Industrial Crops & Products 125 (2018) 220–227

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Antioxidant and antifungal activity of carnauba wax powder extracts

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ARTICLE INFO

Keywords:
Arecaceae
Dermatophytosis
Phenolic acid
Flavonoid

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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https://doi.org/10.1016/j.indcrop.2018.09.004
Received 30 May 2018; Received in revised form 28 August 2018; Accepted 4 September 2018
Available online 10 September 2018
0926-6690/

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

results are expressed as the means ± SD of triplicate experiments.

$$\% \text{ scavenging} = 100 - [(A_S \times 100)/A_{DPPH}] \quad (2)$$

2.5.2. ABTS radical scavenging

$ABTS^+$ radical scavenging activity was evaluated using the method described by Re et al. (1999). $ABTS^+$ radical cation was produced by reacting 7 mM of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt with 2.45 mM potassium persulfate followed by incubation at 25 °C in dark for 16 h. The ABTS solution was then diluted with ethanol to an absorbance of 0.70 ± 0.5 at 734 nm. Then, 20 µL of samples of the extracts at concentrations of 100–2000 µg/mL was mixed with 1.980 mL ABTS solution. The reaction mixture was mixed thoroughly and incubated for 6 min in the dark. ABTS solution without sample was used as blank. ABTS radical scavenging activity was determined by measuring the absorbance at 734 nm using a spectrophotometer. Trolox, quercetin, and gallic acid were used as standards. The results were expressed as the EC_{50}, as described previously for the DPPH assay. Each measurement was performed in triplicate. The results are given as a mean ± standard deviation (SD) of the experiments.

2.6. Phytochemical constituents

2.6.1. Qualitative detection of phytochemical constituents

The active phytochemical constituents of all the extracts were detected using the protocols described by Mattos (2009).

2.6.2. Quantification of total phenolic content

Total phenolic contents of the extracts were determined using the Folin-Ciocalteu phenol reagent method described by Kim et al. (2006), with minor modifications. Briefly, 0.2 mL of each extract (0.25 mg/mL) was placed in a test tube and 1 mL of Folin-Ciocalteu phenol reagent (10% v/v in distilled water) was added. Then, after 2 min, 0.8 mL of 7.5% sodium carbonate was added to each tube and the mixtures were mixed using a vortex. Each test tube was then kept in a water bath at 37 °C, in the dark, for 30 min. After cooling for 10 min, the absorbance spectra were recorded at 765 nm. The test was performed in triplicate and the total phenolic content was calculated using the calibration curve ($y = 0.013x + 0.0664$; coefficient of determination $[R^2] = 0.9988$) using gallic acid (5–100 µg/mL) as a standard, and the results were expressed as milligram of gallic acid equivalent per gram (mg GAE/g) of the lyophilized extract.

2.6.3. Quantification of total flavonoid content

The total content of flavonoids was analyzed using the aluminum chloride colorimetric method (Meda et al., 2005), with minor modifications. Briefly, 1 mL of 2% aluminum trichloride in methanol was mixed with the same volume of extract (0.5 mg/mL). After 20 min, an absorption reading was taken at 415 nm against a blank sample without aluminum trichloride using a UV-VIS spectrophotometer. The total flavonoid content was calculated from the calibration curve ($y = 0.0359x + 0.0179$; $R^2 = 0.9999$), using quercetin methanolic solution as the standard (2.5–40 µg/mL) and expressed as milligram of quercetin equivalents per gram (QE/g) of the lyophilized extract.

2.6.4. Quantification of flavanols

Flavanol content was measured using the *p*-dimethylaminocinnamaldehyde (DMACA) assay, as previously described (Arnous et al., 2002). Briefly, to 0.2 mL of sample (0.25 mg/mL) was added 1.0 mL of DMACA solution (0.1% in 1 N HCl in ethanol). The mixture was vortexed and allowed to react at 25 °C for 20 min. Then, the absorbance at 640 nm was read against a blank prepared similarly without DMACA and the flavonol concentration was estimated from a calibration curve ($y = 0.0558x + 0.0214$; $R^2 = 0.9987$), constructed by plotting known solutions of catechin (1–16 µg/mL). The amount of total flavanol was expressed as milligram catechin equivalent per gram (CTE/g) of lyophilized extract.

2.6.5. HPLC analysis

Phenolic compounds were identified using an HPLC system (Knauer Smartline, Berlin, Germany) equipped with a quaternary pump and a diode array detector (DAD). A 250 mm × 4.6 mm C18 analytical column (Knauer) with a 5 µm particle diameter was used in this study. The mobile phase consisted of 0.1% phosphoric acid (H_3PO_4) aqueous solution (solution A) and HPLC-grade methanol (solution B) run at a flow rate of 1 mL/min, and the oven temperature was set to 30 °C. The injection volume was 50 µL and the elution was carried out in gradient mode as follows, 0–5 min, 20–27% B; 5–18 min, 27–47% B; 18–24 min, 47–61% B; 24–25 min, 61–100% B; 29 min, 20% B until the end of the analysis (35 min). The freeze-dried aqueous and ethanolic extracts (5 mg/mL) were dissolved separately in a solution of 20% aqueous HPLC grade methanol, and filtered through a 0.22 µm nylon membrane filter. The chromatogram profiles of the extracts were acquired at a wavelength of 280 nm. For phenolic compound identification, 13 standard phenolic compounds (gallic acid, catechin, chlorogenic acid, ferulic acid, *p*-coumaric acid, rutin, ellagic acid, trans-cinnamic acid, quercetin, kaempferol, myricetin, naringin, and hesperidin) were dissolved in methanol and analyzed under the same conditions described above.

2.7. Antifungal activity

2.7.1. Inoculum preparation for antifungal susceptibility tests

The inoculum was prepared from strains grown in Sabouraud agar for 5 days at 35 °C and yeast strains grown for 35 h at 48 °C. The standardized inocula of *C. albicans* (2.5–5 × 10^3 CFU/mL), *M. canis*, and *T. rubrum* (5 × 10^4 CFU/mL) were also prepared using turbimetry. Sterile saline solution (0.9% NaCl) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *M. canis* and *T. rubrum* and *C. albicans*. The conidal suspensions of the hyphal fragment of *M. canis* and *T. rubrum* and the blastoconidia suspension of *Candida* were transferred to sterile tubes, the volumes were adjusted to 4 mL, set aside for 5 min at 28 °C, and the density of each sample was read at 530 nm, followed by adjustment to 95% transmittance. The suspensions were diluted with RPMI 1640 medium with L-glutamine without sodium bicarbonate (Sigma-Aldrich) to ratios of 1:2000 for *C. albicans* and 1:500 for *M. canis* and *T. rubrum*, and buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS, Sigma-Aldrich), to obtain inoculum concentrations of approximately 2.5–5 × 10^3 CFU/mL and 5 × 10^4 CFU/mL, respectively.

2.7.2. Broth microdilution method

Because of difficulty in solubilizing EtA and EtB samples in saline solution and 5% DMSO, the antifungal activity was examined only for the aqueous extracts. To this end, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of AqA and AqB against the dermatophyte and yeast strains were determined using the broth microdilution method using 96-well plates according to the Clinical and Laboratory Standards Institute (CLSI) M27A2 standard (NCCLS M27A, 2002).

The compounds were prepared in sterile saline solution at a concentration of 40 mg/mL, diluted in 100 µL RPMI 1640 medium, and tested at a concentration range of 0.156–10 mg/mL. Then, 100 µL inoculum was added to the wells to a final volume of 200 µL/well. The antifungal agents, ketoconazole for dermatophytic strains and amphotericin B for yeast strains, were used as controls at concentrations of 0.05–16 µg/mL. The microplates were incubated at 37 °C and read visually after 5 days. The MIC was defined as 100% inhibition of the fungi while the MFC was determined after transferring 100 µL of the contents of the well without turbidity into tubes containing potato agar at 28 °C.

The MFC against dermatophytes was calculated in accordance with fungal growth in the culture medium after 5 days whereas that of the yeasts was determined after 24 h. Each experiment was run in duplicate.

3. Results and discussion

The cuticular waxes in the leaves, flowers, and fruits of terrestrial plants are secreted to protect them against biotic and abiotic stresses (Oliveira et al., 2003; Shepherd and Griffiths, 2006). Thus, in addition to the physical barrier, waxes can be sources of bioactive compounds with diverse biological activities. In this study, we quantified the antioxidant potential of aqueous and ethanolic extracts of carnauba wax powder and revealed antifungal activity of the aqueous extracts. Discovering new and safe antioxidants from natural sources is of great interest for use as natural antioxidants, functional foods, and nutraceuticals (Diem et al., 2014). Furthermore, extraction is one of the key steps in the investigation of antioxidants (Fan et al., 2015). Various factors influence the extraction process such as solvent polarities, extraction time, and temperature (Metrouh-Amira et al., 2015).

The effect of extraction time on the antioxidant activity of aqueous and ethanolic carnauba wax powder extracts was investigated. It is noteworthy that most previous studies on carnauba wax extracts and isolated compounds used apolar solvents (Almeida et al., 2016, 2017; Freitas et al., 2016), which differs from the current study that focused on aqueous and ethanolic extracts. Water and ethanol, two solvents considered GRAS (Generally-Recognized-As-Safe), were used as the extraction solvents in this study due to their low cost, good safety profile, and acceptability for human consumption (Rodriguez-Rojo et al., 2012).

The results showed that the extraction time (2, 4, 8, or 12 h) did not influence the antioxidant activity of the extracts in the DPPH assay ($p > 0.05$), and 2 h was sufficient to achieve the maximum antioxidant activity using water or ethanol as solvents (Fig. 1). The reduction in extraction time is important, since some studies have reported that prolonged extraction can lead to oxidation or enzymatic degradation of metabolites in the crude extract, causing the loss of bioactive properties (Karling et al., 2017; Mokrani and Madani, 2016). Thus, a reduction in extraction time could be advantageous in preserving the chemical stability of the extracted compounds, in addition to time and cost savings.

For extraction efficiency, ethanol was the most effective extraction solvent for both tyA and tyB wax powders (Table 1). This may be attributable to the higher solubility of the compounds in the wax powder in ethanol than in water. Indeed, solvents with different polarities have varying solubilities properties for particular groups of compounds, which may influence the metabolite extraction yield (Diem et al., 2014; Machado et al., 2013).

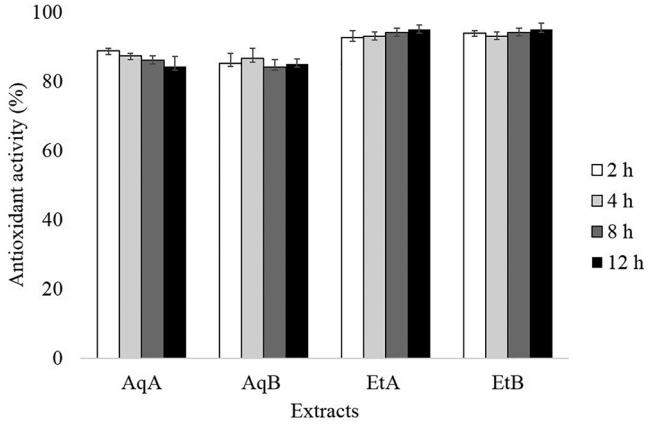

Fig. 1. Effect of extraction time on antioxidant capacity of aqueous and ethanolic extracts of carnauba wax powder.

Table 1
Yield of ethanol and water solvent extracts of carnauba wax powder.

Extract	Yield (%)[a]
AqA	2.4 ± 0.43
AqB	3.5 ± 0.72
EtA	17.9 ± 1.1
EtB	24.2 ± 3.3

[a] Results are means ± standard deviation (SD) of triplicate experiments. AqA and AqB, aqueous extracts of type A and B wax powders (tyA and tyB), respectively; EtA and EtB, ethanol extracts of tyA and tyB, respectively.

Table 2
Antioxidant activities (EC_{50}) of aqueous and ethanolic extracts of carnauba wax powder assessed using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical assays.

Samples	DPPH EC_{50} (μg/mL)	ABTS$^{\bullet+}$ EC_{50} (μg/mL)
AqA	665 ± 28	610 ± 18
EtA	942 ± 54	900 ± 17
AqB	450 ± 26	355 ± 7
EtB	365 ± 7	317 ± 6
Gallic acid	23 ± 2	16 ± 2
Quercetin	46 ± 4	30 ± 2
Trolox	39 ± 2	28 ± 3

EC_{50}, concentration of extract or standard (μg/mL) required to scavenging 50% of DPPH or ABTS radicals.

The antioxidant potentials of the aqueous and ethanolic carnauba wax extracts were quantified as the concentration (μg/mL) required to scavenge 50% of DPPH or ABTS radicals (EC_{50}) in the respective assays (Table 2). These two methods have been widely used in the determination of antioxidant activities of plant and food components because they are quick, sensitive, and simple to perform (Moon and Shibamoto, 2009).

In general, the extracts of tyB wax possessed higher antioxidant activity than the extracts of tyA. For tyA, the water extract possessed greater antioxidant activity than the ethanol extract based on the lower EC_{50} value of the water extract. In contrast, the tyB ethanol extract possessed greater antioxidant activity than the aqueous extract. The antioxidant activity of all analyzed extracts showed similar patterns when measured using the DPPH and ABTS^{+} methods. The antioxidant activity of extracts (EC_{50}) was in following increasing order of magnitude: EtB > AqB > AqA > EtA. In all cases, the scavenging effects of the extracts were concentration-dependent, especially at concentrations < 1 mg/mL (Figs. 2 and 3).

The higher antioxidant activity of wax secreted by mature leaves than that of wax secreted by young leaves indicated that the bioactive compounds of carnauba wax are modified during plant development. The effects of the maturation stage on plant organs and of environmental conditions on the chemical composition and antioxidant activity have already been reported (Petropoulos et al., 2018; Salem et al., 2011).

Antioxidant activity of plant extracts can be attributed to secondary metabolites such as flavonoids and other plant phenolics (Soobrattee et al., 2005). The antioxidant activity of phenolics is mainly attributable to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Carocho and Ferreira, 2013). Polar solvents such as ethanol or aqueous mixtures containing ethanol are frequently used for the recovery of phenol compounds from plant tissue (Dai and Mumper, 2010). Thus, phytochemical screening is one of the methods frequently used to explore antioxidant compounds in plants. The phytochemical screening of carnauba wax powder extracts revealed the presence of different

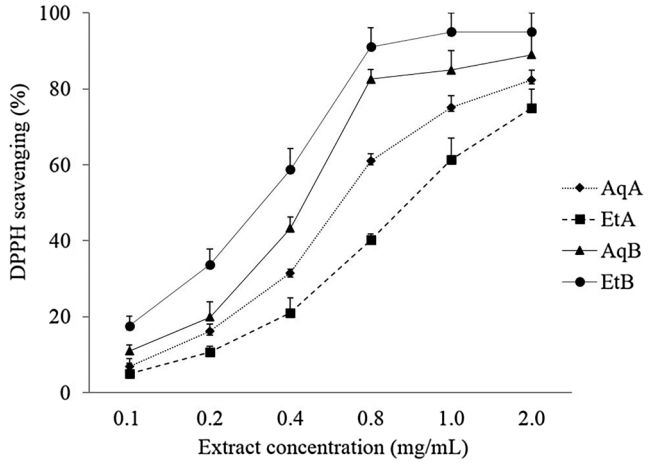

Fig. 2. Scavenging effect of different carnauba wax powder extract concentrations on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

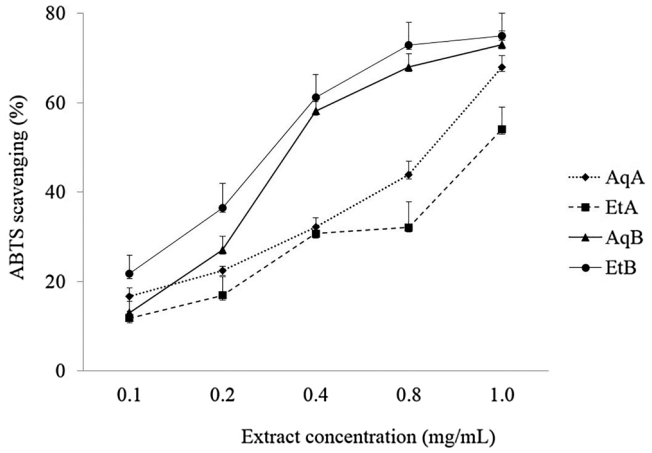

Fig. 3. Scavenging effect of different carnauba wax powder extract concentrations on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical.

Table 3
Preliminary phytochemical screening of carnauba wax extracts.

Phytochemical Constituents	Extracts			
	AqA	EtA	AqB	EtB
Phenols and tannins	+	+ +	+ +	+ + +
Total flavonoids	+ +	+	+ +	+ + +
Flavones, flavanols, xanthones, flavanones	+ +	+	+ +	+ + +
Saponins	+ +	–	+	–

(-) not detected; (+) low presence; (+ +) moderate presence; (+ + +) strong presence. AqA and EtA, aqueous and ethanolic extracts of type A wax, respectively. AqB and EtB, aqueous and ethanolic extracts from type B wax, respectively.

chemical groups (Table 3).

The chemical composition of epicuticular waxes varies greatly among different plant species, organs of the same plant, developmental stage, and environmental factors (Borisjuk et al., 2014; Mackova et al., 2013). Oliveira et al. (2003) evaluated the effects of varying contents and chemical compositions of the foliar epicuticular waxes of species of the Caatinga and Cerrado and showed that the waxes of the Caatinga species were generally more effective against water loss than those of the Cerrado species. Moreover, the chemistry of the wax constituents was shown to be an important factor in determining the degree of resistance to evaporation.

Table 4
Total phenolic, flavonoid, and flavanol contents in carnauba wax powder extracts.

Extracts	Total phenolic (GAE)/g extract	Flavonoids (QE)/g extract	Flavanols (CTE)/g extract
AqA	116.89 ± 3.69	16.39 ± 1.18	12.66 ± 0.73
EtA	114.06 ± 4.45	5.34 ± 0.12	9.41 ± 0.37
AqB	191.75 ± 2.55	18.49 ± 0.38	21.1 ± 1.39
EtB	280.73 ± 4.85	24.59 ± 0.45	32.36 ± 0.93

GAE/g, gallic acid equivalent per gram of lyophilized extract; QE/g, quercetin equivalent per gram of lyophilized extract; CTE/g, catechin equivalent per gram of lyophilized extract.

Among the phytochemicals quantified in this study, the amount of total phenolics varied widely in the extracts, ranging from 114.06 ± 4.45 mg GAE/g of lyophilized extract in EtA to 280.73 ± 4.85 mg GAE/g of lyophilized extract in EtB (Table 4). The EtB and EtA extracts contained the greatest and least amount of flavonoids and flavanols, respectively. In several plant species, phenolic compounds including flavonoids, have been reported in wax composition (Kranjc et al., 2016; Tomasi et al., 2017). For example, four flavones were isolated from the wax of the leaves of *Arrabidaea brachypoda*, a bignoniaceous vine shrub native to the Brazilian Cerrado (Alcerito et al., 2002), and these compounds have been implicated in protecting the plant against pathogens and UV radiation (Kunst and Samuels, 2003).

Unlike the extraction yield pattern observed where the ethanol produced a higher yield, the total phenol, flavonoid, and flavanol contents were not dependent on the polarity of the solvent. However, as observed for the antioxidant activity, tyB wax possessed higher levels of phenolic compounds, flavonoids, and flavanols than tyA wax extracts did. Some studies have demonstrated a positive linear correlation between the total phenolic content and the antioxidant capacity of plant extracts (Vasco et al., 2008; Zugic et al., 2014). The results of our studies showed a higher antioxidant activity in the extracts with higher levels of phenols, flavonoids, and flavanols.

The highest levels of flavonoids and flavanols were observed in the tyB wax extracts. These phytochemicals are related to plant UV protection and response against infection (Ferreyra et al., 2012). In this case, the fact that tyB wax is obtained from fully expanded and mature leaves that are more exposed to environmental conditions and the extreme UV radiation in the Brazilian semiarid region, probably explains the greater production of protective compounds.

HPLC analysis was used to identify the secondary metabolites in tyA and tyB wax extracts by comparing their retention times (Rt) with those of standard chromatograms (Fig. 4a). The resultant chromatographic analysis of AqA extract (Fig. 4b) detected peaks for the phenolic compounds gallic acid (Rt: 5.22 min), catechin (Rt: 9.61 min), and chlorogenic acid (Rt: 11.32 min), while in the AqB extract, only catechin (Rt: 9.43 min) and chlorogenic acid (11.22 min) could be detected (Fig. 4c). In both chromatograms, chlorogenic acid was the predominant compound. Trace amounts of gallic acid, a hydroxybenzoic acid widely distributed in plants and found in abundance in skin and grape seeds (Yilmaz and Toledo, 2004), were detected in AqA. Gallic acid has also been identified in wax derived from *Eucalyptus globulus* fruits (Pereira et al., 2005).

a: standards; b: aqueous extracts of carnauba type A wax; c: aqueous extracts of carnauba type B wax. Standard peak identification: 1, gallic acid (retention time [Rt]: 5.15 min); 2, catechin (Rt: 9.55 min); 3, chlorogenic acid (Rt: 11.18 min); 4, *p*-coumaric acid (Rt: 16.84 min); 5, ferulic acid (Rt: 18.04 min); 6, naringin (Rt: 20.64 min); 7, hesperidin (Rt: 20.98 min); 8, rutin (Rt: 23.21 min); 9, ellagic acid (Rt: 23.99); 10, kaempferol (Rt: 24.29); 11, trans-cinnamic acid (Rt: 25.18 min); 12, quercetin (Rt: 27.26 min); 13, myricetin (Rt: 29.07).

Catechins are flavanols found in abundance in the leaves of green

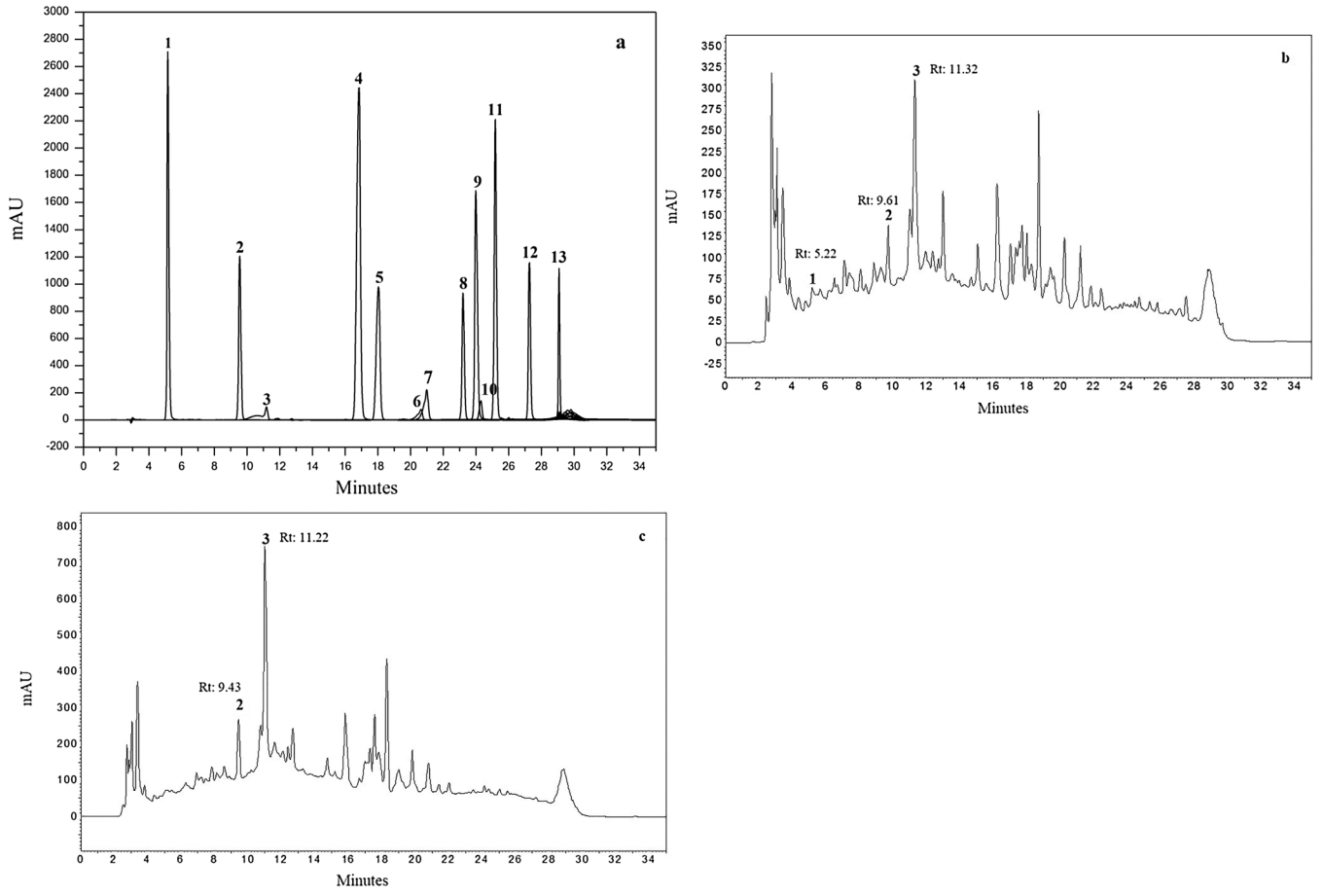

Fig. 4. High-performance liquid chromatography (HPLC) chromatogram of phenolics compounds.

tea, apples, and grape fruits (Rasouli et al., 2017), whereas chlorogenic acid is a hydroxycinnamic acid found in coffee grains and apples (Awad et al., 2000; Monteiro et al., 2007). Studies have demonstrated the antioxidant and antimicrobial potential of gallic acid, catechins, and chlorogenic acid (Martins et al., 2015; Sung and Lee, 2010). While bioactive compounds have been identified and isolated from carnauba wax (Almeida et al., 2016; Filho et al., 2017; Harron et al., 2017), to the best of our knowledge, this is the first report of the presence of gallic acid, catechins, and chlorogenic acid in carnauba wax aqueous extracts.

In both chromatograms, several compounds were eluted after 12 min but were not identified. It was not possible to identify any phenolic compound that appeared in the HPLC results of the ethanolic extracts (data not shown) by comparison with standard profiles using the same analytical conditions.

In this study, AqA and AqB showed antifungal activity against *M. canis* and *T. rubrum*. Holetz et al. (2002) classified the antimicrobial efficacy of plant extracts as good (MIC < 100 µg/mL), moderate (MIC, 100–500 µg/mL), weak (MIC, 500–1000 µg/mL) and inactive (MIC > 1000 µg/mL). According to this classification, AqB showed moderate antifungal activity against *M. canis* and was inactive for *T. rubrum* and AqA had moderate antifungal activity against *M. canis* and *T. rubrum* (Table 5), whereas the extracts had no activity against *C. albicans*. An important focus of modern medicine is the search for novel natural antimicrobial compounds to combat infectious diseases, which present an increasing threat because of increasing bacterial and fungal resistances to antibiotics (Savoia, 2012). Antimicrobial activity of leaf and fruit wax extracts and their constituents have been reported (Alcerito et al., 2002; Johann et al., 2007; Yin et al., 2011) and several groups have demonstrated that the antimicrobial effects of vegetable extracts are due to the presence of flavonoids in their composition (Cushnie and Lamb, 2005).

Studies conducted by Alcerito et al. (2002) showed that flavonoids (3′,4′-dihydroxy-5,6,7-trimethoxyflavone, cirsiliol, cirsimaritin, hispidulin) from the epicuticular wax of the leaves of *Arrabidaea brachypoda* possess antifungal activity against *Cladosporium sphaerospermum*. Further, wax extract and two flavonoids (tangeretin and nobiletin), isolated from citrus peel showed antifungal activity against *Trichophyton mentagrophytes* and *Microsporum canis* (Johann et al., 2007). Compounds isolated from carnauba leaf wax have shown antiprotozoal and antifungal activity (Almeida et al., 2016; Cruz et al., 2002), however, there are no reports in the literature of antifungal activities of the carnauba wax extracts against dermatophytes responsible for skin diseases in humans and several species of domestic animals, especially dogs and cats (Pinheiro et al., 2009).

In conclusion, the results of the present study showed that carnauba wax extracts may be potential sources of natural antioxidant and antifungal agents. Gallic acid, catechin, and chlorogenic acid were reported, for the first time, to be constituents of carnauba wax. The presence of phenolic compounds and flavonoids may be responsible for the antifungal effect of the extracts. However, considering that the studies were performed with crude extracts, it is difficult to identify the compounds exerting the antifungal activity. In this case, only the isolation of constituents in the extracts would enable the elucidation of the antifungal compound. The present results provide a basis for further investigations, particularly of the use of these antioxidants and antifungal compounds. Our study contributes information about the bioactive potential of the compounds present in carnauba wax.

Table 5
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of carnauba wax powder aqueous extracts from type A (AqA) and type B (AqB) wax against *Candida albicans*, *Microsporum canis* and *Trichophyton rubrum*.

Strains	AqA		AqB		Drugs (μg/mL)			
	MIC[a]	MFC[b]	MIC[a]	MFC[b]	amphotericin B		Ketoconazole	
					MIC	MFC	MIC	MFC
Candida albicans								
LABMIC 0201	NI	NI	NI	NI	8.0	16	–	–
LABMIC 0202	NI	NI	NI	NI	1.0	2.0	–	–
Microsporum canis								
LABMIC 0301	310	620	150	310	–	–	4.0	8.0
Trichophyton rubrum								
LABMIC 0101	310	620	2.500	5.000	–	–	4.0	8.0

[a] MIC: minimum inhibitory concentration.
[b] MFC: minimum fungicidal concentration (μg/mL); NI: no inhibition.

Conflict of interests

The authors declare that there are no conflicts of interest.

Fundings

This research study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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