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Content of major classes of polyphenolic compounds, antioxidant, antiproliferative, and cell protective activity of pecan crude extracts and their fractions

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ABSTRACT

Acetonic extracts of pecan kernels and nutshells were fractionated using Sephadex LH-20 and three fractions were obtained from each. Only fraction II of both extracts contained simple polyphenols: catechin, epicatechin and ellagic acid were identified in kernel fraction II, while only epicatechin and ellagic acid were found in shell fraction II. Total gallic and ellagic acids were quantified in acid-hydrolyzed extracts: kernels contained 1.8 and 3.6 mg g^{-1}, respectively, while nutshells contained 2.3 and 0.8 mg g^{-1}, respectively. Kernel fraction III, containing high molecular weight compounds, showed the highest antioxidant activity and better antiproliferative activity in a cancer than a non-cancer cell line. Crude extracts showed cytotoxic activity in cerebellar granule neurons (CGNs), but shell extract was more cytotoxic; in contrast only kernel extract protected CGNs from H_2O_2-induced cell death. Thus, high molecular weight compounds of pecan kernel show noticeable bioactive properties and should be further characterised and analyzed as a rich source of bioactive polyphenols.

1. Introduction

Tree nuts are rich sources of nutrients and bioactive compounds, and have been recognized to play a role in protecting against heart disease (FDA., 2003). More recently, long term nut consumption, as a supplement to a Mediterranean diet, has shown to reduce some features of the metabolic syndrome (Salas-Salvado et al., 2008) and decrease plasma concentration of inflammatory biomarkers (Urpi-Sarda et al., 2012); suggesting nuts may help to maintain health in more ways than previously thought.

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Pecans (*Carya illinoinensis*) are tree nuts of the Juglandaceae family, native to North America and represent some of the major agricultural commodities of some regions in Mexico and the United States. They are among the nuts and, actually, among the food products with highest concentrations of phenolic compounds and antioxidant activity (Wu et al., 2004). Pecans contain phenolic acids (De la Rosa, Alvarez-Parrilla, & Shahidi, 2011), condensed taninns (proanthocyanidins) with various degrees of polymerization (USDA., 2004, 2013), hydrolysable tannins (De la Rosa et al., 2011; Villarreal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007) and anthocyanins (USDA, 2013). The presence of both condensed and hydrolysable tannins, make pecans an interesting and complex source of phytochemicals, since both types of compounds possess strong biological activities (Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009). However, they also make a complete characterization of pecans phenolic profile difficult, which is necessary for fully understanding and take advantage of the potential health benefits of these nuts. Moreover, pecan nutshells also contain high content of phenolic compounds (higher than the edible kernels) and pecan shell infusions have shown neuroprotective properties in animal models with neurological disorders (Trevizola et al., 2011). Nevertheless, characterization of shell phenolic compounds is even less advanced than that for kernels. Hence, it is advisable to test their bioactivity in different model systems before trying to possibly use them for humans. Therefore, in the present paper we had two objectives, first was to achieve a better characterization of kernel and shell phenolic compounds by fractionating extracts and analyzing individual fractions, and, second, to test bioactivity of the crude extracts and their most antioxidative fractions.

2. Materials and methods

2.1. Materials and samples

The LLC-PK1 and HTB4 cells used in this work were purchased from the American Type Culture Collection. Gallic acid, catechin, epicatechin, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), dimethylthiourea (DMTU), pyruvate, thiazolyl blue tetrazolium bromide (MTT), vanillin, sodium chloride, potassium persulphate, Sephadex LH-20gel, trypsin (⩾9000 BAEE units/mg protein), DNAse I (⩾2000 Kunitz Units/mg protein), horseradish peroxidase (221.2 units/mg solid), Basal Eagle Medium and Dulbeccós Modified Eagle Medium (DMEM) were purchased form Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) fetal bovine serum (FBS), penicillin, streptomycin and soybean trypsin inhibitor (⩾7000 BAEE units/mg) were from GIBCO. Trolox (6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid) was from EMD Millipore (Millipore Corporation, Billerica, MA, USA). All other solvents like hydrochloric acid, ethanol, hexane, acetone, acetonitrile and methanol were purchased from J.T. Baker (Avantor Performance Materials, Inc., Center Valley, PA, USA). All chemicals were reactive grade, except acetonitrile and methanol which were HPLC grade.

Pecan nuts from Western cultivar were donated by producers from three different regions of the state of Chihuahua, México: Flores Magón (North), Delicias (Centre) and Jiménez (South). Mature pecans are harvested when the green outer layer (husk) is dry and brown, and it spontaneously opens, exposing the inner seed, which consists of a hard brown shell surrounding the inner kernel. In the present study both kernel and hard brown shell were analyzed. The nuts were obtained approximately 2 weeks after harvest, and were manually cracked and shells and kernels were separated and stored at 4 °C until analysis, no longer than four months.

2.2. Extraction and fractionation of phenolic compounds

Kernels were defatted by blending 25 g of each sample with hexane (1:10, w/v) 3 times for 3 min in a laboratory blender (Waring, Thermo Fisher Scientific, Waltham, MA, USA). The defatted powder was recovered by filtration (Whatman No. 5) under vacuum and allowed to dry for 24 h at room temperature. The shell was grounded in a commercial coffee mill (Mr. Coffee, Cleveland, OH, USA) for 2 min. Once dried and pulverized (60 mesh size), both samples were used for polyphenol extraction. The samples (4.5 g) were extracted in an ultrasonic bath (Fisher Scientific FS220H, Thermo Fisher Scientific, Waltham, MA, USA) with 80% (v/v) acetone (45 mL) for 30 min at room temperature. After that, the suspension was centrifuged (Eppendorff 5810R, Eppendorf Hamburg, Germany) for 10 min at 3000*g* at 4 °C. The supernatant was collected and the pellet was re-extracted under the same conditions. After the two extractions, supernatants were combined and the solvent removed under vacuum by rotary evaporation (BÜCHI Series-114, BÜCHI Labortechnik AG, Flawil, Switzerland). The polyphenolic extracts were lyophilized (LABCONCO Freezone 6, Labconco, Kansas City, MO, USA) and stored at −80 °C in vacuum-sealed plastic bags.

Extracted fractionation were passed through a glass chromatographic column (35 × 2.5 cm), packed with Sephadex LH-20 resin. The packed column was stabilized with 500 mL of 95% (v/v) ethanol, at a flux of 0.8 ± 0.2 mL/min, using a low pressure pump. Crude kernel and shell extracts (150 mg) were dissolved in 3 mL of 95% ethanol and loaded onto the column. Low molecular weight compounds were recovered by passing 100 mL of 95% ethanol. High molecular weight compounds were eluted from the column using 50 mL of 50% (v/v) acetone. Absorbance of each collected tube was read at 280 nm, in order to identify each of the eluted fractions. The different tubes corresponding to one single fraction were mixed and the solvent removed by rotary evaporation. Water was removed by lyophilization for 24 h and the samples were stored at −20 °C until their analysis (less than one month).

2.3. Quantification of major classes of phenolic compounds

2.3.1. Total phenolic compounds

Total phenolic content was determined using the Folin–Ciocalteu reagent according to de la Rosa et al. (2011). Dry extracts were dissolved in methanol (5 mg/mL), and 2.5 mL of the solution were mixed with 1 mL sodium carbonate (7.5%) and 1.25 mL of Folin–Ciocalteu reagent (10% in distilled water). The mixture was incubated for 15 min at 50 °C in complete absence of light and, at the end of the reaction time, the

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