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Biological activities of commercial bee pollens: Antimicrobial, antimutagenic, antioxidant and anti-inflammatory

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ABSTRACT

Bee pollen is considered, since memorable times, a good source of nourishing substances and energy. The present study aimed to evaluate the biological activities of eight commercial bee pollens purchased from the market. The origin of sample A was not specified in the labeling; samples B, C, D and G were from Portugal and the remaining were from Spain. The sample E presented the highest value of phenolics (32.15 ± 2.12 mg/g) and the H the lowest (18.55 ± 095 mg/g). Sample C had the highest value of flavonoids (10.14 ± 1.57 mg/g) and sample H the lowest (3.92 ± 0.68 mg/g). All the samples exhibited antimicrobial activity, being *Staphylococcus aureus* the most sensitive and *Candida glabrata* the most resistant of the microorganisms studied. All the samples exhibited antimutagenic activity, even though some samples were more effective in decreasing the number of gene conversion colonies and mutant colonies. Regarding the antioxidant activity, assessed using two methods, the more effective was sample B. The anti-inflammatory activity, assessed using the hyaluronidase enzyme, was highest in samples B and D. Pearson's correlation coefficients between polyphenols, flavonoids, antioxidant activity and antimicrobial activity were computed. It was also performed a discriminant analysis.

1. Introduction

Bee pollen, commonly referred as the "life-giving dust", results from the agglutination of flower pollens with nectar and salivary substances of the honeybees and is used as food for all the developmental stages in the hive (Almeida-Muradian et al., 2005). The collection of this natural product is a relatively recent development, dependent primarily on the basic concept of scraping pollen off of the bees' legs as they enter the hive (Feás et al., 2012).

The major components of bee pollen are carbohydrates, crude fibers, proteins and lipids at proportions ranging between 13% and 55%, 0.3% and 20%, 10% and 40%, 1% and 10%, respectively (Villanueva et al., 2002). In fact, bee pollen is referred as the "only perfectly complete food", as it contains all the essential amino acids needed for the human organism. However, the composition of bee pollen depends strongly on plant source, together with other factors such as climatic conditions, soil type, and beekeeper activities (Morais et al., 2011). Generic bee pollen composition data were considered sufficient for most purposes, but now the usefulness of bee pollen-specific composition data is increasingly being acknowledged (Nogueira et al., 2012; Bogdanov, 2011).

Bee pollen is considered a health food with a wide range of therapeutic properties, among which: antimicrobial, antifungal, antioxidant, anti-radiation, hepatoprotective, chemoprotective and/or chemopreventive and anti-inflammatory activities (Abdella et al., 2009; Bariliak et al., 1996; Viuda-Martos et al., 2008; Fatrcová-Šramková et al., 2013). In addition, it has been reported to trigger beneficial effects in the prevention of prostate problems, arteriosclerosis, gastroenteritis, respiratory diseases, allergy desensitization, improving the cardiovascular and digestive systems, body immunity and delaying aging (Estevinho et al., 2012). The promotion of tissues' repair, which results from the acceleration on the mitotic rate, has also been lauded (Morais et al., 2011). These therapeutic and protective effects have been related to the content of polyphenols (Almeida-Muradian et al., 2005).

Having into account the European regulations (European Union, 2006) that 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 quantify the bioactive compounds and to determine the biological properties (antimicrobial, antioxidant, anti-inflammatory and antimutagenic) of commercial bee pollens. It is worth mention that, as far as we know, this is the first study on the antigenotoxic activity of bee pollen.

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2. Material and methods

2.1. Chemicals and reagents

All the reagents were of analytical grade purity. Methanol (CH_3OH) and ethanol (CH_3CH_2OH) were supplied by Pronolab (Lisbon, Portugal). The Folin–Ciocalteu reagent chloroform ($CHCl_3$) sodium carbonate (Na_2CO_3) gentaminice and fluconazol were obtained from Merck (Darmstadt, Germany). Gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO, USA). The bovine testicular hyaluronidase (350 units) and the potassium salt of human umbilical cord hyaluronic acid were obtained from Sigma (St. Louis, MO, USA). The culture mediums were purchased from Himedia (Mumbai, India). The TTC solution (235-Triphenyl-2H-tetrazolium chloride) was supplied by Fluka (Buchs, Switzerland). The other chemicals were obtained from Sigma Chemical Co., (St. Louis, MO, USA). High purity water (18 MΩ cm) used in all experiments was obtained from a Milli-Q purification system (Millipore Bedford, MA, USA).

2.2. Pollen samples

Eight commercial pollens (A–H) of different floral sources and geographical origins were purchased from the market and left in the dark at room temperature (±20 °C) until further analysis. The origin of sample A, in which the dominant family was *Cistaceae*, was not specified in the labeling. Samples B (*Fabaceae*), C (*Cistaceae*), D (*Ericaceae*) and G (*Fabaceae*) were from Portugal and E (*Cistaceae*), F (*Ericaceae*) and H (*Boraginaceae*) were from Spain. It is worth mentioning that this botanical classification of the samples was achieved in a previous study of the research team (Nogueira et al., 2012). The preparation of the extracts was performed as described in Morais et al. (2011), by mixing the bee pollen with methanol (1:2) (w/v). After maceration, the extract was evaporated in a vacuum evaporator. The dried bee pollen extract was kept in the dark at room temperature until further analysis.

2.3. Total phenolics and flavonoids

The total phenolic content of the extracts was recorded using the Folin–Ciocalteu method as described by Moreira et al. (2008). Briefly a dilute solution of each bee pollen in MeOH (MeOH-bee pollen; 500 μL of 1:10 g/mL) was mixed with 500 μL of Folin–Ciocalteu reagent and 500 μL of Na_2CO_3 (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 MeOH + sample) using a Unicam Helios Alpha UV–visible spectrometer (Thermo Spectronic, Cambridge, UK). Galic Acid standard solutions (0.01×10^{-3} to 0.08×10^{-3} M) were used for constructing the calibration curve ($y = 1.99813x + 0.0018$; $R^2 = 0.9997$). Total phenols content were expressed as mg of Galic Acid equivalents per g of bee pollen (GAEs).

For flavonoids' contents the aluminium chloride method was used. In briefly MeOH-bee pollen (250 μL) was mixed with 1.25 mL of distilled H_2O and 75 μL of a 5% $NaNO_2$ solution. After 5 min 150 μL of a 10% $AlCl_3{\cdot}H_2O$ solution was added. After 6 min 500 μL of 1 M NaOH and 275 μL of distilled H_2O were added to the mixture and vortexed. The solution was well mixed and the intensity of pink colour was measured at 510 nm. Catechin standard solutions (0.022×10^{-3} to 0.34×10^{-3} M) were used for constructing the calibration curve ($y = 1.0421x - 0.0093$; $R^2 = 0.9918$). Total flavonoids content were expressed as mg of catechin equivalents per g of bee pollen (CAEs).

2.4. Anti-inflammatory activity – hyaluronidase assay

The anti-inflammatory activity was assessed indirectly by measuring the inhibitory effect of bee pollen on the reactions catalysed by hyaluronidase, using de method described by Silva et al. (2012). The reaction mixture is constituted by 50 μL of bee pollens' extract and 50 μL (350 units) of hyaluronidase enzyme (Type IV-S: bovine testes) was incubated at 37 °C for 20 min. Then calcium chloride was added (1.2 μL 2.5×10^{-3} M/L) to activate the enzyme and the mixture was incubated at 37 °C for 20 min. To start the reaction 0.5 mL of hyaluronic acid sodium salt (0.1 M/L) were added. The mixture was incubated at 37 °C for 40 min. After this 0.1 mL of potassium tetraborate 0.8 M was added and it was incubated in water-bath at ebullition for 3 min. The mixture was placed at 10 °C and 3 mL of p-dimethylaminebenzaldehyde were added. Afterwards it was incubated at 37 °C for 20 min. Finally the absorbance was measured at 585 nm using water as control. All the tests were performed in triplicate.

2.5. Antimicrobial activity

In the present study it were used microorganisms isolated from biological fluids, collected in the Hospital Centre and identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança and reference strains obtained from the authorised distributor of ATCC (LGC Standards S.L.U., Barcelona). The microorganisms were *Staphylococcus aureus* ATCC 6538™, *S. aureus* ESA 159, *Pseudomonas aeruginosa* ATCC 15442™, *P. aeruginosa* ESA 22, *Escherichia coli* ATCC 25922™, *E. coli* ESA37, *Candida glabrata* ATCC 66032™, *C. glabrata* ESA 123. The isolates were stored in Muller–Hinton medium plus 20% glycerol at −70 °C before experimental use. The inoculum for the assays were prepared by diluting cell mass in 0.85% NaCl solution adjusted to 0.5 MacFarland scale confirmed by spectrophotometrical reading at 580 nm for bacteria and 640 nm for yeasts. Cell suspensions were finally diluted to 10^4 CFU/mL in order to use them in the activity assays. Antimicrobial tests were carried out according to Morais et al. (2011), using Nutrient Broth (NB) or Yeasts Peptone Dextrose (YPD) on microplate (96 wells). Bee pollen extracts were diluted in dimethylsulfoxide (DMSO) and transferred into the first well and serial dilutions were performed. The inoculum was added to all wells and the plates were incubated at 37 °C for 24 h (bacteria) and 25 °C for 48 h (yeast). Fluconazol and gentamicine were used as controls. In each experiment a positive control (inoculated medium) and a negative control (medium) and DMSO control (DMSO with inoculated medium) was introduced. Antimicrobial activity was detected by adding 20 μL of 0.5% TTC solution. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of bee pollen extract that inhibited visible growth as indicated by the TCC staining (dead cells are not stained by TTC). All the tests were performed in triplicate ($n = 3$) and the results are expressed as mg/mL.

2.6. Antimutagenic activity

The determination of the antimutagenic activity of bee pollen extracts was performed using yeast cells (D7 diploid strain of *Saccharomyces cerevisiae* ATCC 201137) according to the recommended by Zimmermann (1984). Prior to each experiment the *S. cerevisiae* D7 strain (MATa/MATa, ade2-40/ ade 2-119, trp 5-12/ trp 5-27, ilv 1-92/ilv 1-92) was tested for the frequency of spontaneous convertants at the tryptophan (trp) locus and revertants at the isoleucine (ilv) locus. Cells from a culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28 °C until they reached the stationary growth phase. The yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain a final mixture of 2×10^8 cell/mL. The mixture (4 mL) contained 1 mL of cell suspension, potassium phosphate buffer and methanolic extracts of bee pollen, in order to reach final concentrations of 0.00, 0.25, 0.50 and 0.75 mg/L. The mixture was incubated under shaking for 2 h at 37 °C. Then the cells were plated in complete and selective media to ascertain survival, trp convertants and ilv revertants. Ethyl methanesulfonate (EMS), a mutagenic compound, was used as control.

2.7. Antioxidant activity

2.7.1. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Livers were obtained from pigs with an approximate body weight of 150 kg, homogenised with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4), in order to produce a 1:2 (w/v) tissue homogenate, centrifuged at 3000*g* for 10 min. The bee pollen extract (0.2 mL) was added to 0.1 mL of the supernatant, and incubated in the presence of $FeSO_4$ (10 lM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2% w/v, 0.38 mL). The obtained mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde–TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the formula: Inhibition ratio (%) = $[(A-B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration (Ferreira et al., 2009). Butylated hydroxyanisole was used as standard.

2.7.2. Scavenging of DPPH·

The scavenging of DPPH radical was assayed following the method described by Morais et al. (2011). Various concentrations of extracts of bee pollen (300 μL) were mixed with 2.7 mL of a MeOH solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left in the dark, until stable absorption values were obtained. The reduction of the DPPH radical was measured by continuously monitoring the decrease of absorption at 517 nm. The radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical scavenging activity (EC_{50}) was calculated by interpolation from the graph of RSA percentage against extract concentration. The standards used were BHA and a-tocopherol.

2.8. Statistical analysis

Each bee pollen sample was analysed in triplicate. Results are shown as arithmetic mean values ± standard deviation. In each parameter the differences between the samples were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD. P values less than or equal to 0.05 were evaluated as statistically significant. In addition, it were computed Pearson's correlation coefficients between

the phenolic compounds, flavonoids, antioxidant activity (assessed by TBARS and DPPH methodologies) and the values of minimum inhibitory concentration for the microorganisms under study. These treatments were carried out using SPSS version 21.0. In order to determine which characteristics would be more useful to classify the pollen samples a discriminant analysis was performed using the quadratic different covariances and the stepwise variable selection methods. The efficiency of the discriminant power of the models selected was assessed by the test of the Wilks' lambda value. Results were analysed in terms of the absolute assignment of individuals to the pre assigned group, the variance explained by each canonical likelihood, and by the analysis of the standardised scoring coefficients. Statistical analysis was performed using the statistical package JMP Pro 10.

3. Results

3.1. Total phenolics and flavonoids

In Table 1 it are presented the contents of phenolic compounds and flavonoids of the different bee pollen samples. Highly significant differences ($P < 0.001$) were found using the Tukey test for both parameters. Sample E presented the highest value of phenolic compounds (32.15 ± 2.12 mg GAE/g pollen) and sample H the lowest (18.55 ± 0.95 mg GAE/g pollen). Regarding the amount of flavonoids, the highest value was obtained for sample C (10.14 ± 1.57 mg CAE/g pollen) and the lowest for sample H (3.92 ± 0.68 mg CAE/g pollen), even though this sample was not significantly different from samples A, B, F and G.

3.2. Anti-inflammatory activity

Fig. 1 shows the anti-inflammatory activity of the eight bee pollen samples, where the highest was registered for sample D (25.17 ± 3.18%), followed by sample B (23.60 ± 2.17%). It were not found significant differences for these two samples ($P < 0.05$). The lowest percentage of inhibition was obtained for sample H (12.20 ± 1.40%), even though the anti-inflammatory activity of this bee pollen did not differ significantly from the obtained for samples A, C, E and F.

Table 1
Concentration (mg/g) of phenolics and flavonoids in pollen extracts from different samples ($n = 24$).

Sample	Phenolics (mg GAEs/g pollen)	Flavonoids (mg CAEs/g pollen)
A	23.14 ± 0.65^{dc}	4.36 ± 0.28^{c}
B	28.83 ± 2.59^{ab}	4.63 ± 0.73^{c}
C	25.31 ± 1.20^{bc}	10.14 ± 1.57^{a}
D	28.26 ± 0.77^{abc}	9.25 ± 0.86^{ab}
E	32.15 ± 2.12^{a}	7.51 ± 0.96^{b}
F	24.10 ± 2.72^{bc}	3.71 ± 0.25^{c}
G	27.82 ± 2.80^{abc}	4.91 ± 0.83^{c}
H	18.55 ± 0.95^{d}	3.92 ± 0.68^{c}
P-value	<0.001	<0.001

a, b, c, d – means with different superscripts are significantly different for each attribute ($P = 0.05$).

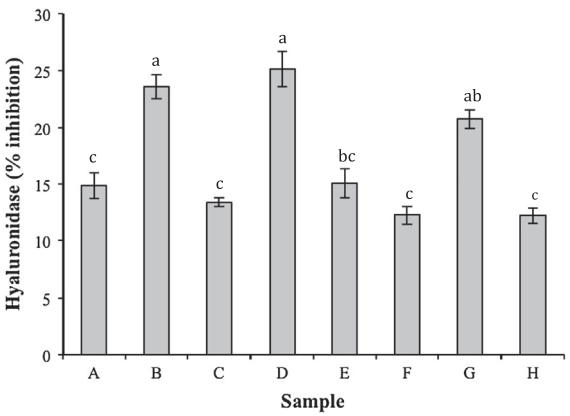

Fig. 1. Anti-inflammatory activity of the bee pollen samples.

3.3. Antimicrobial activities of pollen samples

The minimum inhibitory concentrations (MIC) obtained for the bacteria and yeasts, both isolated from biological fluids and reference strains, are presented in Table 2. It can be observed that the antimicrobial activity varied significantly with the pollen sample, for all the strains under study. For all the cases, bacteria were much more sensitive to the bee pollens' action. The most sensitive microorganisms were both strains of *S. aureus*, one of the most representative bacteria involved in respiratory diseases, particularly when it was used sample H, with MIC values of 1.81 ± 0.29 mg/mL (*S. aureus* ATCC 6538™) and 2.58 ± 0.63 mg/mL (*S. aureus* ESA 159). For this microorganism the less efficient bee pollen was sample E. Regarding *P. aeruginosa*, the most efficient pollen sample was H, with MIC values of 3.71 ± 0.72 and 5.23 ± 0.37 mg/mL, for the reference and isolated strain, respectively. *E. coli* appeared as the most resistant of the assayed bacterial strains, for all the tested extracts. The MIC for this bacteria ranged from 4.08 ± 0.38 to 5.65 ± 0.61 mg/mL, for the reference strain, and from 6.19 ± 0.73 to 9.42 ± 0.52 mg/mL, for the isolated strain. Also for this microorganism, the most efficient pollen sample was H. Concerning the assays carried out with *C. glabrata* the pollen with higher inhibitory action was sample E, with values of 16.00 ± 1.32 mg/mL for the reference strain and 22.67 ± 2.25 mg/mL for the isolated one.

In all the microorganisms under study it was observed that the reference strains were more sensitive than the isolated from biological fluids. As expected, the controls gentamicine (antibacterial) and fluconazol (antifungal) presented lower MIC than the pollen extract.

3.4. Antimutagenic activity

In the present study, the anti-genotoxic effects of bee pollen on *S. cerevisiae* were tested up to 0.75 mg/mL in a 0.5% fixed ethyl methanesulfonate (EMS) concentration, which produces random mutations in genetic material by nucleotide substitution, being an alkylating agent. Samples D and F had the smallest value of survivals, with percentages of 63.58 ± 1.74% and 69.76 ± 1.56%, respectively. It was also observed that the higher the concentration of pollen, the lower the percentage of survival, what is related to its antifungal effect. The results obtained in the present study for the antimutagenic activity reveal that all the samples had substantial antigenotoxic activity, since they all decreased the frequencies of gene conversion (Table 3). The highest number of gene conversion colonies obtained was equal to $4.00 \times 10^6 \pm 3.17 \times 10^5$, corresponding to sample A, and the lowest was obtained for sample H, with a value of $3.5 \times 10^4 \pm 2.0 \times 10^3$. However, only two samples (D and H) significantly reverted the mutations over the entire range of concentrations used.

3.5. Antioxidant activity of pollen samples

The antioxidant activity ranged from 0.35 ± 0.02 (B) to 3.70 ± 0.00 (D) mg/mg extract, for the DPPH method, and from 2.98 ± 0.47 (B) to 6.69 ± 0.75 (D), using TBARS (Table 4). Significant differences were obtained, for both methodologies, between the eight analysed samples.

Table 2
Minimum inhibitory concentration (mg/mL) for the studied microorganisms.

Sample	*S. aureus* ATCC 6538™	*S. aureus* ESA 159	*P. aeruginosa* ATCC™	*P. aeruginosa* ESA 22	*E. coli* ATCC™	*E. coli* ESA 37	*C. glabrata* ATCC™	*C. glabrata* ESA 123
A	3.07 ± 0.72^{ab}	4.01 ± 0.44^{ab}	5.69 ± 0.74^{a}	6.43 ± 1.05^{ab}	5.23 ± 0.70^{ab}	6.75 ± 0.75^{bc}	25.23 ± 5.11^{a}	28.76 ± 1.56^{abc}
B	2.06 ± 0.13^{ab}	3.12 ± 0.38^{ab}	3.99 ± 0.13^{ab}	5.04 ± 0.07^{b}	4.27 ± 0.40^{ab}	6.92 ± 1.18^{bc}	20.83 ± 1.44^{abc}	33.92 ± 1.88^{a}
C	2.88 ± 0.42^{ab}	3.54 ± 0.84^{ab}	4.01 ± 1.24^{ab}	5.62 ± 0.58^{ab}	4.73 ± 0.40^{ab}	7.50 ± 0.90^{abc}	24.17 ± 1.44^{a}	28.23 ± 1.95^{bcd}
D	2.64 ± 0.87^{ab}	4.28 ± 0.38^{a}	4.78 ± 0.30^{ab}	6.96 ± 0.17^{a}	4.97 ± 0.41^{ab}	8.98 ± 0.78^{ab}	24.33 ± 1.61^{a}	33.42 ± 1.28^{ad}
E	3.55 ± 0.23^{a}	4.19 ± 0.48^{a}	4.95 ± 0.28^{ab}	6.67 ± 0.39^{a}	4.17 ± 0.52^{b}	7.42 ± 0.80^{abc}	16.67 ± 1.44^{bc}	24.38 ± 1.66^{cd}
F	2.41 ± 0.61^{ab}	3.74 ± 0.28^{ab}	4.88 ± 0.36^{ab}	6.82 ± 0.40^{a}	5.57 ± 0.39^{a}	8.33 ± 0.63^{abc}	22.50 ± 2.50^{abc}	32.50 ± 2.50^{ab}
G	2.84 ± 0.76^{ab}	3.59 ± 0.64^{ab}	3.74 ± 0.60^{b}	6.37 ± 0.69^{ab}	5.65 ± 0.61^{a}	9.42 ± 0.52^{a}	16.00 ± 1.32^{c}	22.67 ± 2.25^{d}
H	1.81 ± 0.29^{b}	2.58 ± 0.63^{b}	3.71 ± 0.72^{b}	5.23 ± 0.37^{b}	4.08 ± 0.38^{b}	6.19 ± 0.73^{c}	23.33 ± 1.44^{ab}	32.50 ± 2.50^{ab}
P-value	0.036	0.021	0.014	0.006	0.004	0.002	0.001	<0.001

a, b, c, d – means with different superscripts are significantly different for each attribute ($P = 0.05$).

Table 3
Effect of bee pollen extract on the percentage of survival of yeast cells and antigenotoxicity (mutagenesis and gene conversion).

Sample	Pollen concentration (mg/L)	EMS (%)	Survivals (%)	Gene conversion colonies/10^5	Mutants colonies/10^6
–	0.00	0	100.00 ± 0.00	0.80 ± 0.05	0.35 ± 0.04
A	0.00	0.50	88.18 ± 1.60^{a}	52.55 ± 3.19^{b}	380.56 ± 7.67^{b}
	0.25	0.50	81.00 ± 4.61^{ab}	35.71 ± 4.50^{a}	323.60 ± 18.12^{a}
	0.50	0.50	81.81 ± 2.11^{ab}	37.33 ± 1.27^{a}	321.58 ± 17.92^{a}
	0.75	0.50	77.64 ± 2.38^{b}	40.01 ± 3.17^{a}	410.66 ± 1.68^{b}
B	0.00	0.50	85.67 ± 1.74^{a}	53.75 ± 2.62^{b}	369.32 ± 6.49^{a}
	0.25	0.50	84.44 ± 2.31^{a}	33.52 ± 2.30^{a}	390.63 ± 10.87^{ab}
	0.50	0.50	81.45 ± 2.04^{a}	35.20 ± 3.75^{a}	417.67 ± 5.33^{bc}
	0.75	0.50	80.22 ± 3.99^{a}	34.15 ± 2.63^{a}	439.15 ± 13.49^{c}
C	0.00	0.50	88.20 ± 2.11^{a}	51.37 ± 2.24^{b}	379.63 ± 8.76^{b}
	0.25	0.50	88.17 ± 5.33^{a}	32.89 ± 2.26^{a}	308.98 ± 8.97^{a}
	0.50	0.50	84.97 ± 1.86^{a}	34.65 ± 4.22^{a}	314.55 ± 4.17^{a}
	0.75	0.50	85.36 ± 5.29^{a}	39.92 ± 3.20^{a}	403.28 ± 11.40^{b}
D	0.00	0.50	83.07 ± 9.07^{b}	57.15 ± 4.51^{b}	408.87 ± 8.38^{b}
	0.25	0.50	74.85 ± 5.39^{ab}	25.46 ± 1.91^{a}	68.44 ± 4.54^{a}
	0.50	0.50	69.51 ± 2.70^{ab}	24.24 ± 3.06^{a}	68.62 ± 2.26^{a}
	0.75	0.50	63.58 ± 1.74^{a}	16.88 ± 1.37^{a}	67.96 ± 2.91^{a}
E	0.00	0.50	91.04 ± 2.63^{a}	54.74 ± 3.79^{b}	376.51 ± 9.16^{a}
	0.25	0.50	86.57 ± 2.88^{a}	33.97 ± 4.56^{a}	405.91 ± 4.14^{ab}
	0.50	0.50	85.14 ± 3.90^{a}	36.62 ± 4.54^{a}	388.73 ± 8.30^{ab}
	0.75	0.50	80.77 ± 4.59^{a}	32.04 ± 3.29^{a}	418.70 ± 10.67^{b}
F	0.00	0.50	84.22 ± 6.29^{b}	56.41 ± 4.53^{b}	384.88 ± 19.28^{b}
	0.25	0.50	76.56 ± 3.86^{ab}	0.42 ± 0.02^{a}	328.54 ± 10.67^{a}
	0.50	0.50	71.28 ± 3.10^{ab}	0.44 ± 0.04^{a}	380.45 ± 10.07^{ab}
	0.75	0.50	69.76 ± 1.56^{a}	0.51 ± 0.03^{a}	411.12 ± 16.26^{b}
G	0.00	0.50	89.23 ± 3.11^{a}	51.0 ± 4.02^{b}	380.03 ± 14.63^{a}
	0.25	0.50	85.96 ± 1.95^{a}	0.44 ± 0.03^{a}	378.96 ± 18.27^{a}
	0.50	0.50	85.14 ± 2.37^{a}	0.46 ± 0.04^{a}	398.11 ± 14.08^{a}
	0.75	0.50	78.87 ± 6.19^{a}	0.49 ± 0.03^{a}	408.83 ± 13.24^{a}
H	0.00	0.50	85.89 ± 4.40^{a}	52.34 ± 3.94^{b}	407.23 ± 16.70^{b}
	0.25	0.50	83.05 ± 5.93^{a}	0.34 ± 0.04^{a}	80.51 ± 5.25^{a}
	0.50	0.50	86.60 ± 4.81^{a}	0.38 ± 0.02^{a}	72.39 ± 5.91^{a}
	0.75	0.50	85.04 ± 3.77^{a}	0.35 ± 0.02^{a}	71.82 ± 2.68^{a}

a, b, c, d – means with different superscripts are significantly different for each attribute ($P = 0.05$).

3.6. Correlation and discriminant analysis

Pearson's correlation coefficients between the phenolic compounds, flavonoids, TBARS, DPPH, anti-inflammatory activity, antioxidant activities (TBARS and DPPH) and MICs for the microorganisms studied were computed and are presented in Table 5. The phenolic compounds had a significant and positive correlation with the anti-inflammatory activity and a significant and negative correlation with the antimicrobial activity obtained for *C. glabrata* ATTC. Scatter plot of the first two canonical variables, of eight pollen samples considered (Fig. 2) showed that the groups were discriminated with great accuracy.

4. Discussion

4.1. Total phenolics and flavonoids

According to the literature, phytochemicals such as phenolic compounds are considered beneficial for human health since they decrease the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation (Silva et al., 2004). The high ability of the phenolic compounds to neutralize the active oxygen species is strongly associated with their structure such as the conjugated double bonds and the number of hydroxyl groups in the aromatic ring mostly attributed to flavonoids and cinnamic acid derivatives (Leja et al., 2007). Flavonoids

Table 4
Antioxidant activity of pollen samples.

Sample	TBARS (mg/mg extract)	DPPH (mg/mg extract)
A	1.59 ± 0.27^{bc}	6.63 ± 0.59^{a}
B	0.35 ± 0.02^{d}	2.98 ± 0.47^{d}
C	1.08 ± 0.01^{c}	4.88 ± 0.50^{bc}
D	3.70 ± 0.00^{a}	6.69 ± 0.75^{a}
E	2.16 ± 0.03^{b}	5.14 ± 0.58^{a}
F	1.57 ± 0.48^{c}	4.44 ± 0.79^{bcd}
G	3.34 ± 0.19^{a}	6.69 ± 0.56^{a}
H	1.01 ± 0.10^{c}	3.32 ± 0.56^{cd}
P-values	<0.001	<0.001

a, b, c, d – means with different superscripts are significantly different for each attribute ($P = 0.05$).

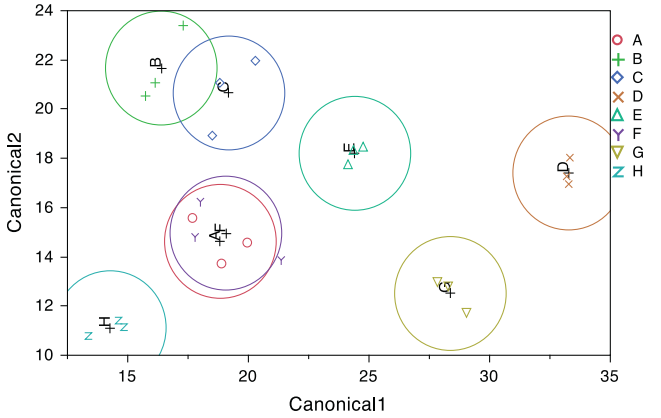

Fig. 2. Scatter plot of the first two canonical variables, of eight groups considered.

have a wide range of biological activities, including antibacterial, antiviral, anti-inflammatory, antiallergic, as well as vasodilatory actions (Abdella et al., 2009). In addition, flavonoids inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and the activity of enzyme systems including cyclo-oxygenase and lipoxygenase (Estevinho et al., 2008; Viuda-Martos et al., 2008). The results obtained in the present study were higher than the observed by Morais et al. (2011), who obtained concentrations of polyphenols between 10.5 and 16.8 mg GAE/g for honeybee-collected pollen from Portuguese Natural Parks. In the other hand, our results are in agreement with the obtained by Kroyer and Hegedus (2001) in pollen collected in Vienna; and are slightly superior to the results of Campos et al. (2003), who analysed samples from New Zealand and Portugal. Carpes et al. (2009) obtained higher values in Brazilian pollens, ranging from 19.28 to 48.90 mg GAE/g of pollen. Higher flavonoid contents were obtained by Carpes et al. (2009). According to the later study, the discrepancies between the polyphenols' and flavonoids concentration might be related to the differences on the botanical and geographical origins.

4.2. Anti-inflammatory activity

The inflammation process involves production and/or release of mediators from neurons or damaged tissues, which are responsible for different responses, including pain. Scavenging of free radicals, generated by neutrophils, is the principal mechanism of conventional drugs (Paulino et al., 2003).

Hyaluronic acid is a polyanionic high molecular mass polysaccharide found in the extracellular matrix, sensitive to oxidant-mediated fragmentation (Gao et al., 2008). The degradation of the hyaluronic acid by the hyaluronidase enzyme may cause bone loss, inflammation and pain. As consequence, the determination of the hyaluronidase enzyme is an indirect way to assess the anti-inflammatory activity (Silva et al., 2012). The lack of studies concerning this biological activity in this specific matrix hampers comparisons. However, the activity of bee pollen is lower than the obtained, using the same methodology, for propolis (Silva et al., 2012), what might be related with the lower amount of polyphenols.

4.3. Antimicrobial activities of pollen samples

Very similar MIC values were obtained by Morais et al. (2011), who analysed bee pollen from Portugal. In previous studies it has been observed that the most sensitive microorganism to the action of poppy pollen ethanolic extract was also *S. aureus* (Fatrcová-Šramková et al., 2013). The results hereby reported were slightly superior to the obtained by Morais et al. (2011), what may be related with the different microorganism origin, since in the present study the bacteria was isolated from biological fluids and in the previous study it was collected from food products. As reported in the literature it has been observed that the Gram-negative bacteria were more resistant to the pollen action than the Gram-positive bacteria, what may be related with the additional outer layer membrane, impermeable to most molecules, that consists of phospholipids, proteins and lipopolysaccharides (Silici and Kutluca, 2005). In all the microorganisms under study it was observed that the reference strains were more sensitive than the isolated from biological fluids, what has already been reported in other beehive products (Silva et al., 2012). This suggests that bee pollen could be used combined with antibiotics, since, as far as we know; there are not microorganisms capable of acquiring resistance to bee pollen.

Table 5
Pearson's correlation coefficients between phenolic compounds, flavonoids, antioxidant activity (determined using the methods TBARS and DPPH) and MIC the microorganisms under study.

	Phenolics	Flavonoids	TBARS	DPPH	Hyal. inhibition
Phenolics	1				
Flavonoids	0.369	1			
TBARS	0.338	0.302	1		
DPPH	0.083	0.286	**0.730****	1	
Hyal. inhibition	**0.477***	0.205	**0.420***	0.221	1
S. aureus ATCC	0.391	0.333	0.303	0.352	−0.149
S. aureus ESA	0.401	0.263	**0.486***	0.372	0.232
P. aeruginosa ATCC	0.143	0.033	0.094	0.184	−0.112
P. aeruginosa ESA	0.259	0.203	**0.652****	**0.434***	0.047
E. coli ATCC	0.004	−0.177	0.388	0.265	−0.002
E. coli ESA	0.169	0.179	**0.723****	**0.454***	0.126
C. glabrata ATCC	−**0.555****	0.144	−0.237	−0.058	−0.221
C. glabrata ESA	−0.303	−0.143	−0.346	−**0.495***	0.151

* corresponds to values <0.05 and ** to values <0.001.

4.4. Antimutagenic activity

Recently, a considerable emphasis is being laid down on the use of dietary constituents as chemoprotective measure for control of neoplastic and genetic diseases (Abdella et al., 2009). Some studies have lauded the antimutagenic properties of diverse beekeeping products against the influence of some chemical and physical mutagens (Bariliak et al., 1996). Most of the protective agents react directly with the mutagen or interfere with the free radicals, free oxygen species produced or inhibit cytochrome P450-mediated metabolism. The specific mechanism of protection of the bee pollen extract is reported to involve the scavenging of potentially toxic and mutagenic electrophiles and free radicals and the modification of antioxidant pathways (Ohta et al., 2007).

4.5. Antioxidant activity of pollen samples

The determination of the antioxidant activities is strongly influenced by the experimental conditions and heterogeneity of matrix, reason by which it is desirable to assess this biological activity using two methodologies (Sakanaka and Ishihara, 2008). In the present study it were used two methods: scavenging of DPPH$^{\cdot}$ and inhibition of lipid peroxidation (TBARS). Silva et al. (2006) reported that the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is one of the few stable organic nitrogen free radicals, being widely used to determine the free radical scavenging ability of diverse samples, either natural or synthetic. In the first method, results are expressed as the ratio percentage of the absorbance decrease of the DPPH radical solution in the presence of the extract. Regarding the second method, the reaction of malondialdehyde, which is formed by the degradation of fatty acids by free radicals, with 2-thiobarbituric acid is one of the most widely used estimators of oxidative stress (Liu et al., 1997). The results obtained with these methods were presented as EC_{50}, which is the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (Table 4). The results obtained were identical to the reported by Morais et al. (2011). These authors found EC_{50} values that ranged from 2.16 to 5.87 mg/mg extract in pollen samples collected from five Portuguese Natural-Parks. Almaraz-Abarca et al. (2004) verified that the antioxidant activity, *in vitro* and *in vivo*, is related to the amount of flavonoids present. However, our results, as well as the obtained by Mărghitaş et al. (2009), revealed that there is no strong relation between the phenolic compounds and the antioxidant activity.

4.6. Correlation and discriminant analysis

Since *C. glabrata* ATTC was the only microorganism whose MIC was significantly correlated with polyphenols, while in the other microorganisms it was related with the antioxidant activity, further studies must be conducted to elucidate the action mechanisms of bee pollen in the cells, both prokaryotic and eukaryotic.

TBARS was significantly and positively correlated with DPPH, suggesting that both methods may be applied to determine the antioxidant activity of bee pollen. Another interesting correlation was found between antioxidant and anti-inflammatory activities. Indeed, according to the literature oxidants play a significant role in the pathogenesis of many disorders among which inflammation (Geronikaki and Gavalas, 2006).

The first canonical dimension explained 53.85% of the total variance and the second 18.14% and a total of 72.2% of variance were accounted for by the two canonical variables. The model accepted one more canonical variable ($P < 0.0001$) and more 17.21% of total variance were accounted reaching 89.40% of the total explained variance for by the three canonical variables. The 98.19% of total variance explains was obtained with a fourth canonical variable ($P < 0.0001$) and all individuals of each group were assigned to the corrected group with 100% of classified correctly. The fourth canonical variables discriminated all 8 groups with great accuracy and the more useful characteristics to classify the pollen samples were TBARS, phenolics, flavonoids and anti-inflammatory activity.

5. Conclusions

All the bee pollen samples had substantial antimicrobial activity, being Gram-positive bacteria the most sensitive. In addition, the reference strains were more sensitive than the isolated from biological fluids. It was also demonstrated the antimutagenicity of the eight commercial bee pollens against EMS using a *S. cerevisiae* D7 strain, even though only two samples significantly reverted the mutations. Finally, the results obtained in this study demonstrated that bee pollen possesses good antioxidant activity, suggesting that it could be useful in prevention of diseases in which free radicals are implicated. Further *in vivo* studies must be conducted in order to elucidate the action mechanisms underlying these beneficial biological properties and to determine the functional significance of the present results.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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