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Antioxidant and antifungal activity of carnauba wax powder extracts

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ABSTRACT

Carnauba wax is a commercially valuable product derived from epicuticular wax powder (CWP) from the leaves of *Copernicia prunifera* (Arecaceae), a palm tree native to northeastern Brazil. CWP exists as a fine powder on both young, upright, closed leaves (type A wax), and mature, pendant, open leaves (type B wax). The aim of this study was to evaluate the effect of extraction time on aqueous and ethanolic extracts of carnauba wax powder and quantify their antioxidant potential. Furthermore, we determined the total phenols, flavonoids, and flavonols in the extracts, and tested the effects of the extracts on the dermatophyte fungi *Trichophyton rubrum* and *Microsporum canis*. Antioxidant capacity was evaluated by measuring the scavenging effect of the extracts on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radicals. Overall, higher extract yields were obtained using ethanol as a solvent, rather than water. All extracts showed antioxidant activity and the extraction time did not influence the antioxidant activity of the extracts; the strongest activity was observed for the ethanolic extract of type B wax powder (EtB) with an EC_{50} values of 365 ± 7 μg/mL (by DPPH assay) and 317 ± 6 μg/mL (by ABTS). Total phenolic, flavonoid, and flavanol contents varied from 280.73 ± 4.85 to 114.06 ± 4.45 gallic acid equivalents (GAE), 24.59 ± 0.45 to 5.34 ± 0.12 quercetin equivalents (QE), and 32.36 ± 0.93 to 9.41 ± 0.37 catechin equivalents (CTE), respectively. Qualitative high-performance liquid chromatography (HPLC) analysis revealed the presence of gallic acid, catechin, and chlorogenic acid in type A and type B wax aqueous extracts. The aqueous extracts showed antifungal activity against dermatophytes *M. canis* and *T. rubrum*. These results provide evidence that carnauba wax powder extracts might be potential sources of natural antioxidant and antifungal agents.

1. Introduction

Plant wax is the general term used to describe the lipid components of the cuticle, a substance that covers the outer surface of aerial plant tissues (Samuels et al., 2008). These waxes protect plants from various stresses such as water loss by transpiration, prolonged exposure to a dry atmosphere, excessive solar radiation, and UV radiation, and help to defend plants from pathogen attacks and herbivores (Buschhaus and Jetter, 2011).

Carnauba wax is a product of commercial value used in large quantities in cosmetics, polishes, lubricants, and surface coatings, and has many other applications (Machado et al., 2012; Mehyar et al., 2012; Milanovic et al., 2010). It is obtained by processing the epicuticular wax powder (CWP) found on the leaves of the *Copernicia prunifera* (Miller) H.E. Moore (Arecaceae) tree, a palm tree native to northeastern Brazil (Lorenzi et al., 2010). CWP, which forms a thick layer of solid material on the surface of the leaves, can be obtained from fully expanding and mature leaves (named “pó de palha”), which form the crown of the carnauba, or the closed and young leaves (named “pó de olho”), which constitute the apical region of the tree (Alves and Coelho,

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ANOVA, analysis of variance; AqA, aqueous extract of tyA; AqB, aqueous extract of tyB; CWP, epicuticular wax powder; DMACA, *p*-dimethylaminocinnamaldehyde; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EtA, ethanolic extract of tyA; EtB, ethanolic extract of tyB; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; MOPS, morpholine propanesulfonic acid; QE, quercetin equivalents; tyA, type A wax powder; tyB, type B wax powder

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2008; European Food Safety Authority-EFSA, 2012).

Brazilian technical regulations define the “pó de olho” and “pó de palha” wax powder as type A and type B, respectively (Brazilian Department of Agriculture, Livestock and Food Supply, 2004). Since the apical leaves have a lower percentage of chlorophyll, type A wax has a coloration that varies from white to light yellow, and has a higher economic value than type B does, which has a greenish-gray coloration.

The chemical composition of carnauba wax is a complex mixture of long-chain fatty acids, esters, free alcohols, aliphatic acids, aromatic acids, hydroxy acids, triterpene diols, cinnamic acids, and proteins (Almeida et al., 2017; Cruz et al., 2002; Cysne et al., 2006; Harron et al., 2017; Koonce and Brown, 1944; Wang et al., 2001). The inorganic compounds present include aluminum, calcium, copper, iron, manganese, magnesium, sodium, and zinc (Dantas et al., 2013).

Research into antimicrobial and antioxidant compounds derived from plants has increased in recent years because they possibly act as antioxidants in food, preservatives in fruits and cosmetics, and as antifungal, antibacterial, and antiviral therapeutics (Brewer, 2011; Porter and Bode, 2017; Savoia, 2012). However, considering that carnauba wax has a diverse chemical composition including many potentially antioxidant molecules, and it is widely used in industrial processes including the manufacturing of food, pharmaceuticals, and cosmetics, surprisingly few studies have investigated its biological activity.

It is known that a *p*-methoxycinnamic acid isolated from type A wax has hypoglycemic, antioxidant, and hypolipidemic properties (Filho et al., 2017; Freitas et al., 2016; Rodrigues et al., 2014). Triterpenoids, crude hexane, and ethanol extracts of carnauba wax have also shown antiprotozoal activity against intracellular amastigotes of *Leishmania infantum* and trypomastigote forms of *Trypanosoma cruzi* (Almeida et al., 2016). Furthermore, chitinase and a β-1,3-glucanase isolated from type B wax, have shown antifungal activity against phytopathogenic fungi (Cruz et al., 2002).

However, to the best of our knowledge, polar compounds found in the ethanol or water extracts of carnauba wax powder have not been analyzed for potential *in vitro* free radical scavenging or antifungal activities against dermatophyte fungi.

Therefore, the goal of this study was to quantify the antioxidant potential and the total phenol, flavonoid, and flavanol content of the aqueous and ethanolic extracts of carnauba wax powder, and to evaluate whether these extracts possess antifungal activity again *Candida albicans* and the dermatophyte fungi *Trichophyton rubrum* and *Microsporum canis*.

2. Materials and methods

2.1. Chemical compounds

2,2-Diphenyl-1-picryl hydrazyl (DPPH•), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade phenolic standards, gallic acid, catechin, chlorogenic acid, ferulic acid, p-coumaric acid, rutin, ellagic acid, trans-cinnamic acid, quercetin, kaempferol, myricetin, naringin, and hesperidin were purchased from Sigma-Aldrich Co.

2.2. Plant material

Carnauba wax powder (type A and B) were kindly provided by producers in the town of Granja, state of Ceará, northeastern region, Brazil. The wax powder came from the harvest of 2016. All material was free of dirt. In this study, we designated the type A and B wax powders as tyA and tyB, respectively.

2.3. Fungal strains

Strains of *Microsporum canis* (LABMIC 0301), *Trichophyton rubrum* (LABMIC 0101), and *Candida albicans* (LABMIC 0201 and 0202) were stored in the fungal collection of the Laboratório de Microbiologia-LABMIC (Universidade Estadual Vale do Acaraú, Ceará, Brazil), where they were maintained in saline (0.9% NaCl) at 28 °C. For the analysis, an aliquot of each suspension was collected and inoculated into potato dextrose agar (Difco, Detroit, USA) and then incubated at 28 °C for 2–10 days.

2.4. Crude extracts

The tyA and tyB wax were passed through a 35 mesh sieve to obtain a uniform particle size and remove possible leaf residues. Then, 10 g each of tyA and tyB was placed in separate containers, and mixed with distilled water or ethanol (99% pure) at a proportion of 1:20 (w/v). To determine the effect of the extraction time on the antioxidant activity of extracts, the mixture was agitated for different times (2, 4, 8, and 12 h), at 25 °C. Then, the extracts were filtered through filter paper and centrifuged at 6000 × *g* for 10 min to obtain a clear crude extract, which was used directly for estimation of antioxidant activity via DPPH assay. All experiments were performed in triplicate and the results are expressed as mean ± standard deviation (SD). The statistical analyses were performed using a one-way analysis of variance (ANOVA) and a $P < 0.05$ was considered significant.

After identifying the optimal extraction time, the ethanolic crude extract was concentrated in a rotary vacuum evaporator at 50 °C and lyophilized. The aqueous crude extracts were lyophilized without prior evaporation, the dried extracts were weighed to calculate the extract yield, expressed as a percentage (%), and then stored at 4 °C for subsequent analysis. The lyophilized extracts were named AqA (aqueous extract of tyA), AqB (aqueous extract of tyB), EtA (ethanolic extract of tyA), and EtB (ethanolic extract of tyB). The extraction processes and all sample analyses were conducted in triplicate.

2.5. Antioxidant activity

2.5.1. DPPH free radical scavenging

The DPPH radical scavenging activity was evaluated using the method described by Brand-Wiliams et al. (1995) with minor modifications. Initially, the crude extract (0.1 mL) was mixed with 2.4 mL of 100 mM DPPH dissolved in ethanol. The reaction mixture was mixed thoroughly and incubated for 30 min in the dark. A control containing all reagents without the sample was used for comparison. DPPH radical scavenging activity was then determined by measuring the absorbance at 515 nm using an ultraviolet-visible (UV-VIS) spectrophotometer. The decrease in absorbance wasconverted to percentage radical scavenging antioxidant activity (Eq. (1)).

$$\text{DPPH}^{\cdot}\text{radical scavenging (\%)} = [A_{DPPH} - (A_S - A_{SO})]/(A_{DPPH})] \times 100 \quad (1)$$

Where A_{DPPH}, A_S, and A_{SO} are the absorbance values of the DPPH solution with ethanol or water instead of sample, with the sample; and the absorbance of the sample with ethanol instead of DPPH solution, respectively.

To express the values in terms of the concentration of extract (μg/mL) required to scavenge 50% of the DPPH radicals (EC_{50}), the freeze-dried extracts (AqA, AqB, EtA, and EtB) were dissolved in water or ethanol to concentrations of 100–2000 μg/mL and subjected to a DPPH assay as described above. The EC_{50} value was calculated using a linear equation based on the extract concentrations and the respective percentages of radical DPPH sequestration (Eq. (2)). Antioxidative capacities of the extracts were compared with those of Trolox (5–100 μg/mL), quercetin (20–150 μg/mL), and gallic acid (10–100 μg/mL). The