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Secondary metabolites from *Eucalyptus grandis* wood cultivated in Portugal, Brazil and South Africa

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ABSTRACT

The composition of the lipophilic and phenolic fractions of the wood of *Eucalyptus grandis* cultivated in Portugal, Brazil and South Africa, was studied. The lipophilic fraction of the studied *E. grandis* wood is mainly composed of sterols, fatty acids and phenolic compounds. Three triterpenic acids were detected for the first time in the wood extracts from Brazil. *E. grandis* wood from Portugal presents the largest lipophilic content (1.67 g kg^{-1} of dry wood), followed by South Africa (1.56 g kg^{-1} of dry wood) and Brazil (1.05 g kg^{-1} of dry wood). 51 Phenolic compounds were identified in *E. grandis* wood MeOH:H_2O extracts, from which 11 are reported for the first time as *E. grandis* constituents and 4 phenolic compounds are firstly reported as *Eucalyptus* genus components. *E. grandis* wood from Brazil shows the highest phenolic content (~2.36 g kg^{-1} of dry wood), followed by South Africa (~1.90 g kg^{-1} of dry wood) and Portugal (~1.30 g kg^{-1} of dry wood), demonstrating the influence of the geographic origin over *E. grandis* wood extractives composition and abundance, as well as on the antioxidant activities of the phenolic fractions. The detailed knowledge of these *E. grandis* extracts can contribute on the one hand to prevent their impact in the bleaching process, and, on the other demonstrates the potential of this species as a source of bioactive phytochemicals for nutraceutical applications.

1. Introduction

The genus *Eucalyptus,* from the angiosperm family *Myrtaceae*, includes over 700 species (Brooker, 2000), occupying a planted area of nearly 20 million ha spread over the world (Myburg et al., 2014). Although being native from Australia region, *Eucalyptus* species are the most extensively cultivated hardwood trees by pulp and paper companies due to their adaptability to different edaphoclimatic conditions, fast growing and excellent wood properties for pulp and paper production (Grattapaglia and Kirst, 2008), and have therefore an important economic impact worldwide. Furthermore, despite many environmental concerns, the high-productivity of eucalypts forests contributes to diminish the industrial pressure over tropical forests and associated biodiversity (Grattapaglia and Kirst, 2008; Myburg et al., 2014).

Among the multitude of species, *Eucalyptus grandis* (Hill ex Maiden) is one of the most cultivated for industrial purposes, particularly in South Africa and Brazil (Ugalde and Pérez, 2001). In this scenario, the detailed study of its chemical composition, and in particular of the less abundant and also less studied components *viz.* extractives, is a relevant issue. This knowledge can contribute to prevent pulp production problems associated with their presence, e.g. pitch deposition and consumption of bleaching reagents (Gutiérrez et al., 2001). On the other hand, theses extracts might be valuable sources of added value compounds that might be extracted prior to pulping. In fact, the presence of bioactive secondary metabolites in different *Eucalyptus* tissues, namely bark (Domingues et al., 2011a,b; Santos et al., 2012), wood (Barbosa et al., 2005; Freire et al., 2006; Kilulya et al., 2014; Rencoret et al., 2007; Santos et al., 2013a), and leaves (Kulkarni et al., 2008; Singh et al., 1998; Umehara et al., 1998) from *E. grandis* has already been reported. Particular attention has been devoted to lipophilic and phenolic compounds, with bark extracts being mainly composed

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by triterpenic acids, such as ursolic, betulinic and oleanolic acids (Domingues et al., 2011a,b), epicatechin and quercetin-glucuronide (Santos et al., 2012); and the leaves extracts by euglobals (Singh et al., 1998; Umehara et al., 1998) and pyrogallol, among others (Kulkarni et al., 2008).

Regarding *E. grandis* wood, hexadecanoic and 9,12-octadecadienoic acids, β-sitosterol and stigmasterol are the main lipophilic components (Kilulya et al., 2014; Rencoret et al., 2007), whereas phenolic acids and derivatives, flavonoids, ellagic acid derivatives, galloylglucose derivatives and hydrolysable tannins are the major phenolic compounds identified so far (Santos et al., 2013a).

The inter- and intra-species and geographic variability in the chemical composition is well-known in *Eucalyptus* genus. However, despite the study of Kilulya et al. (2014) about the effect of soil composition on the amount of lipophilic extractives present in *E. grandis* from South Africa, there is no systematic studies regarding the variation of the lipophilic and phenolic composition of *E. grandis* wood with geographic location. Additionally, and to the best of our knowledge, only a single study has been reported concerning the phenolic fraction of *E. grandis* wood (Santos et al., 2013a).

In this vein, a detailed study of the dissimilarities in the lipophilic and phenolic fractions of *E. grandis* wood extractives from three different geographic origins, namely Portugal, Brazil and South Africa, using gas chromatography-mass spectrometry (GC–MS) and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC–MS) is reported. In addition, the antioxidant properties of the phenolic extracts were assessed by the DPPH• and ABTS+• scavenging.

2. Material and methods

2.1. Chemicals

Dichloromethane (99%), pyridine (99%), trimethylchlorosilane (99%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (99%), tetracosane (99%), octadecanoic acid (99%), triacontan-1-ol (99%), β-sitosterol (99%), coniferyl alcohol (98%), gallic acid (>97.5%), quercetin (>98%), isorhamnetin (>99%), Folin- Ciocalteu's phenol reagent, 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (>99%), 2,2-diphenyl-1-picrylhydrazyl hydrate radical (DPPH•), and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied by Sigma Chemical Co (Madrid, Spain). Ursolic acid (98%) was purchased from Atkin Chemicals (Chengdu, China). Protocatechuic acid (>97%), caffeic acid (>95%), naringenin (98%) and trolox (97%) were obtained from Aldrich Chemical Co (Madrid, Spain). Sodium carbonate (99.9%) was supplied by Pronalab (Lisbon, Portugal). Formic acid (>98%), catechin (>99%), ascorbic acid (>99.5%), methanol (>99.8%) and ellagic acid (96%) were purchased from Fluka Chemie (Madrid, Spain). HPLC-grade methanol, water and acetonitrile were supplied by Fisher Scientific Chemicals (Loures, Portugal) and further filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA).

2.2. Sample preparation

E. grandis wood samples, as prepared for the pulp mill industries, were taken from several 8–12 years- old trees randomly harvested from plantations cultivated in Portugal (Alentejo region), Brazil (state of Espirito Santo) and South Africa (Mpumalanga province). *Eucalyptus* wood samples were chipped, air-dried at room temperature until a constant weight was achieved and within less than two months all samples were ground to a granulometry lower than 2 mm and submitted to extraction.

2.3. Lipophilic and phenolic compounds extraction

Three ground samples (30 g) of each wood were Soxhlet extracted during 6 h with dichloromethane (600 mL), which was used for being a fairly specific solvent for lipophilic extractives isolation for analytical purposes. The solvent was evaporated to dryness, the lipophilic extracts were weighed and the results were expressed in percent of dry material (% DW).

Subsequently, the solid residues from the dichloromethane extraction were suspended (m/v 1:100) in a methanol/water (MeOH:H_2O, 50:50 v/v) mixture, at room temperature for 24 h under constant stirring. The suspension was then filtered, MeOH removed by reduced pressure evaporation, and the extracts freeze-dried.

2.4. Total phenolic content

The total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method (Singleton and Rossi Jr., 1965), with some modifications (Vilela et al., 2014). Briefly, 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 MeOH:H_2O corresponding to concentration ranges between 0.05 and 0.1 mg of extract per mL. Each mixture was kept for 5 min at 50 °C and after cooling the absorbance was measured at 760 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The total phenolic content was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (0.02–0.08 mg mL^{-1}) and expressed as mg of gallic acid equivalent (GAE) per g of extract. The analyses were carried out in triplicate and the mean was calculated in each case.

2.5. Antioxidant activity of the phenolic extracts

2.5.1. DPPH• assay

The antioxidant activity of the extracts was determined by DPPH• scavenging, following a procedure described before (Santos et al., 2013a; Sharma and Bhat, 2009). The extracts concentration ranged between 1.25 and 12.5 μg mL^{-1}. The absorbance at 517 nm was measured on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Ascorbic acid and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were used as reference compounds. The antioxidant activity was expressed as IC_{50} values (inhibitory concentration of the extract necessary to decrease the initial DPPH• concentration by 50% and are expressed in μg mL^{-1}), as well as in g of ascorbic acid equivalents per kg of wood (g AAE kg^{-1} of wood).

2.5.2. ABTS assay

The ABTS assay is based on the scavenging of the ABTS+• converting it into a colorless product. In this test, ABTS+• cation was generated by reacting ABTS 7 mM solution with potassium persulfate 2.45 mM, following the originally method described by Re et al. (1999), with minor adjustments (Touati et al., 2015). This mixture was then incubated in the dark at room temperature for 16 h. Before usage, the ABTS+• solution was diluted with methanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm. 30 μL of extracts or standard compounds were mixed with 3 mL of ABTS+• solution, obtaining final concentrations of 0.99–3.47 μg mL^{-1} and 0.99–4.95 μg mL^{-1}, respectively. The absorbance was measured at 734 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The ABTS+• scavenging capacity of the extracts was compared with that of trolox. Triplicate measurements were done. The antioxidant activity was expressed as IC_{50} values (the extract concentration providing 50% inhibition), as well as in mg of trolox equivalents per g of dry wood (mg TE g^{-1}dry wood).

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