

CHEMICAL COMPOSITION OF CUTICULAR WAXES AND CUTIN IN *EUCALYPTUS GLOBULUS* LEAVES

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ABSTRACT

In leaves, epidermal cells and cuticle, including epicuticular and intracuticular waxes, and cutin, are frontiers to the environment. The cuticle of *Eucalyptus globulus* was previously studied only in isolated cuticles and this work used a whole leaf approach to characterize cuticular waxes and cutin in juvenile and adult leaves. Cuticular waxes were extracted by dichloromethane (DCM) in complete leaves first to extract epicuticular waxes, and subsequently to extract intracuticular waxes. Internal DCM solubles were removed by 6h-soxhlet extraction, and cutin determined by methanolysis. Composition of cuticular waxes, internal DCM solubles, and cutin were analysed by GC-MS. Juvenile and adult leaves contained respectively 2.3% and 1.8% total cuticular waxes (157 and 205 $\mu\text{g}/\text{cm}^2$), with epicuticular waxes representing 44.4% and 31.3% of total waxes, and 15.6% and 16.9% cutin. Internal DCM solubles amounted to 4.2% and 3.9%. Epicuticular and intracuticular waxes showed no significant compositional differences, containing mostly terpenic compounds (average 81.5% of total compounds, mainly ursolic and oleanolic acids and β -eudesmol). Cutin contained 73.1% ω -hydroxyacids, 7.7% carboxylic acids and 2.1% α,ω -diacids largely with C18 chain length. Proportion and composition of *E. globulus* cuticles allow considering them as a chemicals' source, and integrating leaves as a resource for a biorefinary-based valorization.

KEYWORDS: epiderm, cuticle, cutin, waxes, terpenoids

INTRODUCTION

The epidermal cells of plant leaves and their overlaying and connected cuticle are frontiers to the environment. Leaf cuticle is a boundary membrane involved in the regulation of non-stomatal water loss and gas exchange, and confers resistance to several biotic and abiotic attacks, therefore at the forefront of potential responses to adverse or changing environmental conditions [1-3]. The cuticle consists of cutin, an insoluble glyceridic polyester matrix of hydroxyl fatty acids mostly with C16–C18 chain lengths, and of cuticular waxes soluble in low polarity solvents composed of intracuticular waxes, embedded in the cutin matrix, and epicuticular waxes deposited as an external layer, including triterpenoids and long-chain aliphatic hydrocarbons, carboxylic acids and esters [4,5].

Despite the functional and protective roles of cuticles in leaves, relatively few studies have characterized them in trees even in species of high commercial importance. This is the case of *Eucalyptus globulus*, a major pulpwood species with extensive areas in temperate regions producing a valued industrial fibre material for pulping and paper production.

E. globulus leaves have a high heteroblastic development: juvenile leaves are broad, generally oriented horizontally, dorsiventral, with stomata only on the abaxial surface, while adult leaves are oblong, thick, petiolated, generally oriented vertically, isobilateral with stomata on both sides [6]. Juvenile and adult leaves have distinct functional properties regarding photosynthesis, transpiration and isoprene and monoterpene emissions [7,8].

The cuticle of *E. globulus* has been studied at anatomical and chemical levels in isolated cuticles from the adaxial surface [9]. The waxes represented 37.3% of the cuticle, composed by terpenes and terpenoids (45%), followed by phenolics (21%), while cutin represented 53.6 % of the cuticle and was chiefly composed of aliphatic ω -hydroxyacids (69%) and α,ω -diacids (8%) with a C18/C16 ratio of 3.1. A few other studies, although not directly targeted to characterize the cuticle, give information on amount and composition of waxes and cutin, although their results are frequently not coincident. For instance, the dichloromethane 8h-soxhlet extraction of ground leaves that may be considered as corresponding to removal of the total cuticular waxes, yielded 3.4% of dry leaf mass, including mostly triterpenic acids (3.1%) with small amounts of fatty acids and alcohols (0.3%) [10]. The petroleum ether/acetone extraction by rapid immersion, which may be considered to correspond to epicuticular waxes, extracted 0.7% and 0.9% of the dry leaf biomass from juvenile and adult leaves respectively, and included mostly triterpenoid compounds (0.5% of dry leaf mass) with a small proportion of fatty acids and alcohols [11]. Another study reported yields obtained by rapid immersion in petroleum ether/acetone and n-hexane/acetone show 5.7% and 5.2% respectively, mainly composed of triterpenic acids, while the subsequent dichloromethane soxhlet extraction yielded 2.4% and 2.9% respectively, therefore giving an approximation to the proportion of epicuticular and intracuticular waxes respectively [12].

The present work is targeted to study the amount and composition of cuticular waxes and cutin of *E. globulus* juvenile and adult leaves on both adaxial and abaxial sides, using a whole leaf approach and expressing the results on a dry leaf mass basis (mg/g) and as a leaf coverage (μg per cm^2 of leaf area). The cuticular waxes were solubilised with dichloromethane in a two step process: first with a short contact time at ambient temperature to extract mostly the epicuticular waxes, and second with a 3 h near boiling point extraction of the intracuticular waxes. The dewaxed leaves were shredded and exhaustively extracted with dichloromethane to remove all internal solubles prior to cutin determination after depolymerization by methanolysis. The composition of cuticular waxes, cutin and internal leaf solubles was analysed by GC-MS and here reported grouped in their main chemical families.

EXPERIMENTAL

Juvenile and adult leaves of *Eucalyptus globulus* were sampled, taking only whole leaves without any fracture or damage of the blade. Dichloromethane (DCM) was used for cuticular waxes extraction (Simões et al. 2022), using a shallow rectangular glass vessel (30 cm x 20 cm x 5 cm) where the leaves were immersed flat in the solvent. The leaves were first digitalized and analysed using WinSEEDLETM 2011 for area determination, and oven-dried at 60°C to a constant mass and the total dry mass per leaf was determined.

The epicuticular waxes were solubilized by a short 5 min immersion at ambient temperature (23°C) in 500 ml of DCM. The solvent was taken, evaporated in a rotavapor, and kept for chemical analysis. The extracted leaves were dried at 60°C overnight and weighted. The intracuticular waxes were extracted with 500 ml of DCM using the same glass vessel in a water bath at 36°C during 3 h. The solvent was taken, concentrated, and kept for chemical analysis. The leaves were dried at 60°C overnight. With this experimental procedure, the cuticular waxes were extracted from both surfaces of the leaves. The amount of the solubilized cuticular material was expressed on a dry weight basis (g/100 g dry leaf mass) and on a leaf surface area (as $\mu\text{g}/\text{cm}^2$), corresponding to a leaf surface coverage, with the area being the two-sided leaf surface area.

The leaves were shred into about 1 cm^2 pieces and soxhlet extracted with DCM during 6 h to determine internal lipids here called leaf internal DCM solubles. The solvent was evaporated and kept for analysis, and the leaf pieces dried at 60°C. The depolymerization of cutin was carried out by transesterification with a sodium methoxide (NaOMe)-catalyzed methanolysis applied to the extracted leaves (Pereira, 1988; Simões et al., 2021). Approximately 1.5 g extracted leaves were refluxed during 3 h with 100 mL of a methanolic 3% NaOCH₃ solution, filtrated, washed with CH₃OH, refluxed again with 100 mL CH₃OH during 15 min, filtrated, the combined filtrates acidified to pH 6 with 2 M H₂SO₄, and

evaporated to dryness. The residue was suspended in 50 mL of water and extracted three times with DCM (50 mL each). The combined DCM extracts were dried over anhydrous Na₂SO₄ and evaporated to dryness.

Cutin was quantified by the mass loss by methanolysis after drying and weighing the leaves residue, and was expressed in percent of the initial dry weight basis (g/100 g dry leaf mass) and on a leaf surface area (as $\mu\text{g}/\text{cm}^2$), corresponding to a leaf surface coverage, the area being the two-sided leaf surface area.

The extracts of epicuticular and intracuticular waxes, the leaf internal DCM solubles, and the cutin monomers obtained by transesterification of the extracted leaves were solubilized in DCM and derivatized into silylated derivatives for GC–MS analysis. The procedure follows that described by Simões et al. (2020). The compounds were identified as TMS derivatives by comparing with the spectral library Wiley, NIST, and with published data. The peak area in the GC–MS total ion chromatograms was integrated, and each peak was quantified in area proportion of the total chromatogram area.

RESULTS AND DISCUSSION

The mass proportion of cuticular waxes and cutin the juvenile and adult leaves of *E. globulus* are given in Table 1. Cuticular waxes represent respectively 5.2% and 5.8% of o.d. mass of juvenile and adult leaves, of which epicuticular waxes correspond to 2.3% and 1.8%. These determinations were made on complete leaves, therefore corresponding to the surface solubilization of the lipophilic compounds (epicuticular and intracuticular waxes) from both upper and lower sides of the leaves. When extraction of lipophilic compounds is allowed to assess the internal part of the leaves, by using comminuted leaves (internal DCM solubles) a further removal of 4.2 and 3.9% of od mass was obtained.

A direct comparison with the few reported results is difficult due to the different methodologies e.g. solvent, extraction time and temperature, leaf particle size, and the fact that neither study was targeted to characterize the cuticle in mass proportion and leaf coverage, as it was the case here.

While taking into account these aspects, namely by considering the assignment of the extracts yields to the cuticular waxes only as a rough approximation, some reported values are the following: total cuticular waxes of 3.4% [10] or 7.3% [12], epicuticular waxes of 0.7% and 0.9% of juvenile and adult leaves respectively [11] or 5.2-5.7%, and 2.4-2.9% for intracuticular waxes [12].

Cutin corresponded to a substantial mass proportion of the leaves of 15.6% and 16.9%, respectively in juvenile and adult leaves (Table 1). Cutin therefore represents 75% of the cuticle (including the cuticular waxes and cutin), conferring the leaves a strong protective layer of this macromolecular matrix. The only available comparative value is the 54% of cutin in isolated cuticles of the adaxial leaf surface of *E. globulus* [9].

When analysing the cuticle development in terms of leaf coverage, a significant difference was found between juvenile and adult leaves, in line with their different development [6], giving adult leaves nearly a two fold higher cuticular waxes and cutin coverage (Table 1). In juvenile and adult leaves, respectively, leaf coverage of cuticular waxes was $347 \mu\text{g}/\text{cm}^2$ and $647 \mu\text{g}/\text{cm}^2$ and of cutin was $1054 \mu\text{g}/\text{cm}^2$ and $1890 \mu\text{g}/\text{cm}^2$. To our knowledge these are first time reported cuticular leaf coverage values. These values are high compared to values of other tree species, e.g. of *Quercus suber* [5,13].

The high proportion of cuticular waxes and cutin in the leaves of *E. globulus* not only in mass proportion but also in relation to the leaf coverage indicates that the leaves have a substantial membrane complex that will confer enhanced environmental protection and interaction as well as an improved physical resilience, especially in adult leaves.

Table 1. Chemical characterization of juvenile and adult leaves of *Eucalyptus globulus*, regarding epicuticular and intracuticular waxes, and cutin (mg/g of dry leaves and mg/cm²), leaf internal dichlorometane (DCM) solubles (mg/g of dry leaves) are also reported. Mean and standard deviation.

Leaf features	Juvenile	Adult
Epicuticular waxes (mg/g)	23.2 ± 1.1	18.2 ± 2.0
Epicuticular waxes (µg/cm ²)	156.9 ± 9.7	203.2 ± 14.7
Intracuticular waxes (mg/g)	28.9 ± 2.4	39.9 ± 12.2
Intracuticular waxes (µg/cm ²)	190.5 ± 13.4	443.5 ± 151.6
Total (mg/g)	52.2 ± 1.3	58.1 ± 10.3
Total (µg/cm ²)	347.4 ± 3.7	646.7 ± 136.9
Cutin (mg/g)	155.9 ± 1.8	169.3 ± 7.5
Cutin (µg/cm ²)	1054.4 ± 12.2	1890.2 ± 83.7
Internal DCM solubles (mg/g)	42.2 ± 0.9	38.9 ± 8.8

The chemical composition of cuticular waxes is given in Table 2 by chemical families and Table 3 reports their triterpenic composition. Juvenile and adult leaves have a similar composition. Epicuticular waxes include mostly terpenoids that represent on average 80.9% of all compounds, constituted by the triterpenes ursolic acid and oleanolic acid, Intracuticular waxes are also dominated by terpenic compounds (82.1% of all compounds) constituted by ursolic acid, echynocystic acid and oleanolic acid.

The predominance of triterpenic acids in DCM extracts of *E. globulus* leaves has been reported in the other studies [10-12]).

Table 2. Composition by chemical class of epicuticular and intracuticular waxes of juvenile and adult leaves of *Eucalyptus globulus* (% of the total chromatogram peak areas). Mean and standard deviation.

Chemical class	Epicuticular waxes		Intracuticular waxes	
	Juvenile	Adult	Juvenile	Adult
n-Alkanols	1.7 ± 0.3	4.2 ± 1.6	3.9 ± 0.0	1.9 ± 0.0
n-Alkanes	0.3 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Fatty acids	2.0 ± 0.8	1.8 ± 0.0	5.0 ± 0.9	2.1 ± 0.6
Ketone	4.6 ± 3.7	1.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Diacids	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Aromatic compounds	0.5 ± 0.3	0.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
Sterols	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.3	2.0 ± 0.0
Terpenes	79.4 ± 3.0	82.4 ± 1.1	78.9 ± 3.1	85.3 ± 3.1
Sugars	0.00 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	1.4 ± 0.9
Others	1.7 ± 1.4	0.9 ± 0.8	0.1 ± 0.0	0.2 ± 0.1
Total identified compounds	90.2 ± 0.9	91.3 ± 1.2	92.4 ± 1.6	93.3 ± 1.4

Table 3. Triterpenic composition of epicuticular and intracuticular waxes of juvenile and adult leaves of *Eucalyptus globulus* (% of the total chromatogram peak areas). Mean and standard deviation.

Triterpene	Epicuticular waxes		Intracuticular waxes	
	Juvenile	Adult	Juvenile	Adult
Ursolic acid	53.1 ± 1.0	49.7 ± 2.2	46.1 ± 1.2	54.6 ± 6.4
Oleanolic acid	22.2 ± 1.4	26.7 ± 1.0	11.8 ± 1.7	9.0 ± 2.3
Echynocystic acid	-	-	18.5 ± 2.3	13.8 ± 0.5

The chemical composition of cutin is given in Table 3 by chemical families showing similar values for juvenile and adult leaves. The cutin monomers are mostly ω -hydroxyacids (73.1% of all compounds) with also carboxylic acids (7.7%) and α,ω -diacids (2.4%). The main cutin monomers were 9-epoxy-18-hydroxyoctadecanoic acid (36.0%) and 10,16-dihydroxyhexadecanoic acid (16.8%). This is accordance with the cutin composition previously reported with these two compounds also as the main monomers [9].

The cutin extract also contained some terpenic compounds that still remained in the leaves after the soxhlet extraction of the internal DCM solubles, and were solubilized during the methanolysis procedure. The composition of the internal DCM solubles (Table 5) is dominated by terpenic compounds (75.7%) but differing from the cuticular waxes by including monoterpenes (1,8-cineol, α -terpineol, geranyl acetate), sesquiterpenes (aromadendrene, globulol, β -eudesmol) and triterpenes (ursolic acid).

Table 4. Composition by chemical class of cutin of juvenile and adult leaves of *Eucalyptus globulus* (% of total chromatogram peak area). Mean and standard deviation

Chemical class	Juvenile	Adult
Fatty acids	7.9 \pm 0.9	7.5 \pm 0.4
α, ω - Diacids	2.3 \pm 0.2	2.5 \pm 0.7
ω - Hydroxyacids	74.8 \pm 0.5	71.3 \pm 1.7
Aromatic compounds	0.2 \pm 0.0	0.2 \pm 0.0
Terpenes	2.6 \pm 0.2	4.3 \pm 0.1
Others	4.1 \pm 0.6	5.9 \pm 0.9
Total identified compounds	92.0 \pm 0.5	92.3 \pm 0.5

Table 5 Composition by chemical class of the leaf internal dichlorometane (DCM) solubles after extraction of cuticular waxes (epicuticular and intracuticular) of juvenile and adult leaves of *Eucalyptus globulus* (% of total chromatogram peak areas). Mean and standard deviation.

Chemical class	Juvenile	Adult
n-Alkanols	0.7 \pm 0.3	0.2 \pm 0.1
n-Alkanes	0.1 \pm 0.0	0.0 \pm 0.0
Fatty acids	1.0 \pm 0.3	0.6 \pm 0.3
Diacids	0.0 \pm 0.0	0.1 \pm 0.0
Aromatic compounds	0.7 \pm 0.1	1.5 \pm 0.0
Sterols	4.6 \pm 1.2	1.9 \pm 0.3
Terpenes	72.6 \pm 0.7	78.7 \pm 0.1
Sugars	0.2 \pm 0.2	1.0 \pm 0.2
Total identified compounds	79.9 \pm 1.1	83.9 \pm 0.8

E. globulus leaves showed a substantial surface coverage with cuticular waxes, particularly of coverage was very high. These characteristics may be associated with an enhanced capacity to control water loss and an improved surface structural resilience. Apart from the physiology-related role of the cuticle and the planet-environment interactions that this interface membrane allows, the *E. globulus* cuticles may be considered as a chemical source of terpenic compounds and of fatty acids and related aliphatic compounds. Figure 1 exemplifies the formula of the main terpenic compounds that are present in *E. globulus* leaves. The large potential of plant cuticles has already been proposed given their availability and composition [14]. This enlarges the valorization of *E. globulus* foliage, at present mostly restricted to the hydrodistillation of essential oils. Therefore a biorefinery integrated approach to use eucalypt

leaves has potential to be considered using cuticles, essential oils and other extractable compounds, namely phenolics, and further the lignocellulosic residual biomass.

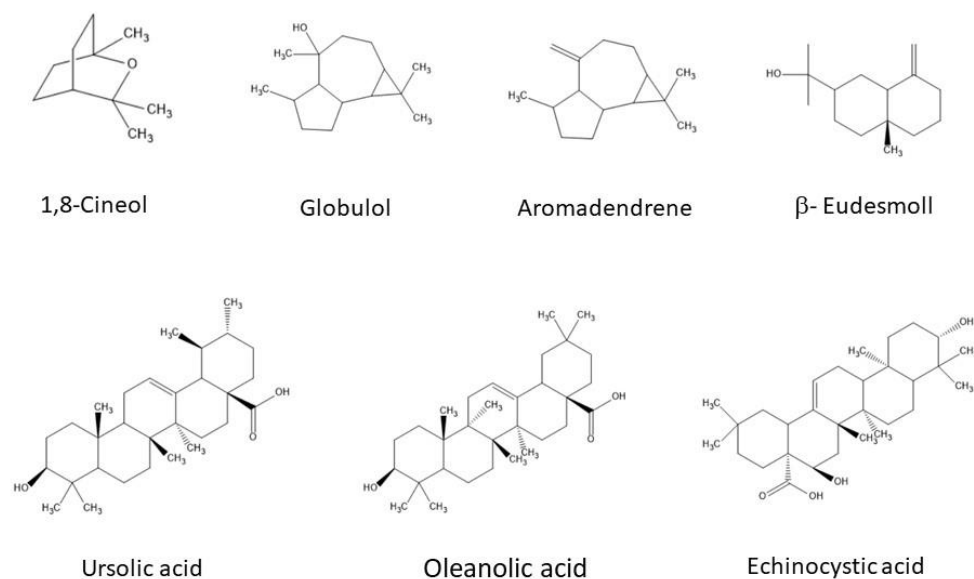


Figure 1. Chemical structure of the main terpenic compounds present in *E. globulus* leaves

CONCLUSIONS

The quantification and chemical composition of the cuticle components – epicuticular and intracuticular waxes, and cutin – of *Eucalyptus globulus* juvenile and adult leaves, allows the following conclusions.

The mass proportion of cuticular waxes and cutin is similar in juvenile and adult leaves, but the distinct heteroblastic development leads to a higher leaf surface coverage of waxes and cutin in adult leaves. Cutin content is particularly high suggesting an enhanced resilient behavior of leaves. Overall the substantial *E. globulus* cuticle indicates an effective performance as a plant-environment frontier membrane.

The chemical composition of waxes and cutin is similar in juvenile and adult leaves. The major components of cuticular waxes are triterpenic acids, mainly ursolic acid. Cutin is characterized by ω -hydroxyacids as major monomers, with carboxylic acids and diacids present in smaller proportions.

E. globulus foliage has potential as a chemical source for an integrated valorization.

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