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Qualitative and Semi-quantitative Analysis of Phenolics in *Eucalyptus globulus* Leaves by High-performance Liquid Chromatography Coupled with Diode Array Detection and Electrospray Ionisation Mass Spectrometry

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ABSTRACT:
Introduction – *Eucalyptus* species are widely cultivated in Mediterranean regions. Moreover, plants of this family have been utilized for medicinal purposes. A number of studies have been devoted to the identification of eucalypt phenolics, all of them have focused on specific families of compounds, and no exhaustive profiling has been reported in leaves of this plant.
Objective – To develop methods that allows the identification and quantification of different classes of phenolics in *Eucalyptus globulus* leaf.
Methodology – Acetonic extract was fractionated by chromatography on a Sephadex LH-20 column using consecutive elution with ethanol, methanol and aqueous acetone (60%). High-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS) were applied to determine the structure of different compounds. Quantities were evaluated from peak areas in the HPLC profile, using external calibration curves.
Results – Fractionation of acetonic extract yielded three fractions: F1, F2 and F3. In total 39 phenolic compounds are detected. Among them: 16 hydrolyzable tannins, 3 terpenyl derivatives, 12 ellagic acid derivatives, 5 flavonols, 2 hydroxybenzoic acids and 1 formylated phloroglucinol. 26 compounds described in this study have not previously detected in leaves of this plant and this is the first report of quercetin 3-*O*-*β*-galactoside-6″-*O*-gallate and cypellogin A and B, in *E. globulus* plant. Quantitatively, ellagic acid derivatives and sideroxylonal A or B are largely predominant.
Conclusion – Fractionation of crude extract by chromatography on Sephadex LH-20 was efficient to separate different molecular weight compounds. HPLC-DAD-ESI/MS enabled detection of gallotannin, ellagitannin and flavonol derivatives, in leaves of *E. globulus*. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: HPLC–DAD–ESI/MS; Sephadex LH-20 column; phenolic compounds; *Eucalyptus globulus* leaves

Introduction

Phenolic compounds are a group of plant secondary metabolites that are synthesised during normal development and in response to stress such as infection, wounding and UV radiation (Nicholson and Hammerschmidt, 1992). These compounds occur ubiquitously in plants and are a diversified group of phytochemicals (Scehovic, 1990). Phenolic compounds include several classes, such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and tannins. These compounds have attracted a great deal of scientific interest because of their biological activities (Shrikhande, 2000; Santos *et al.*, 2011).

Different chromatographic techniques have been developed for analysis of phenolic compounds. The HPLC method is preferred to others because it offers higher sensitivity and greater efficiency than thin-layer chromatography, and enables the analysis of these polyphenols without the derivatisation necessary for gas chromatography. Reversed-phase HPLC columns are the most appropriate for the separation of polyphenols, hence the frequent use of the C_{18}-column. Combining HPLC with MS has provided a powerful tool in the analysis of polyphenols from crude and purified extracts by detecting negative ions produced by electrospray ionisation (ESI) (Barry *et al.*, 2001).

Eucalyptus plants belonging to the Myrtaceae family are known to be rich sources of biologically active terpenoids, tannins, flavonoids and phloroglucinol derivatives (Ito *et al.*, 2000; Takahashi *et al.*, 2004). Because of their antioxidant activity,

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leaf extracts of *E. globulus* have been proposed as food additives (Amakura *et al*., 2009). Therefore, this species might be a good candidate for further development as a nutraceutical. However, detailed information has not been published about the phenolic composition of leaves of this species. We previously reported the characterisation of 55 phenolic constituents in fruits of *E. globulus*, including gallic acid, hydrolysable tannins and flavonols (Boulekbache-Makhlouf *et al*., 2010). Among them, seven had never been detected in any eucalypt species. In the present study, as a continuation of our studies on phenolic constituents of *E. globulus*, we have studied extracts of leaves. Phenolic compounds from leaves of *E. globulus* were characterised by HPLC–DAD–ESI/MS following fractionation by chromatography on a Sephadex LH-20 column. We report the identification of 39 phenolic compounds including 26 compounds that have not previously been detected in *E. globulus* leaves. Semi-quantitative data on the major families is also provided by HPLC–DAD.

Experimental

Plant material and phenolic standards

Leaves of *E. globulus*, Myrtaceae family, were obtained from field-grown plants. They were taken from 25-year-old trees (10 trees) randomly harvested from the arboretum of Derguinah; Bejaia in northeast Algeria (36°31'13.56"N, 5°17'18.43"E), in February 2008. Formic acid was from Sigma-Aldrich (Saint Louis, MO, USA). Gallic acid and quercetin 3-*O*-glucoside were from Extrasynthese (Genay, France). Methanol, ethanol, acetone, hexane and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) and ellagic acid were purchased from Fluka (Buchs, Switzerland). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Pure water Milli-Q was delivered by water purification system Millipore (Bedford, MA, USA).

Preparation of extracts

The leaf samples were cleaned, dried in an oven at 40 °C over 4 days, and ground to obtain a thin powder (250 μm diameter). One gram was extracted with l00 mL of 70% aqueous acetone containing 0.5% acetic acid to prevent oxidation. The process of extraction continued for a week at room temperature in the dark, using a magnetic stirrer. The extract was filtered through a Whatman filter paper No.4 and concentrated to dryness under reduced pressure in rotary evaporation at 40 °C. The residue obtained was treated with hexane (25 mL ×3) to remove lipids, concentrated under reduced pressure, and lyophilised to obtain 0.247 g of *E. globulus* leaf extract.

Column chromatography fractionation of the crude extract

Sephadex LH-20 gel was used for fractionation by column chromatography. The crude extract was dissolved in 75% aqueous ethanol and after sonication for 20 min, the solution was applied to a column (length 30 cm, internal diameter 1.6 cm) filled with Sephadex LH-20 and fractionated by consecutive elution with ethanol, methanol and 60% aqueous acetone, at a flow rate of 1.7 mL/min. Five fractions (5 mL each) were eluted with 100% ethanol, two fractions (10 mL each) were eluted with 100% methanol and nine fractions (10 mL each) were collected after elution by 60% aqueous acetone. After evaporation of solvents under vacuum at 40 °C, all fractions were reconstituted with ethanol and then their absorbance was measured with a spectrophotometer at 280 nm (UVmc^2, SAFAS, Monaco). Based on the absorbance data, subfractions were pooled in three fractions (F1 to F3). Solvent was evaporated to dryness under vacuum at 40 °C. The recovery of the crude extract material in the different fractions was calculated as the percentage weight of the crude extract. Dried fractions were dissolved in 1 mL of ethanol:water:formic acid (75:24.5:0.5, v/v/v) and analysed by HPLC–DAD/MS.

HPLC–DAD–ESI/MS analysis

Separations were performed using a Waters Millenium HPLC–DAD system (Milford, MA, USA), on a (250 × 2 mm i.d., 5 μm) Atlantis dC$_{18}$-column (Waters, Milford, MA, USA) with a guard column, operated at 30 °C. Mobile phase consisted of water:formic acid (98:2, v/v) (eluent A) and water:acetonitrile:formic acid (18:80:2, v/v/v) (eluent B). Flow rate was 0.25 mL/min. The elution program was as follows: isocratic for 2 min with 0% B, 0–2% B (2–5 min), isocratic with 2% B (5–12 min), 2–3% B (12–15 min), 3–8% B (15–25 min), 8–20% B (25–40 min), 20–25% B (40–45 min), isocratic with 25% B (45–55 min), 25–65% B (55–70 min) and isocratic with 65% B (70–75 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 effluent was directed to an LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA, USA). Experiments were performed in negative ion mode, scanning from *m/z* 100 to 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. The MS was carried out using helium as the target gas, and collision energy was set at 30%. 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 discussed below.

The phenolic recovery yield was calculated as the ratio of total HPLC peak areas at 280 nm measured for each fraction to that of the crude extract.

The quantity of each compound was evaluated from peak areas in the HPLC profile, using external calibration curves established with gallic acid (at 280 nm) for gallic acid, terpenyl derivatives and gallotannins, ellagic acid (at 253 nm) for ellagic acid and its derivatives, and with 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 mg/g of crude extract (CE). Quantification of sideroxylonal was achieved on the basis of its peak area at 280 nm using a response coefficient calculated from that of epicatechin, with a correction factor corresponding to the ratio between the extinction coefficients of epicatechin (3988; Kennedy and Jones, 2001) and of sideroxylonal (6764; Neve *et al*., 1999), measured in methanol at 280 nm.

Results and discussion

HPLC–DAD–ESI/MS analysis of crude extract

The HPLC analysis of the crude *E. globulus* leaf extract yielded a complex profile, with numerous poorly resolved peaks (Fig. 1A). We thus proceeded with fractionation of the extract, using Sephadex LH-20, which had proven successful for characterisation of the fruit phenolic composition (Boulekbache-Makhlouf *et al*., 2010).

Sephadex LH-20 column chromatography of crude extract

Fractionation of crude extract yielded 16 fractions that were pooled into three fractions (F1, F2, F3), according to the absorbance readings at 280 nm (Fig. 2). The weights recovered in F1, F2 and F3 represented 30%, 20% and 45%, respectively, of that of the crude extract.

Identification of chromatographic peaks of the three fractions

HPLC–DAD–ESI/MS analysis of each of these fractions showed a large number of compounds. However, the profiles of the three fractions appeared different, and some compounds eluting at

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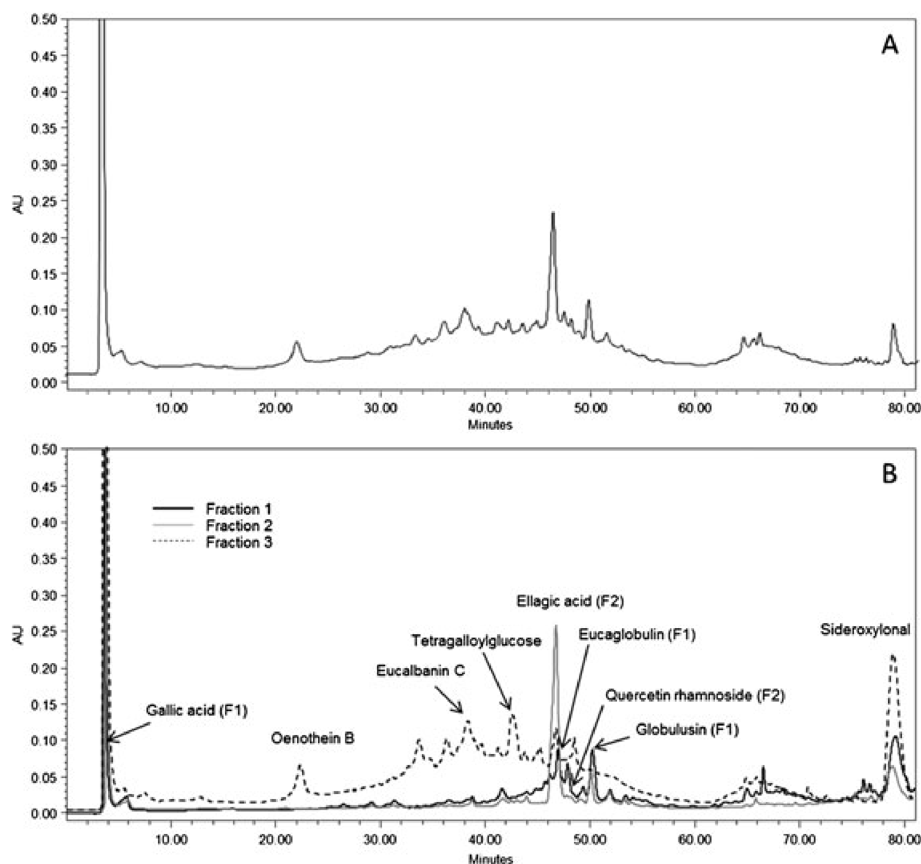

Figure 1. (A) The HPLC chromatogram at 280 nm of crude extract and (B) the three fractions recovered after Sephadex LH-20 chromatography of leaves of *Eucalyptus globulus*.

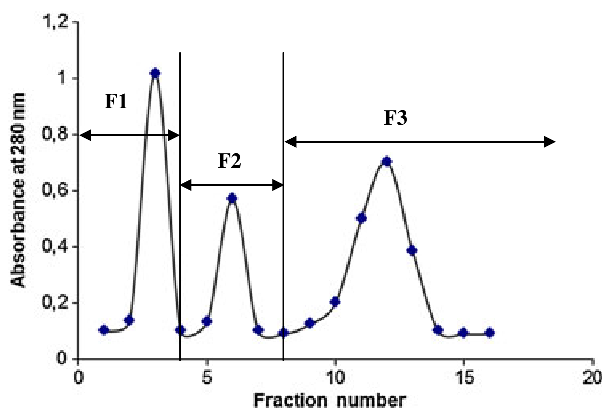

Figure 2. Sephadex LH-20 chromatography profile of the extract of *Eucalyptus globulus* leaves.

the same retention time showed different UV–vis and MS spectra in different fractions (e.g. peaks eluted at 50.26 min and 50.21 min, respectively in F1 and F2), confirming the efficiency of the fractionation procedure (Fig. 1B). The recovery of phenolic compounds determined by HPLC analysis in each fraction was about 9.7, 6.6 and 35.4% for F1, F2 and F3, respectively.

Three groups of compounds could be distinguished on the basis of their UV–vis spectra, resembling that of ellagic acid (with two absorbance maxima around 253 and 366 nm), of gallic acid (maximum around 272 nm) and of flavonols (two absorbance maxima around 250–260 and 350–360 nm), respectively, as described earlier for fruit phenolic composition (Boulekbache-Makhlouf *et al*., 2010).

Each compound was further analysed by MS. Identifications were confirmed by comparison of the retention time and spectral data with those of reference compounds when available (i.e. for gallic acid, quercetin 3-*O*-glucoside and ellagic acid) (Fig. 3, **1**, **5** and **7**). Data obtained for all peaks, including retention times, UV–vis spectra, pseudomolecular ion and fragmentations obtained by MS^2 experiments, are given in Table 1. In total 33 different phenolic compounds were detected; their tentative identification is presented below for each fraction.

Fraction 1

Seven compounds (F1-1 to F1-7) were detected in fraction 1, which was eluted with ethanol from the Sephadex LH-20 column.

Gallic acid (F1-1; Fig. 3, **1**) was identified from comparison of its retention time, UV–vis spectrum and MS signal (*m/z* 169) with those of an authentic standard. Two compounds (F1-2 and F1-3) detected at *m/z* 497 provided fragment ions at 169 and 313 amu that can be interpreted as a galloyl moiety and a galloylglucose group. The neutral loss of 184 amu is characteristic of oleuropeic acid (Hasegawa *et al*., 2008). These MS spectra correspond to those of eucaglobulin (Fig. 3, **2**; Hou *et al*., 2000; Hasegawa *et al*., 2008) and globulusin B (Fig. 3, **3**; Hasegawa *et al*., 2008). The signal detected at *m/z* 519 (F1-7), showing the loss of an oleuropeic acid ($[M-H-184]^-$) could be attributed to cypellocarpin C

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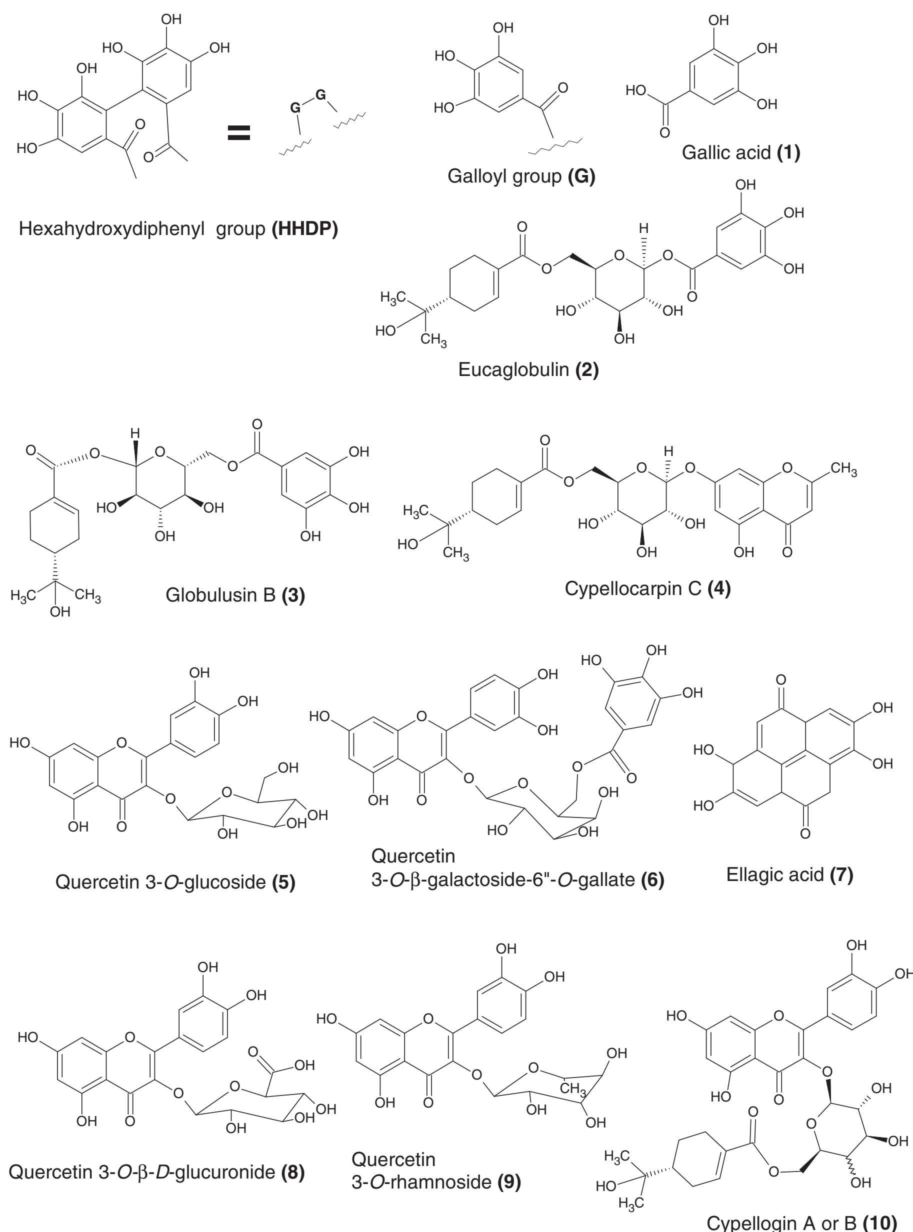

Figure 3. Chemical structures of phenolic compounds detected in *Eucalyptus globulus* leaves.

(Fig. 3, **4**), which is a monoterpene compound conjugated with gallic acid.

Three mass signals detected in this fraction could be attributed to ellagic acid derivatives. The peak at *m/z* 447 (F1-4) yielded a fragment ion at *m/z* 315 through loss of 132 amu, corresponding to a pentosyl residue. It could thus be assigned to methyl ellagic acid pentoside. Peak F1-5 detected at *m/z* 461 with a fragment at *m/z* 315 ($[M-H-146]^-$) corresponds to methylellagic acid, loss of 146 amu corresponds to a rhamnosyl residue, thus, this compound could be assigned to methylellagic acid rhamnoside, presumably 3-*O*-methylellagic acid 3′-α-rhamnoside. Peak F1-6 detected at *m/z* 503 was characterised by its fragment ions at *m/z* 443 (loss of 60 amu, corresponding to acetic acid) and a fragment at *m/z* 315 (loss of 188 amu, corresponding to an acetyl−rhamnosyl moiety), and could thus be assigned to methylellagic acid-acetyl-rhamnoside. 3-*O*-methylellagic acid 3′-α-rhamnoside and three isomers of its acetyl derivative have been reported in stem bark of *E. globulus* (Kim *et al*., 2001).

Gallic acid, eucaglobulin and globulusin B have been detected previously in leaves and fruits of *E. globulus* (Hou *et al*., 2000; Hasegawa *et al*., 2008; Boulekbache-Makhlouf *et al*., 2010). Compounds [Cypellocarpin C, methyl ellagic acid, 3-*O*-methylellagic acid 3′-α-rhamnoside and methylellagic acid-acetyl-rhamnoside] have been reported in fruits (Guo and Yang, 2006; Boulekbache-Makhlouf *et al*., 2010) and bark (Kim *et al*., 2001; Santos *et al*., 2011) of *E. globulus*, but they have not been reported in leaves.

Fraction 2

Twelve compounds were detected in this fraction eluted by ethanol–methanol. They could be distinguished into three

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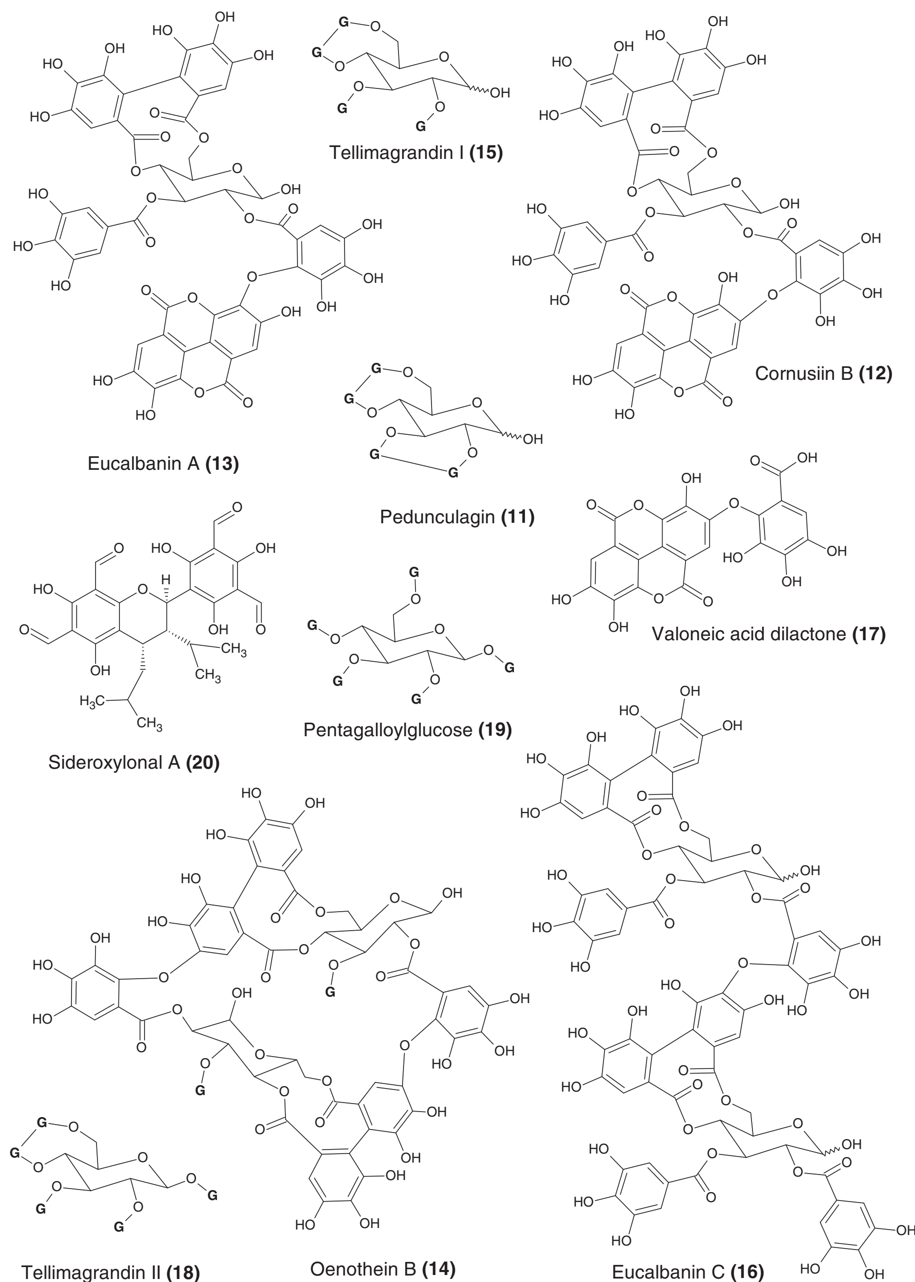

Figure 3. (Continued)

groups, corresponding to gallotannins, ellagic acid and flavonol derivatives.

Compound F2-1 showed a λ_{max} at 262 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 (−170) and of another fragment of 212 amu, characteristic of galloylglucose derivatives. It could be assigned to digalloylglucose. Five mass signals detected in fraction 2 could be attributed to ellagic acid and its derivatives. The presence of free ellagic acid (Fig. 3, **7**) (F2-7) in this fraction was confirmed by its retention time (46.83 min) and MS data (*m/z* 301). The peak at *m/z* 463 (F2-2) with a fragment at *m/z* 301 has been assigned to ellagic acid hexoside. The two peaks F2-4 and F2-6, showing similar UV–vis spectra with two absorbance maxima around 253 and 366 nm, were also postulated to be isomers of ellagic acid derivatives. MS analysis of these compounds 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). A compound at *m/z* 491 (F2-9) and its fragment at *m/z* 329 [M − H − 162, loss of a hexose] can be interpreted as a glucoside of dimethylellagic acid (Pakulski and Budzianowski, 1996).

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 (F2-3) (i.e. 301 + 162 amu, corresponding to an hexoside residue), was assigned to quercetin 3-*O*-glucoside (Fig. 3, **5**), identified from the comparison of its retention time, UV–vis spectrum and MS spectra with those of an authentic standard. Compounds detected at *m/z* 477 (F2-8) and *m/z* 447 (F2-10) were tentatively identified as quercetin 3-*O*-β-*D*-glucuronide (Fig. 3, **8**) and quercetin-3-*O*-α-rhamnoside

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Table 1. HPLC–DAD–ESI/MS Data for phenolic *compounds* in leaves of *Eucalyptus globulus*

Fraction	Compound	t_R (min)	λ (nm)	$[M-H]^-$/ $[M-2H]^{2-}$	MS^2 ions (*m/z*)	Hypothesized phenolic compound
F1	F1-1	5.81	169	ND	ND	Gallic acid **(1)** (coinjection)
	F1-2	47.89	281	497	437-169-211-313-331	Eucaglobulin **(2)**
	F1-3	50.26	291	497	479-437-211-169	Globulusin B **(3)**
	F1-4	51.96	251-366	447	315	Methylellagic acide pentoside[a]
	F1-5	53.42	251-366	461	315-298	3-*O*-methylellagic acid 3′-α-rhamnoside[a]
	F1-6	59.90	251-366	503	443-225-315-167-183-197	Methylellagic acid-acetylrhamnoside [a]
	F1-7	62.78	298-318 sh	519	335-353-233	Cypellocarpin C[a] **(4)**
F2	F2-1	32.9	262	483	271-331-313	Digalloylglucose[a]
	F2-2	42.53	253-363	463	301	Ellagic acid hexose[a]
	F2-3	43.14	355	463	301	Quercetin 3-*O*-glucoside **(5)**
	F2-4	44.02	251-363	477	315	Methylellagic acid hexoside[a]
	F2-5	44.86	265-353	615	463-283-373-301	Quercetin 3-*O*-β -galactoside-6″-*O*-gallate **(6)**
	F2-6	46.60	253-366	477	315	Methylellagic acid hexose isomer[a]
	F2-7	46.83	254-363	301	229	Ellagic acid **(7)** (coinjection)
	F2-8	47.11	255-355	477	301	Quercetin 3-*O*-β-*D*- glucuronide **(8)**
	F2-9	48.28	258-361	491	313-329	Glucoside of dimethylellagic acid[a]
	F2-10	50.21	265-348	447	300-301	Quercetin 3-*O*-α-rhamnoside **(9)**
	F2-11	51.96	251-371	315	300	Methylellagic acid
	F2-12	61.83	260-348	629	463-301-445	Cypellogin A[a] or B[a] **(10)**
F3	F3-1	5.19	267	633	301-275-249-185	HHDP galloylglucose[a]
	F3-2	7.55	267	633	301-275-249-579	HHDP galloylglucose[a]
	F3-3	10.28	265	783	301-481-275	Pedunculagin[a] **(11)**
	F3-4	12.84	265	783	301-481-275	Pedunculagin[a] **(11)**
	F3-5	19.71	266	951	907-783-605	Trisgalloyl HHDP glucose[a]
	F3-6	34.90	253-368	1085	633-739-765-783	Cornusiin B **(12)** or eucalbanin A[a] **(13)**
	F3-7	22.43	267	1567/783	765-935-917-755- 615	Oenothein B **(14)**
	F3-8	33.81	785	785	301 483 633 615 463 419	Tellimagrandin I **(15)**
	F3-9	36.50	258-368	1085	765-783-933-739-597-469-407	Cornusiin B **(12)** or eucalbanin A[a] **(13)**
	F3-10	38.48	270-366	1569/784	1506-784-765	Eucalbanin C **(16)**
	F3-11	38.74	255-364	469	425	Valoneic acid dilactone or one of its isomers[a] **(17)**
	F3-12	39.76	255-364	469	425	Valoneic acid dilactone or one of its isomers[a] **(17)**
	F3-13	42.68	279	787	617-635	Tetragalloylglucose[a]
	F3-14	42.87	280	937	767-741-465-301	Tellimagrandin II[a] **(18)**
	F3-15	39.91	255-364	469	425	Valoneic acid dilactone or one of its isomers[a] **(17)**
	F3-16	43.93	276	787	617-635	Tetragalloylglucose[a]
	F3-17	45.34	276	787	617-635	Tetragalloylglucose[a]
	F3-18	48.65	281	939	769-787-617	Pentagalloylglucose **(19)**
	F3-19	50.15	253-351	629	477-315-301	Galloyl ester of methylellagic acid glucose
	F3-20	79.14	237-279-343	499	249	Sideroxylonal A or B **(20)**

[a]New compounds in leaves of *E. globulus*.

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Table 2. Contents of phenolic compounds in the crude extract of *Eucalyptus globulus* leaves

Fraction	Compounds	Concentration (mg/g)	Concentration per family (mg/g)
F1	Gallic acid	0.84[a]	
	Eucaglobulin	2.11[a]	
	Globulusin B	3.67[a]	Terpenyl derivatives: 6.25
	Cypellocarpin C	0.47[a]	
	3-*O*-methylellagic acid 3′-α-rhamnoside	0.58[b]	Ellagic acid derivatives: 1.25
	Methylellagic acid-acetylrhamnoside	0.25[b]	
	Methylellagic acid 3-*O*- pentoside	0.42[b]	
	Total	8.34	
F2	Digalloylglucose	0.12[a]	Gallotannins: 0.12
	Ellagic acid hexose	0.16[b]	
	Methylellagic acid hexose	0.45[b]	
	Methylellagic acid hexose	7.08[b]	
	Ellagic acid	11.51[b]	Ellagic acid and derivatives: 20.10
	Glucoside of dimethylellagic acid	0.34[b]	
	Methylellagic acid	0.56[b]	
	Quercetin 3-*O*-glycoside	0.05[c]	Flavonols: 7.52
	Quercetin 3-*O*-β-*D*-glucuronide	2.65[c]	
	Quercetin 3-*O*-β -galactoside-6″-*O*-gallate	0.07[c]	
	Quercetin 3-*O*-rhamnoside	4.39[c]	
	Cypellogine A/B	0.36[c]	
	Total	27.74	
F3	Tetragalloylglucose	2.17[a]	
	Tetragalloylglucose	0.29[a]	Gallotannins: 3.78
	Tetragalloylglucose	0.66[a]	
	Pentagalloylglucose	0.66[a]	
	Pedunculagin	0.37[b]	
	Pedunculagin	0.61[b]	
	HHDP galloyl glucose isomer	0.51[b]	Ellagitannins: 12.77
	HHDP galloyl glucose isomer	1.08[b]	
	TrisHHDP galloyl glucose isomer	0.04[b]	
	Oenothein B	3.01[b]	
	Cornusiine ou eucalbanine A	0.02[b]	
	Cornusiine ou eucalbanine A	0.23[b]	
	Eucalbanine	4.58[b]	
	Tellimagrandin I	1.43[b]	
	Tellimagrandin II	1.50[b]	
	Galloyl ester of methylellagic acid glucose	1.33[b]	Ellagic acid and derivatives: 2.61
	Sideroxylonal	77.02	Formylated phloroglucinol: 77.02
	Total	95.51	
Total F1 + F2 + F3		131.59	

[a]Gallic acid equivalent;
[b]ellagic acid equivalent;
[c]quercetin 3-*O*-glucoside equivalent.

(Fig. 3, **9**). The peak detected at *m/z* 615 (F2-5) with its fragments at *m/z* 463 [M − H − 152, loss of galloyl group]⁻, and *m/z* 301 [M − H − 162, loss of hexosyl group]⁻, was tentatively assigned to quercetin galloylhexoside, presumably quercetin 3-*O*-β-galactoside-6″-*O*-gallate (Fig. 3, **6**). These flavonols derivatives have already been described in *Eucalyptus* species (Cadahia *et al*., 1997; Conde *et al*., 1997; Santos and Waterman, 2001a, 2001b; Atoui *et al*., 2005; Amakura *et al*., 2009; Boulekbache-Makhlouf *et al*., 2010).

Finally, compound F2-12 may correspond to cypellogin A or B (Fig. 3, **10**), which are quercetin glucoside and galactoside acylated with oleuropeic acid (Kasajima *et al*., 2005).

Quercetin 3-*O*-β-galactoside-6″-*O*-gallate and cypellogins A and B have been formally identified, respectively, in leaves of *E. ovata* (Santos and Waterman, 2001b) and *E. cypellocarpa* (Kasajima *et al*., 2005) but this is the first report of these three compounds in *E. globulus*. Some flavonoids (8-desmethyl-sideroxylin, chrysin, eucalyptin, 8-desmethyl-eucalyptin) that have been reported previously in leaf waxes (Wollenweber and Kohorst, 1981), as well as in fruit waxes (5-hydroxy-7, 4′-dimethoxy-6-methyl-flavone; Pereira *et al*., 2005) of this species, were not detected in our extract; this is probably due to the extraction method used as these compounds were probably removed with the apolar phase (hexane).

All other compounds detected in this fraction have been reported in several *Eucalyptus* species (Barry *et al*., 2001; Kasajima *et al*., 2005; Yang and Guo, 2007; Santos *et al*., 2011) but not in leaves of *E. globulus*.

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Fraction 3

Twenty compounds could be classified as gallotannins and ellagitannins, on the basis of their UV spectra and characteristic mass fragmentations.

The signals at *m/z* 633 (F3-1, F3-2) were attributed to hexahydroxydiphenoyl acid (HHDP) galloylglucose isomers on the basis of their molecular ion and fragment ions at *m/z* 301, corresponding to HHDP (loss of 332, which indicated the presence of a galloylglucose unit) (Lee *et al*., 2005). F3-3 and F3-4 detected at *m/z* 783 (F3-3, F3-4), yielding fragment ions at *m/z* 301 (ellagic acid; M − 482, loss of HHDP glucose) and at *m/z* 481 (deprotonated HHDP glucose; M − 302, loss of HHDP) correspond to di-HHDP glucose, presumably pedunculagin or pedunculagin isomers (Fig. 3, **11**). Peak F3-5 with *m/z* 951 could be assigned to trigalloyl HDDP glucose. Loss of 44 amu from the $[M - H]^-$ ion, is consistent with a free carboxyl group. Compounds detected at *m/z* 1085 (F3-6 and F3-9) with fragment ion at 633 corresponding to the HHDP galloylglucose (M − 452, loss of trigalloyl group) were assigned to eucalbanin A (Fig. 3, **13**) or its isomer cornusiin B (Fig. 3, **12**).

The signal at *m/z* 937 (F3-14), 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 (Fig. 3, **18**). Compounds at *m/z* 787 (F3-13, F3-16 and F3-17) are assigned to tetragalloylglucose isomers, which are characterised 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. The compound at *m/z* 939 (F3-18) was identified as pentagalloylglucose (Fig. 3, **19**) (loss of a galloyl residue and of gallic acid, yielding fragment ions at *m/z* 787 and at *m/z* 769, respectively). Peaks at *m/z* 469 (F3-11, F3-12 and F3-15) with UV–vis absorption spectra similar to that of ellagic acid give a fragment ion at *m/z* 425 (valoneic acid dilactone with loss of CO_2). These compounds were tentatively identified as valoneic acid dilactone (Fig. 3, **17**) and its isomers.

Compound F3-7 yielded two ions at *m/z* 1567 and *m/z* 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 (Lee *et al*., 2005) and 935 ($[M - H]^-$ ion of galloyl-*bis*-HHDP-glucose), allowed us to assign it to a dimer of tellimagrandin I, linked by two valoneoyl groups, oenothein B (Fig. 3, **14**). The mass signal detected at *m/z* 785 (F3-8), with fragment ions at *m/z* 301 (loss of digalloylglucose) and *m/z* 483 (loss of HHDP), corresponds to digalloylglucose, presumably tellimagrandin I (Fig. 3, **15**).

Another compound, detected at *m/z* 629 (F3-19), showed fragments at *m/z* 477 (methylellagic acid hexoside, loss of a galloyl group) and at *m/z* 315 (methyl ellagic acid, loss of 314 amu, corresponding to a galloylglucose) and was thus identified as a galloyl ester of a methylellagic acid glucose.

Compound F3-10 yielded two ions at *m/z* 1569 and *m/z* 784, corresponding to the monocharged and doubly charged ions of a compound with a molecular weight of 1570. Fragment ions at *m/z* 784, loss of 785 amu, which correspond to HHDP digalloylglucose, can be assigned to eucalbanin C (Fig. 3, **16**).

The compound at *m/z* 499 (F3-20), with fragment ion at *m/z* 249 and UV spectrum at 279–345 nm, is assigned to sideroxylonal A or B (Fig. 3, **20**). This tentative identification is consistent with the low polarity of compound F3-20, recovered in F3 and eluted at the end of the reversed phase chromatographic profile. However, its extraction for quantitative analysis is always described with apolar solvent.

Oenothein B, tellimagrandin I and eucalbanin C have been detected previously in leaves and fruits of *Eucalyptus*. species (Yoshida *et al*., 1992; Hou *et al*., 2000; Barry *et al*., 2001; Santos and Waterman, 2001a; Cruz *et al*., 2005; Amakura *et al*., 2009; Boulekbache-Makhlouf *et al*., 2010). HHDP galloylglucose isomers, pedunculagin, tetra galloylglucose, pentagalloylglucose and valoneic acid dilactone have been reported in fruits (Boulekbache-Makhlouf *et al*., 2010) of *E. globulus* and other *Eucalyptus*. species (Yoshida *et al*., 1992; Barry *et al*., 2001; Santos and Waterman, 2001a; Santos *et al*., 2011), but have not been reported in *E. globulus* leaves.

Sideroxylonal is very well described in *E. globulus* leaves (O' Reilly-Wapstra *et al*., 2004) and in fruit or leaves of other *Eucalyptus* species (Eschler and Foley, 1999; Neve *et al*., 1999; Close *et al*., 2001; Wallis *et al*., 2003).

Semi-quantification of major compounds by HPLC–DAD

The concentrations of the major compounds present in different fractions were evaluated from the HPLC profiles of each fraction (Table 2). Most of the phenolic compounds were recovered in F3 (72.60%) and F2 (21.07%), while F1 contained only 6.33% of the assayed phenolic compounds.

F1, eluted with ethanol, was dominated by terpenyl derivatives (74.94%); F2 eluted with ethanol–methanol was dominated by ellagic acid and its derivatives (72.46%); F3 eluted with aqueous acetone, was dominated by sideroxylonal (80.57%).

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