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# High value triterpenic compounds from the outer barks of several *Eucalyptus* species cultivated in Brazil and in Portugal

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ABSTRACT

The chemical composition of the lipophilic extracts of the inner and outer bark fractions of *Eucalyptus grandis and Eucalyptus urograndis* (*E. grandis* × *Eucalyptus urophylla*) cultivated in Brazil and *Eucalyptus maidenii*, cultivated in Portugal was studied by gas chromatography–mass spectrometry. The extracts were shown to be mainly composed of triterpenic compounds (along with mono and sesquiterpenes in *E. maidenii*) followed smaller amounts of fatty acids, fatty alcohols, and aromatic compounds.

Triterpenic acids (mainly ursolic, betulinic and oleanolic acids), are particularly abundant in outer barks representing 5.2 g/kg, 5.7 g/kg and 9.3 g/kg in *E. urograndis*, *E. grandis* and *E. maidenii* outer barks, respectively. Although these compounds were found in considerably smaller amounts than those previously reported for *Eucalyptus globulus*, the total amounts of bark generated every year in South American pulp mills using *E. urograndis and E. grandis*, as well as the growth potential of *E. maidenii* plantations, the bark residues from these species are obvious candidates for the extraction of valuable triterpenic compounds.



## 1. Introduction

*Eucalyptus* spp. are the most important fiber sources for pulp and paper production in southwestern Europe (Portugal and Spain), South America (Brazil and Chile), South Africa, Japan and other countries (Rencoret et al., 2007). The increasing interest on many *Eucalyptus* species as wood sources for pulp production is related with their rapid growth, their behavior during pulping and bleaching and the excellent properties of the final pulps (Freire et al., 2002a, 2005; Gutierrez et al., 1999; Rencoret et al., 2007).

In Iberian countries *Eucalyptus* plantations (predominantly *Eucalyptus globulus*) occupied by 2008 approximately 1.29 million ha, whereas in South America *Eucalyptus grandis* and *Eucalyptus urograndis* (*E. grandis* × *Eucalyptus urophylla*) are among the preferred species, representing approximately 3.75 million ha of plantations (Trabado and Wilstermann, 2008). It is estimated that about 2010, these two world regions together will produce around 14.7 million ton of *Eucalyptus* spp. pulp, representing 81% of the *Eucalyptus* spp. pulp produced worldwide (BRACELPA, 2009). Furthermore, South America is playing an increasingly important role in the production of eucalyptus wood and pulp. Brazil, for example, jumped from the 7th position as pulp producer in the world (and first in *Eucalyptus* pulp production) by to 2000, to become presently the 4th world pulp producer (ABRAF, 2010; Mora and Garcia, 2000). Furthermore in the last five years the *Eucalyptus* spp. planted area increased by 41% in this country (ABRAF, 2010), demonstrating the growing tendency on the exploitation of these species in South America.

The renewed interest in the integrated exploitation of plants biomass as sources of materials, chemicals, fuels and energy within the biorefinery concept (Fernando et al., 2006; Kamm et al., 2006; Ragauskas et al., 2006) has attracted the interest of agro-forest industries in the perspective of taking the maximum value out of their crops. This approach has also triggered the interest of pulp and paper industries as this sector produces considerable amounts of residues such as bark, normally removed in the mills and burned for energy production, but also leaves, branches and fruits from harvesting and logging operations which are either left in the forest for nutrition or burned in the biomass boiler.

Some of these residues can have high value components in their composition, which can be exploited without affecting the current most important outputs of the existent mills (pulp and power), while minimizing the waste streams. For instance, the exploitation of valuable extractives, such as phytosterols, namely β-sitosterol

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(Fernandes and Cabral, 2007; Hamunen, 1983; Niemela, 1990), lignans (Pietarinen et al., 2006; Willfor et al., 2004a,b) and triterpenoids (Kolomitsyn et al., 2007; Krasutsky, 2006) from by-products of the industrial processing (e.g. bark, knots, pulping liquors), is a strategy already implemented in some pulp industries and can be considered among the most successful examples of the implementation of the biorefinery concept integrated in the pulp mills (Huang et al., 2008; Kamm et al., 2006).

In the case of *Eucalyptus* ssp., bark is among the most interesting residues (Domingues et al., 2010; Freire et al., 2002b). It has been reported that the lipophilic extracts of *E. globulus* outer bark contain high amounts of several triterpenic acids with lupane, ursane and oleanane skeletons, namely, betulonic, betulinic, 3-acetylbetulinic, ursolic, 3-acetylursolic, oleanolic and 3-acetyloleanolic acids (see Fig. 3 bellow) (Domingues et al., 2010; Freire et al., 2002b). These triterpenic acids are recognized as promising compounds for the development of new multi-targeting bioactive agents (Dzubak et al., 2006; Laszczyk, 2009; Sultana and Ata, 2008; Yogeeswari and Sriram, 2005). For example, oleanolic and ursolic acids show significant anti-tumor (Li et al., 2002) and anti-angiogenic (Cardenas et al., 2004; Sogno et al., 2009) properties; betulinic acid is also known for its antitumoral properties (Tolstikova et al., 2006), and as precursor for anti-HIV drugs, such as *bevirimat* (Lee et al., 1997), which soon will be a phase III drug (Smith et al., 2007).

In this vein, the search for new biomass sources of these valuable triterpenes represents a very important issue (Jager et al., 2009).

The composition of the lipophilic extractives from *E. grandis* and *E. urograndis* wood, used in South America, has already been described in the literature, with particular emphasis in their role on formation and deposition of pitch on pulps and equipments during the wood processing for pulp and paper production (Freire et al., 2006; Miranda and Pereira, 2001; Rencoret et al., 2007; Silverio et al., 2007a,b). However, to the best of our knowledge there is no information about the composition of the lipophilic fractions of barks from these species, which based on the production figures mentioned above should originate huge amounts of bark residues annually. It is therefore of high interest to evaluate their potential as sources of valuable compounds in order to develop possible extraction and purification strategies.

*Eucalyptus maidenii* is not so widely explored as a wood source for pulp production as the three *Eucalyptus* species mentioned so far, but it is already used in considerable amounts (Rencoret et al., 2007) demonstrating very interesting characteristics and potential for forest breeding and new papermaking opportunities (Kibblewhite et al., 2001) and, to the best of our knowledge there is still also no information concerning the analysis of the lipophilic bark extractives.

In this context, the aim of the present paper is to evaluate the potentialities of *E. grandis*, *E. urograndis* and *E. maidenii* bark as a source of valuable triterpenoids, pointing out new ways to up-grading bark, one of the main *Eucalyptus* spp. pulp industry by-products, within the context of the biorefinery integrated in the pulp mills. Thus, the dichloromethane extracts of the outer and inner barks from the referred *Eucalyptus* spp. were prepared and analyzed by gas chromatography–mass spectrometry (GC–MS), and the potential of these species as sources of high value triterpenic compounds is discussed.

## 2. Materials and methods

### 2.1. Samples

*E. urograndis* and *E. grandis* bark samples were taken from a 5-year-old and 10-year-old tree, respectively, randomly sampled from clone plantations cultivated in Alfredo Chaves, state of Espírito Santo, Brazil (20°38′08″ S, 40°44′57″ W), while *E. maidenii* bark was obtained from a 10-year-old tree randomly sampled in a clone plantation cultivated in Odemira, southwestern region of Portugal (37°33′04″ N, 8°38′43″ W).

The two different morphological regions of the bark, inner and outer bark, were handly separated as described elsewhere (Freire et al., 2002b) and analyzed in separate. Representative samples of each bark fractions were air dried until a constant weight was achieved and ground to a granulometry lower than 2 mm prior to extraction.

### 2.2. Extraction

All samples of inner and outer bark (15 g) were Soxhlet extracted with dichloromethane for 7 h. The solvent was evaporated to dryness, the extracts were weighed and the results were expressed in percent of dry bark. Dichloromethane was chosen because it is a fairly specific solvent for lipophilic extractives.

### 2.3. Alkaline hydrolysis

20 mg of each extract were dissolved in 10 ml of 1 M KOH in 10% aqueous methanol. The mixture was heated at 100 °C, under nitrogen atmosphere in caped reaction vessels, for 1 h. The reaction mixture was cooled, acidified with 1 M HCl to pH ~ 2 and then extracted three times with dichloromethane. The solvent of the combined organic fractions was evaporated to dryness.

### 2.4. GC–MS analysis

Before GC–MS analysis, nearly 20 mg of each dried sample were converted into trimethylsilyl (TMS) derivatives according to the literature (Ekman, 1983; Freire et al., 2002b). GC–MS analyses were performed using a Trace Gas Chromatograph 2000 Series equipped with a Thermo Scientific DSQ II mass spectrometer, using helium as carrier gas (35 cm $s^{-1}$), equipped with a DB-1 J&W capillary column (30 m × 0.32 mm i.d., 0.25 mm film thickness). The chromatographic conditions were as follows: initial temperature: 80 °C for 5 min; temperature rate of 4 °C $min^{-1}$ up to 260 °C; 2 °C $min^{-1}$ till the final temperature of 285 °C; maintained at 285 °C for 10 min; injector temperature: 250 °C; transfer-line temperature: 290 °C; split ratio: 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan $s^{-1}$ over a range of $m/z$ 33–700. The ion source was maintained at 250 °C.

For quantitative analysis, GC–MS was calibrated with pure reference compounds, representative of the major lipophilic extractives components (namely, palmitic acid, nonacosan-1-ol, β-sitosterol, betulinic acid, ursolic acid and oleanolic acid), relative to tetracosane, the internal standard used. The respective multiplication factors needed to obtain correct quantification of the peak areas were calculated as an average of six GC–MS runs. For aromatic compounds, monoterpenes and sesquiterpenes a response factor of 1.0 was assumed. Compounds were identified, as TMS derivatives, by comparing their mass spectra with the GC–MS spectral library, with data from the literature (Budzikiewicz et al., 1963; Burnoufradosevich et al., 1985; Elaissi et al., 2010; Freire et al., 2002b; Gutierrez et al., 1999; Pelillo et al., 2003; Pereira et al., 2005a; Rencoret et al., 2007; Silverio et al., 2007b) and in some cases, by injection of standards.

Two aliquots of each extract were analyzed. Each aliquot was injected in triplicate. The presented results are the average of the concordant values obtained for each part (less than 5% variation between injections of the same aliquot and between aliquots of the same sample). Only the results of the GC–MS analysis of the extracts after alkaline hydrolysis will be presented and discussed, on the

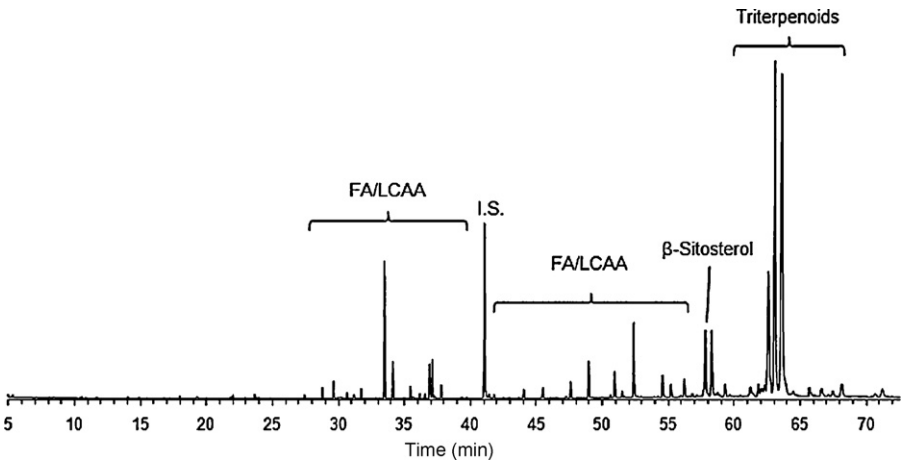


**Fig. 1.** GC–MS chromatogram of the dichloromethane extract of *E. grandis* outer bark after alkaline hydrolysis. FA, fatty acids; LCAA, long chain aliphatic alcohols; and IS, internal standard (tetracosane).

one hand because the major components (triterpenic acids) are mainly detected in the free form and, on the other hand, to simplify the discussion. However, (as will be discussed below) considerable increases in the amounts of fatty acids and long chain aliphatic alcohols were observed after hydrolysis. Moreover, this procedure also allowed us to identify smaller amounts of other compounds, such as aromatic compounds, only detectable after hydrolysis of the extracts (Freire et al., 2002a).

### 2.5. Chemicals

Nonacosan-1-ol (98% purity) and β-sitosterol (99% purity) were purchased from Fluka Chemie (Madrid, Spain); ursolic acid (98% purity), betulinic acid (98% purity) and oleanolic acid (98% purity) were purchased from Aktin Chemicals (Chengdu, China); betulonic acid (95% purity) was purchased from CHEMOS GmbH (Regenstauf, Germany); palmitic acid (99% purity), dichloromethane (99% purity), pyridine (99% purity), bis(trimethylsilyl)trifluoroacetamide (99% purity), trimethylchlorosilane (99% purity), and tetracosane (99% purity) were supplied by Sigma Chemical Co (Madrid, Spain).

## 3. Results and discussion

### 3.1. Extraction yield

The yields of the dichloromethane extractives from the three *Eucalyptus* bark samples investigated (Table 1) were markedly different between species and between the two morphological regions. Generally, it is observed that the outer bark fractions are richer in lipophilic extractives than inner bark counterparts. In the inner bark samples they ranged from 0.3% in *E. urograndis* to 2.6% (w/w) in *E. maidenii* while in outer bark from 1.3% in *E. grandis* to 6.1% (w/w) in *E. maidenii*.

The lipophilic components extraction yields of the inner bark of *E. urograndis* and *E. grandis* are of the same order of that found in the corresponding wood (Freire et al., 2006; Rencoret et al., 2007; Silverio et al., 2007b) and are in close agreement with previously published results for *E. globulus* inner bark (Freire et al., 2002b). As referred above, the lipophilic extractives contents of the outer bark fractions of *E. urograndis* and *E. grandis* are considerably higher than those of the inner bark fractions as also observed for *E. globulus* (Freire et al., 2002b), however they are lower (∼50%) than in *E. globulus* outer bark in which they accounted for up to 3.9% (w/w).

*E. maidenii* inner and outer barks present considerably higher amounts of lipophilic extractives (Table 1) comparatively with the three *Eucalyptus* species referred above and also with the corresponding *E. maidenii* wood, which contains about 0.5% (w/w) of lipophilic extractives (Rencoret et al., 2007).

**Table 1**
Extraction yields (% w/w) of the different eucalyptus bark fractions characterized.

| Bark sample | Extraction yield (% w/w) | |
|---|---|---|
| | Inner bark | Outer bark |
| *E. urograndis* | 0.3 | 1.7 |
| *E. grandis* | 0.5 | 1.3 |
| *E. maidenii* | 2.6 | 6.1 |

### 3.2. Extracts composition

The chemical composition of the dichloromethane extracts of the three *Eucalyptus* bark fractions investigated varies significantly with the species and the morphological fraction. A chromatogram of the lipophilic extract (after alkaline hydrolysis and as TMS derivatives) of a selected outer bark extract (from *E. grandis*) is presented in Fig. 1 and the detailed qualitative and quantitative compositions of all the hydrolyzed extracts are listed in Table 2.

#### 3.2.1. Outer bark extractives

From a qualitative point of view, the chemical composition of the samples of *Eucalyptus* outer barks extracts studied differs considerably (Table 2 and Fig. 2). The hydrolyzed lipophilic extracts

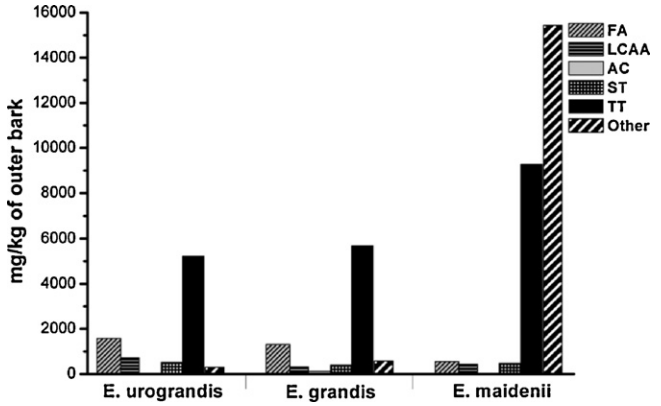


**Fig. 2.** Major families of lipophilic components identified in the dichloromethane extracts of *E. urograndis*, *E. grandis* and *E. maidenii* outer barks after alkaline hydrolysis. FA, fatty acids; LCAA, long chain aliphatic alcohols; ST, sterols; TT: triterpenoids; and AC, aromatic compounds.

**Table 2**
Compounds identified in the dichloromethane extracts of the different eucalyptus bark fractions characterized, after alkaline hydrolysis[a].

| rt (min) | Compound | Content (mg/kg of bark fraction) | | | | | |
|---|---|---|---|---|---|---|---|
| | | *E. urograndis* | | *E. grandis* | | *E. maidenii* | |
| | | Outer | Inner | Outer | Inner | Outer | Inner |
| | *Fatty acids* | 1577.3 | 798.1 | 1310.8 | 676.7 | 548.5 | 671.8 |
| 5.31 | Hexanoic acid | | 5.4 | | | | |
| 14.92 | Nonanoic acid | | 4.5 | | | | |
| 17.99 | Decanoic acid | | 4.6 | | | | |
| 23.67 | Dodecanoic acid | | 6.2 | 11.9 | 6.8 | | 9.2 |
| 28.81 | Tetradecanoic acid | 14.5 | 13.4 | 26.5 | 11.9 | 29.4 | |
| 31.24 | Pentacosanoic acid | | 2.4 | 12.3 | 7.7 | | |
| 33.5 | Hexadecanoic acid | 490.9 | 207.6 | 358.8 | 213.8 | 110.7 | 208.5 |
| 35.74 | Heptadecanoic acid | | 5.8 | | 15.5 | | |
| 36.90 | Linolenic acid | | | | | 57.6 | |
| 36.98 | Linoleic acid | 99.6 | 166.9 | 100.6 | 125.4 | 62.7 | 296.4 |
| 37.19 | Oleic acid | 82.2 | 156.3 | 103.0 | 132.8 | 37.3 | 124.2 |
| 37.26 | *trans*-9-Octadecenoic acid | | | | 6.0 | | |
| 37.87 | Octadecanoic acid | 46.2 | 67.4 | 35.7 | 65.2 | | 33.5 |
| 41.77 | Eicosanoic acid | | 14.5 | | 6.6 | | |
| 43.75 | Heneicosanoic acid | | 3.4 | | | | |
| 45.58 | Docosanoic acid | 38.2 | 9.4 | 33.9 | 4.4 | | |
| 47.34 | Tricosanoic acid | | 5.0 | | | | |
| 49.02 | Tetracosanoic acid | 182.4 | 14.5 | 107.8 | 8.1 | | |
| 50.69 | Pentacosanoic acid | | 6.7 | | | | |
| 52.35 | Hexacosanoic acid | 363.0 | 32.5 | 271.6 | 24.0 | 222.4 | |
| 54.23 | Heptacosanoic acid | | 3.5 | | | | |
| 56.21 | Octacosanoic acid | 89.7 | 14.6 | 76.6 | 17.9 | 28.3 | |
| 60.42 | Triacontanoic acid | 47.0 | 29.1 | 21.0 | 10.5 | | |
| | *ω-Hydroxyfatty acids* | | | | | | |
| 51.59 | 22-Hydroxydocosanoic acid | | 4.3 | 22.3 | | | |
| 55.28 | 24-Hydroxytetracosanoic acid | 52.9 | 5.6 | 57.2 | 6.2 | | |
| 59.29 | 25-Hydroxypentacosanoic acid | 70.8 | 14.7 | 71.6 | 13.9 | | |
| | *Long chain aliphatic alcohols* | 715.5 | 55.1 | 314.1 | 156.7 | 434.5 | 110.7 |
| 6.73 | Octan-1-ol | | 2.9 | | | | |
| 31.75 | Hexadecan-1-ol | 44.5 | 5.1 | 23.4 | 32.6 | | 16.3 |
| 35.48 | (z)-Octadec-9-en-1-ol | 43.3 | 5.9 | 30.8 | 55.9 | 32.1 | 33.7 |
| 36.24 | Octadecan-1-ol | 24.6 | 3.7 | 0.0 | 23.2 | | 12.8 |
| 44.11 | Docosan-1-ol | 44.8 | 4.3 | 33.8 | 2.9 | 78.6 | 0.0 |
| 47.60 | Tetracosan-1-ol | 104.5 | 3.5 | 48.4 | 0.0 | 63.3 | 25.8 |
| 50.98 | Hexacosan-1-ol | 224.1 | 8.3 | 91.0 | 6.8 | 179.2 | 22.1 |
| 54.56 | Octacosan-1-ol | 110.4 | 13.3 | 86.7 | 18.1 | 81.3 | 0.0 |
| 58.58 | Triacontan-1-ol | 119.2 | 8.1 | | 17.2 | | |
| | *Aromatic compounds* | 37.1 | 11.9 | 111.4 | 9.8 | 0.0 | 0.0 |
| 19.35 | Vanillin | | 3.3 | | | | |
| 30.68 | *p*-Coumaric acid | | | 19.5 | 9.8 | | |
| 34.14 | *trans*-Ferulic acid | 37.1 | 8.7 | 91.9 | | | |
| | *Sterols* | 510.9 | 353.2 | 394.4 | 372.3 | 476.5 | 383.3 |
| 58.01 | β-Sitosterol | 510.9 | 319.7 | 366.2 | 333.2 | 476.5 | 383.3 |
| 58.17 | β-Sitostanol | | 33.5 | 28.2 | 39.1 | | |
| | *Triterpenoids* | 5228.7 | 810.1 | 5694.7 | 777.0 | 9282.8 | 498.2 |
| 57.79 | β-Amyrin | | 32.1 | | 38.8 | 219.1 | 415.0 |
| 58.47 | α-Amyrin | | 108.9 | | 115.9 | | |
| 61.37 | Betulonic acid | | | | | 614.8 | |
| 62.69 | Oleanolic acid | 831.1 | 94.4 | 782.3 | 89.0 | 1368.6 | 13.8 |
| 63.21 | Betulinic acid | 1304.8 | 222.6 | 2085.7 | 237.9 | 1746.2 | 16.0 |
| 63.73 | Ursolic acid | 1895.6 | 232.1 | 2154.4 | 210.1 | 3640.3 | 34.0 |
| | Unidentified triterpenoids | 1197.3 | 119.9 | 672.3 | 85.3 | 1693.8 | 19.4 |
| | *Other compounds/unidentified compounds* | 298.1 | 247.3 | 574.8 | 264.8 | 15435.7 | 6416.5 |
| | Monoterpenes | | | | | 2479.4 | 415.4 |
| | Sesquiterpenes | | | | | 6595.7 | 2549.8 |
| | *Total detected compounds* | 8367.6 | 2275.8 | 8400.1 | 2257.4 | 26178.1 | 8080.5 |

[a] Results are the average of the concordant values obtained (less than 5% variation between injections) for the two aliquots of each sample injected in triplicate.

of *E. urograndis* and *E. grandis* are mainly composed of several triterpenoids namely, betulinic, ursolic and oleanolic acids (Fig. 3). β-Sitosterol is also an abundant component of these extracts, followed by minor amounts of fatty acids (C12 to C30), long chain aliphatic alcohols (C16 to C30), and aromatic compounds.

In *E. maidenii* outer bark the main lipophilic components identified are several monoterpenes and sesquiterpenes (detected in the GC–MS chromatograms at rt 4.14–13.69 min and 15.08–24.56 min, respectively), with 1,8-cineole, α-terpineol, aromadendrene, *allo*-aromadendrene and globulol (Fig. 4) as the most abundant components of these families (referred in Table 2 and Fig. 2 as "other compounds"). These compounds are commonly found in the essential oils of the leaves of numerous *Eucalyptus* species (Elaissi et al., 2010; Silvestre et al., 1994, 1997), including E. *maidenii*, and were also identified in the outer bark (Freire et al., 2002b) and fruits (Pereira et al., 2005a,b) of *E. globulus*. However, considering that the aim of this paper is focused mainly in the triterpenic fractions of the extracts and because the methodology followed in this work is not the most suitable for the analysis of this type of compounds the composition of this fraction will not be discussed in detail here.

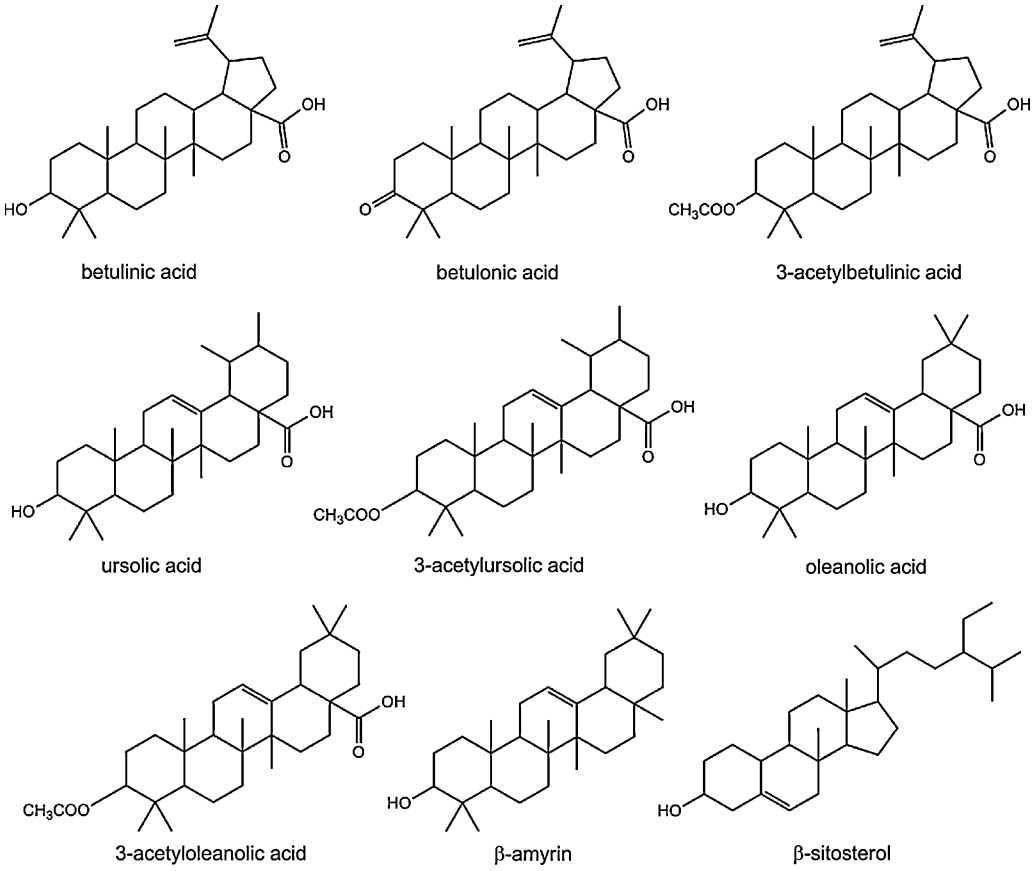


**Fig. 3.** Major triterpenoids identified in *E. urograndis*, *E. grandis* and *E. maidenii* bark.

Nevertheless, triterpenoids also correspond to an important fraction of the *E. maidenii* outer bark extract and, as observed for *E. urograndis* and *E. grandis*, betulinic, ursolic and oleanolic acids are the main components of the triterpenic fraction (Table 2). Betulonic acid and β-amyrin were also detected in this extract, although in smaller amounts (Table 2).

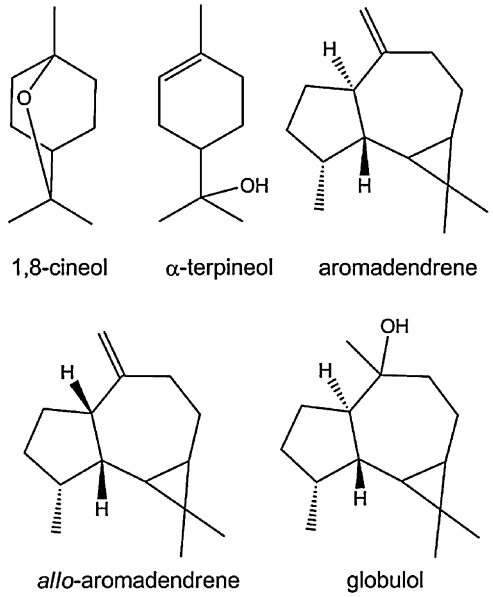


**Fig. 4.** Structures of the main monoterpenes and sesquiterpenes identified in *E. maidenii* bark.

All these triterpenic compounds have already been reported as components of the leaves waxes of several *Eucalyptus* species (Li et al., 1997), *E. globulus* bark (Freire et al., 2002b) and fruits (Pereira et al., 2005a). However, to our knowledge their presence in *E. urograndis*, *E. grandis* and *E. maidenii* barks has not been previously reported.

From a quantitative point of view, the total triterpenoids content range from 5.2 g/kg to 9.3 g/kg (in dry bark basis) in *E. urograndis* and *E. maidenii* outer bark, respectively (Table 2 and Fig. 2) and their relative amount in each extract ranged from about 15% in *E. maidenii* up to 43% in *E. grandis*. As expected, these results show that this group of triterpenic compounds is highly concentrated in the outer layers of the studied barks, although in lower concentrations than those reported in *E. globulus* outer bark, where they account for up to 25 g/kg (Freire et al., 2002b).

Ursolic acid (40% of which was found as 3-acetylursolic acid, on average before alkaline hydrolysis) is generally the most abundant component of all outer bark extracts, varying from 1.9 g/kg in *E. urograndis* up to 3.6 g/kg in *E. maidenii*.

Betulinic acid (1.3–2.1 g/kg) and oleanolic acid (0.8–1.4 g/kg, 48% of which found as 3-acetyloleanolic acid, on average before hydrolysis) were also found among the main components of all outer barks extracts, being betulinic acid more abundant in *E. grandis* and oleanolic acid in *E. maidenii*.

As referred before, betulonic acid (0.6 g/kg) and β-amyrin (0.2 g/kg) were only detected in *E. maidenii* outer bark. This difference in composition should obviously be related with the species, although the effected of the geographic location over the composition of *Eucalyptus* lipophilic extractives is well known (Freire et al., 2002a; Gutierrez et al., 1999) and this species was collected in Portugal whereas the other two were from Brazil.

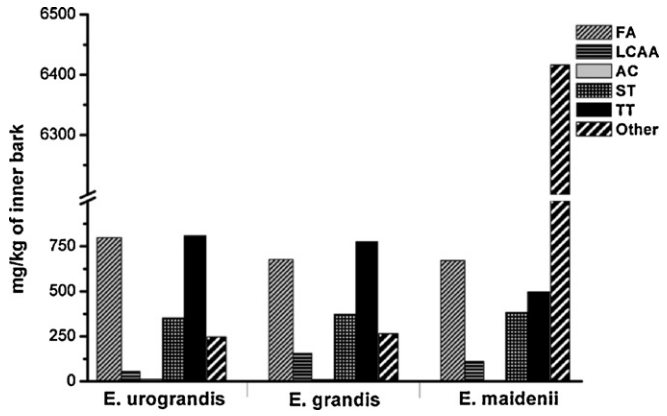


**Fig. 5.** Major families of lipophilic components identified in the dichloromethane extracts of *E. urograndis*, *E. grandis* and *E. maidenii* inner bark after alkaline hydrolysis. FA, fatty acids; LCAA, long chain aliphatic alcohols; ST, sterols; TT triterpenoids; and AC, aromatic compounds.

Finally, a lupane type triterpenic acid was also detected in considerable amounts (0.7 and 1.2 g/kg, respectively) in *E. urograndis* and *E. maidenii* outer bark extracts. The fragmentation pattern of this compound is very similar to that of betulinic acid, with the same [$M^+$] ion at *m*/*z* 600 and the intense peak at *m*/*z* 189, typical of compounds with lupane skeletons (Mathe et al., 2004; Niemela, 1990; Razborsek et al., 2008), as well as typical fragments at *m*/*z* 585 [M−CH3], 510 [M−TMSOH], 483 [M−TMSOCH], 393 [M−TMSOH−TMSOOC] and at *m*/*z* 320, 307, 279, 203 and 73 (Mathe et al., 2004; Niemela, 1990; Razborsek et al., 2008), but showing a different retention time (62.47 min). Although its unambiguous identification could not be achieved, the similarity of the spectra with that of betulinic acid and the concordant elution order with other lupane and 3-*epi*-lupane structures namely, lupeol and 3-*epi*-lupeol (Mathe et al., 2004), suggests the possibility of being a stereoisomer, namely 3-*epi*-betulinic acid.

Sterols fraction (0.4–0.5 g/kg), essentially composed of β-sitosterol, and fatty acids fraction (0.5–1.6 g/kg, 67% of which were present in esterified forms, on average before alkaline hydrolysis), mainly composed of hexadecanoic, oleic, linoleic, tetracosanoic and hexacosanoic acids, and some ω-hydroxyfatty acids (24-hydroxytetracosanoic acid and 25-hydroxypentacosanoic acid) were identified in all outer barks extracts. Smaller amounts of long chain aliphatic alcohols (0.3–0.7 g/kg, 50% of which were present in esterified forms, on average before alkaline hydrolysis), mainly represented by hexacosan-1-ol, were also detected in the studied outer bark extracts.

All the identified sterols, fatty acids and long chain aliphatic alcohols (Table 2) have already been reported in the lipophilic extracts from the wood of *E. urograndis*, *E. grandis* and *E. maidenii* (Freire et al., 2006; Rencoret et al., 2007; Silverio et al., 2007a,b).

Finally, two aromatic compounds, namely *p*-coumaric and *trans*-ferulic acids, were identified in *E. urograndis* and *E. grandis* outer bark extractives after alkaline hydrolysis. Ferulic acid esters have been previously reported in *E. globulus* bark extractives (Freire et al., 2002b) and in several *Eucalyptus* species wood extracts (Freire et al., 2002a; Rencoret et al., 2007). *p*-Coumaric acid esters have also been found in the bark from *E. globulus* (Freire et al., 2002b).

### 3.2.2. Inner bark extractives

Triterpenoids (0.8 g/kg), as ursolic acid (0.2 g/kg, 46% of which found as 3-acetyursolic acid, on average before hydrolysis) and betulinic acid (0.2 g/kg) are also the major components of the hydrolyzed dichloromethane extract of *E. urograndis* and *E. grandis* inner bark (Table 2 and Fig. 5). Considerable amounts of α-amyrin and oleanolic acid as well as smaller amounts of β-amyrin were also detected.

The amounts of triterpenoids found in *E. urograndis* and *E. grandis* inner bark extracts (0.8 g/kg) are in the same range as those found in *E. globulus* inner bark extract (Freire et al., 2002b), however, in the latter case β-amyrin was the most abundant triterpenoid and triterpenic acids were not detected.

The hydrolyzed dichloromethane extract of *E. maidenii* inner bark is qualitatively and quantitatively quite different from those of *E. urograndis* and *E. grandis* (Table 2 and Fig. 5), but relatively similar to the corresponding outer bark extract from a qualitative point of view. Thus, the *E. maidenii* inner bark extract is mainly composed of monoterpenes and sesquiterpenes (once more assigned in the "others" group as referred above), whereas triterpenoids, fatty acids, sterols and long chain aliphatic alcohols represent a smaller fraction of the extract. The triterpenic fraction (0.5 g/kg) is mainly composed of β-amyrin (0.4 g/kg), along with small amounts of ursolic, betulinic and oleanolic acids; this composition is similar to that reported for *E. globulus* inner bark extract (Freire et al., 2002b).

Fatty acids are detected among the main components of the hydrolyzed extract of *E. urograndis* and *E. grandis* inner bark (0.7–0.8 g/kg, with an average increase of 203% after alkaline hydrolysis), with hexadecanoic, oleic and linoleic acids as the most abundant components of this group. These results are in agreement with those reported for *E. globulus* inner bark (Freire et al., 2002b) and for *E. urograndis* and *E. grandis* wood fatty acids (Freire et al., 2006; Rencoret et al., 2007; Silverio et al., 2007b). In *E. maidenii* inner bark fatty acids accounted for 0.7 g/kg (with a 348% increase after hydrolysis), and only a few components, namely hexadecanoic, linoleic, oleic, dodecanoic and octadecanoic acids, were identified.

The amounts of sterols (0.4 g/kg), mainly β-sitosterol (0.3–0.4 g/kg), detected in the inner bark of *E. urograndis*, *E. grandis* and *E. maidenii* are in the same order of those reported in the hydrolysable lipophilic extract of the corresponding woods (Rencoret et al., 2007; Silverio et al., 2007a,b) and in *E. globulus* inner bark (Freire et al., 2002b), although in wood a larger number of sterols has been reported.

Long chain aliphatic alcohols represented only a smaller portion of the total extractives identified in the inner bark of the three *Eucalyptus* species (55.1–156.7 mg/kg). Octacosan-1-ol in *E. urograndis* and (z)-octadec-9-en-1-ol in *E. grandis* and *E. maidenii* were the most abundant alcohols found in these bark samples.

*p*-Coumaric acid was identified, in very small amounts, in the hydrolyzed extracts of *E. grandis* whereas *trans*-ferulic acid and vanillin were detected in *E. urograndis* inner bark extracts.

## 4. Conclusions

The lipophilic fractions of the inner and outer barks of *E. grandis*, *E. urograndis* and *E. maidenii* were shown to be mainly composed of triterpenic compounds (along with mono and sesquiterpenes in *E. maidenii*) followed by smaller amounts of fatty acids, fatty alcohols, and aromatic compounds.

Triterpenic acids (mainly ursolic, betulinic and oleanolic acids), are particularly abundant in outer barks representing 5.2 g/kg, 5.7 g/kg and 9.3 g/kg in *E. urograndis*, *E. grandis* and *E. maidenii*, respectively. These values are considerably smaller than those reported for *E. globulus* outer bark (Freire et al., 2002b) and other biomass residues (Domingues et al., 2010). However considering the total amounts of bark that are generated in South American pulp mills using *E. urograndis* and *E. grandis*, as well as the growth potential of *E. maidenii* plantations, it is obvious that the bark residues from these species represent a tremendous potential for the isolation of these valuable triterpenic compounds.

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## References

ABRAF, 2010. ABRAF Statistical Yearbook—Base Year 2009, Available from: http://www.abraflor.org.br, viewed September 2010.

BRACELPA, 2009. Brazilian Pulp and Paper Industry Performance, Available from: http://www.bracelpa.org.br/eng/estatisticas/, viewed September 2010.

Budzikiewicz, H., Wilson, J.M., Djerassi, C., 1963. Mass spectrometry in structural and stereochemical problems. 32 pentacyclic triterpenes. J. Am. Chem. Soc. 85, 3688–3699.

Burnoufradosevich, M., Delfel, N.E., England, R., 1985. Gas chromatography–mass spectrometry of oleanane-type and ursane-type triterpenes—application chenopodium-quinoa triterpenes. Phytochemistry 24, 2063–2066.

Cardenas, C., Quesada, A.R., Medina, M.A., 2004. Effects of ursolic acid on different steps of the angiogenic process. Biochem. Biophys. Res. Commun. 320, 402–408.

Domingues, R.M.A., Sousa, G.D.A., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., 2010. *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. Ind. Crop. Prod. 31, 65–70.

Dzubak, P., Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., Markova, L., Urban, M., Sarek, J., 2006. Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat. Prod. Rep. 23, 394–411.

Ekman, R., 1983. The suberin monomers and triterpenoids from the outer bark of *Betula-verrucosa* Ehrh. Holzforschung 37, 205–211.

Elaissi, A., Marzouki, H., Medini, H., Khouja, M.L., Farhat, F., Lynene, F., Harzallah-Skhiri, F., Chemli, R., 2010. Variation in volatile leaf oils of 13 *Eucalyptus* species harvested from Souinet Arboreta (Tunisia). Chem. Biodivers. 7, 909–921.

Fernandes, P., Cabral, J.M.S., 2007. Phytosterols: applications and recovery methods. Bioresour. Technol. 98, 2335–2350.

Fernando, S., Adhikari, S., Chandrapal, C., Murali, N., 2006. Biorefineries: current status, challenges, and future direction. Energy Fuels 20, 1727–1737.

Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., 2002a. Identification of new hydroxy fatty acids and ferulic acid esters in the wood of *Eucalyptus globulus*. Holzforschung 56, 143–149.

Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., Cavaleiro, J.A.S., 2002b. Lipophilic extractives of the inner and outer barks of *Eucalyptus globulus*. Holzforschung 56, 372–379.

Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., 2005. Lipophilic extractives in *Eucalyptus globulus* kraft pulps. Behavior during ECF bleaching. J. Wood Chem. Technol. 25, 67–80.

Freire, C.S.R., Pinto, P.C.R., Santiago, A.S., Silvestre, A.J.D., Evtuguin, D.V., Neto, C.P., 2006. Comparative study of lipophilic extractives of hardwoods and corresponding ECF bleached kraft pulps. Bioresources 1, 3–17.

Gutierrez, A., del Rio, J.C., Gonzalez-Vila, F.J., Martin, F., 1999. Chemical composition of lipophilic extractives from *Eucalyptus globulus* Labill. wood. Holzforschung 53, 481–486.

Hamunen, A., 1983. Process for the purification of β-sitosterol isolated from the unsaponifiables in crude soap from the sulphate cellulose process. US Patent 4,422,974.

Huang, H.J., Ramaswamy, S., Tschirner, U.W., Ramarao, B.V., 2008. A review of separation technologies in current and future biorefineries. Sep. Purif. Technol. 62, 1–21.

Jager, S., Trojan, H., Kopp, T., Laszczyk, M.N., Scheffler, A., 2009. Pentacyclic triterpene distribution in various plants—rich sources for a new group of multi-potent plant extracts. Molecules 14, 2016–2031.

Kamm, B., Kamm, M., Schmidt, M., Hirth, T., Schulze, M., 2006. Lignocellulose-based chemical products and product family trees. In: Kamm, B., Gruber, P., Kamm, M. (Eds.), Biorefineries—Industrial Processes and Products: Status Quo and Future Directions. Wiley-VCH, Weinheim, Germany, pp. 97–150.

Kibblewhite, R.P., Johnson, B.I., Shelbourne, C.J.A., 2001. Kraft pulp qualities of *Eucalyptus nitens*, *E-globulus*, and *E-maidenii*, at ages 8 and 11 years. In: 55th Appita Annual Conference Proceedings, Hobart, Australia.

Kolomitsyn, I.V., Holy, J., Perkins, E., Krasutsky, P.A., 2007. Analysis and antiproliferative activity of bark extractives of *Betula neoalaskana* and *B-papyrifera*. Synthesis of the most active extractive component—betulin 3-caffeate. Nat. Prod. Commun. 2, 17–26.

Krasutsky, P.A., 2006. Birch bark research and development. Nat. Prod. Rep. 23, 919–942.

Laszczyk, M.N., 2009. Pentacyclic triterpenes of the lupane. Oleanane and ursane group as tools in cancer therapy. Planta Med. 75, 1549–1560.

Lee, K.H., Kashiwada, Y., Hashimoto, F., Cosentino, L.M., Manak, M., 1997. Betulinic acid and dihydrobetulinic acid derivatives and uses therefor. US Patent 5,679,828.

Li, H., Madden, J.L., Potts, B.M., 1997. Variation in leaf waxes of the Tasmanian *Eucalyptus* species. 1. Subgenus *Symphyomyrtus*. Biochem. Syst. Ecol. 25, 631–657.

Li, J., Guo, W.J., Yang, Q.Y., 2002. Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. World J. Gastroenterol. 8, 493–495.

Mathe, C., Culioli, G., Archier, P., Vieillescazes, C., 2004. Characterization of archaeological frankincense by gas chromatography–mass spectrometry. J. Chromatogr. A 1023, 277–285.

Miranda, I., Pereira, H., 2001. Provenance effect on wood chemical composition and pulp yield for *Eucalyptus globulus* Labill. Appita J. 54, 347–351.

Mora, L.M., Garcia, C.H., 2000. Eucalypt Cultivation in Brazil, first ed. Instituto de Pesquisas e Estudos Florestais, São Paulo.

Niemela, K., 1990. Low-molecular-weight Organic Compounds in Brich Kraft Black Liquor. Helsinki University of Technology, Helsinki.

Pelillo, M., Lafelice, G., Marconi, E., Caboni, M., 2003. Identification of plant sterols in hexaploid and tetraploid wheats using gas chromatography with mass spectrometry. Rapid Commun. Mass Spectrom. 17, 2245–2252.

Pereira, S.I., Freire, C.S.R., Neto, C.P., Silvestre, A.J.D., Silva, A.M.S., 2005a. Chemical composition of the epicuticular wax from the fruits of *Eucalyptus globulus*. Phytochem. Anal. 16, 364–369.

Pereira, S.I., Freire, C.S.R., Neto, C.P., Silvestre, A.J.D., Silva, A.M.S., 2005b. Chemical composition of the essential oil distilled from the fruits of *Eucalyptus globulus* grown in Portugal. Flavour Fragr. J. 20, 407–409.

Pietarinen, S.P., Willfor, S.M., Ahotupa, M.O., Hemming, J.E., Holmbom, B.R., 2006. Knotwood and bark extracts: strong antioxidants from waste materials. J. Wood Sci. 52, 436–444.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R., Tschaplinski, T., 2006. The path forward for biofuels and biomaterials. Science 311, 484–489.

Razborsek, M.I., Voncina, D.B., Dolecek, V., Voncina, E., 2008. Determination of oleanolic, betulinic and ursolic acid in Lamiaceae and mass spectral fragmentation of their trimethylsilylated derivatives. Chromatographia 67, 433–440.

Rencoret, J., Gutierrez, A., del Rio, J.C., 2007. Lipid and lignin composition of woods from different eucalypt species. Holzforschung 61, 165–174.

Silverio, F.O., Barbosa, L.C.A., Maltha, C.R.A., Silvestre, A.J.D., Pilo-Veloso, D., Gomide, J.L., 2007a. Characterization of lipophilic wood extractives from clones of *Eucalyptus urograndis* cultivate in Brazil. Bioresources 2, 157–168.

Silverio, F.O., Barbosa, L.C.A., Silvestre, A.J.D., Pilo-Veloso, D., Gomide, J.L., 2007b. Comparative study on the chemical composition of lipophilic fractions from three wood tissues of *Eucalyptus* species by gas chromatography–mass spectrometry analysis. J. Wood Sci. 53, 533–540.

Silvestre, A., Cavaleiro, J., Delmond, B., Filliatre, C., Bourgeois, G., 1994. The essential oil of *Eucalyptus globulus* Labill. from Portugal. Flavour Fragr. J. 9, 51–53.

Silvestre, A.J.D., Cavaleiro, J.A.S., Delmond, B., Filliatre, C., Bourgeois, G., 1997. Analysis of the variation of the essential oil composition of *Eucalyptus globulus* Labill from Portugal using multivariate statistical analysis. Ind. Crop. Prod. 6, 27–33.

Smith, P.F., Ogundele, A., Forrest, A., Wilton, J., Salzwedel, K., Doto, J., Allaway, G.P., Martin, D.E., 2007. Phase I and II study of the safety, virologic effect, and pharmacokinetics/pharmacodynamics of single-dose 3-O-(3′,3′-dimethylsuccinyl)betulinic acid (bevirimat) against human immunodeficiency virus infection. Antimicrob. Agents Chemother. 51, 3574–3581.

Sogno, I., Vannini, N., Lorusso, G., Cammarota, R., Noonan, D.M., Generoso, L., Sporn, M.B., Albini, A., 2009. Anti-angiogenic activity of a novel class of chemopreventive compounds: oleanic acid terpenoids. In: Senn, H.J., Kapp, U., Otto, F. (Eds.), 5th International Cancer Prevention Conference. St Gallen, Switzerland, 2008.

Sultana, N., Ata, A., 2008. Oleanolic acid and related derivatives as medicinally important compounds. J. Enzym. Inhib. Med. Chem. 23, 739–756.

Tolstikova, T.G., Sorokina, I.V., Tolstikov, G.A., Tolstikov, A.G., Flekhter, O.B., 2006. Biological activity and pharmacological prospects of lupane terpenoids: I. Natural lupane derivatives. Russ. J. Bioorg. Chem. 32, 37–49.

Trabado, G.I., Wilstermann, D., 2008. Eucalyptus Universalis. Global Cultivated Eucalypt Forest Map 2008, Available from: http://www.git-forestry.com/, viewed September 2010.

Willfor, S., Nisula, L., Hemming, J., Reunanen, M., Holmbom, B., 2004a. Bioactive phenolic substances in industrially important tree species. Part 1: Knots and stemwood of different spruce species. Holzforschung 58, 335–344.

Willfor, S., Nisula, L., Hemming, J., Reunanen, M., Holmbom, B., 2004b. Bioactive phenolic substances in industrially important tree species. Part 2: Knots and stemwood of fir species. Holzforschung 58, 650–659.

Yogeeswari, P., Sriram, D., 2005. Betulinic acid and its derivatives: a review on their biological properties. Curr. Med. Chem. 12, 657–666.