HPLC system was 25 μL, and UV−vis detection was performed at 280 and 340 nm. Before the injection in the HPLC, each extract was dissolved in the same solvent used for extraction (HPLC grade), to obtain a final concentration of about 10 mg mL^{-1}, and then filtered through a 0.2 μm PTFE syringe filter.

ESI-QqQ-MS Analysis. The HPLC system was coupled to a Micromass spectrometer (Manchester, U.K.), operating in negative mode, equipped with an electrospray source and a triple-quadrupole (QqQ-MS) analyzer. The cone and capillary voltages were set at −30.0 V and −2.6 kV, respectively. The source temperature was 143 °C, and the desolvation temperature was 350 °C. MS/MS spectra were obtained using argon as collision gas, with the collision energy set between 10 and 45 V. Detection was carried out considering a mass range of m/z 50−1000, with a scan duration of 0.5 s. Data acquisition was performed using the MassLynx data system (Waters, Milford, MA).

ESI-IT-MS-MS Analysis. To gather additional MS information about several chromatographic peaks, these were manually collected following the conditions discussed above. The ensuing HPLC fractions were dissolved in methanol and directly injected into a Linear Ion Trap LXQ (ThermoFinnigan, San Jose, CA), also equipped with an ESI interface by means of a syringe pump, at flow rate of 8 μL min^{-1}. Optimal ESI conditions were as follows: nitrogen sheath gas, 30 psi; spray voltage, 4.7 kV; capillary temperature, 275 °C; capillary voltage, −7.0 V' and tube lens voltage, −71.8 V. CID-MS/MS and MS^n experiments were performed on mass-selected precursor ions using standard isolation and excitation configurations. The collision energy used was in the range of 15−40 (arbitrary units). Data acquisition was carried out with the Xcalibur data system (ThermoFinnigan).

Table 2. Phenolic Compounds Identified in *E. globulus* Bark Extracts and Corresponding MS/MS and MS^n Fragmentation Profiles

					IT-MS^n					
compd no.	t_R (min)	compd name	$[M-H]^-$ (m/z)	QqQ-MS/MS product ions (m/z)	MS^2 ions (m/z)	MS^3 ions (m/z)	MS^4 ions (m/z)	MS^5 ions (m/z)	identification	presence in *Eucalyptus* species[d]
1	3.2	quinic acid[a]	191	173, 111, 87, 85	173, 149, 127, 111, 93, 85				19	RFE
2	3.8	gallic acid	169	125					Co[b]	Egl b, w, l, f, Eca Eru l, Ere b, w [29]
3	5.1	dihydroxyphenylacetic acid[a]	167	123					17	RFE
4	5.7	protocatechuic acid	153	109					Co	Egl b (8) Egl Eca Eru b,[32] l [34]
5	10.1	bis(hexahydroxydiphenoyl)-glucose[a]	783		481,[c] 301	301, 275			29	Egl f, Eni w, Eco Evi l [29]
6	11.2	methyl gallate	183	168, 124					18	Egl b [35]
7	12.4	catechin	289	245, 205, 203, 125, 109					Co	Egl l,[11] b,[10,35] w (6), Ere b, w (6)
8	13.2	chlorogenic acid	353	191					Co	Egl b, w (6) Ecy l [36]
9	13.9	galloyl-bis(hexahydroxydiphenoyl)-glucose [a]	935	935, 633, 301, 300, 275	**633**	481, 299, 275			29, 37	Egl f, Eni w Eal f [29]
10	14.4	galloyl-hexahydroxydiphenoyl-glucose[a]	633	301	615, 481, 463, 421, **301**, 275	257, 229			29	Egl f, Eni w Eco l [29]
11	14.9	caffeic acid[a]	179	135					Co	Ehy l [38]
12	15.6	digalloylglucose	483	**313**, 169	331, 327, 313, 169	271, 211, 193, 169, 125			29	Egl b (10), Egl f, Eni w Eco l [29]
13	19.3	isorhamnetin-hexoside[a]	477	**315**	315	**300**	271, 272, 244		26	RFE
14	19.8	ellagic acid	301	229, 185, 173, 157, 146					Co	Esp b, f, l, w [29]
15	20.7	taxifolin	303	285, 177, 151, 125					23	Egl b [8,32,35]
16	21.7	quercetin-hexoside[a]	463	301, 300	**301**, 300	179, 151			27	Egl f, l, Eca Eru l Egu h [29]
17	22.9	methylellagic acid-pentose[a]	447	315	**315**	**300**			29	Egl f [29]
18	23.8	myricetin-rhamnoside[a]	463	317					24	RFE
19	24.9	isorhamnetin-rhamnoside[a]	461	315	**315**	**300**	272, 244		39	RFE
20	26.6	aromadendrin-rhamnoside	433	287, 269, 259, 180, 179, 151	**287**, 269	**259**	241, 215, 125		23	Egl b [35]
21	28.4	mearnsetin[a]	331	316	**316**	**287**, 271	259		25	RFE
22	29.4	phloridzin[a]	435	273	**273**	**167**	123		28	RFE
23	30.9	mearnsetin-hexoside[a]	493	331	**331**	**316**	**287**, 271, 244	259	25	RFE
24	33.1	eriodictyol	287	151, 135, 107					23	Egl b [8,35]
25	33.8	B-type proanthocyanidin dimer[a]	577		451, 425, 407, **289**, 287	245, 205			22−24	RFE
26	34.8	luteolin[a]	285	175, 151, 133					Co	Egl Eca l,[34] Eca b [32]
27	35.2	quercetin	301	179, 165, 151, 121, 107					Co	Egl b [8,35]
28	37.7	isorhamnetin	315	300, 271					26	Egl b [8,10]
29	40.5	naringenin	271	177, 151, 119, 107					Co	Eca b,[32] Eru l [34]

[a] Compounds identified for the first time in *E. globulus* bark. [b] Co, co-injection of the authentic standard. [c] m/z in bold was subjected to MS^n analysis; [d] Egl, *E. globulus*; Eca, *E. camaldulensis*; Eru, *E. rudis*; Ere, *E. regnans*; Eni, *E. niteus*; Eco, *E. consideniana*; Evi, *E. viminalis*; Ecy, *E. cypellocarpa*; Egu, *E. gunnii*; Eal, *E. alba*; Ehy, *Eucalyptus* hybrids; Esp, *Eucalyptus* species; b, bark; l, leaves; f, fruits; w, wood; h, hook; RFE, reported for the first time in *Eucalyptus* species.

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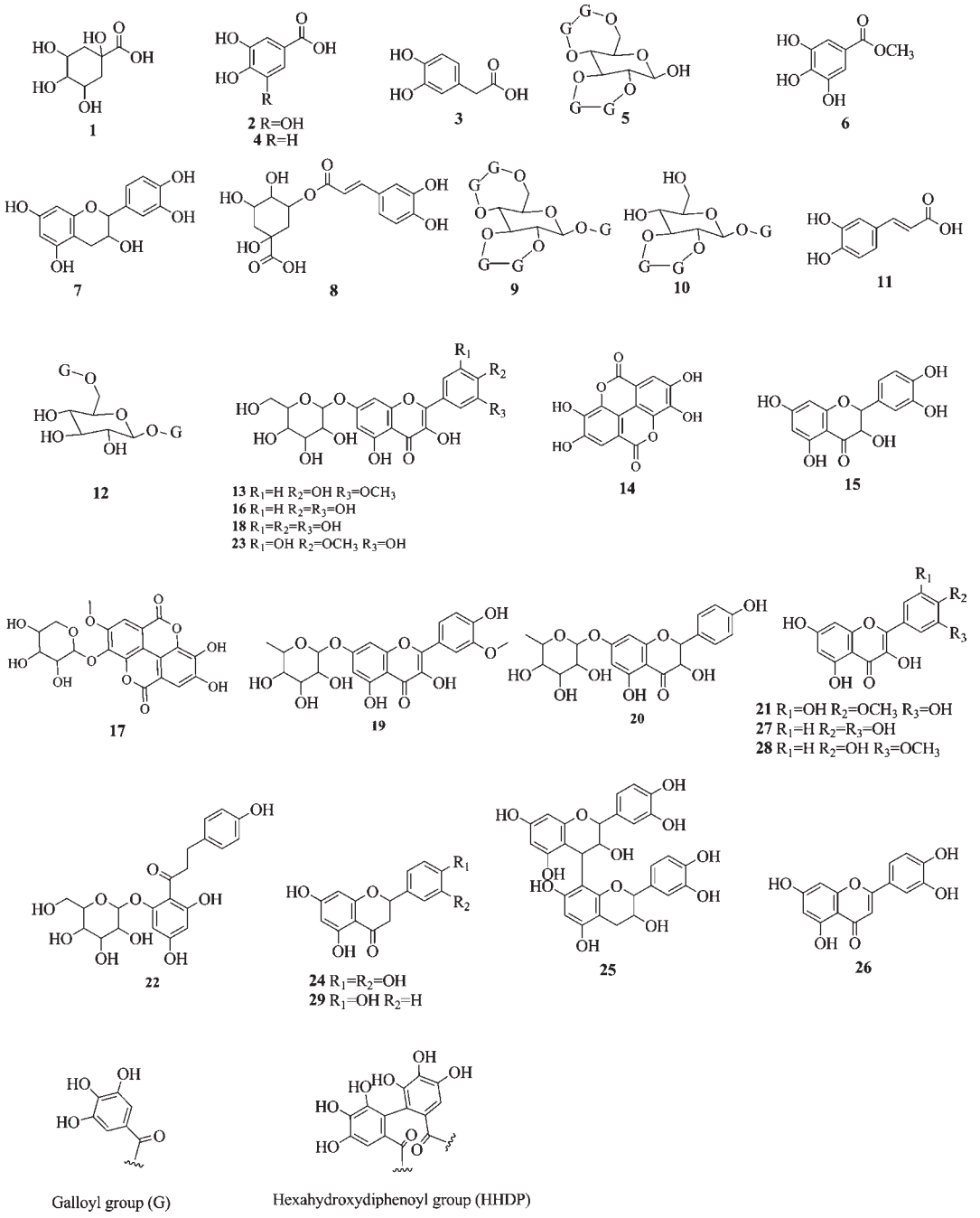

Figure 1. Structures of the phenolic compounds identified in *E. globulus* bark extracts. G, galloyl group; G-G, hexahydroxydiphenoyl (HHDP) group.

HPLC-UV Quantification. HPLC-UV calibration curves were obtained by injection of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, quercetin, and naringenin standard solutions in MeOH, with five different concentrations between 0.01 and 1.20 mg mL^{-1}. The relevant data for obtaining the calibration curves is shown in Table 1. Quantification of individual compounds (Table 3) was obtained using the calibration data of the most similar standard. Three aliquots of the extract were injected in triplicate, and compound concentrations were the average value calculated in each case.

RESULTS AND DISCUSSION

Extraction Yields and Total Phenolic Content. The extraction yield of *E. globulus* bark obtained with MeOH/H_2O (9.28%) is lower than the sum of the extraction yields for MeOH (8.24%) and water (1.93%). The MeOH and MeOH/H_2O extraction yield values reported here are considerably higher than those recently published for *E. globulus* bark extracts;[16] however, the extraction conditions applied were different, including the temperature, time of extraction, and solid/liquid ratio. The total phenolic contents of the three extracts of *E. globulus* bark, determined by Folin−Ciocalteu method, accounted for 115.3 ± 0.50, 409.7 ± 2.76, and 413.8 ± 5.27 mg GAE g^{-1} in water, MeOH, and MeOH/H_2O extracts, respectively, demonstrating that MeOH and MeOH/H_2O extracts have similar TPCs and that, jointly, in the sequential extraction with MeOH followed by water, this last solvent adds 20% more TPC in comparison to the single-step

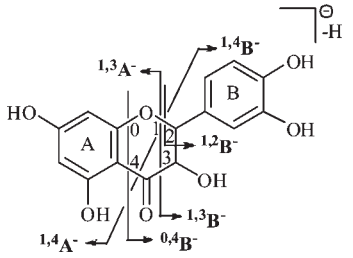

Figure 2. Fragmentation nomenclature for $[M - H]^-$ flavonoids (adapted from refs 20 and 21). The superscripts on the left of the A or B ring indicate the broken C-ring bonds.

extraction with MeOH/H_2O. Finally, the reported phenolic contents are in the range of those previously reported for *E. globulus* bark extracts.[16]

Identification of Phenolic Compounds. The identification of the components of the MeOH, H_2O, and MeOH/H_2O extracts was carried out by HPLC-UV, HPLC-MS/MS, and, in some cases, MS^n using distinct equipment, as described above. Table 2 summarizes the phenolic compounds characterized in each extract, their retention time, the molecular ion $[M - H]^-$, and the main product ions obtained by HPLC-MS/MS and in some cases by MS^n. Compounds were identified by comparing their fragmentation profiles with reference compounds run under the same experimental conditions, or, when standards were not available, their identifications were corroborated with the literature as indicated in Table 2 and discussed bellow.

Phenolic Acids and Esters. Compounds **1**, **3**, and **6** were identified as quinic acid, dihydroxyphenylacetic, and methyl gallate (Figure 1), by comparing their MS/MS profiles with published data.[17−19] Compounds **2**, **4**, **8**, and **11** were identified as gallic, protocatechuic, chlorogenic, and caffeic acids, respectively (Figure 1), by comparing their retention times and fragmentation pathways observed in the MS/MS spectra with those of the corresponding reference compounds.

Flavonoids. Flavonoid fragmentation pathways are recognized by the typical retro-Diels−Alder fissions,[20] the main product ions of which observed in this study are shown in Figure 2, using the nomenclature adapted from that proposed by Ma et al.[21]

Flavanols: Compound **7** corresponds to catechin, as confirmed by co-injection with standard.

Compound **25** was identified as a B-type proanthocyanidin dimer (Figure 1), on the basis of the MS/MS product ions, characteristic of the retro-Diels−Alder fission,[22] and also the two product ions at m/z 289 and 287, which correspond to the two flavanol monomeric units.[23,24] Furthermore, the MS^3 of the ion at m/z 289 generates the characteristic product ions of catechin.

Flavonols: Compounds **21**, **27**, and **28** were identified as mearnsetin, quercetin, and isorhamnetin, respectively, on the basis of the matching of the retention time as well as the fragmentation pattern with those of an authentic sample for compound **27** and by comparison with published data for compounds **21**[25] and **28**.[26]

Flavones: Compound **26** was identified as luteolin (Figure 1), on the basis of its $[M - H]^-$ and MS/MS product ions, which matched those of an authentic sample.

Flavanones: Compounds **15** and **24** were identified as taxifolin and eriodictyol, respectively, on the basis of their characteristic $[M - H]^-$ and MS/MS product ions,[23] whereas compound **29** was identified as naringenin after comparison of its $[M - H]^-$ and MS/MS product ions and retention time with those of an authentic standard.

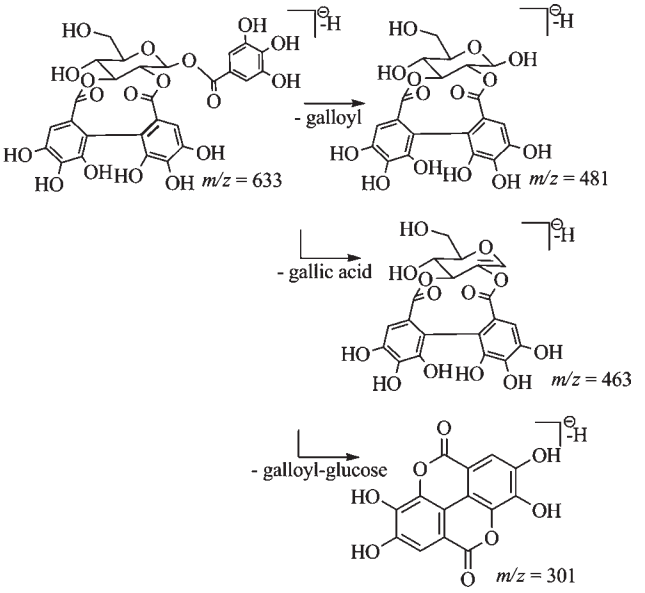

Figure 3. Fragmentation pathway of an isomer of galloyl-HHDP-glucose **10**.[29]

Flavonoid Glycosides. Compounds **13**, **16**, **18**, **19**, **20**, **22**, and **23** were identified as isorhamentin-hexoside, quercetin-hexoside, myricetin-rhamnoside, aromadendrin-rhamnoside, phloridzin, and mearnsetin-hexoside (Figure 1), respectively, on the basis of their characteristic $[M - H]^-$ and MS^n fragmentation profiles,[23−28] including the detection of the corresponding aglycone ions and typical fragmentations, as discussed above. In the case of compounds **13**, **16**, and **23**, the hexose moiety could be a glucose or galactose unit.

Although some authors reported the possibility of differentiating flavonoid glycoside positional isomers on the basis of mass spectrometry data,[12,27] such differentiation was not unambiguously possible in the present study. However, the most typical 7-*O*-glycosidic unit linkage[12] was assumed for the structures presented in Figure 1.

Ellagic Acid and Derivatives. Compound **14** was identified as ellagic acid (EA) after comparison of its retention time and fragmentation pathways with those of an authentic standard.

Compound **17** shows an $[M - H]^-$ ion at m/z 447, which, on the basis of the molecular weight, could be either a methyl-EA-pentose conjugate, an EA-rhamnose, or an isorhamnetin-pentose. However, the presence in the MS^2 and MS^3 spectra of the ions at m/z 315 (−132 Da, −pentose) [29] and at m/z 300 (−15 Da, $-CH_3$), respectively, allow the identification of this compound as methyl-EA-pentose conjugate (Figure 1). Thus, the identification is also confirmed by the absence of the product ions at m/z 271 (which excludes the isorhamnetin algycone) and at m/z 229 and 185 (which exclude ellagic acid).

Galloylglucose Derivatives and Ellagitannins. Compound **12** was assigned to digalloylglucose isomer, with $[M - H]^-$ at m/z 483 showing product ions at m/z 313 (loss of a galloyl moiety) and at m/z 169 (gallic acid deprotonated ion). In this case, as well as for compound **10**, one of the galloyl units should be linked to C-1, as only a single HPLC peak is observed.[30]

Compound **5** showed the characteristic fragmentation pathway of an ellagitannin with a $[M - H]^-$ at m/z 783m and the MS^2 spectrum showed ions at m/z 481 (loss of HHDP) and at m/z 301 (loss of HHDP-glucose). This allows the compound to be

Table 3. Abundance of Phenolic Components Identified in *E. globulus* Bark Extracts[a]

			phenolic content (mg g^{-1} of extract)			phenolic content (mg kg^{-1} of bark)		
compd no.	compd name	λ (nm)	MeOH	H_2O	MeOH/H_2O	MeOH	H_2O	MeOH/H_2O
1	quinic acid[b]	280	1.46	2.47	1.50	120.34	47.64	139.51
2	gallic acid[b]	280	3.41	3.52	8.83	280.84	67.86	819.48
3	dihydroxyphenylacetic acid[b]	280		0.38			7.29	
4	protocatechuic acid[c]	280	1.62	2.80	2.09	133.10	53.96	194.14
5	bis(hexahydroxydiphenoyl)-glucose[b]	280	0.68	0.83	1.02	56.35	16.06	94.30
6	methyl gallate[b]	280	0.68	2.43	1.50	56.18	46.95	139.25
7	catechin[d]	280	6.57	15.94	14.23	541.56	307.75	1320.59
8	chlorogenic acid[e]	280	5.98	5.24	13.36	492.92	101.04	1239.46
9	galloyl-bis(hexahydroxydiphenoyl)-glucose[b]	280	7.23	4.99	4.85	595.89	96.34	450.37
10	galloyl-hexahydroxydiphenoyl-glucose[b]	280	9.27	9.04	6.86	764.15	174.42	637.04
11	caffeic acid[f]	280		5.03			97.02	
12	digalloylglucose[b]	280	17.95	6.35	17.77	1479.42	122.64	1648.91
13	isorhamnetin-hexoside[g]	340	1.53	tr[j]	1.08	126.38	tr	99.89
14	ellagic acid[h]	340	4.95	tr	5.08	407.99	tr	471.04
15	taxifolin[i]	280	1.48		7.78	121.66		721.58
16	quercetin-hexoside[g]	340	0.15		0.63	12.16		58.77
17	methylellagic acid-pentose[h]	340	tr		tr	tr		tr
18	myricetin-rhamnoside[g]	340	0.20		0.14	16.41		13.31
19	isorhamnetin-rhamnoside[g]	340	9.79	0.17	10.00	806.57	3.29	927.64
20	aromadendrin-rhamnoside[i]	280	tr		0.79	tr		73.52
21	mearnsetin[g]	340	0.34		0.38	27.76		35.30
22	phloridzin[i]	280	tr		0.75	tr		69.89
23	mearnsetin-hexoside[g]	340	1.07		1.30	88.10		121.12
24	eriodictyol[i]	280	6.90[k]	tr	7.91[k]	568.53[k]	tr	733.86[k]
25	B-type proanthocyanidin dimer[i]	280						
26	luteolin[g]	340	2.31[k]		3.66[k]	190.08[k]		340.00[k]
27	quercetin[g]	340						
28	isorhamnetin[g]	340	3.98		4.65	327.80		431.63
29	naringenin[i]	280	0.79		0.76	65.17		70.92
	total (mg g^{-1} of extract/mg kg^{-1} of bark)		88.34	59.18	116.93	7279.34	1142.26	10851.52

[a] Results correspond to the average value estimated from the injection of three aliquots analyzed in triplicate (standard deviation <5%). [b] Calibrations curve used: gallic acid. [c] Calibration curve used: protocatechuic acid. [d] Calibration curve used: catechin. [e] Calibration curve used: chlorogenic acid. [f] Calibration curve used: caffeic acid. [g] Calibration curve used: quercetin. [h] Calibration curve used: ellagic acid. [i] Calibration curve used: naringenin. [j] tr, traces. [k] Sum of the phenolic content by partial overlapping.

identified as bis(HHDP)-glucose.[29] Identification was corroborated by the MS^3 spectrum (481 → 301), confirming the loss of a glucose unit from this precursor ion.

Compound **9** was identified also as an ellagitannin, a galloyl-bis(HHDP)-glucose, because its $[M - H]^-$ ion at m/z 935 is typical for ellagitannins with this structure. Furthermore, the MS/MS spectrum shows the ions at m/z 633 (−302 Da, −HHDP) and at m/z 301 $[HHDP - H]^-$, due to the loss of the HHDP and galloyl and glucose units. This identification was corroborated by the MS^3 (633 → 481), obtained in the ion trap, which shows the loss of a galloyl group.[29]

Compound 10 was presumed to be an isomer of galloyl-HHDP-glucose (Figure 1). The fragmentation of the molecular ion $[M - H]^-$ at m/z 633 yields the product ions at m/z 481, due to the loss of the galloyl group, and at m/z 301, corresponding to the HHDP unit after lactonization to ellagic acid.[29] The fragmentation pathway of a galloyl-HHDP-glucose isomer is illustrated in the Figure 3.

To the best of our knowledge a total of 16 compounds are reported here for the first time as *E. globulus* bark components, namely, quinic **1**, dihydroxyphenylacetic **3** and caffeic **11** acids, bis(HHDP)-glucose **5**, galloyl-bis(HHDP)-glucose **9**, galloyl-HHDP-glucose **10**, isorhamentin-hexoside **13**, quercetin-hexoside **16**, methyl-EA-pentose conjugate **17**, myricetin-rhamnoside **18**, isorhamnetin-rhamnoside **19** mearnsetin **21**, phloridzin **22**, mearnsetin-hexoside **23**, luteolin **26**, and a proanthocyanidin B-type dimer **25**. Although some of these compounds, namely, bis(HHDP)-glucose **5**, galloyl-bis(HHDP)-glucose **9**, galloyl-HHDP-glucose **10**, quercetin-hexoside **16**, and methyl-EA-pentose conjugate **17** have already been reported as constituents of other morphological parts of *E. globulus*, such as the leaves and fruits,[29] no reference has been made to the bark. Furthermore, proanthocyanidins were already referenced as constituents of *E. globulus* bark;[9] however, B-type dimers have never been reported. In addition, Kim[31] and Yazaki[6] identified six ellagic acid derivatives in *E. globulus* bark, but none of them as a methyl-EA-pentose conjugate.

Among the phenolic compounds identified for the first time in *E. globulus* bark, eight are reported for the first time as components of the *Eucalyptus* genus (Table 2), namely, quinic acid **1**, isorhamnetin-hexoside **13**, myricetin-rhamnoside **18**, isorhamnetin-rhamnoside **19**, mearnsetin **21**, phloridzin **22**, mearnsetin-hexoside **23**, and a B-type proanthocyanidin dimer **25**.

Finally, to our knowledge, the detailed phenolic composition of the MeOH/H_2O (50:50) extract of *E. globulus* bark is reported here for the first time, although its total phenolic content and antioxidant activity have been previously reported.[16]

HPLC Quantification of Phenolic Compounds. The phenolic content of each extract quantified by HPLC is shown in Table 3, expressed in mg/g of extract and in mg/kg of bark. In relation to the extracts composition, the single MeOH/H_2O extraction step shows abundances of the identified compounds in the extracts globally higher than those obtained with MeOH, but lower than the total obtained in the sequential extraction with MeOH followed by water. However, the total amount of identified compounds per kilogra, of bark is clearly higher in the case of the MeOH/H_2O extract (~10.9 g/kg) when compared to the sum of the other two (~8.4 g/kg), demonstrating clearly the advantage of this single-step extraction.

This study showed that digalloylglucose **12** is the main compound in the MeOH and MeOH/H_2O extracts, with values of 17.95 and 17.77 mg $g^{-1}$of extract, respectively, followed by isorhmanetin-rhamnoside **19** (9.79 mg g^{-1} of extract) and galloyl-HHDP-glucose **10** (9.27 mg g^{-1} of extract) in the MeOH extract and by catechin **7** (14.23 mg g^{-1} of extract) and chlorogenic acid **8** (13.36 mg g^{-1} of extract) in the MeOH/H_2O extract. It should be highlighted that the same compounds were detected in both MeOH and MeOH/H_2O extracts.

The water extract was found to have considerably lower amounts of phenolic compounds, with catechin **7** (15.94 mg g^{-1} of extract), galloyl-hexahydroxydiphenoyl-glucose **10** (9.04 mg g^{-1} of extract), and digalloylglucose **12** (6.35 mg g^{-1} of extract) as the major components. These results allow verification of the MeOH/H_2O extract as showing the most promise to extract phenolic compounds from *E. globulus* bark as, in general, it allows in a single step higher extraction yields of identified compounds per mass of bark.

In contrast with the results obtained, ellagic acid has been previously reported as the major component on the ethyl ether extracts after MeOH/H_2O (80:20) extraction of *E. globulus* bark,[8,32] however, in lower quantities than detected in this study for MeOH and MeOH/H_2O (~5 mg g^{-1} of extract). In fact, the abundance of this compound is considerably lower than those mentioned above for the more abundant compounds detected. These quantitative differences in chemical composition could be derived from the well-known variability of *E. globulus* extractives composition[9,33] with geographic origin, age of the tree, and part of the tree from which the bark was collected, among others, and also the differences in extraction procedures and analytical methodology.

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