EUCALYPTUS BIOMASS RESIDUES FROM AGRO-FOREST AND PULPING INDUSTRIES AS SOURCES OF HIGH-VALUE TRITERPENIC COMPOUNDS

R. M. A. DOMINGUES, D. J. S. PATINHA, G. D. A. SOUSA,*
J. J. VILLAVERDE, C. M. SILVA, C. S. R. FREIRE, A. J. D. SILVESTRE and
C. PASCOAL NETO

CICECO and Department of Chemistry, University of Aveiro, Portugal
**RAIZ – Forest and Paper Research Institute, Quinta de S. Francisco, Eixo, Portugal*

Received June 8, 2011

A comparative study on the triterpenic acids composition of the outer barks of several *Eucalyptus* species (*E. globulus*, *E. grandis*, *E. urograndis*, *E. maidenii* and E. *nitens*) is reported. The contents of the main triterpenic acids identified in the five species varied between 4.5 g/kg in *E. urograndis* and 21.6 g/kg in *E. nitens*. It has been observed that, out of these *Eucalyptus* species outer barks, those from temperate and Mediterranean zones, namely *E. nitens* and *E. globulus*, are richer in triterpenic acids than the species from sub-tropical and tropical regions. Furthermore, *E. globulus* outer bark is clearly the richest in ursane acids, while *E. nitens* outer bark is the richest in oleanane and lupane acids.

Keywords: biorefinery, *Eucalyptus nitens*, *Eucalyptus grandis*, *Eucalyptus urograndis* (*Eucalyptus grandis x Eucalyptus urophylla*), *Eucalyptus maidenni*, bark, triterpenic acids, ursolic acid, oleanolic acid, betulinic acid

INTRODUCTION

The fast-growing commercial plantations of *Eucalyptus* species have nowadays an important role in the fulfillment of the worldwide increasing demand for pulpwood.[1] By 2008, the total area of *Eucalyptus* plantations, mainly distributed in about one dozen countries spread worldwide,[2] exceeded 19 x 10^6 ha.[3]

In fact, the *Eucalyptus* species are the most important fiber sources for pulp and paper production in South-West Europe (Portugal and Spain), South America (Brazil and Chile), South Africa, Japan and other countries.[4] In the temperate and Mediterranean zones, *E. globulus* and *E. nitens* are the most common planted species while, in sub-tropical and tropical zones, *E. grandis*, *E. urophylla* and their hybrid (*E. urograndis*) are among the preferred ones.[1,2,4] The increasing interest for several *Eucalyptus* species as wood sources for pulp production is related not only to their rapid growth, but also to their behavior during pulping and bleaching, as well as to the excellent properties of the final pulps.[4-7]

During the last decade, a re-emerging interest for the integrated exploitation of plant biomass as sources of materials, chemicals, fuels and energy has been registered, within the biorefinery concept.[8-10] This renewed interest has drawn the attention of agro-forest industries, concerned with taking maximum values out of their crops.

Wood exploitation for pulp production generates large amounts of biomass residues and by-products, which can be important renewable raw materials for the production of high-value chemicals and materials.[11] The biomass residues resulting from *Eucalyptus* pulp mill operations are mainly 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 and by-products can be sources of valuable compounds – such as phytosterols,[12,13] lignans[14-16] and triterpenoids.[17,18] The integrated exploitation of some of these compounds in pulp mills is viewed as one of the most successful examples of the biorefinery concept implementation in this industrial branch.[9,19]

Some previous studies were devoted to the lipophilic composition of bark in some of the most important *Eucalyptus* species used by pulp industry worldwide, namely *E. globulus*,[20,21] *E. grandis*, *E. urograndis* and *E. maidenii*,[22] as well as other biomass residues obtained from *E. globulus*.[20] It was shown that, for technical and economical reasons, bark is among the most interesting residues for possible exploitation in an integrated way.[20] Furthermore, it has been reported that the lipophilic extracts present in the outer barks of all these *Eucalyptus* species, particularly *E. globulus*, contain high amounts of triterpenic acids with lupane, ursane and oleanane skeletons (Fig. 1), namely betulonic, betulinic, ursolic, 3-acetylursolic, oleanolic and 3-acetyloleanolic acids,[20-22] making this fraction of bark the residue from which these compounds can be efficiently extracted in an integrated way with the existing kraft pulp mills.

The triterpenic acids show promising nutraceutical and pharmacological properties, due to their antitumoral[23,24] and anti-angiogenic[25] properties, or as precursors for anti-HIV drugs, some of them already in clinical trial phase.[26] Considering the future perspectives for these triterpenic molecules, the search for biomass sources in which they can be abundantly found becomes an important issue.[27]

In this context, the present paper studies comparatively the triterpenic composition of the outer barks of *E. globulus*, *E. grandis*, *E. urograndis* and *E. maidenii*.[20-22] The lipophilic composition of *E. nitens* bark was also studied, since this species is the preferred *Eucalyptus* pulpwood cultivated in cool-temperate regions (mostly in Chile, Australia and South Africa),[3,28] and is reported here for the first time. In this way, the authors intend to access the potential of several *Eucalyptus* species as possible sources of triterpenic acids, particularly *E. nitens*, within the context of biorefinery integrated in pulp mills.

MATERIALS AND METHOD

Samples

The *E. urograndis* and *E. grandis* bark samples were taken from a 5 and a 10 year-old tree, respectively, randomly sampled from the clone plantations cultivated in Alfredo Chaves, state of Espírito Santo, Brazil (20º38'08'' S, 40º44'57'' W), while the *E. globulus* bark samples were taken from three 12 year-old trees randomly sampled from a clone plantation cultivated in Arouca, region of Aveiro, Portugal (40º55'44'' N, 8º14'37'' W), and the *E. maidenii* bark was obtained from a 10 year-old tree randomly sampled from a clone plantation cultivated in Odemira, southwestern region of Portugal (37º33'04'' N, 8º38'43'' W), as reported elsewhere.[21,22]

The bark samples of *E. nitens* were taken from several 10 year-old trees, randomly sampled from a first rotation of clone plantation cultivated near Paredes, region of Viseu, Portugal (40º29'58'' N, 8º16'03'' W).

The two different morphological regions of bark, the inner and the outer ones, were manually separated, as described elsewhere,[21] and analyzed separately. Representative samples of each bark fractions were air-dried until constant weight, and ground to a granulometry lower than 2 mm, prior to extraction.

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 selected as a fairly specific solvent for lipophilic extractives.

GC-MS analysis

Prior to GC-MS analysis, nearly 20 mg of each dried sample were converted into trimethylsilyl (TMS) derivatives, according to literature.[21,29] GC-MS analyses were performed on 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 μm film thickness). The chromatographic conditions were as follows: initial temperature – 80 °C for 5 min; temperature rate – 4 °C min^{-1} up to 260 °C and 2 °C min^{-1} till the final temperature of 285 °C; keeping at 285 °C for 10 min; injector temperature – 250 °C; transfer-line temperature – 290 °C; split ratio – 1:50. The MS was operated in electron impact mode with an electron impact energy of 70 eV, and data were collected at a 1 scan s^{-1} rate, 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, used as an internal standard. The respective multiplication factors necessary for obtaining a correct quantification of the peak areas were calculated as an average of six GC-MS runs. For aromatic compounds, a response factor of 1.0 was assumed. The compounds were identified as TMS derivatives, by comparing their mass spectra with the GC-MS spectral library, and data from literature,[4,7,21,30-33] 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).

RESULTS AND DISCUSSION

Extraction yield

The yields of the dichloromethane extractives from *E. nitens* and the other *Eucalyptus* bark previously investigated[20-22] (Table 1) were markedly different between both the species and the two morphological regions. It is observed that the outer bark fractions are generally richer in lipophilic extractives than the inner bark counterparts. In the inner bark samples, the yields ranged from 0.3% in *E. urograndis* and *E. nitens*, to 2.6% (w/w) in *E. maidenii* while, in outer bark, yields ranged from 1.3% in *E. grandis* to 6.1% (w/w) in *E.maidenii*. Furthermore, one may observe that the outer bark of the most common species planted in temperate and Mediterranean zones, *E. globulus* and *E. nitens*, are richer in lipophilic extractives than the species of sub-tropical and tropical zones, *E. grandis* and *E. urograndis*.

Composition of *E. nitens* bark extracts

The chemical compositions of the dichloromethane extracts of *E. nitens* bark vary significantly within the two morphological fractions, as previously reported for the other *Eucalyptus* species.[20-22]

A chromatogram of the lipophilic extract (as TMS derivatives) of the outer bark from *E. nitens* is presented in Figure 2, and the detailed qualitative and quantitative compositions of the outer and inner bark extracts are listed in Table 2.

The lipophilic extracts of outer bark are mainly composed of several triterpenoids (24.6 g/kg), mostly triterpenic acids with lupane, oleanane and ursane skeletons (Fig. 1). Oleanolic (7.3 g/kg), betulinic (6.6 g/kg), ursolic (3.5 g/kg), betulonic (2.4 g/kg), as well as the acetyl derivatives 3-acetyloleanolic (1.1 g/kg) and 3-acetylursolic acids (0.6 g/kg) are the main components of this family of compounds, which also includes minor amounts of the triterpenic alcohols lupeol and *β*-amyrin. *β*-sitosterol (0.4 g/kg) is also an abundant component of this extract, followed by minor amounts of fatty acids (C16 to C28, 0.4 g/kg) and long-chain aliphatic alcohols (C22 to C28, 0.3 g/kg).

In the inner bark extract, triterpenoids (1.2 g/kg) are, as well, among the main components of the analyzable extract, although, in this case, the main components of this family of compounds are *β*-amyrin (0.3 g/kg) and *α*-amyrin (0.1 g/kg), followed by minor amounts of lupane, oleanane and ursane acids and ketones (*β*-amyrenone and *α*-amyrenone). *β*-sitosterol (0.6 g/kg) is the most abundant component of this extract. Fatty acids (C9 to C28, 0.3 g/kg) and minor amounts of long chain aliphatic alcohols (C16 to C28, 37.9 mg/kg) and aromatic compounds (13.9 mg/kg) are also detected.

Table 1
Extraction yields (% w/w) of *E. nitens* and of different *Eucalyptus* bark fractions previously characterized[21,22]

Bark sample	Extraction yield (% w/w)	
	Inner bark	Outer bark
E. nitens	0.3	3.3
E. globules [21]	0.5	3.9
E. urograndis [22]	0.3	1.7
E. grandis [22]	0.5	1.3
E. maidenii [22]	2.6	6.1

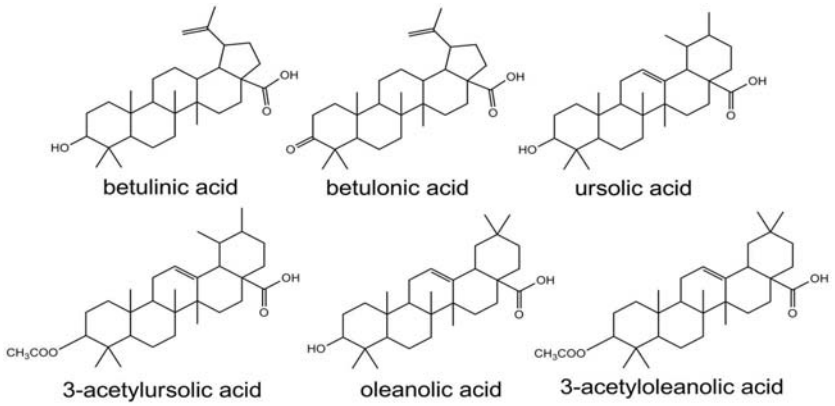

Figure 1: Major triterpenic acids identified in *E. globulus*, *E. urograndis*, *E. grandis* and *E. maidenii* barks

Table 2
Composition of dichloromethane extracts of outer and inner fractions of *E. nitens* bark

Rt (min)	Compound	Content (mg/kg of bark fraction) Outer	Inner
	Fatty acids	419.9	270.5
14.8	nonanoic acid		3.3
17.9	decanoic acid		1.6
23.5	dodecanoic acid		6.8
31.0	tetradecanoic acid		7.4
33.3	pentacosanoic acid		4.5
35.5	hexadecanoic acid	110.2	111.2
36.7	heptadecanoic acid		5.3
31.0	linoleic acid	28.6	29.6
36.9	oleic acid	37.1	26.3
37.1	*trans*-9-octadecenoic acid		4.3
37.6	octadecanoic acid	12.3	21.6
41.6	eicosanoic acid		9.1
43.5	heneicosanoic acid		4.5
45.3	docosanoic acid	36.2	10.8
47.0	tricosanoic acid		5.8
48.8	tetracosanoic acid	103.4	14.6
50.4	pentacosanoic acid		7.6
52.1	hexacosanoic acid	55.3	9.4
55.9	octacosanoic acid	36.9	8.3
	Long chain aliphatic alcohols	310.8	37.9
31.6	hexadecan-1-ol		5.5
36.0	octadecan-1-ol		6.8
43.9	docosan-1-ol	38.6	2.6
47.4	tetracosan-1-ol	65.6	2.6
50.7	hexacosan-1-ol	134.4	5.6
54.3	octacosan-1-ol	72.2	14.9
	Aromatic compounds		13.9
19.2	Vanillin		4.0
26.2	vanillic acid		7.0
29.5	syringic acid		3.0
	Sterols	407.4	620.6
57.6	*β*-sitosterol	407.4	567.7
57.7	*β*-sitostanol		52.9
	Triterpenoids	24606.0	1236.3
55.7	*β*-amyrenone		27.9

Rt (min)	Compound	Content (mg/kg of bark fraction) Outer	Inner
56.5	α-amyrenone		13.4
57.4	β-amyrin	90.9	307.2
58.0	α-amyrin		127.1
58.2	Lupeol	105.9	
61.0	betulonic acid	2436.5	52.3
62.2	oleanolic acid	7250.1	84.7
62.6	betulinic acid	6621.0	83.3
63.1	ursolic acid	3537.1	66.8
63.6	3-acetyloleanolic acid	1101.2	23.4
64.8	3-acetylursolic acid	640.6	22.0
	unidentified triterpenoids	2822.7	428.1
	Other compounds/ unidentified compounds	668.7	164.3
	Total detected compounds	26412.7	2343.5

As expected, these results show that the triterpenic compounds are highly concentrated in the outer layer of *E. nitens* bark, with similar concentrations to those reported[21] in *E. globulus* outer bark, where they account for up to 25 g/kg.

All compounds identified in *E. nitens* bark extracts (Table 2) have been already reported in the lipophilic extracts from the bark and wood of *Eucalyptus* species.[4,5,20-22,34]

Triterpenic composition of *Eucalyptus spp.* pulpwood outer bark extracts

The chemical composition of the outer bark extracts from the studied species is quite similar from a qualitative point of view. The extracts are mainly composed of triterpenic acids, namely betulonic, betulinic, ursolic, oleanolic, 3-acetylursolic and 3-acetyloleanolic acids (Fig. 3, Table 3).

The contents of the main triterpenic acids identified in the five species varied between 4.5 g/kg in *E. urograndis* and 21.6 g/kg in *E. nitens* (Table 3). It is observed that, out of these *Eucalyptus* species outer barks, the ones typical from temperate and Mediterranean zones, particularly *E. nitens* and *E. globulus*, are richer in triterpenic acids than the species from sub-tropical and tropical regions. Furthermore, *E. globulus* outer bark is clearly the richest in ursane acids, while the *E. nitens* outer bark is the richest in oleanane and lupane acids.

Table 3
Major triterpenic components identified in *Eucalyptus* species outer barks (g/kg)

Component	World regions usage for pulpwood				
	Temperate and Mediterranean			Sub-tropical and tropical	
	E. globulus[21]	*E. nitens*	*E. maidenii*[22]	*E. urograndis*[22]	*E. grandis*[22]
Main triterpenic acids	21.3	21.6	8.4	4.5	5.1
betulonic acid	2.6	2.4	1.0	-	-
*oleanolic acid	4.1	8.4	1.7	1.2	0.7
betulinic acid	2.6	6.6	2.0	1.4	2.1
*ursolic acid	12.1	4.2	3.6	1.9	2.4
Other triterpenoids	1.5	3.0	0.2	-	0.1
Total triterpenoids	22.8	24.6	8.5	4.5	5.2

* including 3-acetyl derivative

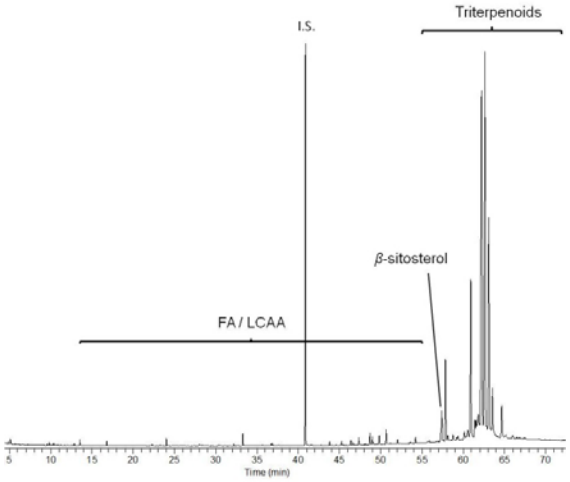

Figure 2: GC-MS chromatogram of *E. nitens* outer bark dichloromethane extract. FA, fatty acids; LCAA, long chain aliphatic alcohols; IS, internal standard (tetracosane)

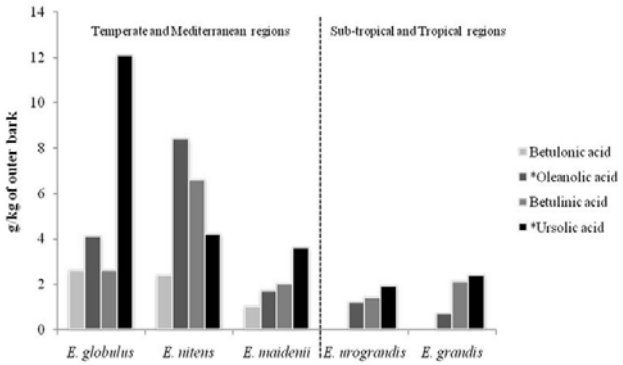

Figure 3: Abundance of the main triterpenic acids in outer barks of the studied *Eucalyptus* species. *Including 3-acetyl derivatives

As to the hypothetical exploitation of bark for triterpenic acids production, *E. globulus* and *E. nitens* outer barks seem to be the most promising raw materials. Considering, as an example, that an *E. globulus* kraft pulp mill with a production capacity of 5.0 x 10^5 tons/year of bleached pulp can generate around 1.0 x 10^5 tons/year of bark, it could, in an integrated way, generate annually around 240 tons of ursolic acid, 80 tons of oleanolic acid, 53 tons of betulinic acid and 50 tons of betulonic acid, among others (based on a inner/outer bark ratio of 8:1).

Although these compounds were found in smaller amounts in the other species here studied, if considering 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, it may be asserted that the outer barks of these species are obvious candidates for the extraction of valuable triterpenic compounds.

ACKNOWLEDGEMENTS: The authors are grateful to the BIIPP project (QREN 11551) and to the AFORE Project (European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No CP-IP 228589-2 AFORE).

REFERENCES

[1] D. I. Forrester *et al*., *Forest Ecol. Manag*., **259**, 9 (2010).
[2] C. Cossalter and C. Pye-Smith, "Fast-wood Forestry: Myths and Realities", edited by C. Cossalter and C. Pye-Smith, Centre for International Forestry Research, 2003.
[3] G. I. Trabado and D. Wilstermann, *Eucalyptus universalis*. Global Cultivated Eucalypt Forest Map 2008, viewed May 2011, available at: http://www.git-forestry.com/.
[4] J. Rencoret, A. Gutierrez and J. C. del Rio, *Holzforschung*, **61**, 2 (2007).
[5] C. S. R. Freire *et al*., *Bioresources*, **1**, 1 (2006).
[6] C. S. R. Freire *et al*., *J. Wood Chem. Technol*., **22**, 1 (2002).
[7] A. Gutierrez *et al*., *Holzforschung*, **53**, 5 (1999).
[8] S. Fernando *et al*., *Energ. Fuel*., **20**, 4 (2006).
[9] B. Kamm *et al*., in "Biorefineries – Industrial Processes and Products: Status Quo and Future Directions", edited by B. Kamm, P. Gruber and M. Kamm, Wiley-VCH, 2006, pp. 97-150.
[10] A. J. Ragauskas *et al*., *Science*, **311**, 5760 (2006).
[11] D. Y. Murzin *et al*., *Chem. Eng. Technol*., **30**, 5 (2007).
[12] P. Fernandes and J. M. S. Cabral, *Bioresource Technol*., **98**, 12 (2007).
[13] A. Hamunen, US Patent 4422974, 1983.
[14] S. P. Pietarinen *et al*., *J. Wood Sci*., **52**, 5 (2006).
[15] S. Willfor *et al*., *Holzforschung*, **58**, 6 (2004).
[16] S. Willfor *et al*., *Holzforschung*, **58**, 4 (2004).
[17] I. V. Kolomitsyn *et al*., *Nat. Prod. Commun*., **2**, 1 (2007).
[18] P. A. Krasutsky, *Nat. Prod. Rep*., **23**, 6 (2006).
[19] H. J. Huang *et al*., *Sep. Purif. Technol*., **62**, 1 (2008).
[20] R. M. A. Domingues *et al*., *Ind. Crop. Prod*., **31**, 1 (2010).
[21] C. S. R. Freire *et al*., *Holzforschung*, **56**, 4 (2002).

[22] R. M. A. Domingues *et al*., *Ind*. *Crop*. *Prod*., **33**, 1 (2011).
[23] M. N. Laszczyk, *Planta Med*., **75**, 15 (2009).
[24] J. Liu, *J*. *Ethnopharmacol*., **100**, 1-2 (2005).
[25] I. Sogno *et al*., *Procs*. *5th International Cancer Prevention Conference*, St. Gallen, Switzerland, 2009, pp. 209-212.
[26] P. F. Smith *et al*., *Antimicrob*. *Agents Ch*., **51**, 10 (2007).
[27] S. Jager *et al*., *Molecules*, **14**, 6 (2009).
[28] C. R. E. Clarke, B. Palmer and D. Gounden, *Southern Forests: a Journal of Forest Science*, **70**, 2 (2008).
[29] R. Ekman, *Holzforschung*, **37**, 4 (1983).
[30] H. Budzikiewicz, J. M. Wilson and C. Djerassi, *J*. *Am*. *Chem*. *Soc*., **85**, 22 (1963).
[31] M. Burnoufradosevich, N. E. Delfel and R. England, *Phytochemistry*, **24**, 9 (1985).
[32] M. Pelillo *et al*., *Rapid Commun*. *Mass Sp*., **17**, 20 (2003).
[33] S. I. Pereira *et al*., *Phytochem*. *Anal*., **16**, 5 (2005).
[34] F. O. Silverio *et al*., *J*. *Wood Sci*., **53**, 6 (2007).