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Carnauba wax *p*-methoxycinnamic diesters: Characterisation, antioxidant activity and simulated gastrointestinal digestion followed by *in vitro* bioaccessibility

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ABSTRACT

The beneficial biological effects of cinnamic acid derivatives and the lack of studies on the antioxidant activity and bioavailability of cinnamic esters from carnauba wax, diesters were extracted from carnauba wax powder. Their structural, physical and morphological characteristics, antioxidant activity and *in vitro* bioaccessibility were measured. *p*-Methoxycinnamic diester (PCO-C) was identified, which has a crystalline, apolar structure and exhibited significant antioxidant activity (107.27 ± 3.92 μM Trolox/g of dry weight) before and after simulated *in vitro* gastrointestinal digestion and 32.46% bioaccessibility. In human cells, PCO-C (250 μg/mL) inhibited the production of intracellular reactive oxygen species, with an effect similar to that of Trolox (80 μM). Thermogravimetric analysis showed that PCO-C had high thermal stability and high UV absorption between 250 and 350 nm. These results indicate that this compound is promising as an antioxidant for pharmaceutical and food industry applications, such as the development of active packaging and functional foods.

1. Introduction

Cinnamic acid and its derivatives are phenols that contain phenyl-3-propenoic acid and differ from each other by their aromatic ring substituents (Sharma, 2011). These phenols occur naturally in plants and have low toxicity (Sova, 2012). They are also precursors for the synthesis of commercially important cinnamic esters that are obtained from various plant sources and used for perfumery, cosmetic and pharmaceutical products (Sharma, 2011).

Several studies have supported cinnamic acid and its derivatives as pharmacologically active substances due to their wide range of biological properties, including antineoplastic (Sova et al., 2013), antibacterial (Rajitha, Nagalakshmi, Devi, & Praneetha, 2011), antioxidant (Kiliç & Yesiloglu, 2013; Li et al., 2014; Marques, Batista de Carvalho, Valero, Machado, & Parker, 2014), antiradical (Avanesyan, Pashkov, Simonyan, Simonyan, & Myachina, 2009), antihyperglycaemic (Yoo, Lee, Lo, & Moon, 2012), antiatherogenic (Lee et al., 2004), and hypocholesterolaemic activities (Yoo et al., 2012).

Thus, cinnamic acid esters have a wide range of biological effects. Several studies have shown that they have a peculiar antioxidant activity (Jakovetic et al., 2013; Menezes et al., 2011). To analyse the effect of esterification on antioxidant activity, Jakovetic et al. (2013) synthesised aliphatic esters from cinnamic acid and found that the antioxidant potential of the esters was greater than that of cinnamic acid. Holser et al. (2008) and Venkateswarlu, Ramachandra, Krishnaraju, Trimurtulu, and Subbaraju (2006) reported that cinnamic acid esters have a protective effect against ultraviolet light and an antibacterial effect, respectively. Qian et al. (2010) developed a novel ester derived from cinnamic acid and metronidazole with powerful inhibitory effects on tumour growth, i.e., a potential anticancer agent.

Therefore, derivatives of cinnamic acid are important, promising compounds with high potential for developing

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pharmaceuticals (Sova, 2012) and for applications in the food industry (Kiliç & Yesiloglu, 2013).

To amplify and potentiate their biological activities, several derivatives of cinnamic acid have been synthesised and studied (Lee et al., 2004; Li et al., 2014; Rajitha et al., 2011). There are several methods for synthesising these compounds, but all have at least one drawback, such as low catalytic enzymatic activity, low yield, long reaction time, need for large quantities of solvents, use of tetrachloromethane (CCl_4) and handling of sodium metal (Sharma, 2011).

Thus, it becomes necessary to explore natural sources of cinnamic acid esters with effective biological activities that can be obtained by simple extraction processes with low environmental impact. In this context, carnauba wax is a possible source.

Carnauba wax, which is extracted from the leaves of the carnauba tree (*Copernicia prunifera* (Miller) H. E. Moore), a Brazilian palm, consists of a complex mixture of esters, free alcohols, aliphatic acids, aromatic acids, free ω-hydrocarboxylic acids, hydrocarbons (paraffins) and triterpene diols. Esters are the main component, corresponding to more than 80% of the composition, and mostly consist of aliphatic esters and cinnamic acid diesters (Wolfmeier et al., 2005).

Using carnauba wax powder ('pó olho' – when extracted from young leaves), Guedes et al. (2011) extracted *p*-methoxycinnamic diesters that the authors called PCO-C. After investigating the activity of these diesters on lipid metabolism in dyslipidaemic mice, they observed hypocholesterolaemic, hypolipidaemic and hypoglycaemic effects, which were equal to or sometimes greater than the effects of the reference drug, simvastatin. The authors suggest that this is a compound with great therapeutic potential for preventing and treating diseases linked to lipid and carbohydrate metabolism disorders.

Later, Rodrigues, Guedes, Marques, Da Silva, and Vieira (2014) studied the therapeutic potential of PCO-C for treating mice with alloxan-induced diabetes mellitus. According to the authors, PCO-C induced hypoglycaemic activity similar to or better than that induced by the reference drug (glibenclamide) when used at a dose of 100 or 150 mg/kg body weight.

Although these authors extracted *p*-methoxycinnamic diesters from carnauba wax powder and reported hypolipidaemic and hypoglycaemic effects, the chemical and physical characteristics of these esters have yet to be thoroughly investigated. Additionally, the mechanisms of action for *p*-methoxycinnamic diesters in treating dyslipidaemias and diabetes have not been determined. Thus, the characterisation of *p*-methoxycinnamic diesters derived from carnauba wax is essential to help uncover their mechanism of action and reveal their possible application in pharmaceuticals and foods.

Therefore, this study sought to present a process for extracting *p*-methoxycinnamic diesters from carnauba wax powder ('pó olho'), to define their structural characteristics and to determine, for the first time, their physical and morphological characteristics, antioxidant activity and *in vitro* bioaccessibility.

2. Materials and methods

2.1. Chemical compounds

Tripyridyltriazine (TPTZ), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), pancreatin, pepsin, bile extract, gallic acid and toluene were acquired from Sigma Aldrich (Saint Louis, USA). All other chemicals and reagents were of analytical grade.

2.2. Extraction of p-methoxycinnamic diesters from carnauba wax powder

Carnauba wax powder ('pó olho') was provided by Pontes Indústria de Cera Ltda. Ethyl acetate and hexane were added to 100 g of wax powder at a proportion of 3:7. The mixture was then agitated (20 min) and filtered through qualitative filter paper, and the filtrate was concentrated in a rotary vacuum evaporator, producing a yellowish solid called PCO-C. After drying, the PCO-C was weighed to calculate the extraction yield and was stored at room temperature for later analysis.

All the extracted PCO-C was frozen (−12 °C) for 10 min and then ground and homogenised. The homogenate was divided into three aliquots, which were used for the analyses described below.

2.3. Organic volatile impurities

Organic volatile impurities were measured by gas chromatography-mass spectrometry (GC–MS) using the methods described by European Pharmacopoeia (2005).

2.4. Infrared absorption spectroscopy

The infrared (IR) spectra of the compound were obtained using a VERTEX 70v (Bruker) Fourier transform infrared (FTIR) spectrometer under vacuum. Samples were placed on a diamond crystal, and the spectra were obtained by measuring the absorbance in the 600–4000 cm^{-1} range with a 4 cm^{-1} resolution in the attenuated total reflectance (ATR) mode.

2.5. Nuclear magnetic resonance (^1H NMR)

The ^1H NMR spectra were recorded at 500.13 MHz in an Avance DRX-500 Bruker Spectrometers® using a 5-mm dual probe. The tetramethylsilane (TMS) signal was used as an internal standard, and $CDCl_3$ was used as a solvent.

2.6. X-ray diffraction

Structural analysis was performed by X-ray diffraction using an X-ray diffractometer (D8 Advance, Bruker) with a 2θ range of 5–50° at a rate of 1/2°/min, CuKα radiation (λ = 1.54056 Å), a tube voltage of 40 kV and a tube current of 30 mA.

2.7. Differential scanning calorimetry (DSC)

PCO-C was analysed by DSC in a TA Instruments Q20 mode device, calibrated with an indium standard. Samples (10-mg masses) were analysed in duplicate using a heating range of 25–500 °C, a flow rate of 50 mL min^{-1} of N_2 and a heating rate of 10 °C min^{-1}.

2.8. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed using a TA Instruments Q50 model device with a heating range of 25–500 °C, a heating rate of 10 °C min^{-1}, and a flow rate of 100 mL min^{-1} of N_2.

2.9. Absorbance in the ultraviolet–visible (UV–Vis) range

Absorption spectroscopy in the UV–Vis range was performed at room temperature in a Hewlett–Packard, Diode-Array, model 8453 double beam spectrophotometer with wavelengths between 190 and 1100 nm using a quartz cuvette with a 1-cm optical path length. To perform this analysis, the PCO-C was first solubilised

in toluene at a concentration of 100 μg/mL (Villalobos-Hernández & Müller-Goymann, 2006).

2.10. Scanning electron microscopy (SEM)

The morphologies of the carnauba wax powder (before PCO-C extraction) and of the PCO-C surface were analysed by SEM. Samples of carnauba wax and PCO-C were mounted on "stubs", covered with a thin layer of platinum in an Emitech model K 550 sputter coater and then visualised under a Zeiss model DSM940A scanning electron microscope with an acceleration voltage of 15 kV.

2.11. Hydrolysis and thin layer chromatography (TLC)

For the hydrolysis, PCO-C (1.0 g) was saponified by heating under reflux with isopropanol (8 mL) and potassium hydroxide (0.2 g). After the hydrolysis was completed, the isopropanol was removed. Water was added to the heptane extraction residue, the pH was adjusted to 3, and the mixture was heated to 90–95 °C. After cooling, the waxy precipitate was filtered and washed with water. The filtrate and the washing water were combined, alkalised, concentrated in a rotary vacuum evaporator, acidified and extracted with ethyl ether. Concentration of the ether extract produced a resinous, dark, hard residue, which was recrystallised with ethyl ether and used for silica gel TLC (Vandenburg & Wilder, 1967).

2.12. In vitro simulation of gastrointestinal digestion

In vitro digestions were performed using simulated gastric and intestinal fluids prepared using the method described in Moura and Canniatti-Brazaca (2006). Simulation of gastrointestinal digestion was performed in two steps, reflecting the gastric and intestinal phases. First, 1 g of each PCO-C sample was added to 100 mL of 0.01 mol L^{-1} HCl, and the pH was adjusted to 2 with HCl (2 mol L^{-1}). Next, 3.2 mL of pepsin was added, and the mixture was agitated in a 37 °C water bath for 2 h to simulate digestion in the stomach. To simulate food digestion in the intestine, the sample was titrated with 0.5 mol L^{-1} NaOH until reaching pH 7.5 to simulate the intestinal pH. Then, dialysis membranes (33 × 21 mm, molecular weight: 12,000–16,000, porosity: 25 angstroms – Inlab, Brazil) containing 0.1 mol L^{-1} $NaHCO_3$ equivalent titratable acidity were added to the digesting samples and agitated in a 37 °C water bath for 30 min. Finally, 5.0 mL of bile salt solution and pancreatin were added and agitated in a 37 °C water bath for 2 h. The contents of the membranes (dialysates) were removed, and the samples were stored at −20 °C for later antioxidant activity analysis.

2.13. Cellular antioxidant activity assay

Oxidative stress was measured in the cells using 2′,7′-dichloro fluorescein diacetate (H_2DCFDA) as the fluorescent probe according to the methodology described by LeBel, Ischiropoulos, and Bondy (1992). Human peripheral blood lymphocytes (HPBLs) were obtained from the blood of healthy donors. Cells were washed, resuspended and cultured for 24 h in RPMI-1640 media supplemented with 20% foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2. Phytohaemagglutinin (2%) was added at the beginning of the culture period. Cell suspensions (0.1×10^6 cells/well) were distributed in 24-well plates, added to PCO-C (50 or 250 μg/mL), and incubated in the dark at 37 °C for 30 min with or without 150 μM hydrogen peroxide. In addition to the samples, 0.025% Tween 80, which was used to solubilise the PCO-C, was used as a negative control, and Trolox (80 μM) was used as a standard antioxidant. Next, 20 μM H_2DCFDA was added to the cells, and the plates were incubated for 30 more minutes. After incubation, the cells were centrifuged, washed, resuspended in PBS buffer and immediately analysed. The intensity of the dichlorofluorescein fluorescence was detected by flow cytometry using a Guava EasyCyte Mini (Guava Technologies, Inc., Hayward, CA, USA) and Guava Express Plus software. The intensity of the dichlorofluorescein fluorescence is proportional to the quantity of intracellular ROS formed. The data were expressed as the means ± standard deviation and analysed by one-way analysis of variance (ANOVA), followed by Tukey's test using GRAPHPAD software (Intuitive Software for Science, San Diego, CA, USA).

2.14. Antioxidant activity by ABTS free radical scavenging assay

Antioxidant activity was measured by the ABTS method according to the procedures described by Re et al. (1999), with modifications. ABTS radicals were generated using a reaction of 5 mL of aqueous ABTS solution (7 mM) and 88 μL of 140 mM L^{-1} potassium persulfate solution. The mixture was maintained at room temperature in the dark for 16 h and then diluted with phosphate-buffered saline (PBS) to an absorbance of 0.70 ± 0.05 at 734 nm. Standard solutions were prepared with the antioxidants Trolox from 100 to 1500 μM L^{-1} and gallic acid from 100 to 500 μM L^{-1} to be used as references.

PCO-C was added to Tween 80 (1:1 ratio) and solubilised in boiling water. A 30-μL aliquot of this apolar carnauba wax extract or the standard antioxidants was reacted with 3 mL of the ABTS radical solution (green–blue) in the dark. The decrease in absorbance at 734 nm was measured after six minutes. The results were expressed as the Trolox-equivalent antioxidant capacity (TEAC) in μM Trolox/g of sample and μM gallic acid/g of sample.

The antioxidant activity after digestion was quantified in the dialysate. The percent bioaccessible fraction was calculated in accordance with Briones-Labarca, Venegas-Cubillos, Ortiz-Portilla, Chacana-Ojeda, and Maureira (2011) (Eq. (1)).

$$\% \text{ Bioaccessible} = 100 \times (H/I)$$

H – antioxidant activity of the dialysate (μM g^{-1}), I – antioxidant activity of the sample (μM g^{-1}).

2.15. Antioxidant activity by ferric reducing antioxidant power (FRAP)

The antioxidant activity was measured by FRAP as published in Rufino et al. (2010), with modifications.

PCO-C was added to Tween 80 (1:1 ratio) and solubilised in boiling water. The method is based on direct measurement of antioxidant capacity through the reduction of the Fe^{3+}/TPTZ complex to Fe^{2+}. A standard curve was created by preparing standard solutions of iron sulphate at concentrations from 500 to 1500 μM. The absorbance was read at 595 nm, and the results were expressed in antioxidant capacity equivalent to μM $FeSO_4$/g of sample.

3. Results and discussion

A total of 63 PCO-C extractions from the raw carnauba wax powder ('pó olho') were conducted, with an average yield of 5.13%. This value is consistent with Vandenburg and Wilder (1967), who isolated aromatic acids (*para*-hydroxycinnamic acid and methoxycinnamic acid) from carnauba wax derived from 'pó olho' (type 1) and observed a yield of 4.4%. According to Wolfmeier et al. (2005), type 1 carnauba wax contains 7% *p*-methoxycinnamic diesters. PCO-C was analysed by infrared spectroscopy, ^{1}H NMR and UV–Vis and was confirmed to be

p-methoxycinnamic diester (Table 1), which is consistent with the work described by Vandenburg and Wilder (1967).

3.1. Organic volatile impurities

Organic volatile impurities were not detected in the analysed PCO-C samples.

3.2. Infrared absorption spectroscopy

Infrared absorption spectroscopy analysis of the PCO-C identified the presence of bands characteristic of ester (1738; 1717; 1169 cm^{-1}), unsaturated (1630, 930 cm^{-1}), *p*-substituted aromatic (830 cm^{-1}) and p-methoxy aromatic (1020 cm^{-1}) functional groups. This spectrum is very similar to the one obtained by Vandenburg and Wilder (1967) and is characteristic of *p*-methoxycinnamic diester.

3.3. Nuclear magnetic resonance (^1H NMR)

Analysis of the ^1H NMR spectrum and its extension confirmed that PCO-C is an ester of *p*-methoxycinnamic acid, based on the spectrometric data and structure described in Table 1.

3.4. X-ray diffraction

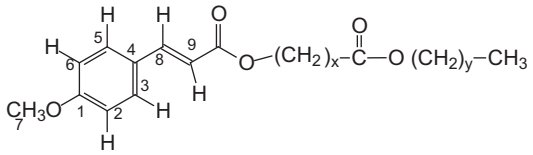
Fig. 1 presents the PCO-C diffractogram, showing a diffraction pattern typical of a crystalline structure with two strong narrow peaks (2θ between 20° and 25°) similar to those described by Villalobos-Hernández and Müller-Goymann (2006) when they analysed carnauba wax.

3.5. DSC

The thermogram in Fig. 2 shows that PCO-C has four endothermic peaks in the heat flow curve located at 66.88 °C and 73.51 °C due to fusion and at 312.46 °C and 446.66 °C due to sample decomposition.

Panteli, Saratsioti, Stamatis, and Voutsas (2010) showed fusion points of 62.56 and 88.64 °C for esters of methyl ferulate cinnamic acid and methyl sinapate, respectively.

Table 1
Chemical shift of *p*-methoxycinnamic acid diester (PCO-C), ^1H NMR spectra and structural diagram of *p*-methoxycinnamic acid diester, with corresponding numbering.



X +Y = 58 mean value

Chemical shift (ppm)	Assignments
7.62	8 (d, 1H, 16 Hz)
7.45	5, 3 (m, 2H)
6.85	6, 2 (m, 2H)
6.33	9 (d, 1H, 16 Hz)
3.83, 3.90	7 (s, 3H)

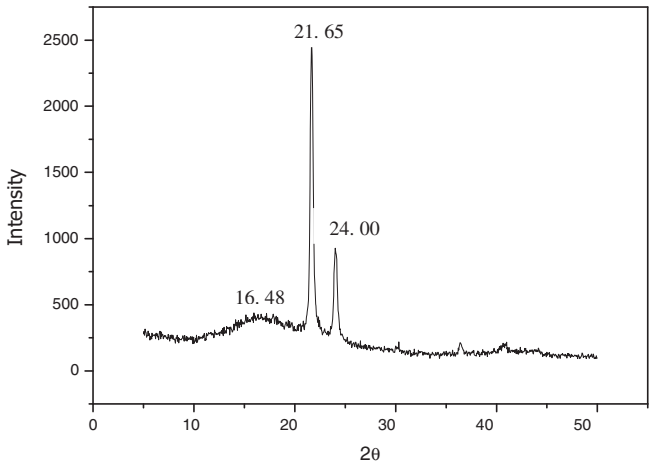


Fig. 1. PCO-C diffractogram.

The literature contains reports of endothermic peaks at approximately 83–90 °C for carnauba wax (Kheradmandnia, Vasheghani-Farahani, Nosrati, & Atyabi, 2010; Villalobos-Hernández & Müller-Goymann, 2006).

Fig. 2 shows the presence of a shoulder next to the main fusion peak. This phenomenon indicates the presence of more than one crystalline structure.

The DSC curve for PCO-C does not show an endothermic peak near 100 °C, indicating that there was no surface water.

3.6. TGA

The thermal stability of PCO-C was analysed by TGA. As seen in Fig. 3, the TGA curve for PCO-C shows that weight loss begins at temperatures around 200 °C.

TGA shows two maximal peaks between 200 and 500 °C, indicating that the largest amount of weight loss in PCO-C occurs between 333.81 and 389.49 °C, corresponding to the PCO-C decomposition process. The nearly complete thermal decomposition of PCO-C in this narrow temperature range demonstrates the purity of the sample.

These results are similar to those obtained by Milanovic et al. (2010), who analysed microcapsules of carnauba wax and found three peak temperatures, with the greatest weight loss at approximately 360 °C.

3.7. UV–Vis absorbance

Despite the large PCO-C dilution in toluene (100 µg/mL), this solution had high absorption values along the UV scanning range of 250–350 nm. This result was similar to that obtained by Villalobos-Hernández and Müller-Goymann (2006), who analysed the UV absorption of carnauba wax solubilised in toluene, and Holser et al. (2008), who recorded strong absorbance between 250 nm and 350 nm for the ester 4-methoxy cinnamoyl glycerol. This finding also confirms the potential of these esters as UV radiation filters. This high UV absorption by methoxy cinnamic esters was also found by Menezes et al. (2011), who recorded absorbances between 226 and 309 nm for *trans*-tetradecyl-3-(4-methoxyphenyl) propenoate. However, PCO-C did not exhibit absorbance in the visible range.

The ability of PCO-C to absorb UV radiation shows its value as a raw material for developing active food packaging. Active packaging is an innovative concept in which the packages that

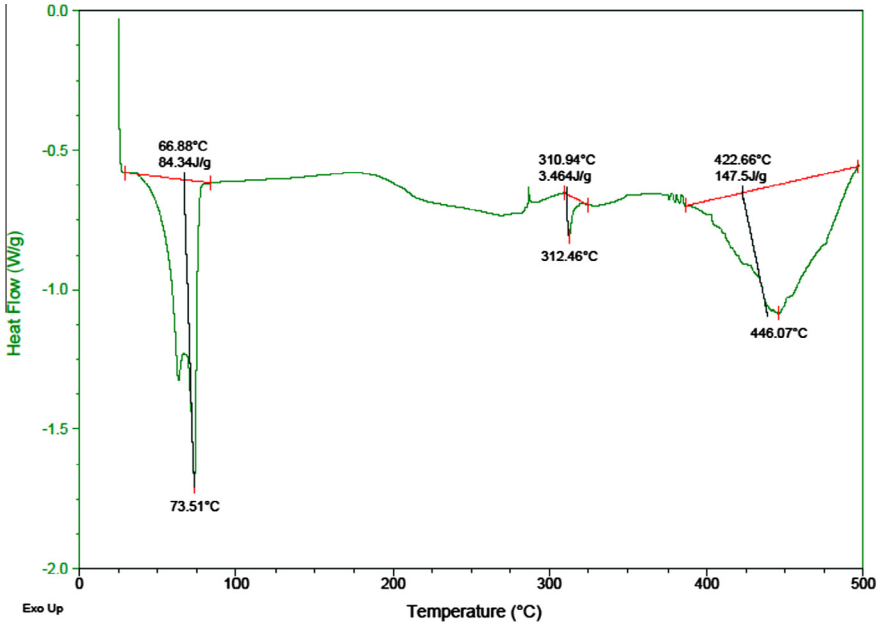


Fig. 2. Thermogram (DSC) for PCO-C.

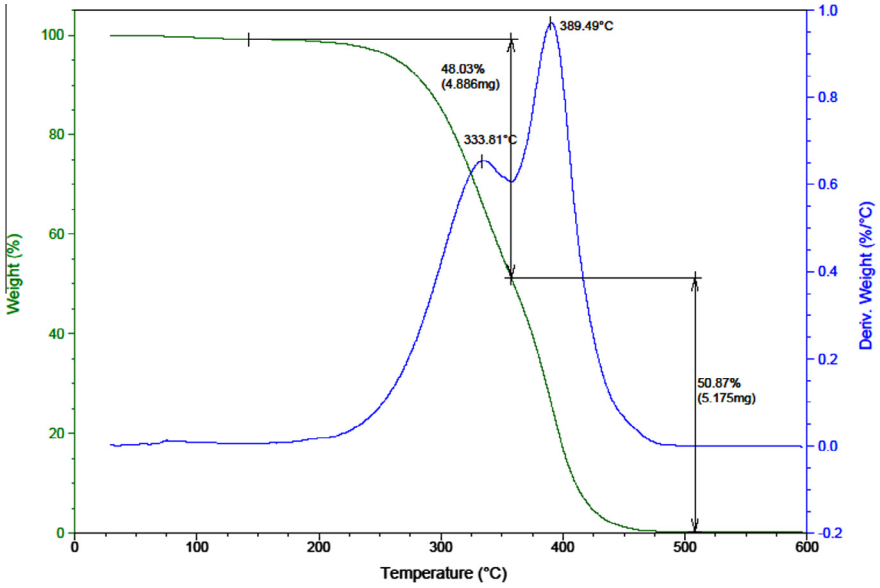


Fig. 3. Thermogram (TGA) for PCO-C.

will contain the food are incorporated with active substances, which are components that release or absorb substances and, thus, interact with the packaged foods or the surrounding environment to prolong shelf life and maintain product quality (Corradini et al., 2013).

Active substances with various mechanisms of action have been studied to confer antioxidant activity to active packaging systems, including substances that scavenge free radicals, scavenge oxygen, chelate metals, absorb ultraviolet (UV) light and suppress singlet oxygen (Tian, Decker, & Goddard, 2013).

Therefore, using PCO-C to absorb UV can potentially provide a light barrier in transparent food packages. This compound is also organically found in nature and, therefore, is completely biodegradable, which is an extremely desirable trait for the packaging industry.

3.8. Scanning electron microscopy (SEM)

Electromicrographs obtained by SEM of the carnauba wax powder samples (before PCO-C extraction) and of the pure PCO-C are shown in Fig. 4.

As shown in Fig. 4A, carnauba wax powder microscopically takes the form of wax sheets with different, randomly distributed sizes and shapes. In Fig. 4B, the cross section of a sheet of smooth wax is shown under 1000× magnification, with a thickness of approximately 6–7 μm. Its surface appears to be compact and non-porous, with an apparently well-organised structure.

Panels C and D in Fig. 4 show that extracted PCO-C is compact, rough and non-porous. At higher magnifications, the presence of small spike-shaped structures suggests the frequent occurrence of polarity in the components of the material, causing the

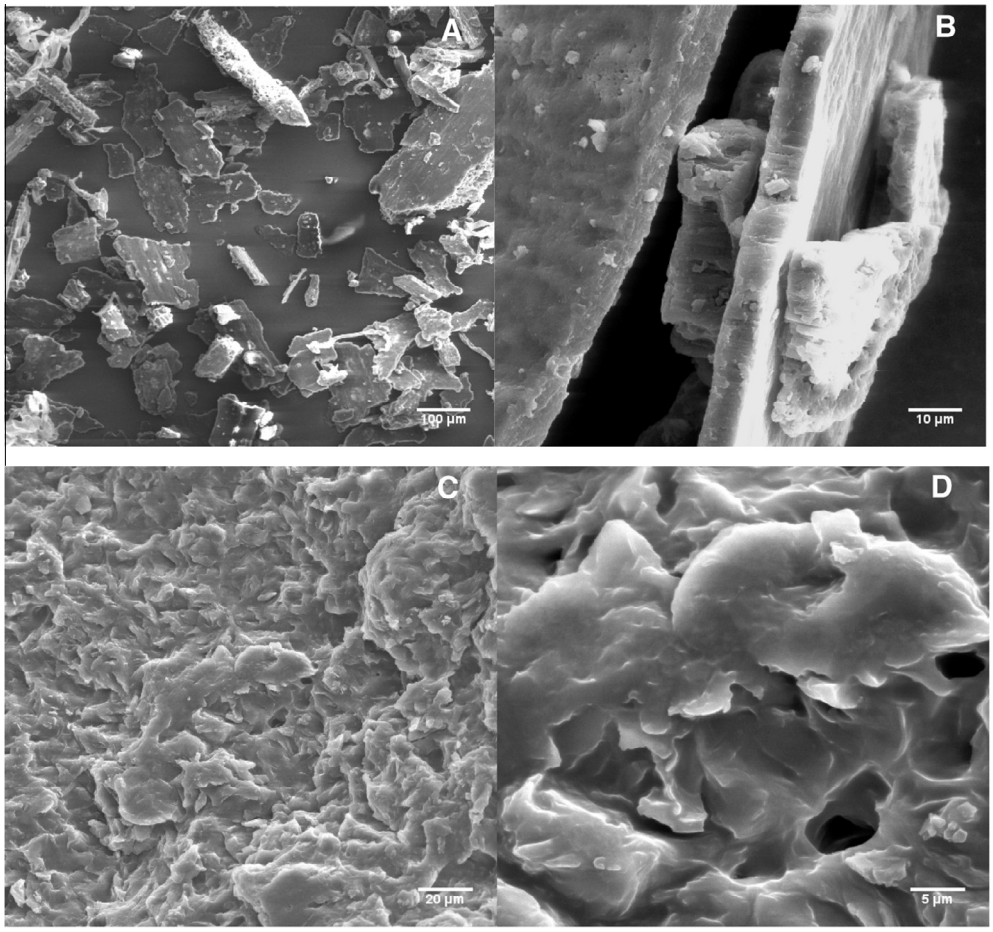


Fig. 4. Scanning electron microscopy (SEM) micrographs of carnauba wax powder samples: (A) wax sheets in the carnauba wax powder before PCO-C extraction, 100× magnification. (B) Detail of the carnauba wax sheets at 1000× magnification. SEM electron micrographs of PCO-C samples: (C) PCO-C at 500× magnification, (D) detail of PCO-C at 2000× magnification.

narrowing of these regions during evaporation of the liquid phase. However, apolar molecular regions are also present, as suggested by the presence of smooth areas on the sample surface.

3.9. Hydrolysis and TLC

Through hydrolysis of PCO-C, the esters were hydrolysed, and the saponifiable material (aromatic acids) was separated from the unsaponifiable material, yielding 0.30 g (30%) of *p*-methoxycinnamic acid. According to the TLC analysis results (Kiejelger 60F254 silica gel plates, 0.20 mm, Merck), the saponifiable material (aromatic acids) had the same retention factor (Rf 0.44) as *p*-methoxycinnamic acid when eluted in heptane/ethyl acetate (1:1) using the developers described by Vandenburg and Wilder (1967).

Recrystallisation of the aromatic acids, following the Vandenburg and Wilder (1967) procedure, generated chromatographically homogeneous white crystals (PF 173 – 175 °C) corresponding to aromatic acids. When comparing the IR absorption spectra (KBr), the recrystallised hydrolysate material (*p*-methoxycinnamic acid) was identical to that found for the *p*-methoxycinnamic acid standard: hydroxyl (3456 cm^{-1}) acid (1685 cm^{-1}, 1176 cm^{-1}), unsaturated (830 cm^{-1}, 980 cm^{-1}), aromatic (1600 cm^{-1}, 1515 cm^{-1}), and *para*-substituted (830 cm^{-1}).

3.10. Antioxidant activity

The antioxidant activity results in Fig. 5 show a significant increase ($p < 0.05$) in intracellular reactive oxygen species (ROS) in cells exposed exclusively to hydrogen peroxide (positive control) compared to the unexposed cells (negative control). However, cells treated with PCO-C alone (50 µg/mL and 250 µg/mL) had low ROS levels, which were not different ($p > 0.05$) from the negative control values. Therefore, PCO-C did not cause cellular oxidation. The lymphocytes that underwent oxidation with H_2O_2 and treatment with PCO-C exhibited a significant reduction in the quantity of ROS formed compared to those of the positive control, and the

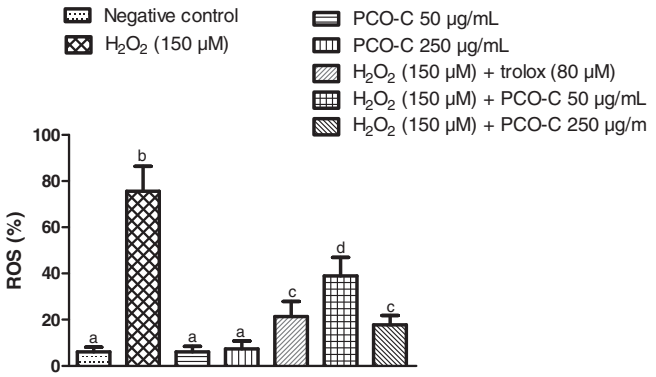


Fig. 5. Inhibitory effect of PCO-C on intracellular ROS production in human peripheral blood lymphocytes. Bars represent the mean ± standard deviation of three independent experiments. Bars with the same letter were not different ($p > 0.05$) by Tukey's test.

relationship was dose dependent. PCO-C had a better antioxidant effect at 250 μg/mL because cells treated with PCO-C at this concentration had an ROS percentage that did not significantly differ from the one obtained for Trolox; i.e., PCO-C (250 μg/mL) had an oxidation inhibition capacity in HPBLs similar to that of Trolox (80 μM).

The antioxidant capacity of PCO-C was also shown by the ABTS and FRAP assays. PCO-C had a total antioxidant activity of 107.27 ± 3.92 μM Trolox/g or 27.96 ± 2.38 μM gallic acid/g by the ABTS method. When analysed by the FRAP method, the results were 73.3 ± 1.83 μM iron sulphate/g. The two assays are indicative of an ability to donate electrons (Benzie & Strain, 1996; Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993) and show considerable antioxidant activity of the studied compound, possibly because it is a derivative of cinnamic acid.

Bioactive phenolic and polyphenol compounds occur naturally and are important components of the human diet due to their antioxidant capacity, which decreases the oxidative stress that induces cell damage associated with severe pathologies, such as cardiovascular and neurodegenerative diseases and cancer (Marques et al., 2014). These results are consistent with those obtained by Jakovetic et al. (2013), Menezes et al. (2011) and Venkateswarlu et al. (2006), who also reported antioxidant activity for various cinnamic acid-derived esters.

The molecular structure of *p*-methoxycinnamic diester extracted from carnauba wax confirms its antioxidant activity. The cinnamoyl fragment is a determining factor for inducing antiradical activity in cinnamic acid derivatives, and the nature and position of substituents on the aromatic ring only increase or decrease this activity. Even when all the benzene carbons are bound to H, there is antiradical activity (Avanesyan et al., 2009). Avanesyan et al. (2009) demonstrated the antioxidant activity of *p*-methoxycinnamic acid. They observed that when all the carbons on the ring are bound to H, cinnamic acid (at 20 μg/mL) had an antiradical activity of 20.4% ± 1.1 (% inhibition), while *p*-methoxycinnamic acid (at 1.2 μg/mL), which has an OCH_3 group in the *para* position of the ring replacing the H, had an inhibition of 32.6% ± 1.5. Taking into consideration that the latter was studied at a low concentration and extrapolating for the same concentration of the former, it can be concluded that the second compound had 26 times greater antiradical activity. In *p*-methoxycinnamic acid, the methoxy group is an electron-donating group (Jakovetic et al., 2013).

Marques et al. (2014) studied several cinnamic acids and identified the structures that contribute to the biological activity of these compounds, including the presence of substituents on the electron-donating aromatic ring (hydroxyl and/or methoxyl) and the carboxylic radical with an adjacent unsaturated C=C double bond, which provides additional attack sites for free radicals and acts as an anchor for the lipid bilayer.

The presence of *p*-methoxycinnamic acid in the form of an ester possibly increases its antioxidant potential, as Jakovetic et al. (2013) reported that esterification increased the antioxidant activity of cinnamic acid and its derivatives.

Moreover, the lipophilicity of this acid in the form of long chain *p*-methoxycinnamic diester is increased, and this compound is more stable due to resonance that favours its electron-donating ability, which may explain its biological activity.

The biological activities of cinnamic acid derivatives are affected not only by their structural characteristics but also by their level of lipophilicity (Mellou et al., 2005) because the lipophilicity of a compound improves its membrane permeability, which in turn affects the behaviour of the antioxidant in biological systems (Zhong & Shahidi, 2011).

Concerning bioaccessibility, the antioxidant activity of PCO-C after *in vitro* simulated gastrointestinal digestion was 34.82 ± 1.6 μM Trolox/g of PCO-C, which is equivalent to a percent bioaccessible fraction of 32.46%. Several studies have confirmed the bioaccessibility of the antioxidant activity of cinnamic acid and its derivatives (Durak, Gawlik-Dziki, & Pecio, 2014; Hemery et al., 2010). However, there are no data in the literature on the antioxidant activity of *p*-methoxycinnamic diesters from carnauba or the antioxidant activity of the bioaccessible fraction.

4. Conclusion

This study presents an efficient extraction process of a cinnamic derivative from carnauba wax powder, resulting in a product without residual solvents. The resulting product was then characterised by IR spectroscopy, ^{1}H NMR, X-ray diffraction and SEM and identified as *p*-methoxycinnamic diester with an apolar, crystalline structure. This compound had relevant antioxidant activity before and after *in vitro* simulated gastrointestinal digestion. It inhibited the formation of intracellular ROS in human cells. Its high thermal stability and UV absorption were confirmed by thermogravimetry and UV spectrophotometry, respectively. Together, these characteristics show that this compound is promising for the pharmaceutical industry and, especially, the food industry. Due to its high stability and antioxidant activity, PCO-C has the potential to be used as an antioxidant in food products. In addition, its apolar nature and UV absorption capacity make it promising for developing active food packaging.

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