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Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of *Eucalyptus globulus* Cultivated in Algeria

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A method based on high-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) following fractionation by chromatography on a Sephadex LH-20 column has been developed to determine the phenolic composition of fruit of *Eucalyptus globulus* growing in Algeria. The presence of 18 gallotannins, 26 ellagitannins, and 2 flavonols was established. Tentative identification is provided for these compounds on the basis of UV−visible spectra and mass spectrometry data. Most compounds described in this study have not previously detected in fruit of *E. globulus*. Moreover, this is the first report of methyl digalloyl diglucose, 3,3′-*O*-dimethylellagic acid 4-*O*-β-glucopyranoside, ellagic acid hexose, methyl ellagic acid pentose, methyltetragalloylglucose, and valoneic acid isomers (sanguisorbic, flavogallic acid dilactone) in the genus *Eucalyptus*. Quantitatively, ellagic acid and its derivatives, including ellagitannins, are largely predominant.

KEYWORDS: *Eucalyptus globulus* fruit; phenolic compounds; Sephadex LH-20 column; HPLC-DAD; HPLC-ESI-MS

INTRODUCTION

The genus *Eucalyptus* is indigenous to Australia, comprises more than 523 species and 138 varieties, and new species and varieties are still being described (*1*). Among all of these species, *Eucalyptus globulus* is the most widely cultivated in subtropical and Mediterranean regions (*2*). It was introduced in Algeria in 1854 by Ramel. There are extensive eucalypt plantations in Algeria: 30000 ha in 1990 and 39000 ha in 1995 (*3*). Its leaves, roots, and fruit have been used as traditional remedies for treatment of various diseases such as pulmonary tuberculosis (*4*), influenza (*5*, *6*), asthma (*7*), and diabetes (*8*−*10*). *E. globulus* is well-known for the volatile terpenoid constituents of its essential oil (*5*, *7*, *11*−*13*), and the phenolic compounds in its leaves and wood have been widely studied (*6*,*8*,*11*,*14*−*17*). According to the published works, the major phenolic compounds of this plant are hydroxybenzoic acids (vanillic, gallic, protocatechuic, ellagic, and gentisic acids) (*14*−*16*, *18*−*20*); hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric, and chlorogenic acids) (*18*, *16*, *20*); flavonols (kaempferol, quercetin, quercetin 3-*O*-rhamnoside, and quercetin 3-*O*-rutinoside) (*14*−*16*); a methyl flavone (naringenin) (*7*, *15*, *11*); a flavan-3-ol ((+)-catechin) (*5*); flavones (luteolin and apigenin) (*15*); hydrolyzable tannins (tellimagrandin, eucalbanin C, eucaglobulin, and pentagalloylglucose) (*5*, *14*, *15*); and condensed tannins (proanthocyanidins) (*14*, *19*−*21*). Phytochemical analysis has established that *E. globulus* contains monoterpene glycosides conjugated with gallic acid (*6*, *5*) and phloroglucinol−sesquiterpene-coupled compounds (*5*). Up to now, little has been done on the phenolic composition of the fruit of this plant. It has been reported to contain gallic acid (*22*, *23*), cypellocarpin C, macrocarpal A, macrocarpal B (*23*), and other ellagic acid derivatives (3,4,3′-*O*-trimethylellagic acid, 3-*O*-methylellagic acid 4′-*O*-(2″-*O*-acetyl)-α-L-rhamnopyranoside, 3-*O*-methylellagic acid, 4′-*O*-α-L-rhamnopyranoside, and 3-*O*-methylellagic acid) (*23*).

Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with diode array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) is frequently used for the separation, detection, and characterization of phenolic compounds from plants (*24*). UV−visible spectra allow identification of the class of compound, whereas ESI-MS data are useful for the structural determination of phenolic compounds (*24*). ESI is a gentle ionization method in MS, generating mainly deprotonated molecules $[M - H]^-$ of the compounds analyzed, when used in the negative ion mode (*25*). Further structural confirmation can be accomplished by tandem mass spectrometry carried out with an ion-trap (IT) mass analyzer, through analysis of the characteristic fragmentation patterns obtained by successive fragmentations of the parent and daughter ions (*24*).

Although a number of studies have been devoted to the identification of eucalypt phenolics, as described above, all of

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them have focused only on specific families of compounds. To our knowledge, no exhaustive profiling has been published about eucalypt fruits.

The recovery of the fruits of this plant to obtain compounds with pharmaceutical or chemical applications, such as phenolic acids, flavonoids, and tannins, could be commercially interesting. The purpose of this study is to enhance knowledge of the phytochemical extract of the fruits of this plant and to highlight specific tracers for the future use of this extract in the industry as was the case for its leaves, which are used as a natural food additive in Japan (*26*). Therefore, we have developed a methodology that consists of combining fractionation using low-pressure chromatography on Sephadex LH-20 with HPLC-DAD-ESI-IT-MS analysis for comprehensive qualitative characterization of the phenolic composition of *E. globulus* fruits. Chromatography on a column filled with Sephadex LH-20 has been widely used for the fractionation of plant extracts (*24*, *27*). Phenolic compounds are separated by gel permeation (molecule sieve effect of the gel) or adsorption (hydrogen bonding), depending on the type of solvent used (*24*). Using column chromatography on Sephadex LH-20 in adsorption mode, nonpolymeric phenols, including phenolic acids, lower molecular weight gallotannins (*27*, *28*), flavonoids (*29*−*32*), and procyanidin oligomers (*24*) are eluted from the gel by ethanol and methanol, whereas hydrolyzable tannins with higher molecular weight are recovered by aqueous acetone (60−70%) (*33*, *34*). Using this approach, in total, 55 compounds have been identified or tentatively identified in *E. globulus* fruits, including phenolic acids, ellagitannins, and flavonols. Among them, 21 compounds had been identified earlier in eucalypt, but had not been described in the fruits, and 7 had never been detected in eucalypt species. Quantitative data on the major families are also provided.

MATERIALS AND METHODS

Chemicals. *E. globulus* fruits, Myrtaceae family, were obtained from their natural habitats. They were collected from the arboretum of Darguinah, Bejaia, in northeastern Algeria, in February 2008. The plant was identified on the basis of its morphological characteristics, and a specimen has been deposited in the 3bs Laboratory (University of Bejaia). Formic acid, tannic acid, and ellagic acid were from Sigma-Aldrich (St. Louis, MO). Gallic acid and isoquercitrin (quercetin 3-*O*-glucoside) were from Extrasynthese (Genay, France). Methanol, ethanol, acetone, hexane, and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Chlorhydric acid (HCl) and ellagic acid were purchased from Fluka (Buchs, Switzerland). Sephadex LH-20 was purchased from GE Heathcare (Uppsala, Sweden). Pure Milli-Q water was delivered by a water purification system from Millipore (Bedford, MA).

Sample Preparation. The sample was cleaned with tap water, dried in the drying oven at 40 °C during 5 days, and reduced to thin powder; 1 g was extracted with 100 mL of acetone/water (70:30, v/v) containing 0.5% acetic acid to prevent oxidation. The process of extraction continued for a week at room temperature in a dark place, using a magnetic blender. The extract was filtered through Whatman filter paper no. 4 and concentrated to dryness under reduced pressure by rotary evaporation at 40 °C. The obtained residue was defatted with hexane (25 mL × 3) to remove lipids, concentrated under reduced pressure, and lyophilized to obtain *E. globulus* fruit extract (EGFE).

Column Chromatographic Fractionation of Crude Extract. Sephadex LH-20 gel was used for fractionation by column chromatography. Crude extract was dissolved in aqueous ethanol (75%); after sonication during 20 min, the mixture was applied to a column filled with Sephadex LH-20 (length = 30 cm, internal diameter = 1.6 cm) and fractionated by consecutive elution with ethanol, methanol, and aqueous acetone (60%), at a flow rate of 1.7 mL/min. Five fractions (5 mL each) were eluted with ethanol, two fractions (10 mL each) were eluted with methanol, and eight fractions (10 mL each) were collected after elution by aqueous acetone (60%). After evaporation of solvents under vacuum at 40 °C, all fractions were reconstituted with ethanol, and then their absorbance was measured at 280 nm using an Agilent spectrophotometer. On the basis of the absorbance data, subfractions were pooled in three fractions (F1−F3). Solvent was evaporated to dryness under vacuum at 40 °C. Dried fractions were dissolved in 1 mL of ethanol/water/formic acid (75:24.5:0.5, v/v/v) and analyzed by HPLC-DAD-MS.

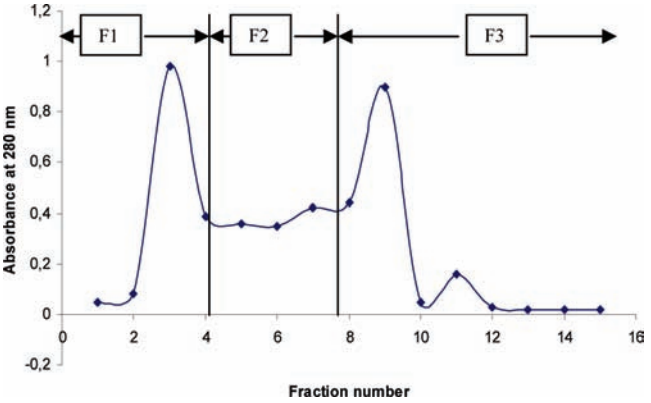

Figure 1. Sephadex LH-20 column chromatography profile of EGFE.

HPLC-DAD and ESI-MS Analysis. The HPLC analysis was carried out on a Waters 2690 HPLC system equipped with a Waters 996 DAD (Waters Corp., Milford, MA) and Empower software (Waters). The separation was performed at 30 °C using a 1.6 cm diameter, 30 cm long column. The solvents were 2% aqueous formic acid (solvent A) and acetonitrile/water/formic acid (80:18:2; solvent B). Gradient conditions were as follows: from 0 to 2% B in 3 min, from 2 to 3% B in 10 min, from 3 to 8% B in 10 min, from 8 to 20% B in 15 min, from 20 to 25% B in 5 min, from 25 to 65% B in 30 min, and then to 80% B in 7 min at a flow rate of 0.25 mL/min. The injection volume was 10 μL, and detection was carried out between 210 and 650 nm. After passing through the flow cell of the DAD, the column eluate was directed to an LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). Experiments were performed in negative ion mode. Scan range was 100−2000. The desolvation temperature was 300 °C. High spray voltage was set at 5000 V. Nitrogen was used as the dry gas at a flow rate of 75 mL/min. MS was carried out using helium as target gas, and collision energy was set at 30%. Identifications were achieved on the basis of the molecular ion mass, fragmentation, UV−visible spectra, and relative retention times or co-injection with standards.

Quantities were evaluated from peak areas in the HPLC profile, using external calibration curves established with gallic acid (at 280 nm), for gallic acid and gallotannins, ellagic acid (at 253 nm) for ellagic acid and its derivatives, and quercetin 3-*O*-glucoside (at 360 nm) for flavonols. Quantities are thus expressed as gallic acid, ellagic acid, and quercetin 3-*O*-glucoside equivalents for each class of compounds in milligrams per gram of powder of initial extract. For gallotannins and ellagitannins, quantities were estimated by calculating the concentration in moles of gallic acid (or ellagic acid) equivalent, dividing by the number of gallic (or ellagic) units in the molecule, and multiplying by the molecular weight.

RESULTS AND DISCUSSION

Sephadex LH-20 Column Chromatography of Crude Extract. Fractionation of EGFE was performed by column chromatography on Sephadex LH-20. Elution with ethanol, methanol, and aqueous acetone (60%), successively, yielded 15 fractions that were pooled in 3 fractions (labeled F1−F3), according to the absorbance readings at 280 nm (**Figure 1**).

Identification of Chromatographic Peaks. HPLC-DAD-MS analysis of each of these fractions showed a large number of compounds eluting throughout the chromatographic profiles. The profiles of the three fractions appeared to be quite different, and most of the compounds were recovered in only one of them (**Figure 2**). Moreover, some compounds eluting at the same retention time in the three fractions showed different UV−visible

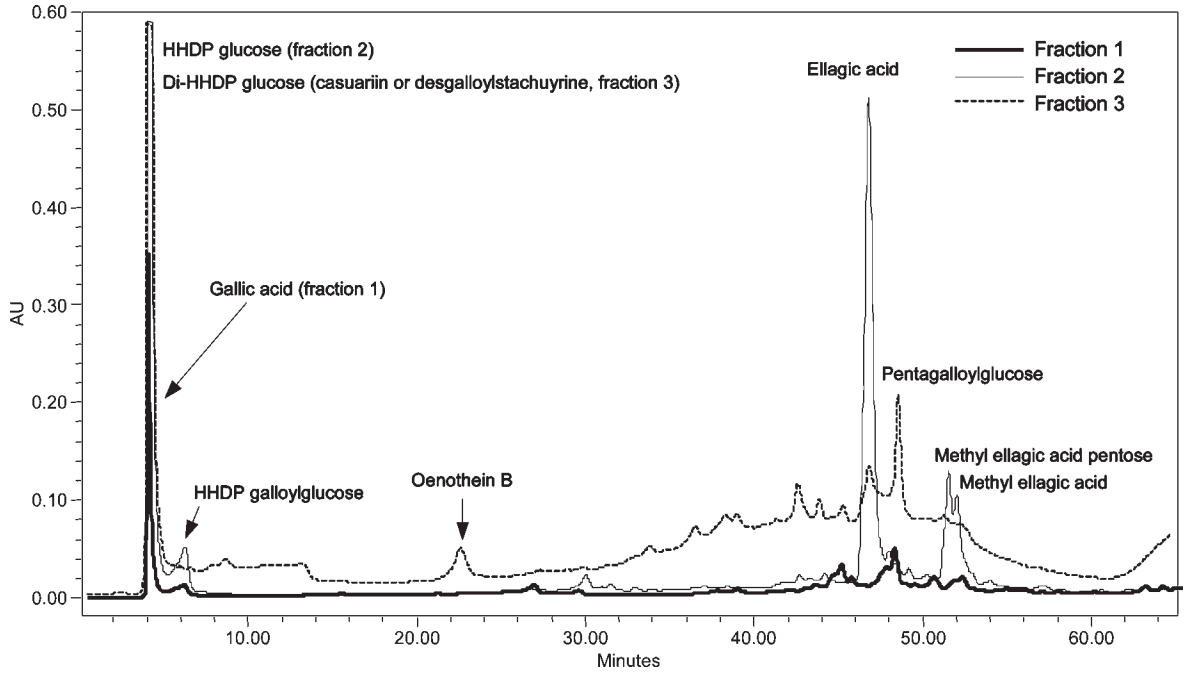

Figure 2. HPLC chromatogram at 280 nm of the three fractions recovered after Sephadex LH-20 chromatography of EGFE.

and MS spectra (e.g., peaks eluted at 5.89, 5.77, and 5.87 min, respectively, in F1, F2, and F3).

The UV−visible spectra, recorded with DAD, allowed three groups of compounds to be distinguished. The two major groups showed spectra resembling those of ellagic acid (with two absorbance maxima around 253 and 366 nm) and gallic acid (single maximum around 272 nm), respectively. The third group is minor and showed spectra that can correspond to those of flavonol derivatives (with two absorbance maxima 250−260 and 250−360 nm). These three groups were thus tentatively attributed to ellagitannins, gallotannins, and flavonol derivatives, respectively. Ellagitannins and gallotannins represent families of hydrolyzable tannins based on a sugar unit (usually D-glucose) multiply acylated with hexahydroxydiphenic acid (releasing ellagic acid (**1**) upon acid hydrolysis) or gallic acid (**2**) (releasing gallic acid upon acid hydrolysis), respectively (**Figure 3a**). Flavonols (e.g., quercetin (**3**)) are a group of flavonoid pigments, often found as glycosides. The λ_{max} values of flavonol derivatives and ellagitannins are the same, but the relative signal intensities at 250 and 360 nm are different, the second maximum being much less intense in the case of ellagitannins (*14*).

Each compound was further analyzed by mass spectrometry. Identifications were confirmed by comparison of the retention time and spectral data with those of reference compounds when available (i.e., for quercetin 3-*O*-glucoside, gallic acid, and ellagic acid). Data obtained for all peaks, including retention times, UV−visible spectra, molecular ion, and fragmentations obtained by MS^2 experiments, are given in **Table 1**. Several minor compounds could not be tentatively identified from their LC-DAD-MS data and are thus not presented.

MS^2 analysis provided $[M - H]^-$ molecular ions and fragmentation patterns that are characteristic of each group of compounds, namely, loss of galloyl groups (−152 amu) and galloyl groups plus water (−170 amu) for gallotannins and/or loss of hexahydroxydiphenoyl (HHDP) groups (−302 amu) for ellagitannins, as described earlier (*36*). Other characteristic fragmentations include loss of hexoside (−162 amu), pentoside (−132 amu) (*37*), rhamnoside (−145 amu) (*24*), and carboxylic function (−44 amu) (*36*). Tentative identifications of all compounds are provided below for each fraction.

Fraction 1. Eight compounds (F1-1−F1-8) were detected in fraction 1, which was eluted with ethanol from the Sephadex LH-20 column. The most polar one was gallic acid (**2**), identified from the comparison of its retention time, UV−visible spectrum, and MS signal (*m*/*z* 169) with those of an authentic standard. Gallic acid anion has been previously detected in fruits of *E. globulus* (*22*, *23*) and in its leaves and wood (*5*, *15*, *19*), in leaves of *E. camaldulensis* and *E. rudis* (*14*), and in bark and wood of *E. regnans* (*20*).

All other peaks were attributed to gallic acid derivatives on the basis of their UV−visible spectra and further characterized by mass spectrometry. Peaks F1-3 and F1-5 detected at *m*/*z* 499 and 535, respectively, were confirmed to be gallotannins on the basis of their characteristic fragment ions (313, 211, and 169). The ion at *m*/*z* 169 corresponds to gallic acid anion and the ion at *m*/*z* 313 to a galloylglucose group [M − H − 18, loss of water] (*37*). The daughter ion at *m*/*z* 211 has also been previously described among fragment ions formed from mono- through pentagalloylglucose (*24*). Mass spectrometric analysis of compound F1-7 revealed a molecular anion at *m*/*z* 659 that gave rise to monogalloyl diglucose (*m*/*z* 493) after loss of 166 amu, corresponding to a methylgalloyl group (*24*); this fragmentation is characteristic of a methyldigalloyldiglucose that, to our knowledge, has never been reported in *Eucalyptus*. Two compounds (F1-4 and F1-6) detected at *m*/*z* 497 provided fragment ions at 169 and 313 amu that can be interpreted as a gallic acid anion and a galloylglucose group, as explained above. They both gave rise to a neutral loss of 184 amu characteristic of an oleuropeic acid moiety (*6*). These MS spectra correspond to those of eucaglobulin (**Figure 3b**, **4**) (*5*, *6*) and globulusin B (**5**) (*6*), which have been already detected in leaves of *E. globulus* (*5*, *6*). This is the first report of these compounds in the fruits of *E. globulus*. These phenol glucosides could be differentiated by their UV absorbance maximum at λ_{max} 291 and 218−279 nm, for globulusin B (*6*) and eucaglobulin (*5*), respectively. The signal detected at *m*/*z* 519 (F1-8), showing the loss of an oleuropeic acid (M − 184), could be attributed to

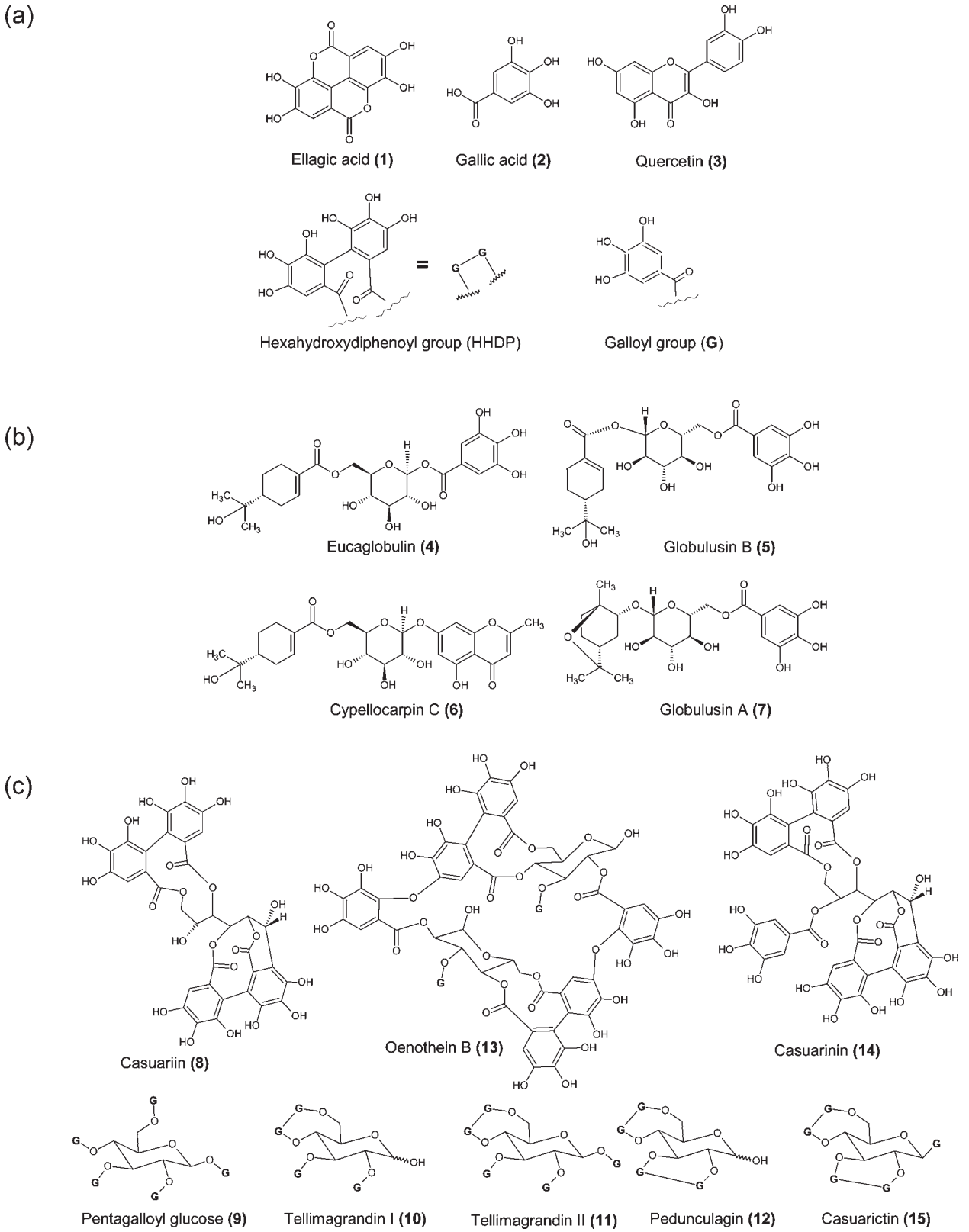

Figure 3. Chemical structures of phenolic compounds detected in EGFE.

cypellocarpin C (**6**), already reported in the leaves of *E. cypellocarpa* (*38*) and fruits of *E. globulus* (*22*).

The mass spectrum of the compound F1-2 showed a molecular anion at *m*/*z* 483, which may correspond to digalloylglucose. However, its UV spectrum (λ_{max} 220 and 276 nm) allowed this hypothesis to be ruled out. According to its UV spectrum and MS data, fragments at *m*/*z* 331 (M − 152, loss of gallic acid) and at *m*/*z* 313 (M − 170, loss of monoterpene moiety: hydroxyl-1,8-cineole or a galloyl group plus water)(*39*), this compound is tentatively identified as globulusin A (**7**, 2-hydroxy-1,8-cineole 2-*O*-*β*-D-(6′-galloyl)glucopyranoside), previously detected in leaves of *E. globulus* (*6*).

Fraction 2. Twenty-three different compounds were detected in fraction 2, eluted with ethanol and then methanol. They could be

Table 1. HPLC-DAD-ESI-MS Data for Phenolic Compounds in EGFE

peak	t_R (min)	λ (nm)	$[M-H]^-$/ $[M-2H]^{2-}$	MS^2 ions (*m*/*z*)	neutral loss	phenolic compound hypothesis (structure no.)	earlier reports in *Eucalyptus* species[a]
F1							
F1-1	5.89	232−272	169	ND[b]	ND	gallic acid (**2**) (co-injection)	E g F (*22*, *23*), E g L B W (*5*, *15*, *19*), E cam, rud L (*14*), E rgn B W (*20*)
F1-2	31.6	220−276	483	331−271−313−439	152−212−170−44	globulusin A (**7**)	E g L (*6*)
F1-3	45.76	282	499	439−313−211−169	60−126−288−330	gallotannin	
F1-4	47−48	218−279	497	169−211−313−437	328−286−184−60	eucaglobulin (**4**)	E g L (*5*, *6*)
F1-5	49.56	239−279	535	313−169	222−366	gallotannin	
F1-6	50.7	291	497	169−479−313−137	328−18−184−60	globulusin B (**5**)	E g L (*6*)
F1-7	60.45	229−279	659	493	166	methyl digalloyl diglucose	first report in genus E
F1-8	63.48	298−318sh	519	335−353−233	184−166−286	cypellocarpin C (**6**)	E g F (*6*), E cyp L (*38*)
F2							
F2-1	3.8	255−300	481	301−275	180−206	HHDP glucose	E nit W (*36*), E alb F (*41*)
F2-2	5.87	232−272	633	301- 463−257−275	170−376−358	HHDP galloylglucose	E nit W (*36*), con L (*40*)
F2-3	7.84	262	633	301−249−275-(481−463)	332−384−358−(152−170)	HHDP galloylglucose	E nit W (*36*), con L (*40*)
F2-4	12	265	633	301−339−421−481−249	332−294−212−152−384	HHDP galloylglucose	E nit W (*36*), E con L (*40*)
F2-5	27	268	633	301−463	332−170	HHDP galloylglucose	E nit W (*36*), E con L (*40*)
F2-6	33.1	269	483	271−331−313	212−152−170	digalloylglucose	E nit W (*36*), E con L (*40*)
F2-7	35	263	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-8	36. 75	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-9	37.17	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-10	38.56	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-11	42.59	253−364	463	301	162	acid ellagic hexoside	first report in genus E
F2-12	43.16	255−355	463	301	162	quercetin 3-*O*-glucoside (co-injection)	E g, cam, rud L (*15*), E g, B (*44*), E gun H (*45*)
F2-13	44	251−364	477	315	162	methyl ellagic acid hexoside	E g F (*23*)
F2-14	44.2	279	787	617−635	170−152	tetragalloyl glucose	E nit W (*36*), E vim L (*40*)
F2-15	47	253−366	301	229	72	ellagic acid (**1**) (co-injection)	E sp (*15*, *26*), E g and E rgn B (*20*)
F2-16	47.1	253−355	477	415−301	62−176	quercetin 3-*O*-glucuronide	E sp L (*15*, *26*), E con L (*40*)
F2-17	48.98	246 −370	491	329−313	162−178	3,3′-*O*-dimethylellagic acid 4-*O*-β-glucopyranoside	first report in genus E
F2-18	50	223−242−255−366	629	477−315−301	152−314−328	galloyl ester of a methylellagic acid hexoside	E g L (*16*)
F2-19	51.7	251−364	447	315	132	methylellagic acid pentose	first report in genus E
F2-20	51.98	246−363	315	ND	ND	methylellagic acid	E g F (*23*)
F2-21	53.19	279	649	497−479	152−170	methyl trigalloylglucose	E nit W (*36*)
F2-22	54.33	239−279	649	479	170	methyl trigalloylglucose	E nit W (*36*)
F2-23	57	220−277	801	631−649	170−152	methyl tetragalloylglucose	first report in genus E
F3							
F3-1	3.77	265	783	301−481−275−765	482−302−508−18	casuariin (**8**)/ desgalloylstachyurin	E nit W (*36*), E con L (*40*)
F3-2	4.53	267	951	907−(783−301)	44−(168−650)	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-3	6.11	269	783	481−765−721−301	302−18−62−482	casuariin (**8**)/ desgalloylstachyurin	E nit W (*36*), E con L (*40*)
F3-4	8−13	265	783	301−481	482−302	pedunculagin (**12**)	E nit W (*36*), E con, vim L (*40*)
F3-5	8−13	265	783	301−481	482−302	pedunculagin anomer	E nit W (*36*), E con, vim L (*40*)
F3-6	14.2	267	951	907	44	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-7	20	267	951	907	44	trisgalloy HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-8	22.59	265	1567/783	765−935	802−632	oenothein B (**13**)	E sp (*40*, *26*), E alb F (*41*)
F3-9	25	267	951	907	44	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-10	27.4	265	935	917−633−783−301	18−302−152−634	casuarinin (**14**)	E nit W (*36*), E alb F (*41*)
F3-11	28.01	281.7	935	917−633−783−301	18−302−152−634	casuarinin anomer	
F3-12	34	270	785	301−483−615	484−302−170	tellimagrandin I (**10**)	E g L (*5*), E ros (*14*), E nit W (*36*), E alb F (*41*), E con, vim L (*40*)
F3-13	34.1	258−366	1085	765−633−473	320−452−612	cornusiin B (**17**) or eucalbanin A (**16**)	E alb F (*41*) or E nit W (*36*)
F3-14	35.7	265	785	ND	ND	HHDP digalloylglucose isomer	E nit W (*36*), E con L (*40*)
F3-15	36.65	258−366	1085	765−783−633	320−302−452	cornusiin B (**17**) or eucalbanin A (**16**)	E alb F (*41*), E nit W (*36*)
F3-16	37.8	253−371	469	425	44	valoneic acid dilactone (**18**) or one of its isomers	first report in genus E
F3-17	38.38	270	785	ND	ND	HHDP digalloylglucose isomer	E nit W (*36*)
F3-18	39.33	266	935	633−301−451	302−634−484	casuarictin (**15**)	E nit W (*36*)
F3-19	39.99	255−366	469	425	44	valoneic acid dilactone (**18**) or one of its isomers	first report in genus E
F3-20	43.5	280	937	767−(741)−465−301	170−(196)−472−636	tellimagrandin II (**11**)	E nit W (*36*), E vim L (*40*)
F3-21	44.21	279	787	617−635	170−152	tetragalloylglucose	E nit W (*36*), E vim L (*40*)
F3-22	45.6	280	787	617−635	170−152	tetragalloylglucose	E nit W (*36*), E vim L (*40*)
F3-23	47.7	239−279	939	769−787	170−152	pentagalloylglucose (**9**)	E g L (*5*), E nit W (*36*), E alb F (*41*)
F3-24	52.78	239−279	2182/1091	939−1285−1787−1935	244−898−396−248	ellagitannin	

[a] Key to simple nomenclature: E, eucalyptus; g, globulus; alb, alba; cam, camaldulensis; con, consideniana; gun, gunnii; nit, nitens; rud, rudis; ros, rostra; rgn, regnans; sp, species; vim, viminalis; cyp, cypellocarpa; L, leaves; , bark; F, fruit; W, wood; H, hook. [b] ND, not identified.

divided into three groups, corresponding respectively to gallotannins, ellagitannins, and flavonols, on the basis of their UV−visible spectra.

The first group contained 14 compounds, showing a λ_{max} around 270 nm and fragment ions characteristic of gallotannin derivatives. Among them, 9 were galloylglucose derivatives, whereas 5 also contained one or two HHDP groups. Compound F2-6 showed a λ_{max} at 269 nm and a molecular anion at m/z 483, yielding fragment ions corresponding to the loss of a galloyl group (−152), of a galloyl group plus a water molecule (−180), and of another fragment of 212 amu, characteristic of galloylglucose derivatives, as explained above. It could thus be assigned to digalloylglucose previously detected in *E. nitens* wood (*36*) and *E. consideniana* leaves (*40*). Similarly, the mass signals at m/z 635 (F2-7−F2-10) with their characteristic fragment ions at m/z 483 and 465 corresponded to trigalloylglucose isomers, detected earlier in *E. nitens* wood (*36*), leaves of *E. consideniana* (*40*), and *E. rostra* leaves (*14*). F2-14, present in a small amount in fraction 2, could be attributed to a tetragalloylglucose. This compound was more abundant in fraction 3 (F3-21) and will be described below along with the other tetragalloylglucose isomers present in this fraction.

The ions detected at m/z 801 and 649 (F2-21, F2-22, and F2-23) also showed fragments corresponding to losses of 152 and 170 amu, indicating gallotannin structures. The MS data (+ 14 amu compared to the molecular ions of tri- and tetragalloylglucose) suggest that these compounds are methylated tetragalloylglucose and trigalloylglucose, respectively. The latter has been previously detected in *E. nitens* wood (*36*).

The $[M - H]^-$ at m/z 481 (F2-1) with absorbance maxima 255−300 nm and fragment ions at m/z 301 (loss of 180, loss of a galloyl group plus water) has been assigned to an HHDP glucose, reported earlier in the wood of *E. nitens* (*36*) and identified as 2,3-(*S*)-HHDP-D-glucose in *E. alba* fruits (*41*).

The signals detected at m/z 633 (F2-2, F2-3, F2-4, and F2-5) were attributed to HHDP galloylglucose isomers on the basis of their molecular ion and fragment ions at m/z 301 (loss of 332, which indicated the presence of a galloylglucose unit) (*42*), corresponding to HHDP, and at 463 (loss of a galloyl group plus water). These compounds have been previously detected in *E. nitens* wood (one isomer) (*36*) and *E. consideniana* leaves (three isomers) (*40*).

Seven mass signals detected in fraction 2 could be attributed to ellagic acid and its derivatives. The presence of free ellagic acid (**1**) in fraction F2 was confirmed by its retention time (47 min) and MS data (m/z 301) (F2-16). The peak eluted at 51.98 min (F2-20) with m/z 315 and UV−visible spectrum (λ_{max} 246 and 363 nm), characteristic of ellagic acid, was tentatively identified as methylellagic acid, which has been previously detected in fruits of *E. globulus* and identified as 3-*O*-methylellagic acid (*23*). The two peaks F2-13 and F2-19, showing similar UV−visible spectra with two absorbance maxima at 251 and 364 nm, were also postulated to be ellagic acid derivatives. MS analysis of the former showed an intense molecular anion at m/z 477, which yielded a fragment ion at m/z 315 attributed to methylellagic acid, through loss of a hexosyl group (m/z 162). This compound is thus a methylellagic acid hexoside, presumably 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside identified earlier in *E. globulus* fruits (*23*). The peak at m/z 463 (F2-11) with a fragment at m/z 301 has been assigned to ellagic acid hexoside. The peak at m/z 447 (F2-19) yielded a fragment ion at m/z 315 through loss of 132 amu, corresponding to a pentose residue. It could thus be assigned to methylellagic acid pentoside as described in raspberry fruits (*37*). To our knowledge, this is the first report of these two compounds in the genus *Eucalyptus*. Another compound detected at m/z 629 (F2-18) showed fragments at m/z 477 (methylellagic acid hexoside, loss of 152 amu, corresponding to a galloyl group) and at m/z 315 (methyl ellagic acid, loss of 314 amu, corresponding to a galloylhexoside) and was thus identified as a galloyl ester of a methylellagic acid hexoside. This compound has been already detected in *E. globulus* leaves (*16*). Finally, a compound at m/z 491 (F2-17) and its fragment at m/z 329 $[M - H - 162]^-$, which corresponds to loss of a hexose, can be interpreted as dimethylellagic acid hexose (*43*). This is the first report of this compound in the genus *Eucalyptus*. Several methylellagic acid derivatives, namely, 3-*O*-methylellagic acid, 3,4,3′-*O*-trimethylellagic acid, 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside, and 3-*O*-methylellagic acid 4′-*O*-(2″-*O*-acetyl)-α-L-rhamnopyranoside, have been identified earlier in the fruits of *E. globulus* (*23*). Methylellagic acid derivatives have also been previously detected in eucalypt species such as leaves of *E. globulus*, *E. camaldulensis*, and *E. rudis* (*15*) and stem bark of *E. globulus* (*20*, *44*).

The last group of compounds detected in this fraction was flavonols, showing the same fragment ion at m/z 301, which could be attributed to quercetin. One of them, detected at m/z 463 (F2-12) (i.e., 301 + 162 amu, corresponding to a hexoside residue), can be identified as a quercetin hexoside, presumably quercetin 3-*O*-glucoside, the presence of which has been reported in the leaves of *E. globulus*, *E. camaldulensis*, and *E. rudis* (*14*−*16*, *26*), in the bark of *E. globulus*, and in the hook *E. gunnii* (*45*) and *E. consideniana* (*40*). Further argument in favor of this identification is provided by coelution with the corresponding standard. The other compound, detected at m/z 477 (F2-16) and yielding the aglycone fragment ion at m/z 301 through loss of a glucuronide substituent (−176 amu), was tentatively identified as quercetin 3-*O*-glucuronide, which has been already described in *Eucalyptus* species (*15*, *40*, *45*). This is the first report of flavonols in *E. globulus* fruits.

Fraction 3. Twenty-four compounds were detected in this fraction. They could be classified as gallotannins and ellagitannins on the basis of their UV spectra and characteristic mass fragmentations.

Compounds at m/z 787 (F3-21 and F3-22) are assigned to tetragalloylglucose isomers, which are characterized by fragment ions at m/z 635 and at m/z 617, corresponding to loss of a galloyl residue (M − H − 152) and of a gallic acid group (M − H − 170), respectively, as described by Barry and co-workers (*36*). These compounds have been previously detected in wood of *E. nitens* (*36*) and in *E. viminalis* leaves (*40*).

The compound at m/z 939 (F3-23) was identified according to its UV−visible and mass spectra data (loss of a galloyl residue [M − H − 152] and of gallic acid [M − H − 170]) as pentagalloylglucose (**9**), yielding fragment ions at m/z 787 and at m/z 769, respectively (*46*). Pentagalloylglucose has been described in wood of *E. nitens* (*36*) and in *E. alba* fruits (*41*).

Other compounds present in the fraction showed a loss of 302 amu, characteristic of ellagitannin structures (*36*). The mass signal detected at m/z 785 (F3-12, F3-14, and F3-17), with fragment ions at m/z 301 (loss of digalloylglucose) and m/z 483 (loss of HHDP), correspond to digalloylglucose, presumably tellimagrandin I (**10**), which has been previously identified in the leaves of *E. globulus* and the wood of *E. nitens* (*5*, *36*), in *E. rostra* leaves (*14*), in *E. alba* fruits (*41*), and in *E. consideniana* leaves (*40*). The signal at m/z 937 (F3-20), yielding fragment ions at m/z 767 (M − 170, loss of gallic acid) and m/z 465 (losses of HHDP and gallic acid groups) can correspond to tellimagrandin II (**11**), which has been detected earlier in *E. nitens* wood (*36*) and *E. viminalis* leaves (*40*). This compound differs from tellimagrandin I by the presence of one additional galloyl unit at the anomeric center of the glucopyranosyl core. Tellimagrandin II is produced by enzymatic transformation of pentagalloylglucose (*47*).

Another group of compounds with λ_{max} around 270 nm showed mass signals characteristic of HHDP glucose derivatives, with molecular ions detected at m/z 783 (F3-1, F3-3, F3-4, and F3-5), yielding a fragment ion at m/z 301, which corresponds to ellagic acid (M − 482, loss of HHDP glucose) as well as loss of ions at m/z 481, which correspond to deprotonated HHDP glucose (M − 302, loss of HHDP). These compounds can correspond to di-HHDP glucose. F3-1 and F3-3 yielded a fragment at m/z 765 (M − 18, loss of water). An abundant loss of 18 amu is characteristic of C-glycosidic ellagitanins (*36*), which are presumably casuariin (**Figure 3c**, **8**), and its anomer desgalloylstachyurin. Casuariin has been previously detected in *E. nitens* wood (*36*) and *E. alba* fruit (*41*). F3-4 and F3-5 can correspond to pedunculagin anomers (2,3;4,6-di-HHDP glucose, **12**) or pedunculagin isomers, as reported by Barry and co-workers (*36*). Three isomers of this compound have been previously detected in *E. nitens* wood (*36*), whereas only one was found in *E. consideniana* leaves (*40*).

Compound F3-8 yielded two ions at m/z 1567 and 783, corresponding to the monocharged and doubly charged ions of a compound with a molecular weight of 1568. Fragment ions at m/z 765, loss of 802 amu, which is reported to be ellagitannin (*42*), and 935 ($[M - H]^-$ ion of galloyl-bis-HHDP-glucopyranose), allowed assignment to a dimer of tellimagrandin I, linked by two valoneoyl groups, oenothein B (**13**) identified in leaves of eucalyptus species (*26*, *40*) and in fruits of *E. alba* (*41*).

On the basis of molecular weight and MS/MS data, the four peaks F3-2, F3-6, F3-7, and F3-9 with m/z 951 would appear to be ellagitannins. Loss of 44 amu from the $[M - 1]^-$ ion is consistent with a free carboxyl group. These compounds could be assigned to trigalloyl HDDP glucose isomers, which have been already detected in *E. nitens* wood (*36*). These consist of an HDDP glucose and a trisgalloyl group such as valoneic acid dilactone (**18**) (*46*) or one of its isomers (tergallic acid dilactone (**19**)), sanguisorbic acid, or flavogallic acid dilactone. Three peaks at m/z 935 (F3-10, F3-11, and F3-18) are also detected; they could be attributed to casuarinin (**14**) and its anomer and casuarictin (**15**); these two isomers could be distinguished by their retention times and also by their MS/MS spectra. The MS/MS spectra of casuarinin and of its anomer showed an ion resulting from a loss of water, owing to its open glucose ring structure. Casuarinin and casuarictin have been already detected in *E. nitens* wood (*36*). Compounds detected at m/z 1085 (F3-13 and F3-15) with fragment ion at 633 corresponding to the HHDP group (M − 452, loss of trigalloyl group) were assigned to eucalbanin A (**16**) or its isomer cornusiin B (**17**), which has been already detected in the fruit extract of *E. alba* (*41*).

The peak at m/z 469 (F3-16 and F3-19) with UV−visible absorption spectra similar to that of ellagic acid gives a fragment ion at m/z 425 (valoneic acid dilactone with loss of CO_2). This compound was tentatively identified as valoneic acid dilactone (**18**) (*46*) or one of its isomers (tergallic acid dilactone (**19**), sanguisorbic acid, or flavogallic acid dilactone). This is the first report of this compound in *Eucalyptus* species. Valoneic acid dilactone can be a product of hydrolysis of cornusiin B or of oenothein B and tergallic acid dilactone a product of its isomer eucalbanin A (*46*).

Co-occurrence of most compounds detected in this fraction can be explained by the proposed biosynthetic pathway for the formation of the hydrolyzable tannins in oak leaves (*48*). Consecutive galloylation steps of glucose lead to the formation of pentagalloylglucose. Oxidative coupling between galloyl groups at C-4 and C-6 produces tellimagrandin II, which yields casuarictin after oxidative coupling between galloyl groups at C-2 and C-3. Cleavage of the galloyl group at C-1 of this compound leads to the formation of casuarinin. Furthermore, it has been suggested that the C-glycosidic ellagitannins are formed from pedunculagin, through formation of casuarinin as intermediate.

HPLC-DAD-ESI-MS/MS enabled detection of a series of gallotannins and ellagitannins and of two flavonols in the fruits of *E. globulus*. Fractionation by low-pressure chromatography on Sephadex LH-20 proved to be efficient to separate lower and higher molecular weight compounds and enabled separation of compounds coeluted in the HPLC procedure.

The concentrations of the major compounds present in EFGE were evaluated from the HPLC profiles of each fraction (**Table 2**). It should be emphasized that these values are indicative, as they are calculated using external calibration with a limited number of standards. Moreover, some changes induced in the relative amounts of the various compounds may have occurred during the extraction and fractionation procedures, because hydrolyzable tannins can be modified by desiccation or thermal treatments and easily undergo partial hydrolysis under acidic conditions.

Most of the phenolic compounds were recovered in F3 (109.4 mg/g of EGFE) and F2 (97.5 mg/g of EGFE), whereas F1 contained only small amounts (7.3 mg/g of EGFE) (**Table 2**).

F1, eluted with ethanol, contained gallic acid, terpenyl derivatives of galloylglucose (tentatively identified to eucaglobulin, globulisin B) and of noreugenin glucoside (tentatively identified as cypellocarpin C), and derivatives of lower molecular weight gallotannins in small amounts (**Table 2**).

F2 contained high levels of ellagitannins (HHDP glucose, HHDP galloylglucose, trigalloylglucose, methyltrigalloylglucose, and methyltetragalloylglucose), ellagic acid, and ellagic acid derivatives (methylellagic acid, ellagic acid hexose, methylellagic acid hexose and pentose) (**Table 2**), which is in agreement with previous studies reporting that ellagic acid and its derivatives are the major phenolic class that characterizes *E. globulus* fruits (*22*, *23*). However, the large amount of ellagic acid and HHDP glucose could be due to hydrolytic reaction during the extraction procedure. Gallotannins and flavonols (presumably quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide) were present only as minor components in this fraction (**Table 2**).

F3, eluted with aqueous acetone, contains tetra- and pentagalloylglucose and higher molecular weight ellagitannins, as expected from the literature (*24*, *25*, *36*, *46*). Di-HHDP glucose (casuariin or desgalloylstachyurin) is particularly abundant in this fraction but can result from hydrolysis of ellagitannins during the extraction procedure.

Fractionation on Sephadex LH-20 followed by HPLC-DAD-MS analysis proved to be efficient for the identification of phenolic compounds in eucalypt fruit. Ellagic acid derivatives were the major phenolic compounds in the fruit extract, ellagic acid and its smaller molecular weight derivatives being recovered in fraction F2, whereas ellagitannins were distributed in fractions F2 and F3. Among the wide range of phenolic compounds (including structural isomers) detected in this study, gallic acid, ellagic acid derivatives, and cypellocarpin C had been previously identified in the fruits of *E. globulus* (*22*, *23*), whereas quercetin derivatives, globulusins A and B, and hydrolyzable tannins were described for the first time in this material. Among hydrolyzable tannins, methyltrigalloylglucose, di-HHDP glucose, HHDP glucose, HHDP galloylglucose, digalloylglucose, trigalloylglucose, tetragalloylglucose, trisgalloyl HHDP glucose, casuarinin, casuarictin, cornusiin B, eucalbanin A, and tellimagrandin II are reported here for the first time in *E. globulus* plants. These compounds have been detected earlier in the wood of *E. nitens* (*36*). Methyltetragalloylglucose, methyldigalloyldiglucose, methylellagic acid pentose, and valoneic acid dilactone are described for the first time in the genus *Eucalyptus*. It should be emphasized

Table 2. Contents of Phenolic Compounds in EGFE

	concn (mg/g)	correction factor[a]	corrected concn[b] (mg/g)	concn (mg/g) per family
compounds in F1				
gallic acid	5.24[c]	170[c]	5.24[c]	
globulusin A	0.01[c]	484[c]	0.01[c]	terpenyl derivatives: 1.75
eucaglobulin	0.16[c]	498[c]	0.16[c]	
globulusin B	1.24[c]	498[c]	1.24[c]	
cypellocarpin C	0.34[c]	520[c]	0.34[c]	
methyldigalloylglucose	0.06[c]	330[c]	0.03[c]	
total F1	**7.05**		**7.03**	
compounds in F2				
digalloylglucose	0.39[c]	318	0.19[c]	gallotannins: 0.7
trigalloylglucose	0.13[c]	212	0.04[c]	
trigalloylglucose	0.32[c]	212	0.11[c]	
trigalloylglucose	0.02[c]	212	0.01[c]	
trigalloylglucose	0.18[c]	212	0.06[c]	
tetragalloylglucose	0.09[c]	197	0.02[c]	
methyltetragalloylglucose	1.06[c]	200.5	0.27[c]	
HHDP glucose	95.99[c]	240.5	48.09[c]	ellagitannins: 50.6
HHDP galloylglucose	6.77[c]	211.33	2.26[c]	
HHDP galloylglucose	0.71[c]	211.33	0.24[c]	
ellagic acid hexose	0.24[d]	464	0.24[d]	ellagic acid and derivatives: 45.8
methylellagic acid hexose	0.41[d]	478	0.41[d]	
ellagic acid	32.35[d]	302	32.35[d]	
dimethylellagic glucopyranose	0.52[d]	492	0.52[d]	
galloyl ester of methylellagic acid	0.05[d]	630	0.05[d]	
methylellagic acid pentose	8.15[d]	448	8.15[d]	
methylellagic acid	4.05[d]	316	4.05[d]	
quercetin 3-*O*-glucoside	0.25[e]	464	0.25[e]	flavonols: 0.5
quercetin 3-*O*-glucuronide	0.21[e]	478	0.21[e]	
total F2	**149.69**		**96.80**	
compounds in F3				
tetragalloylglucose	1.39[c]	197	0.35[c]	gallotannins: 5.0
tetragalloylglucose	7.55[c]	197	1.89[c]	
pentagalloylglucose	14.02[c]	157.6	2.80[c]	
casuariin/desgalloylstachyurin	377.39[c]	196	94.35[c]	ellagitannins: 101.5
pedunculagin	1.22[c]	196	0.30[c]	
pedunculagin	2.44[c]	196	0.61[c]	
trisgalloyl HHDP glucose isomer	0.17[c]	190.4	0.03[c]	
trisgalloyl HHDP glucose isomer	0.26[c]	190.4	0.05[c]	
oenothein B	18.43[c]	196	2.30[c]	
casuarinin	0.65[c]	187.2	0.13[c]	
casuarinin	0.22[c]	187.2	0.04[c]	
tellimagrandin I	1.46[c]	196.5	0.36[c]	
HHDP digalloylglucose isomer	0.31[c]	196.5	0.08[c]	
HHDP digalloylglucose isomer	3.70[c]	196.5	0.92[c]	
casuarictin	0.33[c]	187.2	0.07[c]	
tellimagrandin II	3.00[c]	187.6	0.60[c]	
cornusiin or eucalbanin	0.49[d]	1086	0.49[d]	
cornusiin or eucalbanin	1.12[d]	1086	1.12[d]	
valoneic acid or isomer	0.11[d]	470	0.11[d]	ellagic acid and derivatives: 2.9
valoneic acid or isomer	0.07[d]	470	0.07[d]	
ellagic acid	2.73[d]	302	2.73[d]	
total F3	**437.06**		**109.42**	
total F1 + F2 + F3	**593.80**		**213.25**	

[a] Correction factor: molecular weight/number of gallic (ellagic, flavonol) unit in the molecule. [b] Calculated using the correction factor. [c] Gallic acid equivalent. [d] Ellagic acid equivalent. [e] Quercetin 3-*O*-glucoside equivalent.

that only tentative identification can be provided on the basis of UV−visible and MS spectra, even when associated with co-injection with standards. Further work should thus be performed to provide formal identification of phenolics tentatively characterized in this study.

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