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Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal

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ABSTRACT

Pollen analysis, total phenols content and antioxidant activity were studied for the first time in Portuguese propolis samples from Bornes and Fundão regions. Total phenols content was determined by colorimetric assay and their amount was of 329 mg/g of GAE in Bornes sample and 151 mg/g of GAE in Fundão propolis. The antioxidant capacity of propolis extracts was assessed through the scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) and reducing power of iron (III)/ ferricyanide complex assays. A concentration-dependent antioxidative capacity was verified in DPPH and reducing power assays. Low values of EC_{50} on DPPH scavenging assay were obtained for Bornes and Fundão propolis (of 6.22 μg/mL and 52.00 μg/mL, respectively). For reducing power the values were 9.00 μg/mL, for Bornes propolis, and 55.00 μg/mL, for Fundão propolis. The high activity of propolis from Bornes could be related with their different pollen composition. The results obtained indicate that Portuguese propolis is an important source of total phenols showing antioxidant properties that could be beneficial for human health.

1. Introduction

Propolis is a product based on resins collected from resinous sprouts and exudates of some plants by bees of *Apis mellifera* specie. When the bees reap the propolis, they mix the resinous substance collected from plants with the 13-glicosidase enzyme of their saliva, causing the hydrolysis of the glucosyl flavonoids, originating flavonoids aglycones (Pereira et al., 2002). In the beehive, the propolis is used by the bees to defend them from the invaders (causing death by asphyxia) and promotes conservation of their bodies, protecting the beehive from the resultant plagues of putrefaction. Another propolis function is the thermal isolation of the beehive, being used to fill eventual cracks or apertures (Bankova et al., 2002).

In the past few years, the suspected toxicity of some synthetic compounds used in food has raised the interest in natural products (Stone et al., 2003). Some industries, such as those related to food additive production, cosmetics, and pharmaceuticals, have increased their efforts in obtaining bioactive compounds from natural products by extraction and purification. Antioxidant compounds can increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage (Halliwell, 1997; Halliwell and Gutteridge, 1999).

Propolis has been used in the traditional medicine since the primordial times of humanity, having acquired popularity between Egyptians Arabs, Greeks, and many other civilizations (Abd El Hady and Hegazi, 2002). Those biological and therapeutic actions were attributed to their phenolic composition (Lahouel et al., 2004). In fact, different works attribute important properties to propolis, namely antibacterial action against different pathogenic bacteria (Kujumgiev et al., 1999), antifungal and anti-inflammatory (Wang et al., 1993), anti-viral (Amoros et al., 1994), curative, anesthetical and anti-tumoural properties (Kimoto et al., 2001; Matsuno, 1995). Recently, Kim et al. (2005) showed that propolis is able to inhibit the action of the enzyme hyaluronidase, allowing slow aging of cells. For all these reasons, this natural product awakened interest in the pharmaceutical industry, mainly in Asian countries, being propolis introduced in different products for human consumption like drinks, foods and cosmetics (Pereira et al., 2002).

The studies reported above describe propolis characterization, their biological properties and the action of their composition. However, no previous studies were reported about this Portuguese hive product. In this work, and for the first time, the Portuguese propolis, from two different regions, were studied regarding their total phenols content, pollen characterization and antioxidant activities. Antioxidant potential was accessed by the reducing power assay and the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals.

2. Materials and methods

2.1. Samples

Two different samples of propolis were analysed. Bornes sample from Serra de Bornes in the Northeast of Portugal and Fundão sample proceed from the Beira Interior Region in the Centre of Portugal (Fig. 1). Between regions, marked differences were registered in terms of climatic conditions and vegetation.

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2.2. Reagents

Absolute alcohol and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich (Germany). Methanol HPLC grade was obtained from Pronolab (Lisboa, Portugal). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Sample preparation

Samples were prepared by mixing propolis with methanol (1:1, v/v) and were left *over-night* in agitation. After this step, the obtained solution was filtered (Whatman n 4 filter paper). Two other methanolic extractions were performed using the same procedures. The combined methanolic extracts were placed at low temperatures and, after 12 h, filtered to remove wax. The methanol was evaporated with a rotary evaporator. The extracts were evaporated by reduced pressure (Rotavapor Buchi RE 111 with a Buchi 461 water-bath, 2002), re-dissolved in the corresponding solvent at a concentration of 50 mg/mL and analysed for their content in total phenols.

2.4. Pollen analysis

The pollen analysis was executed by the methodology described by Barth et al. (1999). The pollen attainment was initiated by mixing of 0.5 g of propolis with 15 mL of absolute alcohol at least 24 h. The mixture obtained after centrifugation was boiled on KOH 10% for 2 min in water-bath. The sediment was washed in distilled water, filtered, and kept in acid ascetic glacial during a night. Then acetolise of pollen sediments was carried out in a mixture 9:1 of ascetic acid and sulphuric acid anhydride, in water-bath until reaching the temperature of 80 °C for about 3 min. After careful sediment washing with water and glycerinate-water, the sediment was mounted in gelatine-glycerinate, with or without courante (fuchsine basic).

2.5. Determination of total phenol content

Total phenols content in the methanolic extract of the different propolis were estimated by a colorimetric assay based on procedures described by Kumazawa et al. (2002) and Singleton et al. (1999) with some modifications. The reaction of 0.5 mL methanolic extract solution mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3 was kept in the dark at room temperature for 1 h, after which the absorbance was read at 700 nm (Unicam UV-Visible Spectrometry Heλios, United Kingdom). Methanolic extract samples were evaluated at the final concentration of 20 mg/mL. Gallic acid standard solutions were used for constructing the calibration curve. Total phenols content were expressed as mg of gallic acid equivalents/g of extract (GAEs).

Fig. 1. Location of propolis samples origin (Bornes and Fundão) on the Portugal map.

2.6. Scavenging of DPPH radicals

The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was assayed following the method of Hatano et al. (1989). Solutions with different extract concentrations were prepared. One millilitre of the extract solution was dissolved in MeOH with 1:1 (v/v) of DPPH solution (0.1 mM). The mixture was shaken vigorously and left to stand for 50 min in the dark at room temperature (until stable absorbance values were obtained).

The reduction of the DPPH-radical was measured by continuous monitoring the decrease of absorption at 517 nm (Unicam UV-Visible Spectrometry Heλios, United Kingdom). DPPH scavenging effect was calculated as a percentage of DPPH discolouration using the equation:% scavenging effect = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in solution.

2.7. Reducing power

The reducing power was determined according to the method described by Shi and Dalal (1991). The propolis extract (2.5 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide.

The mixture was incubated at 50 °C for 20 min. After 2.5 mL of 100 mg/mL trichloroacetic acid were added, the mixture was centrifuged at 650*g* for 10 min (Eppendorf centrifuge 5810 R, Germany). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 1.0 mg/mL of ferric chloride. The mixture absorbance was measured at 700 nm (higher absorbance indicates higher reducing power) in an spectrophotomer (Unicam UV-Visible Spectrometry Heλios, United Kingdom). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance against extract concentration in the solution.

3. Results and discussion

3.1. Pollen analysis

The pollen profile obtained for the two propolis samples are present in Table 1. Marked differences were found among propolis samples of different origin. *Castanea sativa* was the most predominant pollen in Bornes propolis, representing 45% of the total pollen composition, being absent in Fundão propolis. On the other hand, *Populus tremula* was the main pollen in samples of Fundão (50% of the total pollen composition), while in Bornes propolis was the second predominant pollen (30%). *Pinus* sp. was observed only in Fundão samples (Table 1) and corresponds to the second most predominant pollen.

3.2. Total phenolic contents

The total phenols content in propolis extracts was different according to the provenience region (Table 2). Bornes propolis showed the high amount of these compounds, with 329.00 mg/g of GAE, twice the value found in the Fundão propolis (151.00 mg/g of GAE).

Table 1
Pollen composition (%) of different propolis samples

Species	Pollen composition (%)	
	Bornes	Fundão
Populus tremula	30	50
Castanea sativa	45	0
Pinus sp.	0	15
Others[a]	25	35

[a] Some of pollens with predominance less than 5%.