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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 EC₅₀ 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.

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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, anesthetic 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 (fuchsin 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-Ciocalteu re-

agent and 0.5 mL of 10% Na₂CO₃ was kept in the dark at room temperature for 1 h, after which the absorbance was read at 700 nm (Unicam UV-Visible Spectrometry He λ ios, 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).

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 He λ ios, United Kingdom). DPPH scavenging effect was calculated as a percentage of DPPH discoloration 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 (EC₅₀) 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 650g 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 a spectrophotometer (Unicam UV-Visible Spectrometry He λ ios, United Kingdom). Extract concentration providing 0.5 of absorbance (EC₅₀) 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).



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

Table 1
Pollen composition (%) of different propolis samples

Species	Pollen composition (%)	
	Bornes	Fundão
<i>Populus tremula</i>	30	50
<i>Castanea sativa</i>	45	0
<i>Pinus</i> sp.	0	15
Others ^a	25	35

^a Some of pollens with predominance less than 5%.

Propolis is commercialized in different parts of the world and it is recognized as an important source of compounds with pharmacological properties (Fujimoto, 1992; Miyataka et al., 1997). Bornes samples showed the highest values of total phenols in comparison to propolis samples from other countries; high total phenols amounts were also found in Chinese samples from Hebei, 302 ± 4.3 mg/g of GAE (Ahn et al., 2007), and Hubei, 299 ± 0.5 mg/g of GAE (Kumazawa et al., 2004); and Korean propolis from Yeosu, with 212.7 ± 7.4 mg/g of GAE (Choi et al., 2006). Even the total phenols obtained from Fundão samples, from the central region of Portugal, was half the value of total phenols in registered in Bornes samples (151 mg/g of GAE). Its amount was higher than the propolis from Brazil, with 120 ± 3.5 mg/g of GAE (Choi et al., 2006) and Thailand, with 31.2 ± 0.7 mg/g of GAE (Kumazawa et al., 2004).

Some other studies were developed concerning the phenolic composition of propolis samples (Marcucci and Bankova, 1999; Tazawa et al., 2000). The compounds reported by different authors are caffeic acid; *p*-coumaric acid; 3,4-dimethoxycinnamic acid; quercetin; pinobanksin 5-methyl ether; apigenin; kaempferol; pinobanksin; cinnamylidenacetic acid; chrysin; pinocembrin; galangin; pinobanksin 3-acetate; phenethyl caffeate; cinnamyl caffeate; tectochrysin; artepillin C (Marcucci and Bankova, 1999; Medic-Saric et al., 2004; Kumazawa et al., 2004).

3.3. Scavenging of DPPH radicals

The scavenging activity on DPPH radicals has been widely used to determine the free radical-scavenging activity of different matrices by our research group (Pereira et al., 2006; Sousa et al., 2008; Oliveira et al., 2007, 2008). DPPH is a stable free radical that is dissolved in methanol and its purple colour shows a characteristic absorption at 517 nm. Antioxidant molecules scavenge the free radical by hydrogen donation and the colour from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Hatano et al., 1989). In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength.

In this work the pattern of DPPH radicals inhibition showed a concentration-dependent pattern for both propolis samples (Fig. 2). Propolis from Bornes region presents a very high scavenging activity at very low extract concentration: at 0.001 mg/mL of propolis extract the scavenging activity was 33% and at 0.020 mg/mL reaching 94% of scavenging activity. However from Fundão propolis the activity was weak. These propolis only showed a weak scavenging effects at 0.007 mg/mL, with 1% of scavenging activity, while Bornes propolis had 57% of scavenging activity. The same situation was observed at 0.020 mg/mL where extract propolis from Fundão region was only 18% of scavenging activity (Fig. 2).

The EC_{50} values were very low indicating a high antioxidant activity for both propolis extracts. The EC_{50} value for Bornes extract was 0.006 mg/mL, while for Fundão extract was 0.052 mg/mL,

Table 2
Scavenging effect and reducing power EC_{50} values (mg/mL), and total phenol content (mg/g) of propolis extracts from the two regions

Propolis origin	DPPH antioxidant activity (mg/mL)	Reducing power (mg/mL)	Total phenols (GAE) (mg/g)
Bornes	0.006 ± 0.003	0.009 ± 0.001	329.00 ± 0.01
Fundão	0.052 ± 0.003	0.055 ± 0.001	151.00 ± 0.01

approximately 10 times greater than the first value (Table 2). These activity results were in accordance with the results total phenols found in the two different propolis origin. Bornes samples presented a very high concentration of total phenols and also a strong scavenging activity against DPPH radicals. The total phenols content was considered the main responsible from the antioxidant activity of different extracts (Kumazawa et al., 2004). In fact, Pereira et al. (2008) and Sousa et al. (2008) proved that the EC_{50} values were statistically correlated with total phenols content in their analysed samples.

3.4. Reducing power

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form (Fe^{2+}) monitored at 700 nm (Sousa et al., 2008). In this assay, the yellow colour of the test solution changes to green depending on the reducing power of the sample tested. In both analysed propolis samples, a concentration-dependent activity was observed (Fig. 3). When extract concentrations increase the absorbance at 700 nm also increases. At very low extract concentrations, for example 0.1 mg/mL, high values of absorbance were observed, namely 0.950 for Fundão propolis and 1.582 for Bornes sample. The results obtained in our work were much better than the results obtained in Brazilian propolis by Wang et al. (2004). These authors, for the same propolis extract concentration, obtained approximately half of activity, when we compare their results with the ones obtained with Fundão propolis, and approximately 30% of the activity of Bornes propolis.

In the present work, EC_{50} values obtained for methanol extracts were of 0.009 mg/mL, for Bornes propolis samples, and 0.055 mg/mL to the Fundão propolis samples. In general, extracts with high

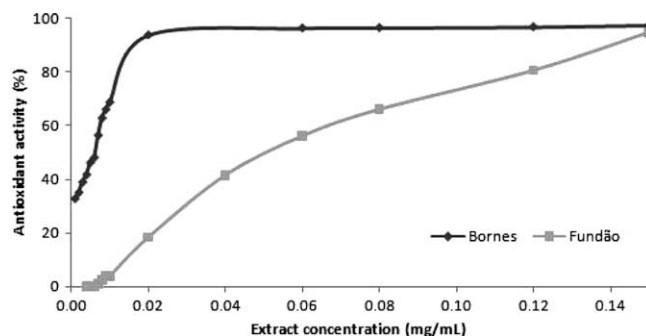


Fig. 2. Scavenging effect of propolis extracts from Bornes and Fundão regions.

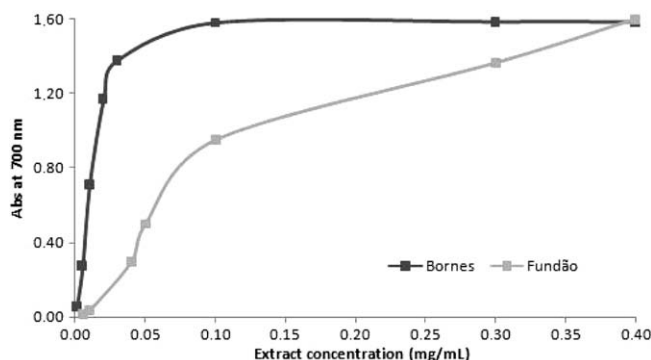


Fig. 3. Reducing power values of propolis extracts from Bornes and Fundão regions.

total phenols content presented lower EC₅₀ values in reducing power assay (Table 2).

The results obtained in our work allow a preliminary pollen characterization of Portuguese propolis. We demonstrate also the high concentration in total phenols and a strong antioxidant activity of the Portuguese propolis samples.

Conflict of interest statement

The authors have declared that there is no conflict of interest.

References

- Abd El Hady, F.K., Hegazi, A.G., 2002. Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. *Z Naturforsch* 57, 386–394.
- Ahn, M., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T., 2007. Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chemistry*, 1383–1392.
- Amoros, M., Lurton, E., Boustie, J., Girre, L., Sauvager, F., Cormier, M., 1994. Comparison of the anti-herpes simplex virus activities of propolis and 3-methylbut-2-enyl caffeate. *Journal of Natural Products* 64, 235–240.
- Bankova, V., Popova, M., Bogdanov, S., Sabatini, A., 2002. Chemical composition of European propolis: expected and unexpected results. *Z Naturforsch* 57c, 530–533.
- Barth, O.M., Dutra, V.M.L., Justo, R.L., 1999. Pollen analysis of some samples of propolis from Southern Brazil. *Ciência Rural* 29, 663–667.
- Choi, Y.M., Noh, D.O., Cho, S.Y., Suh, H.J., Kim, K.M., Kim, J.M., 2006. Antioxidant and antimicrobial activities of propolis from several regions of Korea. *Lwt-Food Science and Technology* 39, 756–761.
- Fujimoto, T., 1992. Qualitative and quantitative characteristics of propolis and its products. *Honeybee Science* 13, 145–150.
- Halliwell, B., 1997. Antioxidants in human health and disease. *Annual Review of Nutrition* 16, 33–50.
- Halliwell, B., Gutteridge, J.M.C., 1999. *Free Radicals in Biology and Medicine*. Oxford University Press, United Kingdom.
- Hatano, T., Edamatsu, R., Mori, A., Fujita, Y., Yasuhara, T., Yoshida, T., Okuda, T., 1989. Effects of the interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-picrylhydrazyl radical. *Chemical and Pharmaceutical* 37, 2016–2021.
- Kim, K.T., Yeo, E.J., Han, Y.S., Nah, S.Y., Paik, H.D., 2005. Antimicrobial, anti-inflammatory, and anti-oxidative effects of water- and ethanol-extracted Brazilian propolis. *Food Science and Biotechnology* 14, 474–478.
- Kimoto, T., Aga, M., Hino, K., Koya-Miyata, S., Yamamoto, Y., Micallef, M.J., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M., 2001. Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. *Anticancer Research* 21, 221–228.
- Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S., 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *Journal of Ethnopharmacology* 64, 235–240.
- Kumazawa, S., Taniguchi, M., Suzuki, Y., Shimura, M., Kwon, M.S., Nakayama, T., 2002. Antioxidant activity of polyphenols in carob pods. *Journal of Agricultural and Food Chemistry* 50, 373–377.
- Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. *Food Chemistry* 84, 329–339.
- Lahouel, M., Boulkour, S., Segueni, N., Fillastre, J.P., 2004. Effet protecteur des flavonoides contre la toxicité de la vinblastine, du cyclophosphamide et du paracetamol par inhibition de la peroxydation lipidique et augmentation du glutathion hépatique. *Pathologie Biologie* 52, 314–322.
- Marcucci, M.C., Bankova, V., 1999. Chemical composition, plant origin and biological activity of Brazilian propolis. *Current Topics in Phytochemistry* 2, 115–123.
- Matsuno, T., 1995. A new clerodane diterpenoid isolated from propolis. *Zeitschrift für Naturforschung* 50c, 93–97.
- Medic-Saric, M., Jasprica, I., Mornar, A., Smolcic-Bubalo, A., Golja, P., 2004. Quantitative analysis of flavonoids and phenolic acids in propolis by two-dimensional thin layer chromatography. *Journal of Planar Chromatography – Modern TLC* 17, 459–464.
- Miyataka, H., Nishiki, M., Matsumoto, H., Fujimoto, T., Matsuka, M., Satoh, T., 1997. Evaluation of propolis I. Evaluation of Brazilian and Chinese propolis by enzymatic and physico-chemical methods. *Biological and Pharmaceutical Bulletin* 20, 496–501.
- Oliveira, I., Sousa, A., Valentao, P., Andrade, P.B., Ferreira, I.C.F.R., Ferreres, F., Bento, A., Seabra, R., Estevinho, L., Pereira, J.A., 2007. Hazel (*Corylus avellana* L.) leaves as source of antimicrobial and antioxidative compounds. *Food Chemistry* 105, 1018–1025.
- Oliveira, I., Sousa, A., Ferreira, I.C.F.R., Bento, A., Estevinho, L., Pereira, J.A., 2008. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. *Food and Chemical Toxicology* 46, 2326–2331.
- Pereira, A.D.S., Seixas, F.R.M.S., Aquino Neto, F.R., 2002. Própolis: 100 years of research and future perspectives. *Química Nova* 25, 321–326.
- Pereira, J.A., Pereira, A.P., Ferreira, I.C.F.R., Valentão, P., Andrade, P., Seabra, R., Estevinho, L., Bento, 2006. Phenolics compounds, antioxidant potential and antimicrobial activity of table olives from Portugal. *Journal of Agricultural and Food Chemistry* 54, 8425–8431.
- Pereira, J.A., Oliveira, I., Sousa, A., Ferreira, I., Bento, A., Estevinho, L., 2008. Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. *Food and Chemical Toxicology* 46, 2103–2111.
- Shi, X., Dalal, N.S., 1991. Antioxidant behaviour of caffeine: efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology* 29, 1–6.
- Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods of Enzymology* 299, 152–178.
- Sousa, A., Ferreira, I.C.F.R., Barros, L., Bento, A., Pereira, J.A., 2008. Effect of solvent and extraction temperatures on the antioxidant potential of traditional stoned table olives 'alcaparras'. *LWT-Food Science and Technology* 41, 739–745.
- Stone, W.L., Leclair, I., Ponder, T., Bags, G., Barret-Reis, B., 2003. Infants discriminate between natural and synthetic vitamin E. *American Journal of Clinical Nutrition* 77, 899–906.
- Tazawa, S., Warashina, T., Noro, T., 2000. On the chemical evaluation of propolis. *Natural Medicines* 54, 306–313.
- Wang, B., Lien, Y., Yu, Z., 2004. Supercritical fluid extractive fractionation – study of the antioxidant activities of propolis. *Food Chemistry* 86, 237–243.
- Wang, L., Mineshita, S., Ga, I., Shigematsu, T., Matsuno, T., 1993. Antiinflammatory effect of propolis. *Japanese Journal of Pharmacological Therapeutics* 24, 223–224.