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ORIGINAL RESEARCH ARTICLE

Brazilian bee pollen: phenolic content, antioxidant properties and antimicrobial activity

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Bee pollen has been promoted as a dietary supplement for humans due to its nutritional and bioactive properties. Sixty-two samples of *Apis mellifera* dehydrated bee pollen collected in Brazil (eight states and Federal District) were analyzed for phenolic compounds, flavonoids, antioxidant activity using DPPH, β-carotene and ORAC methods, and antimicrobial activity. The values obtained for total phenolic compounds ranged from 12.60 to 84.22 mg GAE/g bee pollen while total flavonoids oscillated between 1.90 and 36.85 mg quercetin/g bee pollen. The IC_{50} ranged from 0.35 to 13.42 mg bee pollen/mL of extract. The inhibition percentages ranged from 52.58 to 98.37% of bee pollen extract using the β-carotene method. When measured by the ORAC method, antioxidant activity was between 132.98 and 575.85 μmols eq. Trolox/g bee pollen. Bee pollen efficiently inhibited the growth of all the microorganisms studied. *Candida albicans* was the most resistant, while *Staphylococcus epidermides* was the most sensitive.

Keywords: antimicrobial activity; bee pollen; dpph; orac; total phenolic compounds; **β**-carotene; bleaching assay

Introduction

Since the dawn of human history, bee pollen has been recognized throughout the world for its nutritional and biological properties and consequent beneficial effects on health. Pollen collected by bees is known to be a balanced foodstuff (Mundargi et al., 2016). Studies have recently been undertaken on the physical and chemical composition, the microbiological quality and the identification of the compounds present in bee pollen and great attention has been dedicated to one particular group, the phenolic compounds (Campos et al., 2015; de Arruda et al., 2013a, 2013b; Gabriele et al., 2015; Mărgăoan et al., 2014; Yang et al., 2013).

Researchers worldwide have been dedicating great attention to bee pollen, propolis, and honey, due mainly to the biological properties of these products, such as antibacterial (Basim et al., 2006; Özkalp & Özcan, 2010), antifungal (Özcan et al., 2004), anti-inflammatory (Di Paola-Naranjo et al., 2004), anticarcinogenic (Almas et al., 2001; Gebara et al., 2002), antimutagenic (Pascoal et al., 2014) and immunomodulatory activity (Oršolić et al., 2004).

Beehive products are generally considered to be excellent sources of antioxidant substances; studies such (Almaraz-Abarca et al., 2008) which used bee pollen samples from Mexico, and of Campos et al. (2000) which analyzed Portuguese samples of bee pollen have shown a high antioxidant activity of this product. Pollen collected by bees is normally a mixture of pollens of various botanical origins, each one with an important source of flavonoids in the form of glucosides (Wiermann & Vieth, 1983) and, in some species, of hydroxycinnamic acids. These compounds present species-specific profiles, which suggests that bee pollens collected in different areas or distinct seasons may have different bioactivity (Campos et al., 2015).

Free radical reactive species are generated in the human organism by various endogenous systems, induced by the exposure to different physiochemical conditions and in pathological states. These may attack bio-molecules, among which lipids, proteins, and DNA are supposed to play a crucial role in a wide range of diseases, like cancer and the cerebrovascular problem (Lobo et al., 2010).

The daily consumption of antioxidant substances may exercise effective protective action against the antioxidant processes that occur naturally in the organism. A series of diseases - among them cancer, atherosclerosis, diabetes, arthritis, malaria, AIDS, and heart diseases, may be related to the damage caused by highly reactive forms of oxygen, denominated "reactive oxygenated substances" or simply ROS. These substances are also

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linked to the processes responsible for the aging of the body (Brenna & Pagliarini, 2001; Yıldırım et al., 2001).

Among several techniques for the determination of antioxidant activity, we chose the three main techniques: DPPH; coupled oxidation of β-carotene and linoleic acid; and ORAC. With the use of these three distinct techniques (which have different mechanisms), a complete evaluation of the antioxidant activity of bee pollen from different Brazilian regions was proposed, since in the literature, only DPPH, and β-carotene to evaluate the antioxidant capacity of bee pollen. Besides, it was sought to associate the determination of phenolic compounds and total flavonoids with the results of the antioxidant activity, seeking a possible correlation between them. In this work, samples from several Brazilian regions were evaluated, thus helping to create a database on the composition of bee pollen from various botanical origins and with new data from the application of ORAC (Arruda, 2013).

Considering that regulatory agencies state that any claims of health or nutritional benefits of a food product must be supported by science, the purpose of the present study was to evaluate the content of total phenolic compounds, flavonoids, antioxidant and antimicrobial properties of Brazilian bee pollen (from eight states and Federal District). Furthermore, we emphasize the need for standardization of methods for assessing bee pollen quality and investigating its biological properties, thus adding value to this product.

Materials and methods

Samples

Sixty-two samples of dehydrated bee pollen (from *Apis mellifera* bees) were collected in the years of 2009–2012, from eight Brazilian states (Bahia, Espirito Santo, Sergipe, Sao Paulo, Santa Catarina, Mato Grosso, Rio Grande do Norte, and the Rio Grande do Sul), and the Federal District. The samples were discussed according to the macro-regions of Brazil: Northeast (Bahia, Sergipe and Rio Grande do Norte states), Mid-west (Federal District), Southeast (Espirito Santo and Sao Paulo states), and South (Santa Catarina and the Rio Grande do Sul states). The samples received were placed in appropriate glass containers and stored at $-18\,^{\circ}C$ until analysis. Before the analysis, the samples were homogenized and ground in an analytical mill (Arruda, 2013).

Preparation of the extracts

For the preparation of the sample extracts, it was followed by the method reported by Carpes et al. (2007, 2008), with some minor modifications. In an Erlenmeyer flask, 2 g of dried bee pollen was mixed with 20 mL of 70% ethanol, and the solution was heated for 30 min in the water bath at $70\,^{\circ}C$ by mechanical agitation. After cooling at room temperature, the homogenate was filtered through filter paper and transferred to 25 mL volumetric flasks; the volumes were completed with 70% ethanol and analyzed for total phenolic compounds, total flavanols, and antioxidant activity. All determinations were performed in triplicate ($n = 3$).

Total phenolic compounds

The Folin-Ciocalteu method (Carpes et al., 2008) was used to determine total phenolic content. In brief, an aliquot of 0.50 mL of extract was diluted appropriately in ethanol and then mixed with 2.5 mL of the Folin-Ciocalteau reagent: H_2O (1:10). The mixture was, then, incubated at room temperature for five minutes. Finally, 2 mL of 4% sodium carbonate (Na_2CO_3) was added. After incubation at room temperature, for two hours, in the dark, the absorbance of the reaction mixture was measured at 740 nm against an ethanol blank using a Shimadzu UV 1650 PC spectrophotometer. The total phenolic content was expressed on a dry basis in mg of gallic acid equivalents (GAE)/g of bee pollen.

Total flavanols

The determination of total flavanol content was performed colorimetrically using quercetin as a standard (Carpes et al., 2008). Briefly, the extract was diluted appropriately in ethanol. An aliquot of 0.50 mL of extract solution was mixed with 4.3 mL of 80% ethanol, 0.1 mL of aluminum nitrate at 10%, and 0.1 mL of 1 mol/L of potassium acetate. Similarly, a blank was prepared without aluminum nitrate. The sample and blank absorbances were measured at 415 nm after 40 min in the dark at room temperature (Shimadzu - UV 1650 PC spectrophotometer). The total flavonol content was expressed on a dry basis in mg of quercetin equivalent/g of bee pollen.

Antioxidant activity

Antioxidant properties of bee pollen extracts were evaluated according to ORAC, DPPH, and beta-carotene bleaching (BCB) assays. Indeed, according to Sakanaka and Ishihara (2008) due to the complex nature of bee pollen, it is recommended to access antioxidant activity using more than one methodology in order to avoid possible interferences.

Scavenging of DPPH radicals

The free radical-scavenger activity was determined by the DPPH assay, as described by Carpes et al. (2008), with some modifications. By a dilution series, the antiradical activity of the extracts was evaluated in order to obtain a large spectrum of sample concentrations. This involved the mixing of 1.5 mL of DPPH solution (6.0×10^{-5} M in ethanol) with 0.5 mL of extract solution and 2 mL of ethanol, followed by homogenization. After 30 min, the quantification of the remaining DPPH radicals was recorded by using absorption set at 517 nm (Shimadzu UV 1650 PC

spectrophotometer). A test tube containing 2.5 mL of ethanol and 1.5 mL of DPPH (6.0×10^{-5} M) was used as a negative control. The specific sample blank was determined using 3.5 mL of ethanol and 0.5 mL of the extract solution of each concentration. Results are presented in IC_{50} values, which represent the weight of the sample required to scavenge 50% of the DPPH radicals available. The extract concentration providing 50% of radical scavenging activity (IC_{50}) was calculated by interpolation from the graph of RSA percentage against extract concentration. The standards used were α-tocopherol ($IC_{50} = 16.55$ μg/mL), BHT ($IC_{50} = 17.06$ μg/mL) and BHA ($IC_{50} = 8.47$ μg/mL) tested at a concentration of 40 mg/mL.

β-Carotene bleaching (BCB) assay

The assessment of the antioxidant activity of the extracts of dehydrated bee pollen was carried out following the method described by Ahn et al., (2004) and Carpes et al. (2008). A solution was prepared by dissolving 20 mg of β-carotene in 1 mL of chloroform, and 30μmL was added to 40 mg of linoleic acid and 400 mg of Tween 40 emulsifier. Then the organic solvent was removed under a stream of nitrogen gas, and 100 mL of distilled water was added to the Erlenmeyer. The mixture was shaken, and 3 mL of this emulsion was transferred into different test tubes containing 300 μL of extract diluted in 70% ethanol (1:20). The tubes were shaken and incubated at 50 °C. As soon as the emulsion was added to all tubes, zero-time absorbance was measured at 470 nm. Oxidation of the emulsion was monitored at 15-min intervals over a 120-min period. Control sample contained 300 μL of 70% ethanol in place of the extract. The antioxidant activity was expressed as percent inhibition relative to the control after a 120-min incubation using the eq. (1)

$$AA = \frac{(DR_C - DR_S)}{DR_C} \times 100 \qquad (1)$$

where *AA* is the antioxidant activity, *DRC* is the degradation rate of the control (=ln(*a*/*b*)/120), DR_S is the degradation rate in the presence of the sample (=ln(*a*/*b*)/120), *a* is the initial absorbance at time 0, and *b* is the absorbance at 120 min. The reference standards used were α-tocopherol (97.30%), BHT (95.75%), and BHA (97.89%) tested at a concentration of 40 mg/mL.

Antioxidant activity (ORAC) assay

ORAC procedure used an automated plate reader (Synergi HT, Bio TeK, USA) with 96-well plates (Arruda et al., 2013; Ou et al., 2001). Analyses were conducted in a pH 7.4 (75 mM) phosphate buffer at 37 °C under the condition with a blank sample in parallel. The extracts were diluted with sodium phosphate buffer (75 mM, pH 7.4). The peroxyl radical was generated using 2,2′-azobis (2-amino-propane) dihydrochoride aqueous solution - that was prepared for each run (153 mM). Fluorescein was used as a substrate (40 nM). The ORAC analyzer was programmed to record the fluorescence of FL every minute after the addition of AAPH. All fluorescent measurements were expressed relative to the initial reading. Fluorescence analysis conditions were performed as follows: excitation at 493 nm (filter 485/20) and 515 nm (filter 528/20). The standard curve was linear from 6.25 to 100 μM Trolox®. Results were calculated using the differences of areas under the FL decay curves between the blank and a sample and were expressed as μmol eq. Trolox/g bee pollen.

Antimicrobial activity

The microorganisms *Escherichia coli* ESA37, *Staphylococcus epidermides* ESA7, and *Candida albicans*ESA109 were isolated at the Local Health Units of Bragança and identified at the Laboratory of Microbiology of the School of Agriculture of Bragança (Portugal). The isolates were stored in Muller–Hinton medium plus 20% glycerol at 70 °C. Before experimental use, cultures from the solid medium were subcultivated in liquid media, incubated at 37 °C over night and used as the source of inocula for each experiment. The inocula for the tests were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to 0.5 on the MacFarland scale, confirmed by spectrophotometrical reading at 580 nm for bacteria and 640 nm for yeasts. Cell suspensions were finally diluted to 10^8 for bacteria and 10^5 CFU/mL for yeasts, so they could be used in the activity assays.

Screening of antibacterial activities was performed by measuring the minimal inhibitory concentrations (MICs in mg of extract/mL) which were determined by using Nutrient Broth (NB) or Yeasts Peptone Dextrose (YPD) on a microplate (96 wells), per Duarte et al. (2007), and Morais et al. (2011). Pollen extracts were diluted in 20% DMSO and transferred into the first well, and serial dilutions were performed. Fluconazole (antimycotic) and gentamicin (antibacterial) were used as controls. In each experiment, a positive control (inoculated medium) and negative control (medium), extracts blanks (mediums with compounds), and DMSO control (DMSO with inoculated medium) was introduced. The inoculum (20 μL) was added to all the wells and the plates were incubated at 37 °C for 24 h (bacteria) and 25 °C for 48 h (yeasts). Antimicrobial activity was observed by addition of 20 μL of 0.5% TTC solution. The lowest concentration of bee pollen extract that inhibited visible growth was defined as the minimal inhibitory concentration (MIC), as indicated by the TTC staining.

Statistical analysis

Comparisons between regions were performed by One-Dimensional Analysis of Variance (One-way ANOVA) followed by the Tukey test.

Table 1. Results of the variation of phenolics, flavonoids and antioxidant activity of the ethanol extracts of the samples of dehydrated bee pollen: IC_{50} (mg pollen/mL extract), β-carotene system (%), ORAC (μmol eq.Trolox/g pollen), grouped by region of collection by ANOVA.

Variable	Region	Average	SD	N	p*
Total phenolic compounds (mg GAE/g pollen)	Northeast	19.28	4.42	19	0.002
	Mid-west	23.86	12.52	5	
	Southeast	30.66	15.54	25	
	South	36.94	15.70	13	
	Total	27.94	14.30	62	
Total Flavonoids (mg quercetin/g pollen)	Northeast	5.06	2.77	19	0.025
	Mid-west	4.60	0.61	5	
	Southeast	4.81	2.66	25	
	South	9.95	10.10	13	
	Total	5.95	5.43	62	
DPPH IC_{50} (mg pollen/mL extract)	Northeast	4.18	1.63	19	<0.001
	Mid-west	7.77	5.47	5	
	Southeast	2.74	2.27	25	
	South	1.68	0.62	13	
	Total	3.37	2.72	62	
ORAC (μmol eq.Trolox /g pollen)	Northeast	228.02	49.73	19	<0.001
	Mid-west	255.17	100.06	5	
	Southeast	272.29	88.13	25	
	South	411.39	79.22	13	
	Total	286.51	101.14	62	
β-carotene/ linoleic acid system (%)	Northeast	76.84	10.76	19	0.001
	Mid-west	72.38	13.96	5	
	Southeast	83.39	10.71	25	
	South	90.27	5.10	13	
	Total	81.94	11.34	62	

*Descriptive level of the statistical test.

The existence of associations between the variables was analyzed using the Pearson correlation test and adopting the Pearson's correlation (r) as the parameter to evaluate the nature (directly or inversely proportional) and the intensity of these correlations (0 to 1, with 1 indicating maximum correlation).

The Spearman correlation was adopted for the correlation test; for the comparison of two groups, the Mann-Whitney test was employed, and for more than two groups, the Kruskal-Wallis test was used.

The results were expressed as means of the results ± standard deviation. All statistical analyses were performed using the program STATISTICA 8.0 and adopted the significance level of 5% ($p < 0.05$).

Results

Total PhenolicContent andTotal flavanols

The results obtained for the total phenolics varied between 12.60 and 84.22 mg GAE/g of bee pollen and total flavanols ranged between 1.90 and 36.85 mg of quercetin/g of bee pollen (Table 1).

Antioxidant activity

DPPH radical-scavenging activity

A series of dilutions was undertaken, using the DPPH method, to determine the antioxidant activity of the samples to obtain data for different concentrations (20 to 0.025 mg of pollen/mL of solution). The IC_{50} was determined in the interval which presented linearity, and the antioxidant activity was inversely proportional to the IC_{50}.

β-Carotene bleaching (BCB) assay

When using theβ-carotene method, the antioxidant activity of the samples was between 52.58 and 98.37% (Table 1).

Oxygen radical absorbance capacity (ORAC)assay

When quantified by the ORAC method, the antioxidant activity varied from 132.98 ± 1.58 to 575.85 ± 11.67 μmol eq. Trolox/g pollen (Table 1).

Antimicrobial activity

The results obtained for the antimicrobial activity are presented in Table 2. It may be seen that all the extracts under study possessed antimicrobial activity, with different MIC values for each microorganism. The results were consistent with observed by (Fatrcová-Šramková et al., 2013). *C. albicans* showed the most significant resistance, *S. epidermides* being the most sensitive (Table 2). Gram-positive bacteria (*S. epidermides*) presented MICs oscillating between 0.98 and 2.87% (w/v). For *E. coli*, the values ranged from 1.8 to 7.48% (w/v), while for *C. albicans,* the values were between 10.23 and 18.83% (w/v). There was no average statistically significant difference in the

Table 2. Results of the variation of antimicrobial activity of the ethanol extracts of the samples of dehydrated bee pollen and comparison of the regions of collection by ANOVA. (MIC in mg of extract/mL).

Variable	Region	Average	SD	N	p*
E. coli	Northeast	4.92	1.75	19	0.934
	Mid-west	5.13	1.31	5	
	Southeast	4.78	1.27	25	
	South	5.05	1.34	13	
	Total	4.91	1.42	62	
S. epidermides	Northeast	1.71	0.60	19	0.809
	Mid-west	1.91	0.67	5	
	Southeast	1.79	0.59	25	
	South	1.91	0.58	13	
	Total	1.80	0.59	62	
C. albicans	Northeast	13.22	3.14	19	>0.999
	Mid-west	13.26	2.58	5	
	Southeast	13.20	2.66	25	
	South	13.27	3.42	13	
	Total	13.23	2.90	62	

*Descriptive level of the statistical test.

antimicrobial parameters as between the Brazilian regions ($p > 0.05$).

Discussion

Total phenolic content and total flavanols

Neves et al. (2009) on assessing samples of dehydrated bee pollen from the states of Alagoas, Bahia, Sergipe, and Minas Gerais (Brazil) found values between 6.9 and 13.78 mg GAE/g of bee pollen and 3.46 and 6.87 mg of quercetin/g for total phenolics and total flavonols, respectively. Carpes et al. (2007, 2008) when analyzing dehydrated samples of bee pollen from the South of Brazil found results which varied between 19.28 and 48.90 mg GAE/g for total phenolics and values between 2.10 and 28.33 mg of quercetin/g of pollen for total flavonols, whereas for samples from Paraná and Alagoas (Brazil), respectively, were reported values of 10.90 and 8.10 mg GAE/g for total phenolics. Liberato et al. (2009), when assessing samples produced in the state of Ceará (Brazil), reported values of from 17.79 to 19.52 mg GAE/g of bee pollen for total phenolics and values between 6.90 and 8.26 mg of quercetin/g of pollen for total flavonols. Vecchia and Carpes (2010) analyzed samples originating in the state of Paraná (Brazil), and found values between 5.36 and 42.79 mg GAE/g of dehydrated bee pollen for total phenolics and values between 0.63 and 28.74 mg of quercetin/g of bee pollen for total flavonols, while Meneses et al. (2010) reported values of from 14.31 to 64.14 GAE/g of dehydrated bee pollen for total phenolics in samples produced in the region of Alagoinhas, in the state of Bahia (Brazil).

The composition of bee pollen varies significantly as a result of collection from different geographic regions, the time of collection, and various species of vegetation from which the pollen is harvested by honey bee (Arruda et al., 2013; Campos et al., 2015). The robust antioxidative properties of polyphenols result from both the double bonds and the location of hydroxyl groups on the aromatic ring. Moreover, polyphenols ring structure determines their lipophilic properties, especially in the case of flavonoids. Hydrophobic antioxidants play a protective role in lipid membranes. Polyphenols can scavenge reactive oxygen species and make organic radicals ineffective and can complex metal ions which catalyze oxidation reactions. Bee pollen shows a robust antioxidative activity owing to various compounds contained in it. Additionally, bee pollen is a source of hydrophilic antioxidants which protect against oxidative structure damage to the cytoplasm, inside cell organelles, and in the extracellular fluid.

DPPH

The concentration of 10 mg of bee pollen/mL of solution was observed to demonstrate the highest antioxidant activity for most of the samples. Some samples presented the maximum antioxidant activity in concentrations inferior to 10 mg of pollen/mL of solution, and consequently, a low IC_{50}, suggesting the high antioxidant activity of the compounds present in the ethanol extract. Carpes et al. (2008) used a concentration of 2.7 mg of bee pollen/mL of the solution to determine the antioxidant activity of the extracts; while Vecchia and Carpes (2010) tested extracts at a concentration of 6.6 mg bee pollen/mL of solution. The antioxidant activity obtained by those researchers was identical to that determined by the present study when one used the concentration of 10 mg of pollen/mL of solution. Synthetic antioxidants used as controls presented higher antioxidant activity than that of the samples analyzed. To make more evident the comparison of the results with those obtained in the literature, DPPH was expressed as IC_{50}.

The products of the hive are taken as excellent sources of antioxidants according to the studies of Arruda et al. (2013), Campos et al. (2015), Morais et al. (2011), and Pascoal et al. (2014). The results found for the antioxidant activity of Brazilian bee pollen vary considerably among the samples, the value of IC_{50} oscillated from 0.35 to 13.42 mg pollen/mL of extract. Carpes et al. (2008) that analyzed the antioxidant activity of the ethanolic extracts of bee pollen from the southern region of Brazil, obtained IC_{50} values from 0.81 to 4.69 mg pollen/mL of extract. Campos et al. (2003), using bee pollen samples from Portugal and New Zealand, found IC_{50} values of between 0.04 and 0.5 mg of bee pollen/mL of extract.

β-Carotene bleaching (BCB) assay

This method determines the ability of an antioxidant to protect a lipid substrate (in this case, β-carotene) using the inhibition of free radicals generated during the peroxidation of linoleic acid. These variations may be caused by the differences in their potential and/or in

the concentration of the various reducing substances, mainly the ample class of the phenolic compounds. In the study undertaken by Carpes et al. (2008), who assessed the antioxidant activity of samples of bee pollen from the South of Brazil, using the same method of analysis, observed that the antioxidant activities of the extracts of bee pollen ranged from 69.78 to 93.12% (an average of 83.60%).

ORAC

This method is highly sensitive and tests the scavenging ability of an antioxidant about the formation of a radical-induced from 2,2′–azobis (2-amidinopropane) dihydrochloride (AAPH) at 37 °C. The radical reacts with a fluorescent compound forming a non-fluorescent product. The protective effect of the antioxidant is verified by calculating the area created under the curve of the diminution of the fluorescence of the sample against time, as compared with the control, which does not present antioxidant activity.

The Brunswick Laboratory (16th October 2002) obtained results identical to those ascertained in the present study, even though it is not specified whether bee pollen was dehydrated. Indeed, that laboratory compared data obtained using the ORAC assay of High Desert® bee pollen (CC Pólen Co.) with the values obtained on whole-grain food. A value of 247 μmol eq. Trolox/g of pollen was attributed to High Desert bee pollen.

It was found significant differences ($p < 0.05$) amongst the average values of the antioxidant parameters of the different samples of Brazilian dehydrated bee pollen.

The use of distinct concentrations of the bee pollen extracts in the three methods of evaluation of the antioxidant activity hampers the direct comparison of the results obtained following the different methodologies. This observation was also made by Carpes et al. (2008), who undertake studies with dehydrated bee pollen collected in Brazil (southern region). Each sample presented a different result when evaluated by each of the three methods used for the quantification of their antioxidant activity. These different results can be explained by differences in the methodology reaction mechanisms and depend on the specific free radical being used as a reactant.

Antimicrobial activity

Natural products have been used in traditional medicine throughout the world for thousands of years (Ozan et al., 2007). Indeed, natural substances have demonstrated antibacterial action, mainly because a large part of the vegetable extracts used in alternative medicine consists of flavonoids, cafeic, benzoic and cinnamic acids which act on the bacterial cells in such a way as to break the cytoplasmatic membrane or destroy the cell wall of the microorganism, causing it both functional and structural damage. Also, they can inhibit the activity of many enzymes (Nogueira et al., 2007; Scazzocchio et al., 2006; Uzel et al., 2005).

Morais et al. (2011) observed that Portuguese bee pollen inhibited the growth of bacteria in similar, meager, concentrations. Knazovická et al. (2009) and Kacaniová et al. (2012) attributed the variation in the antimicrobial activity of bee pollen and propolis to their constituent phenolics and the presence of non-volatile compounds in the extracts. Pereira et al. (2007) and Estevinho et al. (2008) demonstrated that the antimicrobial activity of nuts and honey was related to the amount of total phenolic compounds. This fact was not observed in the study carried out by Morais et al. (2011) in which bee pollen extract, with a smaller total phenolic compound, was the most effective against microorganisms. Other factors may be involved; specifically, the nature of the phenolic fraction tested in the study.

In the present study, similarly to Morais et al. (2011), Graikou et al. (2011), Abouda et al. (2011), and Fatrcová-Šramková et al. (2013), Gram-positive bacteria were more sensitive to the extracts of bee pollen than Gram-negative. The reason for this less intense action of the extracts of bee products against Gram-negative than against Gram-positive bacteria is still not well understood; some hypotheses suggest that the resistance of the former microorganisms is related to their possessing a chemically more complex, though flexible, cell wall and because they present a higher lipid content than that observed in Gram-positive bacteria (Morais et al., 2011; Özkalp & Özcan, 2010).

Carpes et al. (2007) undertook a study to determine the antioxidant activity, phenolic compounds, and antibacterial activity of extracts of bee pollen obtained with different concentrations of ethanol (40 to 90%) for samples of dehydrated bee pollen collected in the states of Alagoas and Paraná (Brazil) and observed that the ethanolic extract of bee pollen inhibited *S. aureus* at 90%. The 60% extract of the ethanol solution (sample from Paraná) inhibited the microorganisms *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Klebsiella* spp. (Gondim, 2011) ascertained the antimicrobial effect of bee pollen at a concentration of 5% (in 70% ethanol) on *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis,* and *Lactobacillus casei*, observing that bee pollen was efficient against all these bacteria.

Differently from the results hereby reported and to the described studies mentioned above, Carpes et al. (2009) who assessed thirty-six samples of dehydrated bee pollen from the southern region of Brazil, reported that, despite their high phenolic and flavonoid content, none of the ethanol extracts of bee pollen efficiently inhibited microbial growth, either on the Minimum Inhibitory Concentration (MIC) or Minimal Bactericidal Concentration (MBC) tests. The microorganisms studied in that work were *B. B. subtilis* ATCC 21,332

Table 3. Pearson's correlation between phenols, flavonoids, and antioxidant activity of the ethanol extracts of the samples of dehydrated bee pollen: IC_{50} (mg pollen/mL extract), β-carotene system (%), ORAC (μmol eq.Trolox/g pollen)[1].

Correlation		Total phenols (mg GAE/ g pollen	Total flavonoids (mg quercetin/ g pollen)	DPPH EC_{50} (mg pollen/ mL extract)	ORAC (μmol eq.Trolox /g pollen)
Total Flavonoids (mg quercetin/ g pollen)	r	0.480			
	p	<0.001			
DPPH IC_{50} (mg pollen/ mL extract)	r	−0.436	−0.220		
	p	<0.001	0.086		
ORAC (μmol eq.Trolox /g pollen)	r	0.568	0.539	−0.479	
	p	<0.001	<0.001	<0.001	
Beta (%)	r	0.484	0.055	−0.419	0.436
	p	<0.001	0.671	0.001	<0.001

[1]N = 62; r: Pearson's correlation coefficient; p: probability associated with the test.

P. aeruginosa ATCC 15,442, *Streptococcus mutans Ingbritt*1600, *S. aureus* ATCC 25,923, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens*, *Xanthomonas vesicatoria* pv.vesicatoria, *Xanthomonas axonopodis*pv. vesicatoria and *Pseudomonas syringae*pv. tomato), and the methodology used was the agar diffusion test.

Overall, the results obtained by this, and the other mentioned studies suggest that the botanical origin of the samples directly affects their biological action. The achieved results indicate the need for further studies, particularly regarding the isolation and identification of the main active compounds responsible for the antibacterial activity. These may open doors for their future use by the pharmaceutical industry, given the vast number of microorganisms that have become resistant to existing antibacterial drugs.

In the last 60 years, the resistance of human and animal pathogens to antibiotics has been one of the best-documented cases of biological evolution and constitutes the main problem faced by Public Health in the world, affecting both developed and developing countries (Davies & Davies, 2010). This fact is an inevitable consequence of the indiscriminate use of antibiotics in humans and animals. In Europe and North America, *S. aureus* resistant to methicillin (MRSA), *Streptococcus pneumoniae* not susceptible to penicillin (PNSSP), enterococcus resistant to vancomycin (VRE), and *Enterobacteriaceae* producers of beta-lactamase of an amplified spectrum (ESBL) have emerged and spread in hospitals and communities (Santos, 2004). Baquero and Blázquez (1997) have commented on the danger of a return to a pre-antibiotic era, especially given the fact that no new class of antibiotics has been discovered in recent years, despite the intense research undertaken by the pharmaceutical industry. In the light of the present scenario, the search for new antimicrobial substances on the base of natural sources, including plants and products such as bee pollen, has assumed great importance in pharmaceutical companies. The best of our knowledge, microorganisms with resistance to the action of bee pollen have not been discovered (Pascoal et al., 2014), which further highlights its potentiality as a complement to the conventional antimicrobial agents against resistant pathogens.

Correlations

The study of the botanical origin, involving 62 samples of dehydrated bee pollen, is appropriately discussed in a previous article (Freitas et al., 2013). Only weak and moderate correlations were observed between the pollen types studied and the parameters (total phenolics, total flavonols, DPPH, ORAC, β-carotene, and linoleic acid method, and antibacterial activity) evaluated for the dehydrated samples of bee pollen. Moreover, moderate and weak correlations were also observed between the studied parameters (total phenolics, total flavonols, DPPH, ORAC, β-carotene, and linoleic acid method, and antibacterial activity) (Table 3).

Conclusions

It was demonstrated that bee pollen carries flavonols and phenolic compounds with antioxidant activity. Thereby bee pollen may be useful in the prevention of those diseases in which free radicals are involved. It is, further, notable that bee pollen has promising antimicrobial properties, particularly taking into account the increasing bacterial resistance to the conventional commercial antibiotics. Further study for the isolation of active compounds and *in vivo* assays are necessary, so that the observations presented in this article may be confirmed.

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