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Phenolic composition and antioxidant activity of *Eucalyptus grandis*, *E. urograndis* (*E. grandis* × *E. urophylla*) and *E. maidenii* bark extracts

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ABSTRACT

The phenolic composition of *Eucalyptus grandis*, *Eucalyptus urograndis* (*E. grandis* × *E. urophylla*) and *Eucalyptus maidenii* bark is reported for the first time. High performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) and tandem mass spectrometry (MS^n) analysis of the methanol:water (50:50) extracts allowed to identify thirteen, twelve and twenty-four phenolic compounds in *E. grandis*, *E. urograndis* and *E. maidenii* bark extracts, respectively. Furthermore, ellagic acid-rhamnoside, dihydroxy-isopropylchromone-hexoside and dihydroxy-(methylpropyl)isopropylchromone-hexoside are referenced for the first time as constituents of *Eucalyptus* species. Epicatechin and quercetin-glucuronide are the major phenolic compounds in *E. grandis* and *E. urograndis* bark, followed by ellagic acid-rhamnoside and ellagic acid in *E. grandis* and by galloyl-*bis*-hexahydroxydiphenoyl (HHDP)-glucose and gallic acid in *E. urograndis*. Catechin, chlorogenic acid and methyl-ellagic acid-pentose are the major compounds in *E. maidenii* bark. The phenolic content of the three extracts shows a positive correlation with their antioxidant activities, evaluated by 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging, showing activity values between those of two commercial antioxidants, ascorbic acid and butylated hydroxytoluene (BHT). These results, together with the phenolic composition, confirm the high potential of these species as source of biologically active phenolic compounds.

1. Introduction

Eucalyptus species are the main wood sources for pulp and paper production worldwide, due to their fast growing and short rotation periods as well as to favourable pulping and bleaching hability (Ugalde and Pérez, 2001). *Eucalyptus* plantation areas cover around 19 million hectares worldwide (Trabado and Wilstermann, 2008), with *Eucalyptus grandis* as the most cultivated specie for industrial purposes, particularly in South Africa and Brazil (Ugalde and Pérez, 2001). *Eucalyptus urograndis*, an hybrid between *E. grandis* and *Eucalyptus urophylla*, is produced in Brazil and was developed to conjugate the fast growing properties of *E. grandis* and the high density and superior pulp properties of *E. urophylla* wood (Quilhó et al., 2006), demonstrating the increasing interest on the exploitation of *Eucalyptus* spp. to pulp and paper production in the South America. In fact, in the last 5 years the eucalyptus planted area in Brazil has increased 5.3% per year, being the 6th world pulp producer in 2010 (ABRAF, 2011). *Eucalyptus maidenii* is presently not so widely used as a fibre source for pulp production as others *Eucalyptus* species, nevertheless its potential for forest developing and excellent pulp qualities has also been demonstrated (Kibblewhite et al., 2001).

It is well known that pulp and paper industries generate high amounts of residues, including mostly bark, but also leaves, branches, fruits and knots, which are commonly burned on the biomass boilers or just leaved on the forest for fertilization purposes. However, in the last years, these by-products, as many other agro-forest residues, are seen as promising sources of materials, chemicals, fuels or energy, to take their maximum value out as also a response of the depletion of fossil resources, within the bio-refinery concept (Fernando et al., 2006; Kamm et al., 2006; Ragauskas et al., 2006). In fact, the exploitation of agro-forest residues as a source of valuable compounds is a strategy already applied in some pulp mills and one of the most popular examples of the implementation of the bio-refinery concept (Hamunen, 1983; Pietarinen et al., 2006).

In this perspective, in recent years, the biomass residues of the exploitation *Eucalyptus* species, and particularly outer bark, have attracted much interest namely as sources of high value triterpenic acids (Freire et al., 2002; Domingues et al., 2010, 2011).

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More recently but also in the case of *Eucalyptus globulus* bark as source of phenolic compounds (Santos et al., 2011). However, the information of the phenolic composition of *E. grandis*, *E. urograndis* and *E. maidenii* is scarce and only studies about the presence of these compounds on the leaf litter from *E. urograndis* (Chapuis-Lard et al., 2002) and leaves from *E. grandis* (Kulkarni et al., 2008) and *E. maidenii* (Manguro et al., 1995) have been published. To the best of our knowledge no study has been carried out about the phenolic composition of the barks from these species, despite the well-known wide range of valuable properties assigned to phenolic compounds, such as anti-inflammatory, antioxidant, antibacterial, antimicrobial, anti-trombotic or even anticarcinogenic and anti-HIV-1 agents, among others (Carrol et al., 1999; Hassan Khan et al., 2007).

In this context, and following our interest on the study of the chemical composition and valorization of *Eucalyptus* barks, the aim of this study is to evaluate the potential of *E. grandis*, *E. urograndis* and *E. maidenii* bark as sources of valuable phenolic compounds, analyzing their methanol:water extracts by high-performance liquid chromatography–mass spectrometry (HPLC–MS) and also accessing their total phenolic content by the Folin-Ciocalteu assay and their antioxidant properties determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging.

2. Materials and methods

2.1. Chemicals

Dichloromethane (99% purity), gallic acid (purity higher than 97.5%) and quercetin (purity higher than 98%) were supplied by Sigma Chemical Co. (Madrid, Spain). Protocatechuic acid (purity higher than 97%), chlorogenic acid (purity higher than 95%) and naringenin (98% purity) were obtained from Aldrich Chemical Co. (Madrid, Spain). HPLC-grade methanol, water and acetonitrile, were supplied from Fisher Scientific Chemicals (Loures, Portugal). Formic acid (purity higher than 98%), methanol (purity higher than 99.8%), catechin (purity higher than 96%) and ellagic acid (96% purity) were purchased from Fluka Chemie (Madrid, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

2.2. Raw materials

Bark samples were collected from trees with age and geographic origin representative of their industrial exploitation: *E. urograndis* and *E. grandis* bark samples were taken from a 5-year-old and 10-year-old tree, respectively, randomly harvested 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).

2.3. Phenolics extraction

The total bark of each species was air dried until a constant weight was achieved and ground to granulometry lower than 2 mm giving rise to a compost sample from which about 45 g of each dried bark were submitted to a Soxhlet extraction with dichloromethane for 6 h to remove the lipophilic components. The solid bark residues were then suspended (m/v: 1:100) in a methanol:water (MeOH:H_2O) mixture, 50/50 (v/v), at room temperature for 24 h under constant stirring. The suspensions were then filtered, MeOH removed by low pressure evaporation and the extracts freeze dried (Santos et al., 2011).

2.4. Total phenolic content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965; Santos et al., 2010). 2.5 ml of Folin-Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 ml of aqueous sodium carbonate (75 g l^{-1}) were added to accurately weighed aliquots of the extracts dissolved in 0.5 ml of methanol, corresponding to concentration ranges between 80 and 200 μg of extract ml^{-1}. Each mixture was kept for 5 min at 50 °C and, after cooling, the absorbance was measured at 760 nm, using a UV-Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). TTPC was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (10–85.0 μg ml^{-1}) and expressed as g of gallic acid equivalent (GAE) g^{-1} of extract. The analyses were carried out in triplicate and the average value was calculated in each case.

2.5. Chromatographic procedure

Analyses were carried according to a previously reported procedure (Santos et al., 2011), by using a Hewlett–Packard (HP) 1050 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Rheodyne injector with a 10 μl loop, a quaternary pumping system and a UV detector. It was used a Discovery® C-18 (15 cm × 2.1 mm × 5 μm) column supplied by Supelco (Agilent Technologies, Waldbronn, Germany). The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.2 ml min^{-1}, at room temperature. The mobile phases consisted in water:acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0–3 min: 0% B; 3–10 min: 0–10% B; 10–30 min: 10–20% B; 30–35 min: 20–25% B; 35–50 min: 25–50% B; 50–60 min: 50–0% B; followed by re-equilibration of the column for 10 min before the next run. The injection volume in the HPLC system was 25 μl and the UV–vis detection was performed at 280 and 340 nm. Before the injection in the HPLC each extract was dissolved in MeOH:H_2O (50:50), HPLC grade, to obtain a final concentration of about 10 mg ml^{-1} and then filtered through a 0.2 μm PTFE syringe filter.

2.6. ESI–QqQ–MS analysis

The HPLC system was coupled to a Micromass spectrometer (Manchester, UK), operating in negative mode, equipped with an electrospray source and a triple quadrupole (QqQ–MS) analyzer. The cone and capillary voltages were set at −30.0 V and −2.6 kV, respectively. The source temperature was 143 °C and the desolvation temperature was 350 °C. MS/MS spectra were obtained using argon as collision gas with the collision energy set between 10 and 45 V. The detection was carried out considering a mass range of m/z between 50 and 1000, with a scan duration of 0.5 s. The data acquisition was done by using the MassLynx® data system (Waters, Milford, MA, USA).

2.7. ESI–IT–MS/MS analysis

To gather additional MS information about several chromatographic peaks, these were manually collected following the chromatographic conditions discussed above. The resulting HPLC fractions were dissolved in methanol and directly injected into a Linear Ion trap LXQ (ThermoFinnigan, San Jose, CA, USA), also equipped with an ESI source by means of a syringe pump, at flow rate of 8 μl min^{-1}. The nitrogen sheath gas was 30 psi, spray voltage 4.7 kV and capillary temperature 275 °C. The capillary and tune lens voltages were set at −7.0 V and −71.8 V, respectively. CID-MS/MS and MS^n experiments were performed on mass-selected