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Antioxidants extraction from Pinhão (*Araucaria angustifolia* (Bertol.) Kuntze) coats and application to zein films

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ABSTRACT

Seeds from *Araucaria angustifolia* (Bertol.) Kuntze are consumed after cooking and their coats discarded. Both coats and the cooking water present phenolic compounds, which may be used to improve mechanical properties and provide antioxidant characteristics to films. The objective of this work was to obtain and pinhão coat extracts and to apply these polyphenolic-rich extracts in zein films. Phenolic compounds composition, extraction yield and antioxidant activity (DPPH, ABTS and FRAP) of the extracts were determined. The most abundant molecules present in the hydroethanolic extract were (+)-catechin and an (epi)catechin dimer, whereas protocatechuic acid were predominant in the both cooking water and ethanolic extracts. Glass transition temperature of zein was not found in the extract-loaded films. Morphological changes were also caused by the presence of the extracts yielding smoother surfaces. The extracts added to zein films led to a three-fold increase in tensile strength (from 5.80 MPa to 17.65 MPa) and two-fold increase in the elongation at break (from 1.60% to 3.18%).

1. Introduction

Seeds produced by *Araucaria angustifolia* (Bert.) O. Kuntze (Paraná tree), referred to as "pinhão", are formed by a resistant external tegument (shell or coat) composed of lignocellulosic material rich in tannins and an internal edible pulp (Bello-Pérez et al., 2006; Conforti & Lupano, 2008; Lima et al., 2007; Santos et al., 2013). Seeds are produced seasonally and typically consumed after boiled in water. Although pinhão production is not an organized culture (Conab, 2014), there is an increasing interest in the industrial development of pinhão based products, such as pickled pinhão (Riele Conservas, 2016; WWC, 2016) and beverages such as beer (Cervejaria Campos do Jordão, 2016). In 2007, Brazil generated about 10 ton of pinhão wastes (Lima et al., 2007).

Residual pinhão coats, which represent about 20% of the weight (Conforti & Lupano, 2008; Cordenunsi et al., 2004), has also been attracting interest due to its antioxidant properties and potential biological activity. da Mota et al. (2014) obtained methanolic extracts from the shell and pulp of *A. angustifolia* seeds and identified in both extracts the presence of molecules with free radicals-trapping ability (*e.g.* polyphenolic compounds, flavonoids and proanthocyanins). The biological activity of pinhão extracts was reported by da Silva et al. (2014) who investigated the efficacy of a pinhão coat extract (70% ethanol in water) to decrease the postprandial glycemic levels in rats after starch administration. Authors claimed that the extract may be potentially used to suppress postprandial hyperglycemia in diabetic patients due to its inhibitory properties. In the study developed by Oliveira et al. (2015), a pinhão coat extract proved to be an effective inhibitor of pancreatic lipase and to effectively decrease plasma triglyceride levels in mice after a load of olive oil. Also, Branco et al. (2015) demonstrated that an aqueous extract of *A. angustifolia* bracts presented selective cytotoxicity and pro-apoptotic activity in laryngeal carcinoma HEp-2 cells.

The application of phenolic compounds in film formulations to obtain antioxidant properties, or active packaging materials, has been widely studied. For instance, studies have been published on gelatin-

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based films added with curcuma ethanol extract (Bitencourt, Fávaro-Trindade, Sobral, & Carvalho, 2014), low density polyethylene incorporated with rosemary extract and natural extracts obtained from a brewery residual waste (Barbosa-Pereira, Aurrekoetxea, Angulo, Paseiro-Losada, & Cruz, 2014), starch-based films with propolis extract (De Araújo et al., 2015), and zein and chitosan matrices supplemented with phenolic compounds (ferulic and gallic acids, respectively) (Cheng, Wang, & Weng, 2015).

Zein films may serve as carriers for antioxidant compound in food packaging applications (Arcan & Yemenicioğlu, 2011; Forato, Britto, Scramin, Colnago, & Assis, 2013; Park et al., 2012). In zein films, for instance, the addition of gallic acid, *p*-hydroxy benzoic acid, ferulic acid, flavone, (+)-catechin, and quercetin (Arcan and Yemenicioğlu, 2011), butylated hydroxyanisole and butylated hydroxytoluene (Kleen, Pauda, & Engeseth, 2002), green tea extract (Lee, Lee, & Song, 2004), gallic acid (Neo et al., 2013), and ferulic acid and gallic acid (Cheng et al., 2015) have been reported. However, to the best of our knowledge polyphenolic compounds from pinhão extracts have not yet been applied in films formulation as a carrier of antioxidant substances. In such case, the actual impact of the extracts on the films properties must also be determined. Also, applications involving zein films are worth investigating since zein is an important co-product of maize starch production, as well as a by-product of the bioethanol industry.

In this context, the current work aimed to obtain and to characterize pinhão coat extracts as well as to apply these extracts as a source of natural polyphenolic compounds in zein films. Extracts were obtained from the cooking water extract and also by subsequent ethanolic and hydroethanolic extraction of the residual pinhão coats.

2. Materials and methods

2.1. Materials

Pinhão seeds were acquired from the local market in Brazil in June 2015. Zein, Folin–Ciocalteu reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing/antioxidant power (FRAP) reagent [0.3 M acetate buffer, pH 3.6 and 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ)], Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) and potassium persulfate (dipotassium peroxydisulfate) were purchased from Sigma–Aldrich. Glycerol, calcium carbonate and ethanol (Vetec) were analytical grade. Acetonitrile (99.9%, Fisher Scientific, HPLC grade) was used in the chromatographic analyses. Formic acid was purchased from Panreac Química SLU. (Barcelona, Spain). Phenolic standards were from Extrasynthèse (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Pinhão preparation and antioxidant extraction

Pinhão seeds (526 g) were cooked in water (1 L) for 2 h, using a pressure cooker. Seeds were recovered using a sieve and the extract (cooking water, CW) was frozen (−18 °C).

The cooked pinhão seeds were opened and their coats dried in a forced air convection oven (Cienlab, Brazil) at 40 °C for 24 h. Thereafter, they were crushed in a domestic blender and classified using a vibrating set of sieves (200/+ 400 mesh Tyler). The resultant material was stored in the freezer (−18 °C) until extraction.

The antioxidant ethanolic (EtOH) and hydroethanolic extracts (HA) were prepared using pure ethanol and an 80% (v/v) hydroethanolic mixture, respectively. First, sieved pinhão coats (5 g) and solvent (120 g) were stirred using a magnetic stirrer for 5 h. Extraction yield (%, w/w) was determined gravimetrically. All extraction procedures were carried out in triplicate.

2.3. Antioxidant activities

The antioxidant activity of the extracts was assessed by the ABTS and DPPH radical scavenging assays. The ABTS assay was based on the method of Arnao, Cano, and Acosta (2001), as adapted by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Hawkins Byrne (2006). First, a stock solution was prepared from equal volumes of aqueous solutions of 7.4 mM ABTS and 2.6 mM sodium persulfate. The stock solution was kept in the dark for 12 h to allow the formation of the ABTS radical ($ABTS^{\cdot+}$), then diluted with methanol until an absorbance of 1.100 ± 0.010 units at 734 nm was reached (Ocean Optics, Red Tide USB650 Fiber Optic Spectrometer, USA) to form the working solution. In test tubes, 2.85 mL of the working solution was added to 150 µL of the extract (or 150 µL ethanol as the control). After homogenization, the solutions were stored in the dark for 2 h and the absorbance at 734 nm was determined. All samples were analyzed in triplicate. Results were calculated using a previously obtained Trolox calibration curve (50–500 µM, $y = 1.0595x - 0.0017$; $R^2 = 0.9991$) and expressed as µmol of Trolox equivalent (TE) per 100 g of pinhão coat ($\mu mol_{TE}.100\ g_{pinhão\ coat}^{-1}$).

For the DPPH assay, the procedure described by Mensor et al. (2001) was used, with some modifications. In a test tube, 2.5 mL of the extract was mixed with 1 mL of 0.3 mM DPPH methanol solution (or pure methanol as the control). After 30 min in the dark, the absorbance was determined at 518 nm. All samples were analyzed in triplicate. The antioxidant capacity was calculated using a Trolox standard curve (15 − 75 µM, $y = 1.0728x - 0.0168$; $R^2 = 0.9977$) and the results expressed as $\mu mol_{TE}\ 100\ g_{pinhão\ coat}^{-1}$.

The FRAP assay was performed according to Benzie and Strain (1996), with minor modifications. First, 100 µL of the extract solution (or 100 µL of distilled water, for the blank sample) and 300 µL distilled water were added to a test tube. Then, 3.0 mL of FRAP reagent (10 mM TPTZ in 40 mM HCl, plus 20 mM ferric chloride and 300 mM acetate buffer, pH 3.6, 1:1:10 v/v/v) were added and the solution homogenized and warmed at 37 °C for 30 min in a water bath. Finally, the absorbance of the colored product (ferrous tripyridyltriazine complex) was determined at 593 nm. A Trolox standard curve (50–1000 µM, $y = 0.0013x + 0.0111$; $R^2 = 0.9991$) was prepared and the results expressed as $\mu mol_{TE}\ g_{pinhãocoat}^{-1}$.

2.4. Phenolic compound analyses

The lyophilized extracts were re-dissolved in a 80:20 methanol:-water (v/v mixture and the phenolic profile was determined by high-performance liquid chromatography-diode array detection-electrospray ionization multi-stage mass spectrometry (HPLC-DAD-ESI)/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) as previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira (2016). Double online detection was carried by DAD using 280 and 370 nm as the preferred wavelengths and a mass spectrometer connected to the HPLC system *via* the DAD cell outlet. The MS detection was performed in negative ion mode, using an LTQ XL linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. Positive identification of the phenolic compounds was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra. Also, tentative identification was made based on data reported in the literature. For quantitative analysis, a calibration curve, obtained for each available phenolic standard, was constructed based on the UV signal. For the phenolic compounds identified in the absence of an available commercial standard, quantification was performed based on the calibration curve of the most similar available standard. Results were expressed as $mg\ g_{extract}^{-1}$.