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Short Communication

Biological activities of polyphenols-enriched propolis from Argentina arid regions

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ABSTRACT

Background: Propolis is a bioactive natural product collected by honeybees (*Apis mellifera*) from plant sources.
Purpose: This study was undertaken to determine the effect of propolis extracts from arid region of Argentina, on the activity/expression of pro-inflammatory enzymes, and as potential free radical scavenger, antifungal and anthelmintic agent as well as to get a first insight into the polyphenolic profile of the active fractions.
Study design/methods: Two propolis samples were collected in different time from hives located in Tucumán, Argentina. They are representative of the collection time of the raw material for phytotherapeutical purposes. Ethanolic extracts from both propolis were obtained. The PEEs were analyzed for total polyphenol (TP), non-flavonoid phenols (NFP) and flavonoid (FP) content followed by HPLC-DAD analysis and identification of components by HPLC-MS/MS^n. The potentiality as anti-inflammatory (LOX, COX, iNOS enzymes), antioxidant, antifungal and nematicidal was determined.
Results: PEEs contain high levels of TP, NFP and FP, including cinnamic acid, caffeic acid prenyl ester, caffeoyl dihydrocaffeoate and caffeic acid 3,4-dihydroxyphenethyl ester, liquiritigenin, 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone. The PEEs *in vitro* reduced the activity of LOX and COX-2. Pretreatment of RAW 264.7 cells with PEEs before the induction of inflammatory state, inhibited NO overproduction and the iNOS protein expression was significantly decreased. The PEEs exhibited antioxidant, antifungal (*Candida* sp.) and nematicidal effect (*C. elegans*).
Conclusion: These findings show the potential use of characterized PEEs from arid regions of Argentina as phytomedicine.

Introduction

The propolis are natural products produced by honeybees (*Apis mellifera* L.), they collect resins from bark and leaf bud from plants that grow around of hives. The chemical composition of propolis depend on the vegetation that grow around of hives as well as the time of collection (Bankova et al. 2006; Isla et al. 2005, 2009, 2013; Vera et al. 2011; Solorzano et al. 2012). Several phytogeographical regions with different plant species were found in Argentina. Isla et al. (2013) described at least four different types of propolis in Argentina, based on the chemical composition and botanical origin. Some of them show biological properties, including antibacterial effect against antibiotic-resistant human pathogenic bacteria (Nieva Moreno et al. 1999; 2005; Isla et al. 2009; Vera et al. 2011; Solorzano et al. 2012) and canine pathogenic bacteria (Salas et al. 2014), antifungal activity against dermatophytes and yeasts (Agüero et al. 2010; Isla et al. 2013), free radical scavenging and antimutagenic activities (Isla et al. 2005; Nieva Moreno et al. 2005; Vera et al. 2011; Danert et al. 2014). However, little is known about anti-inflammatory and nematicidal activity of Argentinean propolis. The aim of the present

Abbreviations: PEE, propolis ethanolic extract; TP, total phenolic; NFP, non-flavonoids phenolics; FP, flavonoid phenolics; QE, quercetin equivalents; NE, naringenin equivalents; GAE, gallic acid equivalent; LPS, lipopolysaccharide; PVDF, polyvinylidene fluoride; iNOS, inducible nitric oxide synthase; COX-2, cyclooxigenase-2; PG, prostaglandin; LOX, soy lipooxygenase-1; MTT, thiazolyl blue tetrazolium bromide; DNPH, 2,4-dinitrophenylhydrazine; ABTS, 2,2′-azino-bis-3-ethylbenzothiazolilne-6-sulfonic acid; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene; TLC, thin-layer chromatography; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; NGM, nematode growth medium.

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study was to assess the activity of propolis from the arid region of Tucumán, Argentina, as inhibitor of pro-inflammatory enzymes, antioxidant, antifungal and nematicidal effect as well as to get a first insight into the polyphenolic profile of the samples.

Material and methods

Propolis samples

Two propolis samples were provided by INTA-PROAPI, Argentina, from hives located in the Agrotechnical School, Monte Region, Tucumán (26°35'S, 65°55'W). The samples were collected in December 2012 and March 2013 and are representative of the collection time of the raw material for phytotherapeutical purposes. Samples were weighed and frozen at –20 °C until processing.

Preparation of propolis extracts

20 g of each crude propolis was extracted with 250 ml ethanol:water 80:20 (v/v) at room temperature during 7 days. Each extract was taken to dryness under reduced pressure and lyophilized to afford the propolis hydroethanolic extracts (PEEs). The extracts were labeled as PEE-1 (December sample) and PEE-2 (March sample). Extracts were stored at –20 °C in the dark until analysis.

Phenolics content and distribution

TP concentration in the samples was determined spectrophotometrically according to the Folin–Ciocalteu method (Salas et al. 2014). The NFP and FP content were determined according to Isla et al. (2014) and expressed as mg GAE/g PEE. Total flavone/ flavonol (expressed as mg QE/g PEE) and flavanones/ dihydroflavonol (mg NE/g PEE) content was estimated according to Danert et al. (2014).

Biological activities: Assays using cell systems

Cell viability was assessed by the MTT assay as described by Torres Carro et al. (2015) using a density of 2.0×10^5 RAW 264.7 cells (ECACC, Salisbury, UK) per well with or without PEE (12–100 μg/ml). The cells were cultured in the presence or absence of LPS (1.0 μg/ml) and with or without PEEs (12–50 μg/ml) or dexamethasone (10 μM, 3.92 μg/ml). The iNOS and COX-2 expression and NO production were determined according to Torres Carro et al. (2015).

Inhibition of pro-inflammatory mediators in a cell free system

The inhibitory activity of the PEE (up to 100 μg/ml) on COX-2 was measured using a COX inhibitor screening assay kit following the manufacturer's instructions, based on measuring PG by ELISA (Cayman Chemical). The effect on LOX was determined according to Torres Carro et al. (2015). In all assays, reference compounds were used.

Antioxidant activity

The antioxidant capacity assay was carried out by using the ABTS cation radial and the system β-carotene-linoleic acid (Zampini et al. 2010; Danert et al. 2014). In both antioxidant assays, BHT was used as positive control.

Antifungal activities

To assess the antifungal effect, the following microorganisms were used: *Candida albicans* (A, B), *C. tropicalis* (C, D), *C. krusei* (E), *C. parapsilosis* (F), *C. glabrata* (G), *C. guilliermondii* (H), *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22013. The bioautographic assay for *Candida* was done according to Svetaz et al. (2007). MIC and MFC values were determined by the broth microdilution methods (M27-A3, 2008) in presence of PEEs (50–1000 μg/ml).

Nematicidal activity

The nematicidal assay was determined according to D'Almeida et al. (2015) using the wild-type strain N2 (Bristol) from *Caenorhabditis elegans* (Genetics Center of University of Minnesota, Minneapolis, MN, USA).

Fractionation of the PEE for polyphenols identification

The aim of the PEE fractionation was to identify the most active fractions to allow a better chemical identification of the compounds. A sample of the PEE-2 (1.12 g) was permeated on Sephadex LH-20 using MeOH as mobile phase. Ten fractions were obtained with the following yields: I (78 mg), II (142 mg), III (185 mg), IV (222 mg), V (60 mg), VI (44 mg), VII (14 mg), VIII (9 mg), IX (10 mg) and X (4 mg). The fractions III to VIII showed antioxidant and antifungal activities. Then, fraction III (20 μl of a 50 mg/ml solution) was fractionated by HPLC system coupled to a photodiode array detector (Waters Corporation, Milford, MA, USA) in a semi-preparative C18 column (Phenomenex) using a linear gradient solvent system consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol as follows: 35% A to 20% A over 10 min, 20% A to 0% A from 10 to 20 min, 0% A during 10 min The yields of the more abundant sub-fraction were as follow: SF-I (13 mg), SF-II (14 mg), SF-III (15 mg) and SF-IV (8 mg).

Fraction F-III, the bioactive sub-fractions SF-I to SF-IV as well as fractions F-IV and F-V were analyzed by HPLC using XBridge™ C18 column (Waters) according to Costamagna et al. (2015). The fractions and sub fractions were monitored at 254 nm and UV spectra were recorded from 220 to 500 nm for peak characterization.

Data were recorded on a HPLC-ESI-MS/MS system which consisted of the HPLC HP1100 (Agilent Technologies Inc., Santa Clara, CA-USA) connected through a split to the mass spectrometer Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltonik GmbH, Bremen, Germany) according to Costamagna et al. (2015).

Results and discussion

In Argentina, the collection of propolis for phytotherapeutical preparations is from December to March (late spring to summer time for the southern hemisphere). Two samples of propolis (December and March) from the same production place were collected and compared to characterize the raw material for phytotherapeutic use.

Polyphenolic content

About 50% of the crude propolis constituents are soluble in ethanol:water (80:20). Both PEEs showed high content of TP (593 ± 15 and 587 ± 20 mg GAE/g PEE for PEE-1 and PEE-1, respectively) and NFP (357 ± 13 and 368 ± 10 mg GAE/g PEE followed by FP (236 ± 10 and 219 ± 18 mg GAE/g PEE). The content of flavone/flavonol (276 ± 5 and 343 ± 6 mg GAE/g PEE) was higher than flavanone/ dihydroflavonol content (165 ± 12 and185 ± 15 mg QE/g PEE). No significant differences were found in the polyphenol content between the December and March propolis collection (Supplementary material Table 1).

Effects on pro-inflammatory mediators

PEE-1 was 33% more effective as LOX inhibitor than PEE-2 (IC_{50} values of 94.9 ± 4.7 and 63.9 ± 3.2 μg/ml). Both samples were active on COX-2 with similar IC_{50} values, 100 ± 4 and 106 ± 5 μg/ml for PEE-2 and PEE-1, respectively (Supplementary material Table 1).