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BIOCHEMICAL FEATURES OF ORGANIC EXTRACTIVES FROM *Eucalyptus* AND *Corymbia* WOODS USING ETHANOL AS A SOLVENT

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ABSTRACT

This study aims to evaluate chemical characteristics, antioxidant and antibacterial activities of organic compounds extracted from three *Eucalyptus* wood and *Corymbia maculate* wood using ethanol as a solvent. To obtain the ethanolic extracts, 15 g of a powdered wood sample was mixed with 150 mL of ethyl alcohol 99 % PA by constant mechanical stirring, which was further magnetically mixed at 60 ºC for 24 h. The extractives were analyzed utilizing percent yield, Fourier-transform infrared spectrum, inhibitory index (measured after antimicrobial tests), antioxidant activity, and condensed tannins content. The *Eucalyptus dunnii* extract showed the highest percent yield. The infrared spectra of all the extractives presented similar profiles, with remarkable bands ascribed to the presence of lipophilic extracts, sterols, fatty acids, and other hydroxylated substances, such as carbohydrates and phenolic compounds. In all cases, the higher the concentration of the

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extractive was, the higher the antioxidant activity was. The antioxidant activity of *Eucalyptus saligna* extract stood out since overcame that of the positive control (ascorbic acid). Regarding the condensed tannins content, that extract from *Eucalyptus grandis* excelled.

Keywords: Chemical characteristics, condensed tannins, natural extractives, ethanol extraction, infrared.

INTRODUCTION

Wood is composed of structural macromolecules of high molecular weight, namely cellulose, hemicellulose, and lignin. Aside of them, extractives are low molecular weight substances from wood. They are often hydrophobic or lipophilic secondary metabolites and do not influence in the tree growth (Saha Tchinda *et al.* 2018).

The amount and composition of the wood extractives may vary in function of both radial and axial position in the wooden trunk. Moreover, factors associated with the forest also play significant roles, such as conditions related to both tree growth and wood storage (Saha Tchinda *et al.* 2018). Some wood extractives are soluble in both water and neutral organic solvents and, besides of that, most of them are located in bark (Morais *et al*. 2005).

According to Valette *et al*. (2017), the wood extractives are biosynthetized in trees to avoid injuries attributed to biotic (like fungi and insects) and abiotic (like rain, sunlight, wind, among other) agents. Other authors also reported strong correlations between the content of some wood extractives and the wood durability (Kirker *et al*. 2015, Pometti *et al*. 2009).

Eucalyptus–based woods present several types of extractives, including essential oils, fatty acid esters, as well as small amounts of inorganic substances (Santos *et al*. 2016). The essential oils from *Eucalyptus* woods are widely applied in chemical, cosmetic, and pharmaceutic industries (Albuquerque *et al*. 2017). The high added value of these compounds also may encourage researches on their production and characterization.

Extractives and essential oils obtained from plants are important sources of natural antioxidants (Hayat *et al*. 2010, Luna *et al.* 2010). Some recent studies reported secondary metabolites in the composition of some wood extractives, such as phenols (Jiang *et al*. 2017), terpenoids (Andrew *et al*. 2013), and flavonoids (Takahashi *et al*. 2004). Yamakoshi *et al*. (1992) and Nakayama *et al*. (1990) affirmed that extractives obtained from *Eucalyptus macrocarpa* and *Eucalyptus perriniana* were effective against the proliferation of some Gram-positive bacteria (namely *Staphylococcus aureus* and *Bacillus subtilis*). Moreover, Takahashi *et al*. (2004) studied extractives obtained from *Eucalyptus* leaves and flavonoids from *Corymbia maculate* wood and reported great performances against the growth of microbes and fungi.

For Wu *et al.* (2019), a feasible use of a wood extractive must be done after an effective and cheap extraction process. Ethanol is a commonly used solvent for obtaining several compounds from wood and other vegetable sources, such as extractives and/or essential oils (Hofmann *et al.* 2015). The ethanol soluble compounds from wood include fatty acid esters, long-chain alcohols, steroids, phenolic compounds, and glycosides (Sjöström and Alén 1998, Gullichsen and Paulapuro 1999, Sun and Sun 2002, Morais *et al.* 2005). This solvent can be obtained from renewable sources (like sugar cane) using well-known and cheap routes. This is also commonly combined with other organic solvents, like methanol and benzene, which may allow an improved extraction yield or even a selective extraction of particular substances (Abdul Mudalip *et al.* 2013, Peng *et al.* 2017).

This study aimed to evaluate chemical features and both the antioxidant and antibacterial activities of ethanol extractives obtained from three *Eucalyptus* woods and a *Corymbia maculate* wood.

MATERIALS AND METHODS

Raw materials and ethanol extractions

Fifteen 22-28-year-old exotic trees from *Eucalyptus dunnii, Eucalyptus saligna, Eucalyptus grandis*, and *Corymbia maculate* were felled in homogeneous forests located in Tapes/Brazil. Wood flakes cut from the sapwood of each specie were crushed until pass through a 40 mesh sieve and be retained in a 60 mesh sieve.

A high purity ethanol solution (99 %) was purchased from Sigma Aldrich. 15 g of powdered wood sample and 150 mL of ethanol were placed into 250 mL Erlenmeyer and kept under magnetic stirring at 60 ºC for 24 h. After that, the solvent was evaporated using a Heidolph Rotary Evaporator (Laborota 4002 equipment) and then the flask was hermetically closed and stored under -4 ºC for further analyses. Yield of the ethanol extraction was calculated according to TAPPI T 204 cm-97 (1997), as shown in Equation 1. Besides, chemical groups were evaluated by Attenuated Total Reflection-Fourier transform infrared spectroscopy (ATR-FTIR), in which 32 scans were performed at the 4000 cm^{-1} to 600 cm^{-1} range, 4 cm^{-1} resolution, and 2 $mm \cdot s^{-1}$ scanner velocity, Equation 1.

$$Y = \left(\frac{m_e - m_f}{m_f} \right) x\ 100 \quad (1)$$

Where: Y is the percent yield, m_e is the mass of the flask plus the evaporated extract, m_f is the mass of the flask, and m_w is the mass of the wood sample.

Antimicrobial activity

Test organisms

Antimicrobial activity was evaluated using gram-positive standard strains from *Staphylococcus aureus* (ATCC 25923) and *Enterococccus faecalis* (ATCC 51299), as well as gram-negative standard strains from *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC 14028). These microorganisms were provided by the Oswaldo Cruz Foundation (FIOCRUZ). The evaluated strains were kept in Mueller-Hinton agar at 4 °C and reactivated prior to the antimicrobial evaluation.

The antimicrobial assays with these bacteria were carried out following the broth microdilution method, indicated by Clinical and Laboratory Standards Institute (CLSI 2018). Extractives were diluted in a 0,5 % dimethyl sulfoxide (DMSO) solution and then placed into 96-well microplates (Kasvi®), reaching concentrations that ranged from 0,0078 $mg \cdot mL^{-1}$ to 1 $mg \cdot mL^{-1}$. 100 µL of the DMSO-based solution was used as emulsifier to control the overall sterility and a mixture of DMSO and extractive (ratio of 1:1) was used to control the microbial growth. The bacteria were suspended in a 0,9 % saline solution until reach 0,5 McFarland standard. For that, the optical density was accessed using a UV-VIS spectrophotometer adjusted at 630 nm wavelength until reach an absorbance of 0,08–0,1 range. Then, the bacterial solution was adjusted to a final concentration of 3E4 colony-forming unit mL^{-1} and the microplates were incubated at 37 ºC for 24 h. Afterwards, a 20 mL of 0,02 % Resazurin (acquired from Sigma Aldrich) was used to reveal the microbial growth in each well. The bacteria were indicated in pink colour and its minimal inhibitory concentration (MIC) was determined.

Antioxidant activity

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as free radial, following that methodology described by Brand-Williams *et al.* (1995). Variable concentrations of each extractive were progressively incorporated in a 300 mL of a methanol-DPPH solution with 2,7 mL of methanol and incubated for 15 min in darkness. After that, a UV-VIS spectrum was obtained in a UV-M51 equipment (Bel Ptotonics brand) adjusted for a wavelength of 517 nm. Both a reference sample and two control samples were also prepared and measured. The latter one had 2,7 mL of methanol and 300 µL of DPPH, while both ascorbic acid and rutin (Vetec brand) were used as control samples at the following concentrations: 1,0 mg.mL-1, 0,5 mg.mL-1, 0,25 mg.mL-1 and 0,15 mg.mL-1. All analyses were performed in triplicate. Inhibition of the DPPH radical at different extract concentrations was calculated using Equation 2.

$$\%\ \text{Inhibition} = \left(\frac{A_{DPPH} - (A_{extract} - A_{Blank})}{A_{DPPH}} \right) x\ 100 \quad (2)$$

Where: A_{DPPH} is the absorbance of the DPPH radical without samples, $A_{Extract}$ is the absorbance of extracts mixed with DPPH radical and A_{Blank} is the absorbance of ethanol.

Condensed tannins content

The condensed tannins content was determined according to the vanillin method, described by Morrison *et al*. (1995). Firstly, a vanillin solution was prepared using equal volumes of 1 g of vanillin in 100 mL of

methanol and 8 mL of concentrated HCl in 100 mL of methanol. This solution was incorporated with 0.1 mL of extractive sample (with a concentration of 50 mg·mL^{-1}) and 0,9 mL of methanol. Then, it was left in water bath for 20 min and the absorbance was read at 500 nm. All analyses were performed in triplicate and the corrected spectra were converted to catechin equivalents from standard curves (Missio *et al*. 2017).

Statistical analyses

The data were arranged in a completely randomized design. Normality of the data and homogeneity of variances were verified by the Shapiro-Wilk and Levene tests, respectively. Whenever the null hypothesis was rejected, a Fisher test (at a confidence level of 95 %) was performed to compare the means.

RESULTS AND DISCUSSION

The studied wood species presented a wide range regarding the values of yields of ethanol extractions (Table 1), which are in agreement with a previous work by Gomide *et al.* (2010), that reported that the main chemical compounds from wood may vary among genders, species, parts from a same tree, as well as they are affected by microclimatic factors, soil conditions, tree age, and so on.

Table 1: Values of yields of ethanol extractives for the *Eucalyptus* and *Corymbia maculate* woods.

	Eucalyptus grandis	*Eucalyptus saligna*	*Eucalyptus dunnii*	*Corymbia maculate*
Yield of extractives (%)	2,87	1,64	4,64	1,00

The *Eucalyptus dunnii* presented the highest yield of extractives, which is ascribed to some of its intrinsic features, like anatomical, chemical and macroscopic properties. The obtained values for yield of extractives were higher than those reported by Silvério *et al*. (2006). These authors compared different solvents applied for extractions performed with a *Eucalyptus grandis* wood and reported the following yields: 2,17 %, 0,53 %, 0,55 %, and 2,48 %, which are respective to extractions with acetone, chloroform, dichloromethane, and an ethanol:toluene (1:2) mixture.

Figure 1 presents infrared spectra for all extractives, in which prominent peaks were found at 1040 cm^{-1}, 1089 cm^{-1}, and 1386 cm^{-1}, which represent C-O bonds. A remarkable slope was visualized at 2978 cm^{-1}, which represents C-H bonds. The peak at 3326 cm^{-1} appears in all the spectra and can be ascribed to O-H bonds from some alcohols (like β-sitosterol) commonly found in wood extractives. This also indicates that probably other hydroxylated compounds may be present, like phenolic groups (Gullichsen and Paulapuro 1999, Morais *et al*. 2005). Similarly shaped spectra were reported by Abdul Mudalip *et al.* (2013), who carried out an elucidative study on hydrogen bonds from a pure ethanol solution.

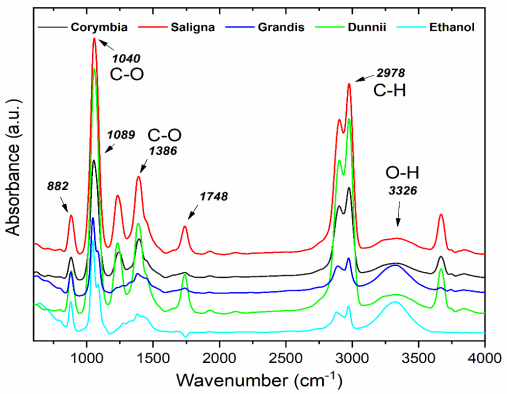

Figure 1: Infrared spectra for the *Eucalyptus* and *Corymbia maculate* woods, as well as the ethanol.

The above mentioned spectra show peaks at 2984 cm^{-1}, 2978 cm^{-1}, 2971 cm^{-1}, 2967 cm^{-1}, 2899 cm^{-1}, 2897 cm^{-1}, and 2879 cm^{-1} correspondent to C-H stretching, as well as CH, CH2, and CH3 groups commonly found in aliphatic compounds, like fatty acids, fatty esters, and long-chain alcohols. Silverstein *et al*. (2002) found the same organic compounds and associated them to peaks at 2954 cm^{-1}, 2919 cm^{-1}, and at 2850 cm^{-1}. Other minor peaks at 1748 cm^{-1}, 1741 cm^{-1}, and 1735 cm^{-1} are associated to C=O bonds from esters (Sjöström and Alén 1998).

Figure 2 presents the different % inhibition values of the extracts from *Eucalyptus* and *Corymbia maculate* in function of their concentrations. The *E. saligna* extract showed the greatest inhibition of the DPPH radical, followed by the extract of *Corymbia maculate*, *E. dunnii* and *E. grandis*. Regarding the positive control, *E. saligna* extract showed greater inhibition than ascorbic acid (except at the concentration of 0,125 $mg \cdot mL^{-1}$), and in relation to the positive control rutin, all extracts showed greater inhibition in all concentrations. Also, the extractive from the *Eucalyptus saligna* presented a higher inhibition than the ascorbic acid, which may be attributed to its high amounts of both phenolic and hydroxyl groups.

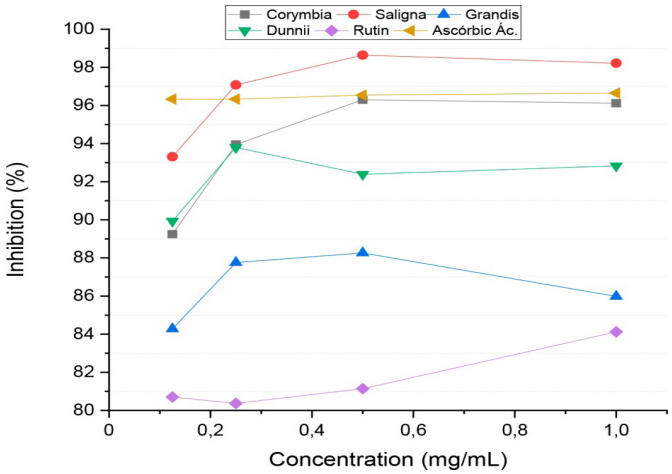

Figure 2: Minimal inhibitory concentration (antioxidant activity) values for the *Eucalyptus* and *Corymbia maculate* woods, as well as the positive controls (rutin and ascorbic acid).

Schumack *et al.* (2018) ascribed significant inhibitory actions to commercial essential oils extracted from *Eucalyptus ssp*. woods against two bacteria, namely *E. coli* and *S. aureus*. Regarding the same bacteria, Estanislau *et al.* (2001) reported similar results for essential oils extracted from both *Eucalyptus grandis* wood and *Eucalyptus saligna* wood. These essential oils were obtained by hydro-distillation and differ from those extracts studied here since wood extractives obtained by ethanolysis do not encompass essential oils (Silveira *et al.* 2012).

It is expected that those extractives with high contents of phenolic, fatty acids, and steroids may present high antioxidant activities. Chang (2000) and (Gomes and Canhoto 2003) reported phenolic compounds for similar wood species. Bio-based antioxidants can replace synthetic antioxidants, when equivalent or superior inhibitions of enzymatic lipid oxidation are reached (Bandoniene and Murkovic 2002). Natural antioxidants can act as inhibitors against free radicals, chelators, and oxygen scavengers and these compounds include flavonoids, phenolic acids, terpenes, tocopherols, phospholipids, and polyfunctional organic acids (Gómez 2003, Ribeiro 2007). Wood extractives with these substances are known due to their antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial characters (Hras *et al*. 2000).

The extractives from *Eucalyptus grandis* wood presented the highest condensed tannin content, followed in a decreasing order by *Eucalyptus saligna* wood, *Corymbia maculate* wood, and *Eucalyptus dunni* wood (Table 2). That result for the *Eucalyptus grandis* wood indicates its high potential for particular applications, when certain features are needed, such as antibacterial, antiviral and protein-binding (Salminen 2018, Zeller 2019). The condensed tannin content is directly related to the amount of phenolic groups. In plants, condensed tannins are obtained by liquid-solid extractions and are composed of sugars, proteins, lipids and some minor phenolic compounds (Brown *et al*. 2017).

Table 2: Values of condensed tannins contents for ethanol extractives of the *Eucalyptus* and *Corymbia maculate* woods.

	Eucalyptus grandis	*Eucalyptus saligna*	*Eucalyptus dunnii*	*Corymbia maculate*
Condensed tannins content (%)	58,62	15,91	0,35	0,53

CONCLUSIONS

Among the wood species, the *Eucalyptus dunnii* wood presented the highest extractives content after the ethanol extractions. All the wood extractives yielded such infrared spectra, which were similar to lipophilic extractives, like sterols, fatty acids, and other hydroxylated substances, such as some carbohydrates and phenolic compounds. The antioxidant activities were directly related to the extractives content and the *Eucalyptus saligna* wood stood out, since its inhibition was higher than that of the ascorbic acid. The wood extractives showed a great potential related to the production of condensed tannins, especially the *Eucalyptus grandis* wood. On the other hand, there were no promise results regarding the reported antimicrobial activities and, in this sense, we recommend further studies dealing with higher concentrations of wood extractives.

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