



Comparative study of different Portuguese samples of propolis: Pollinic, sensorial, physicochemical, microbiological characterization and antibacterial activity

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ARTICLE INFO

Article history:

Received 28 July 2012

Accepted 29 August 2012

Available online 5 September 2012

Keywords:

Antimicrobial

Microbiological quality

Physicochemical characterization

Propolis

Sensorial analysis

ABSTRACT

The aim of this work was to study four propolis samples from Trás-os-Montes region of Portugal. The propolis samples' color was different, which pollen analysis showed to be due to different botanical sources: *Populus* sp., *Pinus* sp., *Quercus* sp. and *Castanea sativa*. The data from physicochemical analysis (moisture, soluble and insoluble solids content, pH, conductivity, ash content, wax, total phenolics and flavonoids content) was treated using multivariate statistical tools as cluster heat map, principal components analysis and linear discriminant analysis with the purpose of classifying the sample accordingly to the botanical/geographical origin. The discriminant analysis was applied with stepwise to select the variables that most contribute to sample identification accordingly to pollinic profile. The cross-validation technique was applied, using the leave-one-out procedure, which showed good prediction capabilities of the samples. Microbiologically, the commercial quality was satisfactory, since the samples didn't contain deterioration or pathogenic microorganisms. All the samples studied presented antimicrobial activity against Methicillin-resistant *Staphylococcus aureus*, in a dose dependent way. The antimicrobial activity was strictly related to the physicochemical composition. This work will allow connecting a particular chemical propolis type to a specific type of biological activity, what is essential for the use in therapeutic applications.

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1. Introduction

Propolis is a substance produced by bees (*Apis mellifera* L.) from harvested exudates of plant which are mixed with the salivary enzyme β -glucosidase. Bees use propolis in their combs as protection, to repair damage, to build aseptic locals and as a thermal insulator (Cardoso et al., 2011). Many studies have reported the efficiency of this product in medical treatments, both human and animal (Pastor et al., 2010; Carvalho et al., 2011). However, these studies have focused mainly the bioactive compounds' analysis and performed clinical assays, in order to ascertain the properties of this beehive product. These biological properties include antioxidant (Krol et al., 1990), anti-inflammatory (Paulino et al., 2008), anti-hepatotoxic (Banskota et al., 2001), anti-tumoral (Valente et al., 2011), anti-HIV-1 (Gekker et al., 2005) and cario-

static (Libério et al., 2009). The antimicrobial activity of propolis against a wide range of bacteria, fungi and viruses has been investigated lately. These studies have reported that *Staphylococcus aureus* is one of the bacterial species that appears to be susceptible to propolis (Kilic et al., 2005). Indeed, the growing number of resistant pathogens is a reminder that the clock is ticking on the lifetime of the usefulness of current antibiotics. In this context, the research on natural products, such as propolis, plays a key role.

It is important to remember that the properties of this beehive product are related with its chemical composition (Silva et al., 2012). The chemical composition of propolis is highly variable mainly due to the variability of plant species growing around the hive, from which the bees collect the exudates (Bankova et al., 2000). According to Kumazawa et al. (2004), in samples from Brazil terpenoids and prenylated derivatives of *p*-coumaric acids predominated whilst the samples from China and Europe mostly contained phenolic acid esters and flavonoids. Bankova et al. (2000) reported that more than 300 different compounds can be found in propolis, being the most important: aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids,

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vitamins and inorganic substances. This is the reason why it is very important to study the composition of propolis from different regions.

In addition, in comparison to other countries, like Argentina, China, Australia and Bulgaria, Portugal is a small producer of propolis, being its production greatly confined to artisans. Propolis from Portugal has only recently begun to be studied; therefore, relatively little is known about its chemical composition, botanical origins, and biological properties. Portuguese propolis is currently being commercialized by less than 50€ per kilogram, whereas the product from other countries costs about 150€ per kilogram (Pereira et al., 2002). However, in order to economically value this product and to make it accepted officially into the mainstream of the health system, it's essential to ensure the chemical standardization, concerning its quality and safety (Bankova, 2005).

The present study was conducted in order to determine the chemical composition, to assess the safety, quality and sensorial characteristics of four samples of Portuguese propolis, obtained in locals with different predominant vegetation. The antimicrobial activity against methicillin-resistant *S. aureus* (MRSA), isolated from different biological fluids, was also assessed. In this context, the scientific research will act like a vector of the development of the national economy, since this study will help to certify the quality and the composition of propolis.

2. Material and methods

2.1. Chemicals and reagents

Ethanol ($\text{CH}_3\text{CH}_2\text{OH}$) was supplied by Pronolab (Lisbon, Portugal). The Folin–Ciocalteu reagent, chloroform (CHCl_3), sodium carbonate (Na_2CO_3), gentamicin and fluconazol were obtained from Merck (Darmstadt, Germany). Gallic acid and catechin were purchased from Sigma (St. Louis, MO, USA). The culture mediums were purchased from Himedia (India). The TTC solution (2,3,5-triphenyl-2H-tetrazolium chloride) was supplied by Fluka (Buchs, Switzerland). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the reagents were of analytical grade purity. The culture mediums were bought from Himedia (India). High purity water (18 M Ω cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Propolis samples

Propolis samples were collected by beekeepers between August and September of 2011 from four places of the Trás-os-Montes region in the extreme northeast corner of Portugal: Mirandela (41°29'N 7°11'W), Mogadouro (41°20'N 6°43'W), Nogueira (41°46'N 6°49'W) and Vinhais (41°49'N, 7°0'W). Considering the geographic differences, the Trás-os-Montes region can be grouped into two areas: the "Terra Fria Transmontana" (Cold Lands) is the northern area, with the higher altitudes, where the landscape is dominated by gentler slopes in the plateau, and the "Terra Quente Transmontana" (Hot Lands), the southern areas, marked by the valleys of the Douro River. These two areas have significant climate differences, which gives a wide diversity of landscapes. The locals Vinhais and Nogueira are in Cold Lands separated by 20 km. Mirandela and Mogadouro are locals separated by 70 km in the Hot Lands.

It were collected three samples per location ($n = 12$) and for each sample it were performed three replicates. They were obtained after the honey extraction by scratching the hive walls and frames. Each sample was inspected in order to find rests of bees, wood, plant, pupa of moth, among others. The major visible impurities were removed from the samples. Each sample was weighed and frozen at -20°C until analysis.

2.3. Sensorial analysis

The propolis samples were submitted to sensorial analysis as recommended by the Brazilian Ministry of Agriculture (Instrução Normativa nº 3, de 19 de Janeiro de 2001), since in Portugal there is no legislation concerning propolis. Panel members ($n = 20$) aged between 18 and 58 years were selected among undergraduate and graduate students, as well as employees and lecturers belonging to the School staff. The parameters analyzed were the color, aroma, consistency and granulometry.

2.4. Pollinic analysis

The pollen morphology identification is very important since it allows characterizing the geographical regions where propolis was obtained, as well as the habitat of forest, fields and crops. The pollen analysis was executed by the methodology described by Barth (1989). The pollen attainment was initiated by mixing of 0.5 g of

propolis with 15 mL of absolute alcohol during 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 glacial acetic acid during a night. Then acetolise of pollen sediments was carried out in a mixture 9:1 of acetic acid and anhydride sulphuric acid, 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 (fuchisine basic).

2.5. Physicochemical analysis

All the tests were performed in triplicate.

2.5.1. Moisture

The determination of moisture was ascertained by the standard method (AOAC Official Method 934.01). Five grams of propolis were dried in a mechanical convection oven at 105°C for 1 h. After this time, it was removed and let cool to room temperature and weight back. The procedure was repeated just to stabilize the weigh. The water content was determined using the equation, in which $A1$ = weigh of sample; $A2$ = weigh of sample dried:

$$\text{Moisture (\%)} = \frac{100 \times [A1 - A2]}{A1}$$

2.5.2. Soluble and insoluble solids in ethanol

According to AOAC (2005), to one gram of each propolis sample, 250 ml of ethanol was added. The mixture was shaken in an automatic mixing machine and after 30 min the solution was filtered and the insoluble solids weighed. The soluble solids (SS) were determined by the difference between sample weigh (SW) and insoluble weigh (IW). The result was expressed in percentage, by applying the equation for soluble solids:

$$\text{SS (\%)} = \frac{\text{SW} - \text{IW}}{\text{SW}} \times 100$$

For insoluble solids:

$$\text{IS (\%)} = \frac{\text{IW}}{\text{SW}} \times 100$$

2.5.3. pH

Propolis pH was measured with a combined pH glass electrode connected to pH-meter Basic 20, in a solution prepared with 10 g of propolis in 75 mL of methanol (NP 1309/1976), using methanol as control. Calibration was performed with three standard buffer solutions.

2.5.4. Conductivity

Electrical conductivity of a propolis solution at 20% (w/v) (dry matter basis) in methanol was measured at 20°C in a Crison 522 conductimeter. Results were expressed as milliSiemens per centimeter (mS/cm).

2.5.5. Ash content

The method used in the experiments to determine the mineral content and other inorganic matter in propolis consisted of the desiccation of an amount of 5 g, for each propolis sample, in a platinum dish (AOAC Official Method 920.181). To do so, they were kept in the thermostat at 80°C for 4 h, after which the samples underwent calcination at 550°C , in an electric laboratory furnace SNOL 8.2/1100-1 (AB "Umega", Utena, Lithuania) to constant mass. Total ash contents, expressed as the percentage of residue left after dry oxidation by weight (%), was calculated from the following equation, where m_1 is the mass of dish and ash, m_2 the mass of platinum dish prior to calcination and m_0 is the mass of the propolis taken:

$$\text{Ash (\%)} = \left[\frac{m_1 - m_2}{m_0} \right] \times 100$$

2.5.6. Wax

It was weighed 250 g of each sample and added 750 ml of methanol. The mixture was placed in a freezer over-night (-20°C). Afterwards, the solution was filtered to obtain the wax. The wax was expressed in percentage (W%) using the sample weigh (SW) and the wax weigh (WW). The equation used was:

$$\text{W (\%)} = \frac{\text{WW}}{\text{SW}} \times 100$$

2.5.7. Determination of the bioactive compounds

2.5.7.1. Extraction. Prior to the extraction, the propolis was grounded and homogenized. The samples were extracted with 80% of ethanol/water (1/10, v/v) at 70°C for 1 h. The resulting mixtures were filtered, and the residues were re-extracted in the same conditions. After the second extraction, the filtrated solutions were combined, concentrated and frozen at -20°C (Silva et al., 2012).

2.5.7.2. Determination of the total phenolics. The total phenolic content in the extracts was evaluated indirectly by relating the reducing capacity of propolis and Gallic acid standard compound using the Folin–Ciocalteu method as described by Moreira et al. (2008). Briefly, a dilute solution of each propolis in EtOH (EtOH–propolis; 500 µL of 1:10 g/mL) was mixed with 500 µL of Folin–Ciocalteu reagent and 500 µL of Na₂CO₃ (10% w/v). After incubation in dark at room temperature for 1 h, the absorbance of the reaction mixture at 700 nm was determined against the blank (the same mixture without the EtOH–propolis) using a Unicam Helios Alpha UV–visible spectrometer (Thermo Spectronic, Cambridge, UK). Galic Acid standard solutions (0.01–0.08 mM) were used for constructing the calibration curve ($y = 2.3727x + 0.0022$; $R^2 = 0.9998$). Total phenols content were expressed as mg of Galic Acid equivalents per g of propolis (GAEs).

2.5.7.3. Determination of flavonoids. To determine the flavonoids it was used the aluminum chloride method. In briefly, EtOH–propolis (250 µL) was mixed with 1.25 mL of distilled H₂O and 75 µL of a 5% NaNO₂ solution. After 5 min, 150 µL of a 10% AlCl₃·H₂O solution was added. After 6 min, 500 µL of 1 M NaOH and 275 µL of distilled H₂O were added to the mixture and vortexed. The intensity of pink color of the reaction mixture at 510 nm is determined against the blank (the same mixture without the EtOH–propolis). Catechin standard solutions (0.022–0.34 mM) were used for constructing the calibration curve ($y = 0.9689x - 0.0092$; $R^2 = 0.9987$). Total flavonoids content were expressed as mg of catechin equivalents per g of propolis (CAEs).

2.6. Microbiological determinations

It were researched the commercial quality parameters: mesophilic microorganisms, yeasts and moulds, indicators of sanitary quality: fecal coliforms and *Escherichia coli*, indicators of safety: sulphite reducing clostridium spores and *Salmonella*. All microbial tests were performed in triplicate.

2.6.1. Sample preparation

Prior to the microbiological assays, 10 g of each propolis samples were aseptically taken and homogenized using a pre-sterilized Stomacher Lab-Blender (Seward type 400, London, UK) for 3 min with 90 mL of pre-chilled (4 ± 0.5 °C) sterile peptone–physiological saline solution (0.1% neutral peptone + 0.85% NaCl in sterile deionized H₂O, pH = 7.0 ± 0.05). Decimal serial dilutions were prepared from this homogenate in the same chilled sterile diluents (1:10 (w/w)).

2.6.2. Enumeration of the total mesophilic microorganisms

The aerobic mesophilic microorganisms were counted by incorporation of 1 mL of each dilution into standard Plate Count Agar (PCA), as recommended in NP-3788 (2002) – Portuguese Legislation. Microbial counts were expressed as colony-forming units per gram of propolis (cfu/g).

2.6.3. Enumeration of yeast and moulds

Moulds and yeasts enumeration was made on DG18 (Himedia) and incubated at 25 °C for 5 days (ISO 21527-2, 2008). Microbial counts were expressed as colony-forming units per gram of propolis (cfu/g).

2.6.4. Enumeration of total coliforms and *Escherichia coli*

Enumeration of coliforms and *Escherichia coli* was done using the SimPlate CEC-Cl method (AOAC® Official Method 2005.03) with multiple test medium (BioControl System), according to the manufacturer's instructions and procedures of Feldsine et al. (2005). One ml from the basic dilution was placed in the centre of the SimPlate plating device, and 9 ml of a mixed nutrient agar with blue color was added at the same spot. The SimPlate was rotated in order to disperse the sample and remove air-bubbles. The SimPlates were stacked and stored at 37 ± 1 °C for 24–28 h. Wells were counted positive for total coliforms on the basis of the color change and counted positive for *E. coli* on the basis of color change and fluorescence under UV light. The coliform and *E. coli* populations were determined on the basis of the number of positive wells correlated with the SimPlate conversion table, which generated a most probable number (MPN) per gram of sample.

2.6.5. Determination of sulphite reducing clostridium spores

For sulphite-reducing clostridia counting, aliquots of 10, 5, 1 and 0.1 mL of the initial suspension were added to an empty tube, thermally treated at 80 °C for 15 min and covered with Differential Reinforced Clostridial Broth (DRCM) (Himedia), and incubated at 37 °C for 5 days. At the end, it were counted the black colonies. The results are expressed as presence of Sulphite-reducing clostridia in 0.01 g (ISO 15213, 2003). Results were expressed as most probable numbers of Sulphite-reducing Clostridium spores per gram of bee pollen (MPN/g).

2.6.6. Determination of *S. aureus*

The detection was performed according to the protocol of NP 4400-1 (2002). Following the existent legislation, serial dilutions of the sample were inoculated in Baird-Parker Broth with Egg Yolk Tellurite and Sulfadimidine Solution (Himedia)

during 24 h (37 °C). After, it were selected 3–5 characteristic colonies, in order to testify the presence of coagulase and catalase. Microbial counts were expressed as colony-forming units per gram of propolis (cfu/g).

2.6.7. Detection of *Salmonella* sp.

The detection of *Salmonella* sp. in the samples was carried out using the immunodiffusion 1–2 test, which is a patented single-use test for *Salmonella* used by analysts worldwide and recognized as the AOAC® Official Method 989.13. Rapid and reliable results are obtained 16–20 h after pre-enrichment in buffered peptone water. The results are interpreted visually by observing the development of an immunoband, a characteristic immobilization pattern of cells.

2.7. Antimicrobial activity assays

The Methicillin-resistant *S. aureus* used in the antimicrobial activity assays were isolated from biological fluids (in the Hospital Centre) and identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança. Microorganism A was isolated from vaginal exudates, B from urine, C from pus, D from expectoration and E from blood culture. Microorganism F is a reference culture (ATCC 6538™), obtained from the authorized distributor of ATCC (LGC Standards S.L.U., Barcelona). A broth microdilution bioassay in sterile 96-well plates (Sarker et al., 2007) was used to evaluate the growth-inhibiting activity of the test materials toward the organisms. In brief, 100 µL of each test material in MHB medium containing 2.5% dimethyl sulfoxide (DMSO) were added to wells of the first rows of plates, and 50 µL MHB medium was added to remain wells. Serial twofold dilutions of the test materials were prepared. The bacterial suspension (50 µL) containing 1×10^5 cfu mL⁻¹ of each test organism was added to all the wells except those used for backgrounds and blanks that included test samples and DMSO-containing medium only, respectively. Then, 50 µL of medium was added into the background and blank wells. Gentamicin served as positive control.

Antimicrobial activity was detected by adding 20 µL of 0.5% TTC solution. Minimum inhibitory concentration (MIC) end-points were interpreted as the lowest sample concentration that remained unstained by TTC (indicating no growth) or the first dilution that changed to slightly purple (equivalent to prominent growth inhibition). The assays were repeated three times.

2.8. Statistical analysis

Results are shown as mean values and standard deviation. Correlation coefficients among the different variables were computed using the Pearson correlation coefficient. Cluster Heat map was done with software R (R Development Core Team, 2012). The results and graphs from the application of principal component analysis and linear discriminant analysis to the physicochemical parameters were obtained using the software JMP Pro 10.0.0. In the microbiological determinations and antimicrobial activity, the differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD. The p values less than or equal to 0.05 were evaluated as statistically significant. This treatment was carried out using SPSS version 18 (IBM Software).

3. Results and discussion

3.1. Sensorial analysis

The results of the sensorial analysis (color, aroma, consistency and granulometry) are presented in Table 1. All propolis samples showed heterogeneity in their constitution. The color differences between samples (the samples from Cold Lands are darker) showed that the main vegetable origin of propolis formulation is different, which is supported by each sample pollinic profile. Indeed, Fontana et al. (2004) claimed that the contribution of the flora to propolis plus the wax content may explain the physical properties of the product. The balsamic aroma is present in three samples, except in Mirandela sample, which has a resinous aroma (*Populus* sp. is the main pollen). Propolis from Mirandela and

Table 1
Sensorial characteristics of propolis samples.

Sample region	Color	Aroma	Consistency	Granulometry
Mirandela	Green	Resinous	Manageable	Heterogeneous
Mogadouro	Red–yellow	Balsamic	Hard	Heterogeneous
Nogueira	Red–Brown	Balsamic	Manageable	Heterogeneous
Vinhais	Brown	Balsamic	Hard	Heterogeneous

Nogueira have manageable consistency (*Populus* sp. and *Quercus* sp. as main pollen, respectively). The samples from Mogadouro and Vinhais presented hard consistency (*Pinus* sp. and *Castanea sativa* as main pollen, respectively).

3.2. Pollinic analysis

The three most predominant pollens and the percentage of number of pollen grains counted in each sample are shown in Table 2. The total percentage of the three main pollens, in each sample, is higher than 82%. In the four samples, the most predominant pollen has percentages between $46.5 \pm 6.6\%$ and $58.0 \pm 7.1\%$, the second predominant pollen in counts are between $17.5 \pm 2.3\%$ and $22.0 \pm 4.0\%$ and the third predominant pollen, lower than 14%. The pollen profile considering the main three pollens is described by five types of pollen: *C. sativa*, *Erica* sp., *Pinus* sp., *Populus* sp., *Prunus* sp. and *Quercus* sp. Considering the main pollen (counts higher than 46.5%), the four propolis samples have different vegetation origin: *Populus* sp. (Mirandela sample), *Pinus* sp. (Mogadouro sample), *Quercus* sp. (Nogueira sample) and *C. sativa* (Vinhais sample). All the samples had great percentages of *Populus* sp. This is in agreement with the reported by Falcão et al. (2010), who analyzed samples from the temperate zones, among which Portugal.

3.3. Chemical composition

In the existent studies on the chemical composition of European propolis it have only been determined and identified the phenolic compounds and flavonoids (Popova et al., 2004; Moreira et al., 2008; Falcão et al., 2010; Salonen et al., 2012; Silva et al., 2012). However, parameters like moisture, total content in insoluble substances and soluble substances, pH, conductivity, ash and wax have never been tested. The results obtained for the physicochemical parameters are shown in Table 3. Globally, the precision of the triplicate assays reflects the samples heterogeneity referred previously. The moisture results varied between $3.4 \pm 0.2\%$ and $5.4 \pm 0.4\%$. High moisture content was obtained for Nogueira and Mogadouro propolis samples and the lower value for the Vinhais sample. These results lead us to infer that the consistency of propolis, referred in sensory analysis, was not directly related to the humidity of the sample. Our values were slightly superior to the ones obtained by Bonvehi et al. (1994), who studied propolis from Uruguay and China.

Total insoluble and soluble substances results showed that, in the triplicate assays, the precision was reasonable since the relative standard error percentages are lower than 7.0%. Mirandela sample has the higher content in soluble substances ($71.1 \pm 2.2\%$), Mogadouro and Nogueira samples have similar results (both have values near 66%) and Vinhais sample the lowest value ($60.7 \pm 5.1\%$). The sum of these two parameters showed to be nearby the expected value, 100%, except in one assay of Nogueira sample where the difference has a high value (this sample presented high variability in total insoluble substances parameter, with relative standard error percentage of 7.0%), maybe due to sample higher heterogeneity within this assay.

The pH values of the propolis samples are in the range of 4.7 ± 0.1 to 5.3 ± 0.2 . Mirandela and Vinhais samples gave similar and higher levels of pH (5.3), while the lowest pH value (4.7) was obtained in the Nogueira sample. Our results are in agreement with the claimed by Sousa et al. (2007). Tikhonov et al. (1978) found slightly higher values of pH, being the mean value equal to 6.20 ± 0.15 .

The conductivity of propolis was analyzed and results were similar and higher in the samples from Nogueira and Mogadouro (2.2 ± 0.2 and 2.4 ± 0.3 mS/cm, respectively) and the lower result (half of the value of the other sample obtained in the same geographic area) was obtained in the sample of Mirandela (1.2 ± 0.1 mS/cm). These results cannot be explained by the composition in minerals of the samples (ash content), since the amounts of ash showed a different trend from that obtained for conductivity. In decreasing order of ash content, the samples are: Vinhais ($2.2 \pm 0.08\%$), Nogueira ($2.0 \pm 0.3\%$), Mirandela ($1.9 \pm 0.1\%$) and Mogadouro ($1.6 \pm 0.02\%$). The order presented shows that this parameter allows to separate the samples obtained in the Cold Lands from the ones of the Hot Lands in the region of Trás-os-Montes. The content in ashes found in this study was similar to the reported in Chinese propolis (Bonvehi et al., 1994).

The quantity of wax is very different between the samples. The results, in mass percentage, varied in the range of $4.8 \pm 0.4\%$ to $16.0 \pm 2.1\%$. These data are coherent with the reported in the literature, since the maximum wax content found was 15 g/100 g (Silva et al., 2012). According to Negri et al. (1998), the composition of propolis wax is similar to that of comb wax, which suggests that propolis waxes are secreted by bees, rather than originating from plants.

The total quantity of polyphenols and flavonoids in propolis are important parameters to evaluate the quality and biological potentialities of the product (Moreira et al., 2008). The specific phenolic composition of propolis is extremely dependent on the plants found around the hive, as well on the geographic and climatic characteristics of the place (Bankova et al., 2000). The results show differences among samples that are in accordance with their local of origin. The samples obtained in the Cold Lands (Nogueira and Vinhais) have higher content of phenolic compounds and flavonoids compounds (mass percentages, $>23.0 \pm 2.0\%$ and $>9.5 \pm 2.0\%$, respectively) than the samples of the Hot Lands (Mirandela and Mogadouro samples), whose quantities are either less than or equal to half of that obtained in previous samples. Our results demonstrate that these bioactive compounds are important constituents of the Portuguese propolis samples. In the same context, Kujumgiev et al. (1999) found flavanoids and phenolic acid esters as main constituents in Bulgarian samples. The amount of polyphenols and flavonoids obtained in this study is in agreement with the reported in the literature. The content of phenolic compounds obtained by Choi et al. (2006) in Korean propolis ranged between 12.0% and 21.2%. Silva et al. (2012) found values between 8.7% and 27.7% for the polyphenols and between 2.5% and 14.2% for flavonoids.

To verify how these results allow classifying and differentiating the four samples, it is presented in Fig. 1 the graph cluster Heat Map that displays the hierarchical cluster structure, simulta-

Table 2
Pollen profile of the propolis samples.

Sample region	Pollen type					
	<i>Castanea sativa</i>	<i>Erica</i> sp.	<i>Pinus</i> sp.	<i>Populus</i> sp.	<i>Prunus</i> sp.	<i>Quercus</i> sp.
Mirandela			11.00 ± 2.80	58.00 ± 7.10	17.50 ± 2.30	
Mogadouro	13.50 ± 1.80		46.50 ± 6.60	22.00 ± 4.00		
Nogueira	20.00 ± 2.00			9.00 ± 1.80		56.50 ± 6.80
Vinhais	49.50 ± 5.20	20.50 ± 3.90		14.00 ± 2.30		

Table 3Physicochemical characteristics of the samples (average \pm standard deviation).

Sample region	Moisture (%)	Insoluble substances (%)	Soluble substances (%)	pH	Conductivity (mS/cm)	Ash (%)	Waxes (%)	Phenolic compounds (%)	Flavonoid compounds (%)
Mirandela	3.4 \pm 0.2	27.0 \pm 1.0	71.1 \pm 2.2	5.3 \pm 0.2	1.2 \pm 0.1	1.9 \pm 0.1	7.6 \pm 0.7	11.1 \pm 1.3	3.1 \pm 0.3
Mogadouro	5.4 \pm 0.4	40.2 \pm 0.9	66.0 \pm 4.1	5.2 \pm 0.2	2.4 \pm 0.3	1.6 \pm 0.02	4.8 \pm 0.4	17.2 \pm 6.9	4.0 \pm 1.0
Nogueira	5.3 \pm 0.4	45.0 \pm 7.0	66.3 \pm 3.2	4.7 \pm 0.1	2.2 \pm 0.2	2.0 \pm 0.3	12.2 \pm 0.9	28.0 \pm 2.0	12.0 \pm 2.0
Vinhais	3.7 \pm 0.2	45.1 \pm 2.0	60.7 \pm 5.1	5.3 \pm 0.1	1.7 \pm 0.1	2.2 \pm 0.08	16.0 \pm 2.1	23.0 \pm 2.0	9.5 \pm 2.0

neously, of the propolis samples and physicochemical variables (rows and columns in the data matrix, respectively). The data matrix individual values are represented as colors being the larger values represented by small dark gray or black squares and smaller values by lighter squares. To avoid the variables dimension of being a factor of importance in the cluster analysis, the variables (columns) were normalized to the same dynamic range (Z-dimension).

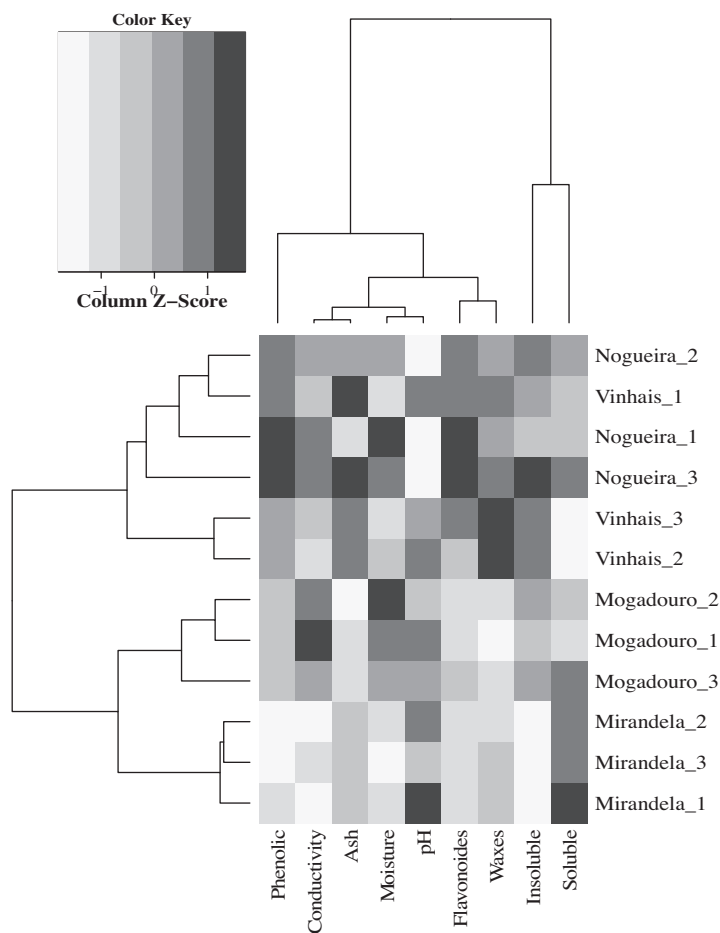
The Fig. 1 shows the samples divided in two groups accordingly to their areas of origin: in the Hot Lands propolis samples there is a separation between the two samples accordingly to its local of origin, as opposed to samples of Cold Lands, where the Vinhais sample was grouped together with Nogueira sample.

The Heat Map graph, for the physico-chemical variables shows three groups: the first consists of the variables of total content of soluble and insoluble substances in the samples; the second group consists of all other variables except the variable of total amount of phenolic compounds, which is the only variable in group 3. In the second group, the presence of the variable total amount of flavonoid

compounds was not expected since the variable of flavonoid compounds have a high correlation with the variable of the phenolic compound (Pearson coefficient correlation, $R = 0.94$).

Another unsupervised multivariate method that can show the natural data pattern is principal component analysis (PCA). After centralize and standardize the data matrix for each attribute, this method is just linear combinations of the original variables. Globally, the first two principal component variables (two dimensions which account for the maximum amount of variance) explain 77.2% of the data variability in the physicochemical variables and the extracted information clearly allows classifying propolis samples accordingly to their local geographic of origin.

Fig. 2 shows the projection of samples (the first plot) and the loadings of the original variables (the second plot) on the first two principal components. The first plot (score plot) shows that the first principal component allows separating the samples of different areas of origin: for higher values of PC1 are present samples of Nogueira and Vinhais (Cold Lands) and for negative values, samples of Mirandela and Mogadouro (Hot Lands). The second main

**Fig. 1.** Cluster Heat Map of the propolis samples and physicochemical parameters.

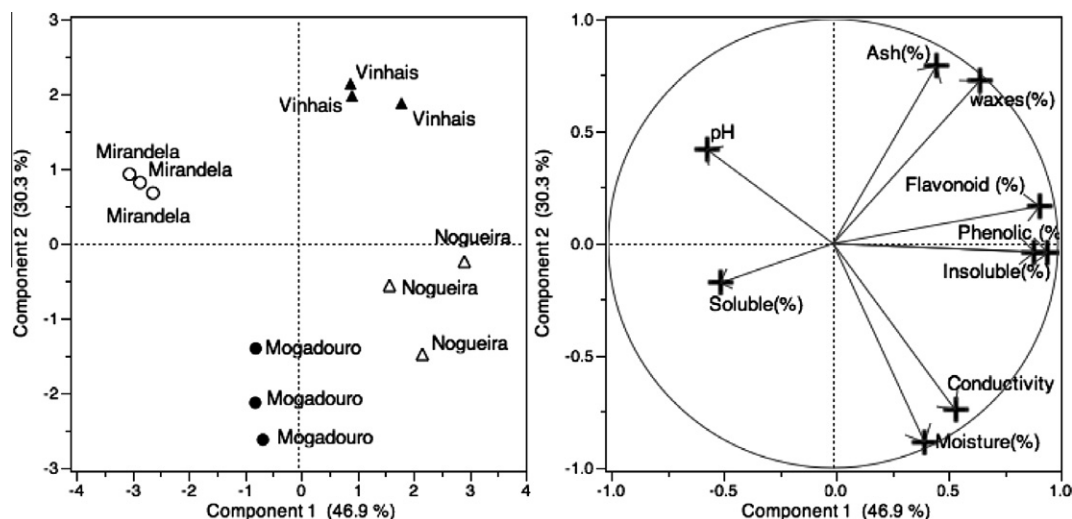


Fig. 2. Score plot (first plot) and the loading plot (physicochemical variables) on the first two principal components. Percentages of variance explained for each principal component are given in parentheses.

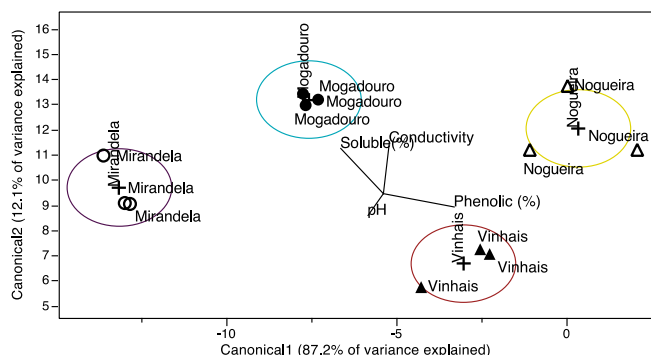


Fig. 3. Propolis samples LDA classification into four groups according to their locals of origin. Percentages of variance explained for each discriminant function are given in parentheses.

component allows identifying each propolis sample accordingly to the local of origin.

The orientation of the physicochemical variables in the second plot show how they contribute in the first two principal components to separate samples. The arrow shows the direction towards where a variable contributes with high values in the principal components space. Considering the extrapolation of the same arrow (in the same direction) but in the reverse way, it is where the variable contributes with low values.

Highlighting only the high values, Fig. 3 shows that: samples from Hot Land show high values of pH and total amount of soluble substances; the sample of Mirandela has higher values over the sample Mogadouro, which has high values of moisture% and conductivity values; for the two samples from the Cold Land it seems that the high values for variables Ash and waxes contribute to the separation of sample Vinhais (also with high pH) while sample Nogueira seems to be differentiated from other samples due to the remaining variables, mainly to high values of phenolic and flavonoid compounds content. The variables that have nearby projections in the loading plot shows similar contribution in the principal components obtained, indicating a possible redundancy in the information provided by these variables.

To determine which physicochemical variables were the most important to classify the four samples of propolis in relation to their local of origin, it was used a supervised method of classification, the

linear discriminant analysis (LDA) with stepwise (based on backward criterion with the probability (p -value) of 0.15 for enter and remove variables).

All independent variables selected by the stepwise procedure of the discriminant analysis were statistically significant according to the Wilks' Lambda test ($p < 0.001$). The stepwise LDA resulted in a discriminant model with three discriminant functions, being only the first two significant ($p < 0.001$ for the Wilks' Lambda test). These two functions explained 99.3% of the variance of the experimental data (the first function explain 87.2% and the second, 12.1%). The model was based in four physicochemical variables: pH, conductivity, soluble substances and phenolic compounds. The variables pH and total content in soluble substances remained important to distinguish samples of locals of Hot Land from those of Cold Land. From the remaining variables only two were selected – the conductivity and the amount of phenolic compounds – as being the most important for identifying the origin of propolis samples in Cold Land; high values of conductivity distinguish the Nogueira sample, as expected, from the Vinhais sample, which as high values of phenolic compounds.

Fig. 3 shows the graph of the first two functions obtained by linear discriminant analysis using stepwise to establish which physicochemical variables allow the identification/separation of the four samples of propolis. The model showed a very satisfactory classification performance allowing to correctly classifying 100% of propolis samples for the cross-validation technique, using the leave-one-out procedure.

These results are consistent with those obtained in principal component analysis in which the assessment of the variable loadings was done. The linear discriminant analysis proved to be advantageous because it allowed obtaining a mathematical model where the variables considered were selected, reducing the redundancy of information, and verify the model predictive ability by using leave-one-out procedure.

3.4. Microbiological determinations

The levels of microbial contamination of propolis are presented in Table 4. Studies about the microbiological contamination of this natural product have never been carried out. However, according to the Brazilian legislation the microbiological quality of propolis must be controlled (Instrução Normativa nº 3, de 19 de Janeiro de 2001).

Table 4

Microbiological analysis of the propolis samples.

Sample	Aerobic mesophiles (cfu g ⁻¹)	Moulds and yeast (cfu g ⁻¹)	Total coliforms (ufc/g)	<i>Escherichia coli</i> (ufc/g)	Sulphite-reducing clostridia (in 0.01 g)	Salmonella (in 25 g)	<i>Staphylococcus aureus</i> (ufc/g)
Mirandela	106.67 ± 23.09 b	63.33 ± 27.54 a	26.67 ± 11.55 b	<1 a	Absent	Absent	Absent
Mogadouro	71666.70 ± 51661.11 a	75.00 ± 18.03 a	113.33 ± 30.55 a	16.67 ± 5.77 a	Absent	Absent	Absent
Nogueira	1096.67 ± 430.16 b	56.67 ± 11.55 a	103.34 ± 28.86 a	<1 a	Absent	Absent	Absent
Vinhais	7933.33 ± 1792.57 b	73.33 ± 15.28 a	<1 b	<1 a	Absent	Absent	Absent

Results are given as the mean ± standard deviation. The letter (a) represents that the honeys are not different by Tukey test with significance of $\alpha = 0.05$.

The results obtained for the aerobic mesophiles were between 71666.70 ± 51661.11 cfu g⁻¹ and 106.67 ± 23.09 cfu g⁻¹ in the samples from Mogadouro and Mirandela, respectively. Significant differences were found between the samples from these origins. Concerning the moulds and yeasts, it were not found significant differences between the different propolis. The total coliforms were found in three of the samples, but in low number. For *E. coli* the obtained values were very small and statistically similar in all the analyzed products. All samples showed negative results for toxigenic species. The propolis with worse microbiology quality were the ones with higher moisture and pH, as expected. These results are in agreement with the reported by Gomes et al. (2010) and Horner and Anagnostopoulos (1973). The microbiological quality of propolis is worse than the quality of honey and bee pollen. This is justified by the almost exclusive dedication of the Portuguese apicultural sector to the production of the latter products. Indeed, the propolis' manipulation techniques are not currently the most adequate. However, since propolis has a great therapeutic potential, it is essential to study and monitor its quality.

3.5. Antimicrobial activity

Pathogenic microorganisms have developed multidrug resistance due to the indiscriminate use of antimicrobial drugs. This

Table 5Minimum Inhibitory Concentration (mg/mL) for Methicillin-resistant *S. aureus* (MRSA) and relation between the same specie (reference culture and isolated microorganisms).

Sample	Microorganism	Mean ± standard deviation	Microorganism's effect (p-value)
Mirandela	A	1.09 ± 0.43 a	* (0.011)
	B	0.88 ± 0.49 ab	
	C	0.65 ± 0.20 ab	
	D	1.04 ± 0.40 a	
	E	0.68 ± 0.19 ab	
	F	0.24 ± 0.17 b	
Mogadouro	A	1.43 ± 0.32 a	** (0.003)
	B	0.91 ± 0.32 ab	
	C	0.82 ± 0.16 ab	
	D	1.04 ± 0.40 ab	
	E	0.88 ± 0.49 ab	
	F	0.52 ± 0.18 b	
Nogueira	A	0.85 ± 0.38 a	NS (0.06)
	B	0.65 ± 0.20 ab	
	C	0.59 ± 0.23 ab	
	D	0.78 ± 0.43 ab	
	E	0.59 ± 0.21 ab	
	F	0.24 ± 0.17 b	
Vinhais	A	0.91 ± 0.32 a	** (0.0014)
	B	0.62 ± 0.26 ab	
	C	0.68 ± 0.20 ab	
	D	0.85 ± 0.38 a	
	E	0.72 ± 0.35 a	
	F	0.24 ± 0.16 b	

The letters a and b symbolise means that are significantly different. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$, NS – non-significant.

has led to the urgent need for new antibiotics to treat infections caused by this group of resistant bacteria. In this context, the research on the natural product with potential antimicrobial activity has mushroomed recently. Several authors have studied the antimicrobial activity of propolis. While some authors have found it active only against Gram-positive bacteria and some fungi (Maccucci, 1995; Kujumgiev et al., 1999), others found also activity against Gram-negative bacteria (Dobrowolski et al., 1991). In the present study it was assessed the antimicrobial activity of four types of propolis against Methicillin-resistant *S. aureus* isolated from different biological fluids. Indeed, according to the Center for Disease Control and Prevention (Atlanta, USA), methicillin-resistant *S. aureus* (MRSA) has been identified as an important nosocomial pathogen. It has also been reported that different strains of *S. aureus* differ in the range of diseases they can cause, including boils and pimples, wound infections, pneumonia, septicemia and food intoxication (Bayat et al., 2011).

The results for the antimicrobial activity of the samples are presented in Table 5. All the propolis samples studied presented antimicrobial activity, but this effect was dependent on the dosage and on the origin of the product. The sample from Nogueira was the most efficient in inhibiting the growth, since it was the one with lower MIC. This ranged between 0.24 ± 0.17 mg/mL for the reference culture and 0.85 ± 0.38 mg/mL for the strain isolated from vaginal exudates. Concerning the propolis from this origin, it weren't found significant differences between the tested microorganisms ($p = 0.06$). For the other propolis, the decreasing order of efficiency is: Vinhais > Mirandela > Mogadouro. In the samples from Mogadouro and Vinhais it were found highly significant differences between the effect of the propolis on the reference culture (ATCC 6538™) and on the *S. aureus* isolated from the vaginal exudates (A). In all the cases, the reference strain was the most sensitive to the propolis action and strain A the most resistant.

According to Koo et al., 2002 the antimicrobial effects of propolis are related with its flavonoids' content and consequently, vary according to the botanical origin of the product. In this study it was found that the products with higher concentration of polyphenols and flavonoids were also the ones with higher antimicrobial activity. These results are corroborated by Silva et al. (2012). Tsuchiya and Iinuma, 2000 suggest that those bioactive compounds increase the permeability of the inner bacterial membrane, nullifying its potential, decreasing the ATP production, the membrane transport and its mobility. Additionally, they inhibit the DNA gyrase which involves in the mechanism of DNA and RNA synthesis of bacteria (Mirzoeva et al., 1997).

4. Conclusions

Pollen analysis showed that the four propolis samples are from different vegetations: *Populus* sp. (Mirandela sample), *Pinus* sp. (Mogadouro sample), *Quercus* sp. (Nogueira sample) and *C. sativa* (Vinhais sample). The color parameter of sensory analysis allowed to distinguish the different samples because the differences show that the main vegetable origin of propolis formulation is different,

which is supported by the sample pollinic profile. The physico-chemical characterization of each sample of propolis, by using simple test procedures, showed to have important information about the samples thus allowing the identification of each sample accordingly to the geographical origin which is associated to a different pollen source, and therefore, to different sensory characteristics. By LDA it was possible to select the variables that most contribute to samples classification accordingly to pollinic profile, which in this work also corresponds to different locals of origin, and to verify that the model with four physicochemical variables has good identification performance by allowing 100% of propolis samples correctly classified. All the propolis studied presented antimicrobial activity, but this effect depended on the dosage and origin of the product. Future studies will be conducted in order to elucidate the mechanisms of action in the cell of this natural product.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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