SUPPLEMENTARY MATERIAL

Phenolic profile and antioxidant activity from non-toxic Mexican *Jatropha curcas* L. shell methanolic extracts

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Abstract

Jatropha curcas seed shells are the by-product obtained during oil extraction process. Recently, its chemical composition has gained attention since its potential applications. The aim of this study was to identify phenolic compounds profile from a non-toxic *J. curcas* shell from Mexico, besides, evaluate *J. curcas* shell methanolic extract (JcSME) antioxidant activity. Free, conjugate and bound phenolics were fractionated and quantified (606.7, 193.32 and 909.59 $\mu\text{g/g}$ shell, respectively) and thirteen individual phenolic compounds were detected by HPLC. The radical-scavenging activity of JcSME was similar to Trolox and ascorbic acid by DPPH assay while by ABTS assay it was similar to BHT. Effective antioxidant capacity by ORAC was found (426.44 ± 53.39 μmol Trolox equivalents/g shell). The Mexican non-toxic *J. curcas* shell is rich in phenolic compounds with high antioxidant activity; hence, it could be considerate as a good source of natural antioxidants.

Keywords: *in vitro* antioxidant activity, phenolic profile, seed shells by-product.

Experimental

Biological material

Seeds of *J. curcas* Puebla (voucher number 2011 IZTA), a non-toxic Mexican genotype were collected from the experimental station of the “Asociación de Agricultores del Río Sinaloa Poniente” located in Guasave, Sinaloa, Mexico (2012). Seed shells were manually separated, milled and sieved (sieve number 35). Powdered shells were stored in the dark at -20 °C until analysis.

Extraction of phenolic compounds from J. curcas shell

Shell powder (1 g) was placed in a flask and was mixed with 20 mL of methanol absolute, sonicated during 30 min, and incubated and shaking during 18 h at 25 °C, 200 rpm, protected from light, then was centrifuged (8000 g) during 10 min and the supernatant was recovered. The residue was re-extracted during 6 h and the combined supernatant was concentrated to dryness using a vacuum rotary evaporator and stored at -20 °C until antioxidant activity analysis. The extractions were carried out by triplicate.

Sample preparations to HPLC analysis of phenolic compounds

The phenolic compounds profile was determined by HPLC. SFP, SCP and BP were extracted as described by Kim et al. (2006) with minor modifications, and then were injected in a HPLC system. Before extraction, powdered shell was defatted using hexane at 5:1 (v/w) ratio during 2 h

at room temperature. The mixture was centrifuged (8000 g) during 15 min and the hexane was completely removed.

Phenolic profile analysis by HPLC

Profile of phenolic compounds was conducted on a HPLC system (Alliance Waters 2695) equipped with a photodiode array detector (PDA, Waters 2998), autosampler and Empower software. The separation was carried out with a Synergi MAX-RP C12 column (250 × 4.6 mm, 4 µm particle size). Chromatographic separation was carried out with 10 µL of extract using two solvent systems: (A) 0.01% (v/v) formic acid in double distilled water and (B) acetonitrile, at flow rate of 1 mL/min. The gradient system was used as follow: solvent (A) 0-34 min, 90%; 35-39 min, 50%; 40-50 min, 90%. Elution of the interest compounds was monitored at 280 nm. The phenolic compounds were identified and quantified using standards for phenolic compounds.

Antioxidant activity

DPPH assay

Antiradical activity of *J. curcas* shell extract was determinate according to Cardador-Martínez et al. (2006) with minor modifications. A solution of 150 µM DPPH[•] was prepared in 80% (v/v) methanol. 20 µL of sample, blank, standard or control and 200 µL of DPPH solution were added to a 96 wells microplate. Absorbance was measured every 10 min from 0-60 min at 515 nm. Different concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 and 4 mg/mL) of standards (Trolox, BHT and ascorbic acid) and sample were used. The antioxidant activity was expressed as % of antiradical activity (% ARA) as described by Cardador-Martínez et al. (2006) and IC₅₀ (mg/mL), the concentration required to cause a 50% DPPH[•] inhibition, was calculated. All determinations were carried out by triplicate.

ABTS assay

This assay was based on the method of Re et al. (1999) with slight modifications for microplate. Different concentrations (0, 0.05, 0.1, 0.2, 0.5 y 1 mg/mL) of standards (Trolox, BHT and ascorbic acid) and sample were used; 20 µL of each standard and sample were mixed with 200 µL of ABTS^{•+} radical solution; absorbance was measured at 734 nm. The antioxidant activity was expressed as % ARA as well as IC₅₀ (mg/mL) in the same way as DPPH. All determinations were carried out by triplicate.

ORAC Assay

The ORAC of *J. curcas* shell ME was measured according Gliwa et al. (2011). For the assay, methanolic extract was diluted at 1:400 (v/v) ratio with ORAC buffer (potassium phosphate buffer, pH 7.4) and a Trolox standard curve (6.25, 12.5, 25, 50 and 100 μ M) was prepared. Rutin (10 μ M) was used as a positive control. The day of analysis a 0.13 μ M fluorescein and 0.16 M AAPH solutions were prepared in ORAC buffer. A 96 wells black microplate was prepared containing 20 μ L of blank (ORAC buffer), standard, control or sample and 120 μ L of fluorescein solution were added. After 20 min of incubation at 37 °C, 60 μ L of freshly prepared AAPH solution were quickly added and data were obtained with the fluorescence reader (FLx800 Multi-Detection Microplate Reader with Gen5 software, BioTek Instruments, Ottawa, ON, Canada) using the following conditions: excitation wavelength of 485 nm and emission wavelength of 525 nm. Readings of fluorescence were taken every minute during 1 h. The result was expressed as μ mol TE per g shell. Determinations were carried out by triplicate.

Statistical analysis

Analysis of variance and a multiple ranges test (Tukey, $p \leq 0.05$) were done with the SAS 7.0 statistic program (SAS Institute Inc., Cary, North Carolina).

Figure S1. HPLC chromatograms of (A) a standard mixture of phenolic acids. Peak1, gallic acid; 2, protocatechuic acid; 3, p-hydroxybenzoic acid; 4, chlorogenic acid; 5, caffeic acid; 6, vanillic acid; 7, syringic acid; 8, p-coumaric acid; 9, sinapic acid; 10, ferulic acid; 11, o-coumaric acid. Detection wavelength of 280 nm. (B) A standard mixture of flavonoids and other phenolic compound. Peak 1, pyrogallol; 2, catechin; 3, epicatechin; 4, rutin; 5, quercetin-3- β -glucoside; 6, epicatechin gallate; 7, myricetin; 8, quercetin; 9, apigenin; 10, kaempferol. Detection wavelength of 280 nm.

Figure S2. HPLC chromatogram of different fractions of phenolic compounds in *J. curcas* shell at 280 nm. **(A)** Soluble free phenolic compounds. Peak 1, *p*-coumaric acid; 2, myricetin; 3, *o*-coumaric acid; 4, quercetin. **(B)** Soluble conjugated phenolic compounds. Peak 1, rutin; 2, *p*-coumaric acid; 3, sinapic acid; 4, ferulic acid; 5, myricetin; 6, quercetin. **(C)** Bound phenolic compounds. Peak 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, caffeic acid; 5, rutin; 6, *p*-coumaric acid; 7, sinapic acid; 8, ferulic acid; 9, *o*-coumaric acid; 10, quercetin; 11, apigenin; 12, kaempferol.

Figure S3. Free radical scavenging activity of *JcSME* and synthetic antioxidants of reference, at different concentrations, determined by DPPH (A) and ABTS (C) assays. Natural logarithm of antioxidant or extract concentration versus antiradical activity against DPPH (B) and ABTS (D). All data are the mean of three replicates.

Experimental section references

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