Industrial Crops & Products 131 (2019) 166–172

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Eucalyptus subgenus *Eucalyptus* (Myrtaceae) trees are abundant sources of medicinal pinocembrin and related methylated flavanones

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ARTICLE INFO

Keywords:
Cryptostrobin
C-methylation
Demethoxymatteucinol
Eucalypt
Flavonoid
Pinostrobin

ABSTRACT

Plant-derived flavanones such as pinocembrin and related methylated forms are of interest for their potential medicinal use. Commercial sources of these compounds are limited, but some trees in genus *Eucalyptus* subgenus *Eucalyptus* (family Myrtaceae) show promise due to their high foliar flavanone content and growth rate, particularly when grown in coppice cultivation. As a first step to developing commercial *Eucalyptus* plantations for flavanone production, this research aimed to identify high-yielding species. Foliar flavanones were quantified in 41 species and subspecies from three major sections within the subgenus. Pinocembrin concentrations were highest in two subspecies of *E. preissiana* with notably high levels of 15 and 18 mg g^{-1} dry weight (DW). The highest concentrations of 7-*O*-methylpinocembrin (12 mg g_{DW}^{-1}) and 5,7-*O*-dimethylpinocembrin (20 mg g_{DW}^{-1}) were found in *E. croajingolensis* and *E. oreades*, respectively. In addition, two *C*-methyl flavanones were identified in *Eucalyptus* for the first time: 6-*C*-methylpinocembrin (7 mg g_{DW}^{-1}) in *E. megacarpa*and 6,8-*C*-dimethylpinocembrin (6 mg g_{DW}^{-1}) in *E. agglomerata*. In general, flavanone profiles for closely related eucalypts such as subspecies were very similar. Moreover, species fit into three distinct categories - those with high concentrations of relatively pure non-methylated pinocembrin, those with highly abundant *O*-methyl flavanones, or those with abundant *C*-methyl flavanones. Saplings of two selected species grown in a trial plot largely reflected adult tree flavanone profiles, albeit at lower levels attributed to ontogenetic effects. In conclusion, we have shown that many species of *Eucalyptus* are abundant sources of promising medicinal flavanones. This work will enable selection of high-yielding species and genotypes for growth-rate assessment trials and the future establishment of commercial plantations.

1. Introduction

Plant-derived flavanones (a class of flavonoids) are of growing interest for their potential therapeutic and pharmacological uses (see recent reviews: (Barreca et al., 2017; Bonetti et al., 2017; George et al., 2017; Testai and Calderone, 2017). One such flavanone, pinocembrin (5,7-dihydroxyflavanone), exhibits a wide range of pharmacological properties, including neuroprotective effects, neutralisation of reactive oxygen species and reduction of inflammation (Lan et al., 2016; Rasul et al., 2013). Pinocembrin is readily absorbed following oral administration in rats (Sayre et al., 2013), and *in vitro* studies show it can pass through the blood-brain barrier in a passive transport process (Yang et al., 2012b). The combination of bioavailability and pharmacological properties in animal models gives pinocembrin the potential for treating a range of disparate human conditions, including cardiovascular disease and ischemic stroke (Rasul et al., 2013), and neurodegenerative disorders (Bonetti et al., 2017).

Commercial production of pinocembrin, like most medicinal flavonoids (Terahara, 2015), is achieved predominantly by extraction from plants: namely from seeds of *Alpinia katsumadai* Hayata, which has a yield range of 0.6 to 2.5 mg g^{-1} dry weight (Li et al., 2012; Yamamoto et al., 2011). Pinocembrin can be chemically synthesized, but the formation of hydrogenation by-products can reduce the efficiency of these processes (Yang et al., 2012a). Further, there is an increasing consumer preference for 'natural products' worldwide (Li and Chapman, 2012), giving plant-derived pinocembrin enhanced commercial appeal.

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https://doi.org/10.1016/j.indcrop.2019.01.050
Received 19 November 2018; Received in revised form 24 January 2019; Accepted 24 January 2019

Despite a lack of systematic screening, pinocembrin has been found in leaf, flower, root and seed extracts of some 50 species from 16 plant families (Rasul et al., 2013). The family Myrtaceae is the most represented, with the compound occurring in extracts of 20 species, including leaves of a *Syzygium* species (Simirgiotis et al., 2008), flowers of a *Corymbia* (Massaro et al., 2014), and leaves of 18 species of *Eucalyptus* (Bick et al., 1972; Goodger et al., 2016; Heskes et al., 2012; Saraf et al., 2015; Tucker et al., 2010). The concentration of pinocembrin in eucalypt leaves is comparable to that in *A. katsumadai*, with 2.0 mg g_{DW}^{-1} in *E. stellulata* (Goodger et al., 2016), 2.4 mg g_{DW}^{-1} in *E. fraxinoides* (Saraf et al., 2015) and 3.4 mg g_{DW}^{-1} in *E. sieberi* (Saraf et al., 2015).

Leaves of some eucalypt species contain even higher concentrations of related flavanones. For example, 7-*O*-methylpinocembrin (pinostrobin or 5-hydroxy-7-methoxy-flavanone) is found at 10 mg g_{DW}^{-1} in *E. oreades* and 5,7-di-*O*-methylpinocembrin (pinocembrin dimethyl ether or 5,7-dimethoxyflavanone) is found at 12 mg g_{DW}^{-1} in *E. nitida* (Goodger et al., 2016). These methylated compounds are also attractive as pharmaceuticals and therapeutics because they can have enhanced biological activities (Wilson et al., 2008), improved intestinal absorption (Wen and Walle, 2006a), and increased metabolic stability (Wen and Walle, 2006b) relative to non-methylated forms. Therefore, these *Eucalyptus* species may be additional targets for commercial production of methylated forms of pinocembrin.

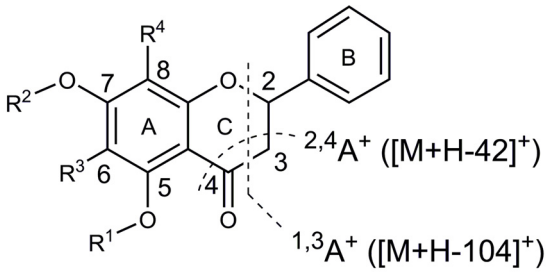
The relatively high foliar flavanone concentrations of some eucalypts, together with their high growth rates, particularly when cultivated in short-rotation coppice plantations (see Goodger et al., 2007b), makes them attractive targets for commercial production systems. The logical first step in establishing a flavanone production system is to identify high-yielding candidate species. The genus *Eucalyptus* is large and contains over 800 species, but interestingly, all 18 known flavanone-containing species belong to the one sub-group: the subgenus *Eucalyptus* (or *Monocalyptus*; Noble, 1989). This subgenus is comprised of some 150 species and sub-species (Nicolle, 2018); thus, foliar flavanone composition has been quantified for a very low percentage of member species. The primary aim of this research is to find suitable candidate species for commercial plantations by quantifying pinocembrin and related methylated forms in a further 41 species and subspecies of *E.* subg. *Eucalyptus*. The research also aims to measure the variability in the quality and quantity of flavanones between individuals of the same species, in this case half-sibling families of two selected species planted in a trial plot.

2. Materials and methods

2.1. Plant material

Fully expanded leaves were collected from a representative tree of each *E.* subg. *Eucalyptus* species growing at Currency Creek Arboretum, South Australia (35°25′45″S, 138°45′46″E) in March 2016 (Table 2), and from saplings growing in an experimental plot at the University of Melbourne's Dookie campus (36°23′3″S, 145°42′52″E) in November 2017. Saplings were maternal half-siblings of *E. nitida* ($n = 12$) and *E. preissiana* subsp. *preissiana* ($n = 12$) propagated from seed purchased from Nindethana Seed Service (Albany, WA, Australia). Seed was germinated in July 2016 and seedlings were grown in a greenhouse at the University of Melbourne (see Goodger and Woodrow, 2010) for potting and greenhouse conditions) and then transplanted to the field in November 2016. Field plants were irrigated daily with 2 L of water until March 2017 and twice weekly thereafter.

Leaves from trees and saplings were harvested using a pole pruner where necessary and immediately sealed in snap-lock plastic bags and returned to the laboratory on dry ice. Leaf samples were stored at −80 °C until analysed. Up to eight leaves per plant (depending on leaf size) were oven-dried at 50 °C for 48 h and then ground in a mortar using a pestle. Ground leaf material was weighed into glass vials to which 4 mL acetonitrile was added and samples were shaken on a rotating shaker at 650 rpm for 48 h.



	R1	R2	R3	R4
pinocembrin	H	H	H	H
7-*O*-methylpinocembrin	H	CH_3	H	H
5,7-di-*O*-methylpinocembrin	CH_3	CH_3	H	H
6-*C*-methylpinocembrin	H	H	CH_3	H
6,8-di-*C*-methylpinocembrin	H	H	CH_3	CH_3

Fig. 1. Chemical structures of five flavanones found in high concentrations in *Eucalyptus* subg. *Eucalyptus* leaves. Dashed lines represent dominant fragmentation patterns observed for flavanones using ESI-MS-MS.

2.2. Chemical analyses

2.2.1. Chromatography and mass spectrometry

Solvent extracts were centrifuged for 3 min at 3000 × *g* and 1 µl aliquots of the resultant supernatants were fractionated using a Shimadzu Prominence High-Performance Liquid Chromatography (HPLC) system with photo diode array UV detection (190–500 nm). The column used was a Gemini C18 (5 µm, 150 × 4.6 mm; Phenomenex) analytical column eluted at a flow rate of 0.8 mL min^{-1}. The eluent system was a gradient of acetonitrile (acidified with acetic acid, 0.1%) from 30 to 50% over 7 min, followed by 50–95% over 7 min and then isocratic at 95% for a further 12 min, and finally a gradient of 95–100% over 0.25 min. Flavanone constituents were identified by retention times and quantified by comparison with an authentic standard series as described in (Goodger et al., 2016). Aliquots (1 µl) of each extract were also analysed using Electro-Spray Ionisation Liquid Chromatography Mass Spectrometry (ESI-LCMS) to confirm constituent identity based on the characteristic fragmentation pattern of flavanones (Fig. 1; Goodger et al., 2016). ESI-LCMS was conducted on an Agilent 6520 QTOF MS system (Santa Clara, CA, USA) with a dual spray ESI attached to an Agilent 1200 series HPLC with a diode array detector using the same column and eluent system as above. The MS was operated in both positive and negative modes. Positive mode was operated using the following conditions: nebulizer pressure 37 psi, gas flow-rate 12 L min^{-1}, gas temperature 350 °C, capillary voltage 4000 V, fragmentor 150 and skimmer 65 V. In negative ion mode, a nebulizer pressure of 45 psi and a capillary voltage of 3500 V were used. Mass spectra were collected in the range of 70–1700 *m/z*. The analytical method comprised two scan experiments: the first was a full scan with a dwell time of 493 ms $spectrum^{-1}$, followed by a second MS/MS experiment with a dwell time of 493 ms $spectrum^{-1}$ where collision energy of 20 V was applied. Analytes were also monitored with diode array UV detection at specific wavelengths of 283 nm and 330 nm with the bandwidth set at 4 nm. Chromatograms and mass spectra were evaluated using MassHunter software (Agilent).

Commercial standards of pinocembrin and 7-*O*-methylpinocembrin (pinostrobin) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 5,7-di-*O*-methylpinocembrin (pinocembrin dimethyl ether) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). In-house standards of 8-*C*-methylpinocembrin (cryptostrobin) and 6,8-di-*C*-methylpinocembrin (demethoxymatteucinol) were purified as

Table 1a
^{1}H NMR (600 MHz) data for pinocembrin and *C*-methylated flavanones extracted from *Eucalyptus* subg. *Eucalyptus* as compared to literature values. Deuterated chloroform was used as solvent.

	Chemical shift δ (ppm); Multiplicity; Coupling constants (Hz)						
Compound	H-2	H-3a	H-3b	H-6	H-8	Me-1	Me-2
Pinocembrin extracted from *Eucalyptus*	5.36 dd 3.0 13.0	2.76 dd 3.1 17.1	3.02 dd 13.1 17.1	5.92 d 2.2	5.94 d 2.2	–	–
Pinocembrin literature values (Rameshkumar et al., 2015)	5.42 dd 3.0 13.0	2.76 dd 3.0 17.1	3.09 dd 12.6 17.1	5.92 d 2.1	5.94 d 2.1	–	–
8-*C*-methylpinocembrin extracted from *Eucalyptus*	5.44 dd 3.0 12.9	2.85 dd 3.1 17.1	3.04 dd 12.9 17.1	–	6.01 s	2.05 s	–
8-*C*-methylpinocembrin literature values (Seligmann and Wagner, 1981)	5.42 dd 5.0 12.0	2.8-3.4 m	2.8-3.4 m	–	6.02	2.05	–
6,8-di-*C*-methylpinocembrin extracted from *Eucalyptus*	5.41 dd 3.0 12.9	2.85 dd 3.1 17.0	3.04 dd 12.9 17.0	–	–	2.079 s	2.084 s
6,8-di-*C*-methylpinocembrin literature values (Solladié et al., 1999)	5.40 dd 3.3 12.1	2.85 dd 3.1 17.1	3.04 dd 12.1 17.1	–	–	2.07 s	2.08 s

described in Section 2.2.3.

2.2.2. Nuclear magnetic resonance spectroscopy

Two unidentified peaks were detected in relatively high abundances in HPLC chromatograms of some species. The peaks were particularly abundant in *E. agglomerata* leaf extracts, and so these were collected for structural elucidation using Nuclear Magnetic Resonance (NMR) spectroscopy. A peak with retention time and mass spectrum matching pinocembrin was also collected from the same species for structural confirmation using NMR spectroscopy. Peaks were collected following fractionation using the HPLC and eluent system described in Goodger et al. (2016), but with a Gemini C18 semi-preparative column (5 µm, 150 × 10 mm; Phenomenex) run at a flow rate of 5 mL min^{-1}. Fractions were collected in several batches and pooled collections of each peak dried under nitrogen. Approximately 1 mg of each dried, semi-purified peak was dissolved in 0.75 mL deuterated chloroform ($CDCl_3$; Cambridge Isotope Laboratories, Tewksbury, MA, USA), filtered, and transferred to a 5 mm NMR tube. ^{1}H and ^{13}C NMR spectra were acquired at 25 °C using a Bruker Avance III 600 MHz spectrometer (Bruker Corporation, Billerica, MA, USA) running the standard Bruker "zg30" pulse sequence (Tables 1a and 1b). $CHCl_3$ was used as an internal reference (7.26 ppm) for all spectra. gHMBC spectra in acetone-d_6 were recorded using a Bruker BioSpin 700 MHz.

3. Results and discussion

The 41 species and subspecies sampled represent three major sections within *E.* subg. *Eucalyptus,* and numerous series and subseries within these sections (Table 2). Species contained varying amounts of up to five abundant flavanones lacking B-ring oxygenation: pinocembrin, 7-*O*-methylpinocembrin, 5,7-di-*O*-methylpinocembrin, 8-*C*-methylpinocembrin and 6,8-di-*C*-methylpinocembrin (Fig. 1). ^{1}H and ^{13}C NMR chemical shifts for the two *C*-methylated flavanones agreed with previous reports (Cannon and Martin, 1977; Massaro et al., 2014; Tables 1a, 1b). The position of the methyl group in 8-*C*-methylpinocembrin was confirmed based on key gHMBC correlations of between C5 and OH5 (Shrestha et al., 2008); C6 and OH5; and C10 and OH5 (Seligmann and Wagner, 1981). Both *O*-methyl flavanones have been reported previously from other members of the genus (Goodger et al., 2016), whereas this is the first report of the two *C*-methylated flavanones from the genus. Nonetheless, they have been reported previously from the Myrtaceae: 6,8-di-*C*-methylpinocembrin was found in leaves, flowers and seed capsules of *Metrosideros* species (Mustafa et al., 2005) and in leaves of *Agonis spathulata* (Cannon and Martin, 1977), and both flavanones were found in seed capsules of *Corymbia torelliana* (Massaro et al., 2014). There was some evidence of other flavonoids such as mono-, di- and tri-methylated flavanones, and flavones in the MS data of particular species, but none of these compounds were in high enough abundance for accurate identification or quantification, nor were any present in amounts comparable to the high levels observed for the five abundant flavanones.

Foliar pinocembrin concentrations were highest in the two subspecies of *E. preissiana* with notably high levels of 15 and 18 mg g_{DW}^{-1} (Table 2, Fig. 2). These levels are much greater than the highest

Table 1b
^{13}C NMR (600 MHz) data for 8-*C*-methylpinocembrin extracted from *Eucalyptus* subg. *Eucalyptus* as compared to literature values. Deuterated chloroform was used as solvent.

			Chemical shift δ (ppm)						
Compound	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
8-*C*-methyl-pinocembrin extracted from *Eucalyptus*	80.25	44.21	197.89	163.53	96.99	166.23	104.66	161.65	103.82
8-*C*-methyl-pinocembrin literature values (Solladié et al., 1999)	79.6	43.6	197.2	161.0/162.9/165.3	96.4	161.0/162.9/165.3	103.3/104.1	161.0/162.9/165.3	103.3/104.1

Table 2
Taxonomic classification and foliar flavanone quantification for 41 *Eucalyptus* subg. *Eucalyptus* species. A representative tree of each species was sampled and a pool of up to eight fully-expanded leaves were extracted from each tree. Species distributions in Australian States are listed in order from most to least common. Species classification is based on (Nicolle, 2018).

Taxonomic classification within subgenus *Eucalyptus*				Natural distribution – State(s)	Foliar flavanones (mg g^{-1} $_{DW}$)				
Section	Series	Sub-Series	Species		PC	7-*O*-PC	5,7-D-*O*-PC	8-*C*-PC	6,8-D-*C*-PC
Eucalyptus	*Regnantes*		*E. regnans*	Tas, Vic	0.59	0.00	0.00	0.00	1.01
	Pachyphloiae		*E. agglomerata*	NSW, Vic	1.37	0.00	0.00	5.71	5.79
	Olsenianae		*E. boliviana*	NSW	0.56	0.00	0.00	0.00	0.04
	Strictae	*Irregulares*	*E. langleyi*	NSW	2.76	0.00	0.51	0.00	0.00
			E. triflora	NSW	1.16	1.83	13.69	0.00	1.25
			E. dendromorpha	NSW	0.04	0.62	7.32	0.00	0.00
			E. burgessiana	NSW	0.08	0.54	4.80	0.00	0.00
			E. spectratrix	NSW	0.00	0.50	7.40	0.00	0.00
			E. stricta	NSW	0.04	0.13	0.02	0.00	0.00
			E. apiculata	NSW	1.52	0.54	2.35	0.00	0.00
		Regulares	*E. cunninghamii*	NSW	0.86	0.78	3.13	0.00	0.00
			E. approximans	NSW	0.51	0.64	3.15	0.00	0.00
			E. microcodon	Qld, NSW	0.31	7.90	0.10	0.00	0.20
	Longitudinales		*E. mitchelliana*	Vic	6.76	0.00	0.00	0.00	0.31
			E. stellulata	NSW, Vic	3.27	3.56	8.99	0.00	0.00
			E. moorei subsp. *'latiuscula'* MS	NSW	1.04	5.40	0.12	0.00	0.00
			E. moorei subsp. *moorei*	NSW	1.16	0.00	0.00	0.00	0.00
			E. moorei subsp. *serpentinicola*	NSW	0.47	0.00	0.00	0.00	0.00
	Radiatae		*E. elata*	Vic, NSW	0.95	3.12	0.00	0.00	0.00
			E. croajingolensis	Vic, NSW	0.37	11.91	0.13	0.00	0.00
			E. dives	NSW, Vic	0.00	0.30	0.00	0.00	0.70
			E. falciformis	Vic, SA	1.79	2.31	8.75	0.00	0.00
			E. pulchella	Tas	0.00	0.26	0.45	0.00	0.46
			E. nitida	Tas	3.93	10.48	0.19	0.00	0.00
	Pauciflorae		*E. gregsoniana*	NSW	0.10	0.00	0.06	0.00	0.00
	Fraxinales		*E. oreades*	NSW, Qld	1.88	4.46	20.21	0.08	0.00
	Psathyroxyla	*Considenianae*	*E. remota*	SA	1.56	4.93	8.58	0.00	0.76
Longistylus	*'Pedaria' MS*		*E. brevistylis*	WA	0.00	0.00	0.00	0.00	0.00
	Jacksoniae		*E. jacksonii*	WA	0.00	0.00	0.00	0.00	0.17
	Occidentales		*E. staeri*	WA	0.00	0.00	0.00	0.03	1.56
	Patentes		*E. patens*	WA	0.00	0.00	0.00	0.08	0.15
'Frutices' MS	*Diversiformae*	*Neuropterae*	*E. diversifolia* subsp. *diversifolia*	SA, Vic	0.69	0.00	0.00	4.81	1.93
			E. diversifolia subsp. *hesperia*	WA, SA	0.52	0.00	0.00	2.36	1.70
			E. todtiana	WA	3.73	0.00	0.00	1.99	1.53
	Suberae		*E. suberea*	WA	0.00	0.00	0.00	0.00	0.00
	Muricatae		*E. sepulcralis*	WA	0.38	0.00	0.00	2.57	1.12
	Pressianae	*Glandulares*	*E. megacarpa*	WA	3.48	0.00	0.00	6.88	4.38
		Piluloculares	*E. preissiana* subsp. *lobata*	WA	17.90	0.11	0.00	1.43	1.72
			E. preissiana subsp. *preissiana*	WA	14.62	0.00	0.00	0.29	1.30
	Insulares		*E. insularis* subsp. *insularis*	WA	1.03	0.00	0.00	4.94	4.74
			E. insularis subsp.*continentalis*	WA	1.12	0.00	0.00	5.26	4.89

Abbreviations (States): Tas, Tasmania; Vic, Victoria; NSW, New South Wales; Qld, Queensland; SA, South Australia; WA, Western Australia; (Flavanones): PC, pinocembrin; 7-*O*-PC, 7-*O*-methylpinocembrin; 5,7-D-*O*-PC, 5,7-di-*O*-methylpinocembrin; 8-*C*-PC, 8-*C*-methylpinocembrin; 6,8-D-*C*-PC, 6,8-di-*C*-methylpinocembrin.

concentration of 2.5 mg g_{DW}^{-1} reported for commercially harvested *A. katsumadai* (Li et al., 2012). Seven species of *Eucalyptus* contained no detectable levels of pinocembrin, four of which were the only species sampled in section *Longistylus*. The highest concentration of 7-*O*-methylpinocembrin was 12 mg g_{DW}^{-1} found in *E. croajingolensis*, and that of 5,7-di-*O*-methylpinocembrin was a remarkable 20 mg g_{DW}^{-1} found in *E. oreades* (Fig. 2). The greatest amounts of the *C*-methyl forms were found in *E. megacarpa*, *E. agglomerata* (Fig. 2) and the two *E. insularis* subspecies (Table 2) with maxima of 7 mg g_{DW}^{-1} for 8-*C*-methylpinocembrin in *E. megacarpa* and 6 mg g_{DW}^{-1} for 6,8-di-*C*-methylpinocembrin in *E. agglomerata*.

Some general trends are evident in the flavanone data presented in Table 2. Firstly, flavanone profiles for closely related eucalypts such as subspecies are very similar. This is perhaps unsurprising given the molecular genetic basis of flavanone biosynthesis (Winkel-Shirley, 2001) and the close genetic relatedness between subspecies. Moreover, species seem to fit into three distinct categories: those with high concentrations of relatively pure non-methylated pinocembrin, those with highly abundant *O*-methyl flavanones, or those with abundant *C*-methyl flavanones (Fig. 3). This is particularly evident in the 12 species from Western Australia that have undetectable or trace amounts of *O*-methyl flavanones, but have high amounts of pinocembrin or *C*-methylated forms (Table 2). This suggests there are two different enzymes responsible for *O*- and *C*-methylation, and that generally at least one of them is absent or relatively inactive in eucalypt species. Alternatively, the *C*-methyl compounds may be derived from the *O*-methyl compounds through an enzymatic transformation that only certain eucalypt species can effect. Flavonoid *O*-methyltransferases have been relatively well characterised from plants (e.g. Itoh et al., 2016), but nothing is known of flavonoid *C*-methyltransferases. Moreover, little is known of *C*-methyltransferases in general, except for those involved in tocopherol biosynthesis in plants (Koch et al., 2003) and enzymes characterised from fungi and marine microbes (Crnovčić et al., 2010; Freeman, 2018). Some of the *Eucalyptus* species reported here could be promising systems in which to study these poorly understood biosynthetic enzymes.

Two species were chosen for inclusion in a trial plot based on their similar total flavanone quantities, but different flavanone profiles: *E. preissiana* subsp. *preissiana*, which is high in pinocembrin, and *E. nitida*, high in 7-*O*-methylpinocembrin (Fig. 2). Saplings of both species

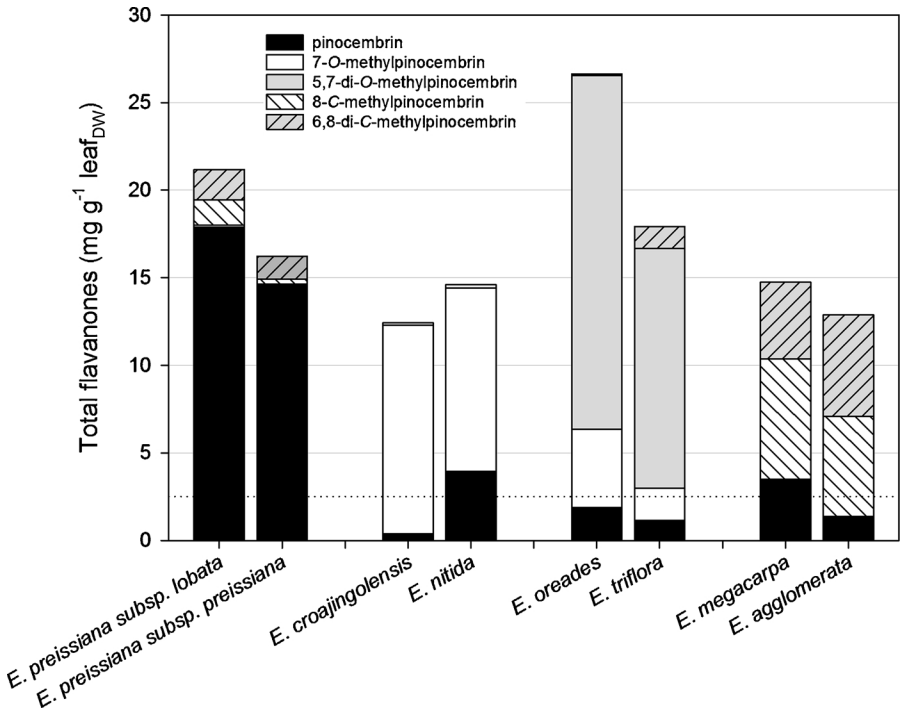


Fig. 2. Quantification of foliar flavanones in *E.* subg. *Eucalyptus* highlighting the two species with the greatest concentration of each of five flavanones. High concentrations were observed in these species, especially when compared with the maximum concentration reported for pinocembrin in *Alpinia katsumadai* (dashed line Li et al., 2012).

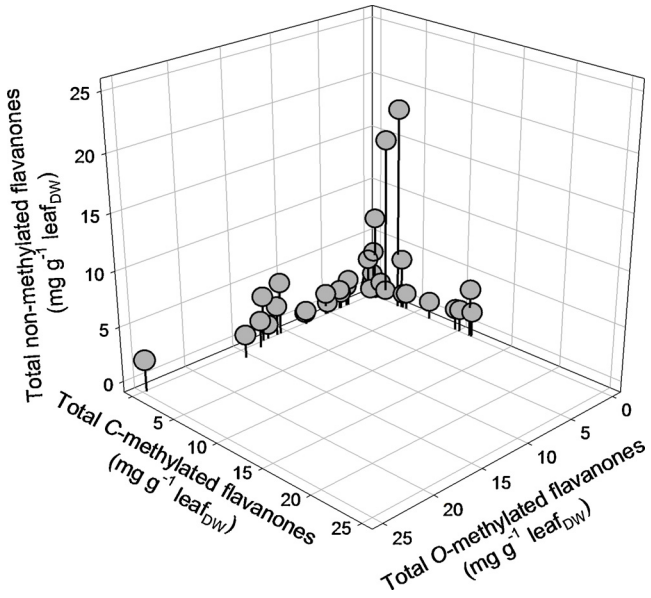


Fig. 3. The relationship between total *O*-, *C*- and non-methylated flavanones for all sampled species of *E.* subg. *Eucalyptus*.

established well in the trial plot, with the large-leaved *E. preissiana* growing to an average height of 1.1 m and the small-leaved *E. nitida* growing to an average height of 1.5 m after 13 months in the field plot (Table 3).

Saplings of *E. preissiana* showed similar flavanone profiles to the adult arboretum tree with pinocembrin the dominant flavanone (70–90% of the total) and only low amounts of mono- and di-*C*-methylated flavanones observed (Fig. 4A). Pinocembrin concentrations in the half-siblings ranged from 3 to 8 mg g_{DW}^{-1} with a mean of 4 mg g_{DW}^{-1}. The di-*C*-methylated flavanone content exceeded that of its mono-*C*-methylated counterpart in all but two individuals, and as with the arboretum tree, no 6-*C*-methylpinocembrin was detected in any individual. In contrast, saplings of *E. nitida* were more variable in their constituent profiles with 7-*O*-methylpinocembrin the dominant flavanone in only five of the twelve half-siblings. The dominant flavanone in the other seven individuals was 5-7-di-*O*-methylpinocembrin (Fig. 4B). Interestingly, five half-siblings contained very low levels of pinocembrin (< 5% of the total), and like the arboretum tree, none showed any trace of 5-*O*-methylpinocembrin.

It is noteworthy that the flavanone concentrations in the saplings were substantially lower than the adult arboretum tree values (Figs. 2 and 4). Pinocembrin is housed in foliar oil glands (Goodger et al., 2016) and an ontogenetic delay to maximum oil gland size and density has been observed in eucalypts (Goodger et al., 2013). Moreover, such ontogenetic delays have been observed for different secondary metabolites in a range of *Eucalyptus* species (Goodger et al., 2007a, 2018; Goodger and Woodrow, 2009). Given this, it can be assumed that the saplings will obtain flavanone concentrations comparable with the arboretum trees as they mature. Future studies are required to determine the optimum time for harvesting to maximise flavanone yields.

Similarly, the variability observed between half-siblings of each species has also been observed for other secondary metabolites in population screens of eucalypts (Goodger et al., 2002; Goodger and

Table 3
Analysis of half-sibling families of two *Eucalyptus* species after 13 months in a trial plot. Saplings were assessed for the most abundant foliar flavanone found in arboretum trees, and for growth and morphological parameters. Data is presented as the mean of 12 saplings per species with one standard error in parentheses.

Species	Most abundant flavanone in arboretum tree samples	Plant height	Average leaf area	Leaf Mass per unit Area
E. preissiana	4.42 (0.51)	1.09 (0.05)	44.3 (3.7)	247 (6)
subsp. *preissiana*	mg pinocembrin g_{DW}^{-1}	m	cm^2	g m^{-2}
E. nitida	0.34 (0.12)	1.49 (0.06)	5.7 (0.6)	183 (53)
	mg 7-*O*-methylpinocembrin g_{DW}^{-1}	m	cm^2	g m^{-2}

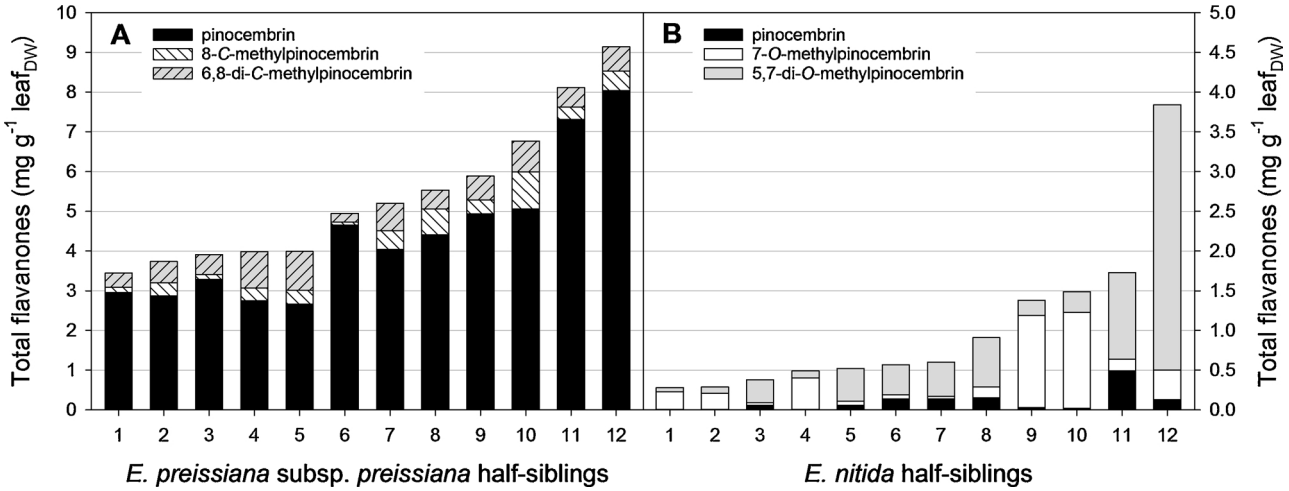


Fig. 4. Assessment of foliar flavanones in families of two *E.* subg. *Eucalyptus* species after 13 months growth in a trial plot. Each half-sibling family is ordered on total foliar flavanones.

Woodrow, 2002). This variation has been attributed largely to genetic factors (Goodger et al., 2004; Goodger and Woodrow, 2012). If we assume a genetic basis to flavanone variability within species, then it is likely that selective breeding and/or the development of clonal seed orchards of selected elite individuals will provide improved seed sources for future commercial plantation establishment.

4. Conclusions

Trees in *E.* subg. *Eucalyptus* show great potential as commercial sources of medicinal flavanones, with many species containing abundant quantities of pinocembrin or related *O*- and *C*-methylated forms of the compound. These results will enable selection of high-yielding species and genotypes for future establishment of commercial flavanone plantations.

Acknowledgements

The authors thank Mr. Alistair Cumming, CEO of Gretals Australia for supporting the research, Metabolomics Australia for LCMS analyses, and Ms. Ros Gall, Director of the Dookie Campus, University of Melbourne for the use of land and facilities for the trial plot. The work was funded by an Australian Research Council grant to IW and JG (LP160101307). The NMR work was supported by Australian Research Council grants to SJW (DP160100597, DP180101957).

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