Journal of Food and Nutrition Research, 2018, Vol. 6, No. 10, 645-654
Available online at http://pubs.sciepub.com/jfnr/6/10/5

DOI:10.12691/jfnr-6-10-5

Physicochemical, Antioxidant and Antimicrobial Properties of selected Portuguese Commercial Monofloral Honeys

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Received September 14, 2018; Revised October 21, 2018; Accepted November 13, 2018

Abstract The physicochemical, antioxidant and antimicrobial properties of selected Portuguese commercial honeys have been characterized aiming at establishing correlations between honey bioactivity and the physicochemical descriptors. All honey samples met the European regulations on honey quality criteria, including moisture and sugar content, free acidity, diastase activity, electrical conductivity, ashes, hydroxymethylfurfural and proline content. Antioxidant activity was evaluated based on phenolic and flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP) and oxygen radical antioxidant capacity (ORAC) assays. The honeys showed antibacterial and antifungal activity with minimum inhibitory concentrations (MIC) between 6.25–25% (w/v) and minimum fungicidal concentrations (MFC) in the range 12.5–50% (w/v), respectively. The bioactivity and physicochemical parameters of honey samples were correlated and depended on the honey floral source. The darkest honey, i.e. heather honey, showed the highest antioxidant and antimicrobial activities, which can be attributed to its higher phenolic, flavonoid and protein content.

Keywords: *portuguese honey, antioxidant, antimicrobial, phenolic compounds, flavonoids*

Cite This Article: João Gonçalves, Isabel Ribeiro, Joana Marçalo, Patrícia Rijo, Célia Faustino, and Lídia Pinheiro, "Physicochemical, Antioxidant and Antimicrobial Properties of selected Portuguese Commercial Monofloral Honeys." *Journal of Food and Nutrition Research*, vol. 6, no. 10 (2018): 645-654. doi: 10.12691/jfnr-6-10-5.

1. Introduction

Honey is a natural product produced from the nectar of flowers by honeybees (*Apis mellifera*) that has been used as a medicine throughout the centuries due to its antioxidant and antimicrobial properties [1,2]. Medicinal-grade honey, such as manuka (*Leptospermum scoparium*) honey from New Zealand and Revamil source honey from the Netherlands are currently employed in wound healing [3]. Honey has a broad-spectrum of antibacterial activity against both Gram-positive and Gram-negative microorganisms, including some drug-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), and also against some fungi and virus [2,4,5]. The bacteriostatic, bactericidal and biofilm growth inhibition properties of honey have been rediscovered mainly due to the emergence and development of bacterial resistance to conventional modern antimicrobial agents, which renewed the interest in this ancient natural remedy.

Honey is mainly a supersaturated solution of sugars (predominantly fructose and glucose) with low water content and minor concentrations of bioactive compounds, such as phenolic acids, flavonoids, carotenoids, amino acids (mainly proline), proteins (including enzymes), vitamins (especially vitamin C and B complex), minerals (e.g. Na, K, Ca, Mg, Cu, Fe, Zn, Mn, P, S), lipids, volatile aroma compounds and pollen grains. The chemical composition, physical properties and bioactivity of honey strongly depend on nectar composition of the flower source, climate, geographical origin, harvesting process and storage conditions [2,6].

Honey main enzymes are diastase (amylase), responsible for the decomposition of starch, invertase (also called sucrose or α-glucosidase), which decomposes sucrose into fructose and glucose, and glucose oxidase (GOx) that catalyzes the conversion of glucose to gluconic acid producing hydrogen peroxide (H_2O_2) in the process. Catalase from pollen origin, which decomposes H_2O_2, can also be present. Hydrogen peroxide content is related with honey antibacterial activity [7] while other factors, such as high osmolarity (due to high sugar content), low water activity (below 0.60), acidic pH (usually between 3.2 and 4.5) and high viscosity also contribute to hinder microbial growth [2,4]. The efficiency of enzymatic production of H_2O_2 increases with honey dilution. However, some

honeys, like manuka honey, have been found to retain their antimicrobial effects after decomposition of H_2O_2 by catalase addition. The non-peroxide antimicrobial activity of these honeys has been associated with specific antibacterial compounds, such as methylglyoxal (MGO) in manuka honey and the antimicrobial peptide bee defensin-1 in Revamil source honey from the Netherlands [3]. Antioxidant phenolic compounds from plant nectar, such as phenolic acids (e.g., gallic, ferulic, caffeic, benzoic, cinnamic, *p*-coumaric, chlorogenic, protocatechuic, ellagic, syringic, abscisic and vanillic) and flavonoids (e.g., quercetin, luteolin, pinocembrin, pinobanksin, chrysin, kaempferol, galagin, apigenin, naringenin and hesperetin) also contribute to the non-peroxide antimicrobial activity of honeys [1,4]. A correlation has been found between antioxidant, antimicrobial, phenolic content and colour of honeys: usually, the darker the honey, the higher the concentration of polyphenols and the higher the antioxidant and antimicrobial activities [8,11].

2. Materials and Methods

2.1. Samples

Commercial Portuguese orange blossom, eucalyptus, lavender and heather monofloral honeys were purchased from a local supermarket in Lisbon in March 2016. All honeys, in their original glass containers, were stored in the dark in a cool and dry place until further analysis. Honey dilutions were performed with double-distilled deionised water (Milli-Q water purification system).

2.2. Chemicals

Solvents, buffers and salts were from Merck as well as hydrogen peroxide 30%, gallic acid, 3,5-dinitrosalicylic acid (DNS), D-glucose and soluble starch. L-Proline, ninhydrin, (+)-catechin, *O*-dianisidine, horseradish peroxidase, 2 N Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-*s*-triazine (TPTZ) and bovine serum albumin (BSA, fraction V) were from Sigma. All reagents were of analytical grade unless otherwise stated.

2.3. Physicochemical Analysis

Physicochemical analysis was performed according to the harmonized methods of the International Honey Commission [12] and the official methods of analysis of the Association of Official Analytical Chemists [13]. Absorbance measurements were performed at room temperature (25°C) in 1 cm path length quartz cells using a UV-visible spectrophotometer (Shimadzu UV-1603), unless otherwise stated. Colour was determined from absorbance measurements at 635 nm of 50% (w/v) honey solutions after conversion to mm Pfund according to: mm Pfund = −38.70 + 371.39×Abs_{635} [14]. Colour intensity (ABS_{450}) was obtained as the difference between the absorbance of 50% (w/v) honey solutions measured at 450 nm and 720 nm expressed as mAU [10]. The pH of 10% (w/v) honey solutions was measured using a pH meter (Metrohm 744) and the free acidity of the solutions was determined by potentiometric titration [13].

The moisture content (% w/w) was determined by refractometric method [13]. The refractive index of pure honey samples was measured at room temperature using the Abbe refractometer (Bellingham-Stanley) and corrected for the reference temperature of 20°C. The correspondent moisture percentage was obtained from the standard conversion table for honey [12]. Electrical conductivity of 20% (w/v) honey solutions (dry matter basis) was obtained from conductance measurements at 20 °C collected at 1 kHz with a Wayne-Kerr B905 automatic precision bridge (WKR, England) using an Ingold conductivity cell type 980-K19/120 with platinum electrodes and a cell constant of 1.15 cm^{-1}. Cell calibration was performed with 0.01 mol/L potassium chloride standard solution. Ash was indirectly determined from electrical conductivity measurements at 20°C using the equation: ash content (%) = [electrical conductivity (mS/cm) − 0.143]/1.743 [15]. The specific rotation was determined according to IHC [12] from optical rotation measurements of 10% (w/v) clarified honey samples (dry matter basis) using a polarimeter (Bellingham-Stanley). Hydroxymethylfurfural (HMF) was determined according to the method of White [13].

The reducing sugar content was determined by the 3,5-dinitrosalycilic acid (DNS) method using glucose as standard [16]. The apparent sucrose content was determined as 95% of the difference in reducing sugar content before and after hydrolysis [12]. Determination of proline content was based on the colour developed with ninhydrin [12]. The protein content of honey samples was measured according to Lowry's methodusing bovine serum albumin (BSA) standard solutions for the calibration curve [12]. Diastase activity was determined as Diastase Number (DN) according to Schade method [13] defined as the amount of enzyme that converts 0.01 g of starch to the prescribed end-point in 1 h, at 40 °C, per gram of honey. The H_2O_2 content was determined according to the colorimetric method of Kwakman et al. [3] using a reagent mixture of *o*-dianisidine (50 μg/mL) and horseradish peroxidase (20 μg/mL) in phosphate buffer, and measuring the absorbance at 510 nm after addition of 6 mol/L sulphuric acid.

2.4. Antioxidant Activity Assays

The total phenolic content (TPC) was evaluated by the Folin-Ciocalteu method [10]. Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of honey. The total flavonoid content (TFC) was evaluated by the aluminium chloride colorimetric method with minor modifications [17]. Results were expressed in mg of catechin equivalents (CE) per 100 g of honey. The free radical scavenging activity (RSA) was determined from the decay of the purple colour of DPPH measured at 517 nm [10] according to: RSA (%) = $[(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100$, where A_{sample} and A_{DPPH} are the absorbance of DPPH in the presence and in the absence of honey (50 mg/mL), respectively. Trolox was used as positive control. The ferric reducing antioxidant power (FRAP) was determined from reduction of ferric TPTZ to the coloured ferrous complex absorbing at 593 nm using ferrous

sulphate as standard solution [16]. Results were expressed in μmol/L of Fe^{2+} equivalents. The ORAC assay using fluorescein as the fluorescent probe was used to measure the capacity of honey to quench peroxyl radicals generated from the thermal decomposition of AAPH [10]. Fluorescence was measured at 37 °C every 3 min for 2 h with excitation and emission wavelengths of 485 nm and 535 nm, respectively, using a microplate fluorescence reader (Zenith Anthos 3100). Fluorescence decay curves were normalized and ORAC values were determined from the net area under the curves (AUC) using Trolox standard solutions for the calibration curve. Results were expressed as μmol of Trolox equivalents (TE) per g of honey.

2.5. *In vitro* Antimicrobial Activity

The antimicrobial activity of honey was evaluated *in vitro* against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ESA1) using broth microdilution method [18]. The microplates were incubated at 37 °C for 24 h (bacteria) or 25°C for 72 h (yeasts) and growth evaluated from optical density at 620 nm. Vancomycin and norfloxacin were used as controls against Gram-positive and Gram-negative bacteria, respectively, and nystatin as control against yeasts. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of honey that prevented the growth of the tested microorganisms. All wells with no apparent growth in the MIC assay were subcultured onto fresh nutrient broth (Mueller Hinton for bacteria and Sabouraud medium for yeasts) and the microplates were incubated at 37°C for 24 h (bacteria) or 25°C for 72 h (yeasts) to determine whether viable microorganisms had persisted. The minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) were determined as the lowest concentration of honey to prevent the survival of viable bacteria or yeasts, respectively.

2.6. Statistical Analysis

All assays were carried out in triplicate and results expressed as mean values and standard deviations (mean±SD). Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey at 95% of confidence level ($p<0.05$) using GraphPad Prism software. Correlation matrix was obtained from Pearson's correlation analysis with significance set at $p< 0.05$ using the same software.

3. Results and Discussion

3.1. Physicochemical Analysis

3.1.1. Sample Identification

Commercial Portuguese honey samples of different botanical and geographical origin varying in colour (Table 1) were analyzed in terms of physicochemical parameters, antioxidant capacity and antimicrobial activity aiming at establishing correlations between physicochemical descriptors and honey bioactivity.

3.1.2. Colour Intensity

Honey colour, which can vary from white to amber and dark amber, is one of the main characteristics for classification of monofloral honeys. The colour of honey and its intensity (measured as ABS_{450}) are related with pollen content, mineral composition and pigments, such as phenolic compounds, flavonoids and carotenoids, being characteristic of floral origin. Several parameters can also influence honey colour, such as ageing (usually honey darkens with age due to formation of Maillard reaction products, caramelisation of fructose and polyphenolic reactions), presence of HMF, exposure to light, high temperatures and contact with metals [16,17]. The ABS_{450} values obtained are between 323 and 1579 mAU (Table 2), in good agreement with literature values reported for other honeys of the same botanical origin, such as honeys from the South of Italy with colour intensities of 310–1260 mAU [19]. Commercial Indian honeys showed slightly higher values, between 524 and 1678 mAU [20] while values in the range 274–1700 mAU have been found for monofloral honeys from arid regions, which included honeys from United Arab Emirates (UAE), Oman and Yemen [15].

3.1.3. Moisture

Moisture content was between 15.7–16.5% (w/w) and well below the limit of 20% (w/w) imposed by European legislation [21] and International standards for honey quality [22]. Moisture content is an important parameter of honey quality that determines honey stability and resistance to spoilage by yeast fermentation upon storage since low water content prevents microbial growth. The moisture contents of the honey samples were in good agreement with the corresponding values found in the descriptive sheets of the main European unifloral honeys [23] but were slightly lower than the ones of 18.1-19.2% obtained for other Portuguese commercial honeys of the same botanical origin [24]. Spanish [25] and Italian honeys [26] showed similar moisture values while Algerian honeys had lower moisture content, between 11.54-14.13% (w/w) [16]. High water content has been reported for some honeys from regions with tropical climate, such as Brazilian [27] and Cuban [8] honeys, as well as in honeys from Indian [20], Argentina [28] and Turkey [29].

3.1.4. pH and Free Acidity

The acidic nature of honey is mainly due to the presence of organic acids, such as gluconic acid, which are in equilibrium with their lactones or internal esters. Honey acidity is responsible for its flavour and stability against microbial growth, contributing to its antimicrobial properties [2,5]. However, unusually high acidity can be the result of deterioration on the account of fermentation of honey sugars due to inappropriate storage conditions and/or ageing. All the studied honey samples were acidic, with pH values between 3.43 and 4.18 and free acidity values below 50 meq/kg (Table 2), which is the maximum limit allowed by international regulations [21,22], an indication of honey freshness. Among the studied samples,

heather honey was the least acidic, and pH values between 3.9 and 4.7 are typical of European heather honeys [23]. The pH and free acidity values of the honey samples were similar to the ones reported for other Portuguese [17,24], Spanish [25] and Italian [26] honeys of the same floral origin.

3.1.5. Electrical Conductivity and Ash Content

Electrical conductivity, which is directly related to the concentration of mineral salts, organic acids and proteins, is a useful parameter for determining the botanical origin of honey. For this purpose, electrical conductivity measurements can replace ash content determination [12]. Electrical conductivity values for the studied honey samples were lower than 0.8 mS/cm (Table 2), suggesting that all samples are monofloral honey as stated. The ash content, which was indirectly determined from electrical conductivity measurements, was always below 0.6% (w/w), typical of monofloral honeys.

Heather honey showed the highest electrical conductivity (0.609 mS/cm) which can be related with a higher amount of minerals, typical of dark honeys [6]. Values higher than 0.8 mS/cm are allowed by the European Union [21] and Codex Alimentarius [22] standards for heather honey, and descriptive sheets of the main European unifloral honeys report electrical conductivity values between 0.49 and 0.97 mS/cm for this type of honey [23]. The light-coloured orange blossom and lavender honeys showed lower and similar electrical conductivity values, which are also related with the amount of plant pollen. The electrical conductivity values obtained were similar to other Portuguese [6,17], Spanish [25] and Italian [26] honeys of the same botanical origin.

A linear correlation (r = 0.965) was found between colour intensity (ABS_{450}) and electrical conductivity (and also ash content), with the darkest (heather) honey showing the highest conductivity (and ash) value. Correlations between colour, mineral content, electrical conductivity and ash content have been previously reported in the literature [10,14].

3.1.6. Sugar Content

The main sugars in honey are the monosaccharides fructose and glucose, which are both reducing sugars. According to the results obtained (Table 2), the reducing sugar content of all the honey samples, corresponding to the sum of fructose and glucose, is between 62.4–71.4% (w/w) and the thus above minimum value of 60 g/100 g imposed to blossom honeys by the European Council directive [21]. These values are similar to the ones reported for other Portuguese [17,24] and also Brazilian [27] honeys of the same floral origin while slightly lower contents were reported for Algerian honeys [16].

Regarding the amount of non-reducing sugars (apparent sucrose), the European regulations [21] establish a maximum amount of 5 g of sucrose per 100 g of honey with some exceptions, for which a higher amount is allowed, such as *Citrus* spp. and *Eucalyptus camadulensis* honeys (up to 10g/100 g) and *Lavandula* spp. honeys (up to 15 g/100 g). The heather and lavender honeys analyzed had an apparent sucrose content below 1%, while orange blossom honey exhibited a higher value (5.5% w/w) and eucalyptus honey had the highest amount (9.4% w/w). Sucrose values in the range 2.76–5.04% (w/w) have been reported for Portuguese commercial honeys of the same botanical origin [24] while sucrose content up to 1.0%, 2.8% and 4.5% (w/w) was observed for Italian heather, eucalyptus and citrus honeys, respectively [26]. The high sucrose content of the studied eucalyptus honey may be an indication that honey is unripe and conversion of sucrose into glucose and fructose by invertase enzyme was incomplete. However, Serrano et al. [25] found sucrose values between 0.16% and 8.74% (w/w) for Spanish (Andalusia region) eucalyptus honeys.

Table 1. Characterisation of the Portuguese Commercial Monofloral Honey Samples

Family	Botanical name	Common name	Local name	Region	Colour
Rutaceae	*Citrus* spp.	Orange blossom	Flor de laranjeira	Algarve	Light amber
Lamiaceae	*Lavandula* spp.	Lavender	Rosmaninho	Serra da Malcata	Light amber
Myrtaceae	*Eucalyptus* spp.	Eucalyptus	Eucalipto	Beira Litoral	Amber
Ericaceae	*Erica* spp.	Heather	Urze	Serra da Estrela	Dark amber

Table 2. Physicochemical Parameters of Portuguese Commercial Monofloral Honeys

Parameters	Orange	Lavender	Eucalyptus	Heather
ABS_{450} (mAU)	323 ± 1^{d}	363 ± 1^{c}	562 ± 1^{b}	1579 ± 1^{a}
Moisture (% w/w)	15.7 ± 0.4^{b}	$15.9\pm0.1^{a,b}$	16.5 ± 0.1^{a}	$16.0\pm0.3^{a,b}$
pH	$3.50\pm0.02^{b,c}$	3.43 ± 0.05^{c}	3.56 ± 0.02^{b}	4.18 ± 0.02^{a}
Free acidity (meq/kg)	37.10 ± 1.37^{a}	33.81 ± 1.37^{a}	33.98 ± 1.37^{a}	15.41 ± 1.37^{b}
κ (mS/cm)	0.256 ± 0.003^{c}	0.215 ± 0.004^{d}	0.385 ± 0.004^{b}	0.609 ± 0.012^{a}
Ashes (% w/w)	0.065 ± 0.003^{c}	0.041 ± 0.004^{d}	0.139 ± 0.004^{b}	0.267 ± 0.012^{a}
$[\alpha]_D^{20}$	-15.1 ± 0.3^{b}	-11.9 ± 0.8^{a}	-15.4 ± 0.6^{b}	-14.8 ± 0.3^{b}
HMF (mg/kg)	7.4 ± 0.1^{d}	24.2 ± 0.1^{c}	27.2 ± 0.1^{b}	28.4 ± 0.1^{a}
Proline (mg/kg)	449.2 ± 4.9^{c}	471.3 ± 4.9^{b}	566.6 ± 4.8^{a}	412.3 ± 4.8^{d}
Protein content (g/100 g)	0.24 ± 0.03^{b}	0.16 ± 0.03^{c}	$0.18\pm0.03^{b,c}$	0.57 ± 0.03^{a}
Reducing sugars (g/100 g)	$68.1\pm2.7^{a,b}$	$67.1\pm2.7^{a,b}$	62.4 ± 2.7^{b}	71.4 ± 2.7^{a}
Apparent sucrose (g/100 g)	5.5 ± 0.1^{b}	0.6 ± 0.1^{c}	9.4 ± 0.1^{a}	0.8 ± 0.1^{c}
Diastase activity (DN)	6.4 ± 1.0^{c}	13.3 ± 1.0^{a}	$11.2\pm1.0^{a,b}$	10.5 ± 1.0^{b}
H_2O_2 content (µmol/L)	16.3 ± 0.6^{c}	141.8 ± 2.0^{a}	11.8 ± 0.5^{d}	31.8 ± 1.2^{b}

Different letters (*a–d*) in a row denote significant differences at $p< 0.05$.

3.1.7. Optical Rotation

The specific rotation is a quality parameter related to the botanical origin of honey and an indicator of honey adulteration. The optical rotation is dependent on sugar concentration and also on the type and relative proportions of sugars in honey, being a useful parameter to distinguish between monofloral honeys and honeydew because usually the former are levorotatory while the later are dextrorotatory [12]. The studied honey samples were levorotatory with specific rotation values between −15.4 and −11.9. Persano Oddo et al. [26] reported similar values for Italian citrus, eucalyptus and heather honeys.

3.1.8. Hydroxymethylfurfural

The HMF content ranged from 7.4 to 28.4 mg/kg, well below the maximum limit of 40 mg/kg (80 mg/kg for honeys from tropical climate regions) imposed by international standards for honey [21,22]. HMF, which results from acid-catalyzed dehydration of hexoses (especially fructose) is an important honey quality parameter. HMF formation is induced by overheating, exposure to high temperatures and high fructose/glucose ratio. HMF is only present in trace amounts in fresh honey, and its concentration increases with storage and prolonged heating of honey. The obtained values were similar to other Portuguese [6], Spanish [25], Algerian [16] and Tunisian [30] honeys. Interestingly, some honeys from tropical climates, including Cuban and Brazilian honeys, showed lower HMF content, between 3.3–15.9 mg/kg [8] and 2.15-4.12 mg/kg [27], respectively. On the other hand, higher HMF content, up to 79.23 mg/kg, was found in honeys from arid regions [15] and up to 91.6 mg/kg in honeys from Córdoba, Argentina [28].

3.1.9. Diastase Activity

Eucalyptus, lavender and heather honeys showed DN above 8, which is the minimum value allowed by European regulations. A lower value, not less than 3, is allowed for honeys with low natural enzyme content, such as *Citrus* sp. honey, and an HMF content of not more than 15 mg/kg [21], which is the case for the studied orange blossom honey. Similar values were obtained for other Portuguese honeys [17,24] and some Italian [26] and Brazilian [27] honeys of the same floral origin. Turkish honeys also showed low diastase activity with values between 6.30–13.20 [29]. Higher values, up to 38.4 and 33.4, were obtained for honeys from Argentina [28] and Cuba [8], respectively. Serrano et al. [25] reported diastase numbers between 1.47–49.42 for eucalyptus honeys and 5.94–35.35 for citrus honeys from Andalusia (Spain). Lavender honey showed the highest diastase number and also the highest hydrogen peroxide content.

3.1.10. Hydrogen Peroxide Content

The hydrogen peroxide content, which has been associated with the antimicrobial properties of many honeys, is produced as a by-product of the conversion of glucose into gluconic acid catalyzed by the enzyme glucose oxidase (GOx) secreted from the hypopharyngeal glands of bees. The levels of GOx in honey can vary according to floral origin and geographical location and several factors are known to influence the H_2O_2 content in honey, such as the presence of catalase, peroxidases and antioxidant compounds (e.g. phenolic acids) as well as light, temperature and oxygen [7]. Moreover, the enzyme activity is inhibited by the low moisture content and high acidity typical of honey but it is activated upon honey dilution. At 25% (w/v) dilution, buckwheat, blueberry and sweet clover Canadian honeys showed H_2O_2 content between 248–2700 μmol/L [7] while Australian honeys derived from native Australian floral sources, namely spotted gum (*Eucalyptus maculata*), red stringybark (*E. macrorrhyncha*) and yellowbox (*E. melliodora*) exhibited H_2O_2 concentrations in the range 151–1017 μmol/L falling to 0–739 μmol/L after heat processing treatment [31]. Heating up to 45 °C is regularly used to increase the rate of filtration for removal of particulate debris from commercial table honeys. However it has been shown that heating above physiological temperature is detrimental to enzyme activity and H_2O_2 production drops after heat treatment relatively to unprocessed samples [31]. Feás, Iglesias, Rodrigues and Estevinho [32] reported H_2O_2 content between 2530–4250 μmol/L for Portuguese raw *Erica* sp. honeys, much higher than the value obtained for the studied commercial heather honey. The low H_2O_2 content of the commercial honeys analyzed can be due to processing and/or storing conditions.

3.1.11. Proline Content

The proline content of honey samples was between 412.3-566.6 mg/kg, above the minimum value of 180 mg/kg recommended for quality honey [12]. Proline content is an indication of honey ripeness and may also serve as an indicator of honey adulteration. This amino acid originates mainly from the salivary secretions of honeybees during the conversion of nectar into honey. Similar proline values (453.09-470.54 mg/kg) were obtained for other Portuguese commercial honey samples of the same floral origin, with a higher value (1044.36 mg/kg) being reported for the heather honey [24]. Proline values between 309 and 1033 mg/kg have been referred for European heather honeys [12]. Spanish eucalyptus and citrus honeys showed proline values between 112.08–986.63 mg/kg and 36.95-417.25 mg/kg, respectively [25]. Higher values, above 2,000 mg/kg, were exhibited by Burkina Fasan [33] and Algerian [16] honeys while proline values lower than 180 mg/kg have been reported for Tunisian honeys [30] and honeys from arid regions [15].

3.1.12. Protein Content

Protein content in honey samples usually ranges from 0.2% to 0.4% (w/w) and consist mainly of enzymes, added by bees during the honey ripening process. The protein concentration in honey varies depending on the botanical or geographical origin and storage time. Protein content for Portuguese honeys has seldom been referred in the literature. The orange blossom, eucalyptus and lavender honeys showed protein contents around 0.2% whereas for heather honey a higher value of 0.6% was found. High protein content is a characteristic of heather honey and has been suggested as a chemical marker for this type of honey [23]. On the other hand, high protein contents, up to 2.24% (w/w), have been found for several Brazilian honeys [27]. Algerian honeys [16] and unifloral honeys from arid regions [15] have protein content

between 0.3–0.4% (w/w) and 0.2–0.6% (w/w), respectively. Lower protein contents, below 0.23%, 0.16% and 0.1% (w/w), were found in Indian [20], Tunisian [30] and monofloral Cuban honeys [8], respectively. The proline and protein contents have been reported to influence the antioxidant properties of honey [16,33].

3.2. Antioxidant Properties

3.2.1. Phenolic Content

The physicochemical composition of honey, namely phenolic acids, flavonoids, ascorbic acid, carotenoids, amino acid and protein content, as well as enzyme activity and Maillard reaction products, can strongly influence the antioxidant activity. The content of phytochemical components is highly dependent on the nectar source [1]. The phenolic content of the studied honey samples ranged from 30.87 to 87.27 mg GAE/100 g (Table 3). The darkest heather honey showed the highest TPC while the lowest TPC was found in the light-coloured orange blossom honey, in good agreement with other commercial Portuguese honey samples of the same botanical origin [24] and also commercial Indian honeys [20]. Lower values, between 2.57-28.57 mg GAE/100 g have been reported for Slovenian honeys [34]. On the other hand, honeys from Burkina Faso [33] and Tunisia [30] showed very high phenolic content, up to 114.75 and 119.42 mg GAE/100 g, respectively.

A wider variety has been found for heather honeys. Most European heather honeys are from *Calluna* while heather honey produced in Portugal (and some regions of Spain and France) is from *Erica* sp. Feás et al. [32] found TPC values of 63.09–71.89 mg GAE/100 g for Portuguese *Erica* sp. honeys while Ferreira et al. [10] reported a value of 72.78 mg/100 g for heather honey from the Portuguese northeastern region. Romanian heather (*Calluna vulgaris*) honeys showed similar TPC, between 57.24 and 72.13 mg GAE/100g [35].

3.2.2. Flavonoid Content

The flavonoid content ranged from 4.85 mg CE/100 g in light-coloured orange blossom to 7.87 mg CE/100 g in dark-coloured heather honey (Table 3). Similar values, in the range 2.71-7.18 mg CE/100 g and 1.7-8.35 mg quercetin equivalents (QE)/100 g, have been reported for Algerian [16] and Burkina Fasan honeys [33], respectively. Honeys from southern Italy showed slightly higher TFC values, between 5.49 and 14.16 mg QE/100 g, but very low phenolic content, between 10.65 and 15.05 mg GAE/100 g [19]. Ferreira et al. [10] reported a TFC value of 58.74 mg CE/100 g for heather honey from the northeastern region of Portugal while Feás et al. [32] obtained TFC values between 45.07 and 67.40 mg CE/100 g for Portuguese *Erica* sp. honey. However, these samples had lower phenolic content than our heather honey sample. High TFC, in the range 29.45–36.21 mg QE/100 g, has also been found in Romanian heather (*Calluna vulgaris*) honeys [35].

3.2.3. DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity (RSA) was in the range 13.9-32.7%. The highest RSA was observed for heather honey, the darkest honey with the highest phenolic and flavonoid contents. Similar results were found for Algerian honeys [16] which showed RSA values between 2.5–44.57%. The RSA is dependent on antioxidant concentration, and highest results for Algerian honeys were obtained at the highest concentration (120 mg/mL) tested [16]. Higher values, between 44–71% and 54.87–78.89%, have been reported for commercial Indian honeys [20] and honeys from South Italy [19], respectively.

3.2.4. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP values ranged from 147.36 to 565.38 μmol Fe^{2+}/L (Table 3), similarly to values of 287.45–403.54 μmol Fe^{2+}/L obtained for Algerian honeys [16]. Higher FRAP values, between 159.74–894.34 μmol Fe^{2+}/L, have been reported for honeys from the South of Italy [19], while the highest value of 1501.4 μmol Fe^{2+}/L has been obtained for strawberry tree (*Arbutus unedo*) honey [14].

Heather honey, which had the highest DPPH scavenging activity, also showed the highest reducing power according to FRAP values, probably associated with its highest phenolic, flavonoid and protein contents. Similarly, Ferreira et al. [10] showed that dark honeys from the Northeast region of Portugal were richer in phenolic compounds and had higher antioxidant activity.

3.2.5. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay, which measures the antioxidant inhibition of peroxyl radical induced oxidations, can detect antioxidant activity due to radical quenching (hydrogen atom transfer), thus complementing the DPPH and FRAP assays that are mainly electron transfer assays [1]. The studied honey samples showed ORAC values in the range 3.22–11.88 μmol TE/g, similarly to other Portuguese honeys of the same botanical origin [24] but slightly lower than the ORAC values of 2.00–21.07μmol TE/g found for some Italian and Burkina Fasan honeys [14]. Once again, the heather honey had the highest ORAC value (although lower than the value of 22.58 μmol TE/g reported by Aazza et al. [24] for Portuguese heather honey), thus confirming its higher antioxidant properties.

3.2.6. Correlation Matrix

A strong correlation has been found between colour (measured as net absorbance ABS_{450}), phenolic content, flavonoid content and the FRAP, DPPH and ORAC values, as shown by Pearson's coefficient analysis (Table 4). The strongest correlations were found between colour intensity and TPC (r = 0.999), and between TFC and FRAP values (r = 0.999). Strong positive correlations between colour parameters, phenolics, and antioxidant capacity have also been observed by Beretta et al. [14] and Bertoncelj et al. [34] for some Italian and Slovenian honeys, respectively, while Ferreira et al. [10] reported similar correlations between color, phenolics, flavonoid content and antioxidant capacity for Portuguese unifloral honeys and honey extracts. Thus, coloured pigments are likely to play a role in the antioxidant properties of honey, as suggested by the strong correlations between ABS_{450}, FRAP, DPPH and ORAC values obtained.

Table 3. Antioxidant Properties of Portuguese Commercial Monofloral Honeys

Assay	Orange blossom	Lavender	Eucalyptus	Heather
TPC (mg GAE/100 g)	30.87±2.88^{c}	35.15±2.88^{c}	43.29±2.96^{b}	87.27±3.00^{a}
TFC (mg CE/100 g)	4.85±0.16^{c}	4.73±0.27^{c}	5.76±0.27^{b}	7.87±0.26^{a}
DPPH RSA (%)	15.9± 0.1^{c}	13.9± 0.1^{d}	17.3± 0.1^{b}	32.7± 0.1^{a}
FRAP (μmol Fe^{2+}/L)	162.0 ±2.4^{c}	147.4 ±4.3^{d}	266.0±4.2^{b}	565.4 ±8.3^{a}
ORAC (μmol TE/g)	4.84±0.25^{c}	3.22±0.24^{d}	6.77±0.25^{b}	11.88±0.26^{a}

Different letters (*a–d*) in a row denote significant differences at $p< 0.05$.

Table 4. Correlation Matrix (Pearson's coefficients) between Physicochemical Descriptors and Antioxidant Properties of Portuguese Commercial Monofloral Honeys

	ABS_{450}	Proline	Protein	TPC	TFC	FRAP	DPPH	ORAC
ABS_{450}	1	−0.487	0.955*	0.999***	0.988*	0.994**	0.992**	0.963*
Proline		1	−0.684	−0.469	−0.366	−0.408	−0.538	−0.347
Protein			1	0.944	0.924	0.939	0.981*	0.921
TPC				1	0.986*	0.992**	0.985*	0.957*
TFC					1	0.999**	0.981*	0.989*
FRAP						1	0.988*	0.986*
DPPH							1	0.972*
ORAC								1

Correlation is significant at the 0.05 (*), 0.01 (**), or 0.001 (***) level (2-tailed).

A strong positive correlation (r = 0.981) has also been found between protein content and radical scavenging activity (Table 4), an indication that protein content also contributes to the antioxidant capacity of the tested honeys. Similar results were reported for Algerian [16] and commercial Indian honeys [20], suggesting that total protein content in honey may be an additional factor contributing to the antioxidant activity. However, no significant correlation could be observed regarding proline content, similarly to other Portuguese honeys of the same botanical origin [24], whereas Burkina Fasan [33] and Algerian honeys [16] showed the highest correlations between antioxidant properties and proline content.

3.3. Antimicrobial Activity

3.3.1. Antibacterial Activity

The antimicrobial properties of Portuguese commercial monofloral honeys were evaluated *in vitro* against representative microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and yeasts (*Candida albicans*, *Saccharomyces cerevisiae*). The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC) have been determined and are summarized in Table 5.

The studied honey samples showed antibacterial activity against all the microorganisms tested (Table 5) with MIC values ranging from 6.25% (w/v) to 25% (w/v) depending on the microorganism. *E. faecalis* was the most sensitive bacteria and *S. aureus* the least sensitive one. All the honeys showed bactericidal activity at high concentrations (MBC of 50% w/v) against Gram-negative bacteria but not against Gram-positive bacteria. However, the MBC/MIC ratio of 4 suggests a bacteriostatic mode of action [5].

Greek and Cypriot honeys showed MIC values of 3.125-25% (v/v) and 6.25-25% (v/v) against *S. aureus* and *P. aeruginosa* [36], respectively, similar to the MIC values obtained for the studied Portuguese honeys against the same microorganisms. Malaysian honeys, including tualang (*Koompassia excelsa*) honey from *A. dorsata*, showed comparable MICs (5-20% w/v) but lower MBC (6.25-25% w/v) against *S. aureus*, *E. coli* and *P. aeruginosa* [37] relatively to the studied Portuguese honeys from *A. mellifera*. On the other hand, Cuban and dark-coloured Polish monofloral honeys showed MIC values between 4-12% (v/v) [8] and 3.12-25% [11], respectively, depending on the tested microorganism, in good agreement with the Portuguese honeys analyzed.

Table 5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal (MBC)/Fungicidal (MFC) Concentration (% w/v) of Portuguese Commercial Monofloral Honeys

Honey	*S. aureus*		*E. faecalis*		*E. coli*		*P. aeruginosa*		*C. albicans*		*S. cerevisiae*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC
Orange blossom	25	>50	6.25	>50	12.5	50	12.5	50	6.25	50	12.5	12.5
Lavender	25	>50	6.25	>50	12.5	50	12.5	50	12.5	25	6.25	12.5
Eucalyptus	25	>50	6.25	>50	12.5	50	12.5	50	12.5	12.5	12.5	12.5
Heather	25	>50	6.25	>50	12.5	50	12.5	50	6.25	25	6.25	12.5

Antibacterial activity of Portuguese heather (*Erica* sp.) honey has been studied before and MIC values of 1.51%, 4.73%, 7.60% and 11.63% (w/v) have been reported against *Bacillus cereus*, *S. aureus*, *E. coli* and *P. aeruginosa*, respectively [32]. Recently, Dezmirean et al. [35] reported values of 5.3%, 2.1%, 7.2% and 6.8% for Romanian heather (*Calluna vulgaris*) honeys against *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa*, respectively. Scottish heather honey exhibited MIC values of 6%, 4%, 6% and below 2% (v/v) against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*, respectively [38]. The Portuguese heather sample analyzed had comparable MIC values against the Gram-negative bacteria and *E. faecalis*, but a higher MIC against *S. aureus*. Although some studies suggest that Gram-positive bacteria, particularly *S. aureus*, are more susceptible to honey than Gram-negative bacteria [9,37], contradictory findings have been reported. Thus, Saudi Arabian lavender honey (which had the same MIC against *S. aureus* as the Portuguese lavender honey analyzed) was also more active against *P. aeruginosa* than against *S. aureus* [39].

3.3.2. Antifungal Activity

All the studied honey samples had strong antifungal activity against *C. albicans* and *S. cerevisiae* with MIC and MFC values in the range 6.25-12.5% and 12.5-50% (v/v), respectively. Australian honeys with different H_2O_2 content showed MIC values of 19-38.3% (w/v) against *C. albicans*, however the antifungal activity did not correlate with the level of H_2O_2 in honeys, suggesting the involvement of non-peroxide factors in the inhibitory effect [31].

Previous studies of Portuguese raw heather (*Erica* sp.) honeys revealed H_2O_2-dependent antifungal activity against *C.albicans*, *C. krusei*, *C. famata* and *Cryptococcus neoformans* with MIC values in the range 14-23% (w/v), [32] slightly higher than MIC value of 6.25% (w/v) obtained for the studied commercial heather honey against *C. albicans*. On the other hand, low antifungal activity was reported for lavender (*Lavandula stoechas*) honey from the North of Portugal when tested against *C. albicans*, *C. krusei* and *C. neoformans* [40]. This behaviour contrasts with the analyzed lavender (*Lavandula* spp.) honey from the south of Portugal (Serra da Malcata), which showed both fungistatic and fungicidal effects against *C. albicans*, with MIC and MBC values of 12.5% and 25% (w/v), respectively. Thus, geographical location can be a determinant factor for the antimicrobial activity of honeys besides floral origin.

3.3.3. Influence of Hydrogen Peroxide Content

Major variances in antimicrobial activity of honey are mainly due to different levels of H_2O_2 and, in some cases, to non-peroxide factors related with floral source, such as phytochemical components and proteinaceous compounds [3,7,31,36,37], or high MGO levels in manuka honey [3]. MIC values are usually obtained with dilute honey solutions (below 25%), thus acidity and osmotic effects are usually of minor importance at these concentrations. Negative correlations between MIC values and honey colour, phenolic content and antioxidant capacity have been reported [8,11,39].

No significant correlations could be established between antimicrobial activity and antioxidant properties or physicochemical parameters since all the studied honeys showed similar antibacterial effects although with different responses (MIC and MBC values) depending on the tested microorganism. However, regarding the antifungal activity some discrimination occurred according to honey's floral origin. Heather and orange blossom honeys (with higher protein content) were the most effective against *C. albicans* while against *S. cerevisiae* lower MICs were obtained with lavender honey, which showed the highest H_2O_2 content, followed by heather honey. Thus, the darkest (heather) honey showed higher antifungal activity, which can be related with its higher antioxidant properties, such as high phenolic content and high protein content in combination with the level of H_2O_2 achieved upon dilution. A combination of factors involving H_2O_2 and synergistic interactions with other honey components has been suggested to modulate honey's antimicrobial action, which also depends on honey floral origin and geographical location [2,4,5].

4. Conclusions

In this work, selected Portuguese commercial monofloral honeys (orange blossom, lavender, eucalyptus and heather honeys) were characterized and correlations were found between physicochemical parameters and biological activity. All honey samples were in compliance with European regulations [21] on honey quality criteria regarding the physicochemical parameters analyzed, namely water and sugar content, free acidity, electrical conductivity and HMF content, which were also in agreement with Codex Alimentarius [22] standards. A strong positive correlation was found between colour intensity and electrical conductivity. The latter is directly related to the concentration of mineral salts and also to the amount of pollen, thus being a useful parameter for determination of the botanical origin of honey.

Strong correlations have also been established between the antioxidant capacity of the studied honeys and colour intensity, TPC, TFC and protein content. The darkest honey, i.e., heather honey, had higher amounts of phenolic compounds, flavonoids and proteinaceous compounds, associated with higher antioxidant activity and also higher antimicrobial activity (lower MIC values). The antibacterial activity of the Portuguese heather (*Erica* spp.) honey was comparable to the antibacterial activity of Scottish heather honey [38] and Romanian heather honey from *Calluna vulgaris* [35].

The antioxidant activity of the studied honeys was strongly dependent on the floral origin and geographical location of honey while antimicrobial activity depended on the tested microorganism and in a lesser extent on the type of honey. The low H_2O_2 levels found in the analysed honeys, which can be due to processing or storage conditions of the commercial samples, suggest that other factors besides H_2O_2 can be involved in the observed antimicrobial activity, such as phytochemical components and protein content. A strong positive correlation was found between radical scavenging activity and protein content, suggesting an important contribution from protein

content to the bioactivity of the studied honeys. The antifungal and fungicidal activities of heather and lavender honeys warrants further investigations with unprocessed (raw honey) samples focusing on the identification of individual phenolic compounds and flavonoids underlying the antimicrobial activity of these honey extracts.

Acknowledgements

The authors acknowledge *Fundação para a Ciência e Tecnologia* (FCT) for financial support [Pest-OE/SAU/UI4013/2014].

Competing Interests

The authors have no competing interests.

List of Abbreviations

AAPH,2,2'-azobis(2-methylpropionamidine) dihydrochloride; BSA, bovine serum albumin; CE, catechin equivalents; DN, Diastase Number; DNS, 3,5-dinitrosalycilic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; GOx, glucose oxidase; HMF, Hydroxymethylfurfural; MBC, minimum minimum bactericidal concentration; MFC, minimum fungicidal concentration; MGO, methylglyoxal; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; ORAC, oxygen radical antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; RSA, radical scavenging activity; TFC, total flavonoid content; TPC, total phenolic content; TPTZ, 2,4,6-tripyridyl-*s*-triazine; VRE, vancomycin-resistant *Enterococcus*.

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