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ORIGINAL RESEARCH ARTICLE

Storage methods, phenolic composition, and bioactive properties of *Apis mellifera* and *Trigona spinipes* pollen

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The aim of this study was to evaluate the influence of lyophilization and drying in stove on phenolic compounds content and the biological activity of *Apis mellifera* and *Trigona spinipes* pollens produced in Brazil. In general, the bee pollen produced by *T. spinipes* presented highest antioxidant activity in dried and fresh samples assessed either by test of plasma ferric reduction capacity (FRAP) and free radical scavenging assay (DPPH) methods. For *A. mellifera* bee pollen the antioxidant activity was higher on the fresh samples. Nevertheless, *β*-carotene bleaching assay (BCB) and linoleic acid content were higher in *T. spinipes* samples, mainly in the fresh ones. Higher antioxidant activity was owing to higher content in phenolic compounds. Lyophilization method was the best for phenolic compounds' conservation for both species. The bee pollen of both species has a high amount of flavonoids: kaempferol-3-*O*-glucoside was the most abundant in *A. mellifera* while for *T. spinipes* the most prevalent was resorcylic acid + epicatechin. All extracts presented antibacterial activity against *Saphylococcus aureus* (ATCC 43300)^{TM}, (ESA 83138150), (ESA 32), *Pseudomonas aeruginosa* (ATCC 15442)^{TM}, *Pseudomonas aeruginosa* (MRC.4) and (MRC.10). The methods used for storage influenced the biological properties of bee pollen from both species. Regarding the content of phenolic compounds, differences were observed amongst the pollen types: for *A. mellifera* these were best preserved with lyophilization, while for *T. spinipes* the three storage methods were equivalent.

Keywords: Honey; HPLC-PDA; *Pseudomonas aeruginosa*; *Pseudomonas* spp; *Staphylococcus aureus*

Introduction

Foods are natural sources of various health promoting substances with important biological activities. Consequently, there is a growing interest on the nutritional and functional properties of food constituents as an important tool for promoting human health. In this context, bee pollen has become a product of interest with increasing investigations regarding its composition and biological activity.

The use of bee pollen as a supplement in diets is recommended because of its bioactivity (Tomás, Falcão, Russo-Almeida, & Vilas-Boas, 2017). Phenolic compounds are the most important bioactive substances of this beehive product and are an important source of secondary metabolites (Bogdanov, 2017). There is increasing evidence that the consumption of a variety of phenolic compounds in natural foods may reduce the risk of serious health disorders due to their antioxidant activity, among other mechanisms (Shahidi & Ambigaipalan, 2015).

Bee pollen has a high nutritional value and contains a wide variety of amino acids, vitamins, minerals, organic acidic proteins, and carbohydrates (Kalaycıoğlu, Kaygusuz, Döker, Kolaylı, & Erim, 2017; Yang et al., 2013). Research has also shown a large amount of biologically active compounds such as flavonoids and polyphenols (Carpes et al., 2013; Kaškonienė, Ruočkuvienė, Kaškonas, Akuneca, & Maruška, 2015) as well as a great range of therapeutic properties including antitumor activity (Wang et al., 2013), antimutagenic (Dias, Tolentino, Pascoal, & Estevinho, 2016), hepato and renoprotective (Huang et al., 2017), antibacterial (Fatrcová-Šramková, Nôžková, Máriássyová, & Kačániová, 2016), anti-inflammatory (Pascoal, Rodrigues, Teixeira, Feás, & Estevinho, 2014), and anti-oxidant (de Florio Almeida et al., 2017; Kalaycıoğlu et al., 2017).

There are several studies that prove the quality of *Apis mellifera* pollen worldwide, but there are still few studies concerning bee pollen from stingless bees, especially *Trigona spinipes*.

Bee pollen has a very variable composition, which depends mainly on the floral source. However, seasonal and environmental factors and processing methods also play an important role (De-Melo et al., 2016). The use

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of intense heat treatments in foods rich in secondary metabolites should be avoided. The objective of this study was to evaluate the effect of the processing methodologies on the phenolic compounds content and the biological activity of bee pollen from *A. mellifera* and *T. spinipes*.

Material and methods

Bee pollen samples

Bee pollen produced by *T. spinipes* Fabricius, 1793 (Arapuá) was collected from May to August 2016 in the territory of Identidade Piemonte do Paraguaçu, Brazil, semi-arid macro-region, under the domination of the caatinga, with characteristics of semiarid climate and has the main hydrographic basin the river Paraguaçu.

The pollen from *A. mellifera* Linnaeus, 1758 was collected in June 2016 by beekeepers of the municipality of Canavieiras (15°41′S, 38°57′W), located in the "South Coast" economic micro-region of the state of Bahia, Brazil, characterized by wide areas of mangrove, but also has large expanses of forest, restinga, and dunes (Dórea, Novais, & Santos, 2010).

All the collected material was stored in containers, previously sterilized, and conditioned in icebox until reception at the laboratory. There the product was homogenized and frozen in a freezer at −18 °C. Each sample was divided into three aliquots, one aliquot was frozen at –80 °C and then dehydrated in a vacuum freeze-dryer (Terroni-Enterprise II-Brazil) for 24 h. The other aliquot was thawed at room temperature and dried in a drying oven with forced air circulation (Tecnal-Brazil) at 42 °C for 48–52 h (De-Melo et al., 2016). The third aliquot was used fresh.

Reagents

Methanol, chloroform ($CHCl_3$), sodium carbonate (Na_2CO_3), sorbitan monooleate (Tween 40), iron (III) chloride hexahydrate, and gentamycin were obtained from Merck (Darmstadt, Germany). Trolox, gallic acid, quercetin, linoleic acid (LA), β-carotene, Folin–Ciocalteu reagent, 2,3,5-triphenyl tetrazolium chloride (TTC), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), and 2,2-diphenyl-1-picrylhydrazyl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). High purity water (18 MX cm) was obtained from a Milli-Q purification system (Millipore, Barnstrad, MA, USA).

HPLC analyses were carried out using HPLC grade methanol and formic acid (Merck, Darmstadt, Germany). The reference standards for phenolic compounds purchased from Sigma-Aldrich (Steinheim, Germany) used in this study were phenolic acids, namely gallic acid (≥99%), protocatechic acid (99.63%), vanillic acid (97%), syringeic acid (≥98%), resorcylic acid (≥97%), 4-hydroxybenzoic acid (≥99%), 4-hydroxybenzaldehyde (98%), ferulic acid (≥ 99%), synapic acid (≥99%), cinnamic acid (≥99%), caffeic acid (≥98%), *p*-coumaric acid (≥98%), chlorogenic acid (>95%), 4-hydroxyphenylacetic acid (98%); (95%), kaempferol (≥98%), myricetin (≥96%), naringenin (98%), rutin (≥94%), pinocembrine (95%), quercetin-3-*O*-glucopyranoside (≥99%), kaempferol-3-*O*-rutinoside (≥98%), kaempferol-3-*O*-glucoside (≥95%); tiliroside (≥98%); the chalcones: dehydrated floridzine (99%) and floretin (≥98.5%); and resveratrol (≥99%).

Hydroethanolic extract of pollen

The extraction process was performed according to de Florio Almeida et al. (2017). Briefly, samples containing 10 g of bee pollen (dry basis) were extracted with 50 mL of 70% ethanol solution (vol/vol) after shaking on a shaker plate for 45 min at room temperature. The extract was filtered through a qualitative filter paper and the supernatants were evaporated on a rotary evaporator (vacuum pressure of 600 mm Hg and 40 °C) until completely dry. The extract was then placed on the drying oven until constant weight was reached. It was stored in refrigerator until subsequent analysis.

β-Carotene bleaching assay

The β-carotene bleaching (BCB) technique, described by Marco (1968), was applied using microplates with slight modifications. Briefly, 4 mg of BCB, 0.5 mL of linoleic acid and 4 g of Tween-40 were dissolved in 20 mL of chloroform, the stock solution was distributed in 1 mL aliquots and the chloroform was evaporated on a rotary evaporator (IKA® RV-USA) at 45 °C for 15 min. The stock solution was resuspended in 25 mL of mili-Q water at 45 °C. For the measurement of the antioxidant capacity, 50 μL of sample was mixed with 250 μL of reagent in a 96-well microplate. The samples were analyzed in triplicate. The absorbance (470 nm) readings were done on Thermo Fisher Scientific microplate reader (Waltham, MA, USA) until BCB was discolored (about 2 h). The antioxidant activity (AA) was calculated as follows:

$$AA = (\text{RD Control} - \text{RD Sample})/\text{RD Control} \times 100$$

RD Control [=ln (a/b)/120] the percentage of BCB without the presence of the antioxidant. Where: ln is the natural logarithm; a = absorbance at time 0; b = absorbance after 120 min. RD Sample [=ln (a/b)/120] the percentage of BCB in the presence of the antioxidant.

Free radical scavenging assay (DPPH)

The antioxidant activity was determined using the 96-well plate assay as previously described by Bobo-García et al. (2015). For that, 20 μL of the diluted sample was added to 180 μL of DPPH solution (150 μmol L^{-1}) in methanol–water (80:20, vol/vol) and shaken for 60 sec in a 96-well microplate. After 40 min in the dark at

room temperature, the absorbance was measured at 515 nm in the Thermo Fisher Scientific microplate reader (Waltham, MA, USA). Trolox was used as the standard at 50–500 μmol L^{-1} to generate a calibration curve.

Test of plasma ferric reduction capacity

The total antioxidant potential of the extracts was determined using a plasma ferric reduction capacity (FRAP) described by Tachakittirungrod, Okonogi, and Chowwanapoonpohn (2007) and LeBlanc, Davis, Boue, DeLucca, and Deeby (2009). The FRAP reagent was freshly prepared: 10 mM 2,4,6-tripyridyl-*S*-triazine (TPZ) in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.3 M sodium acetate buffer and 0.3 M acetic acid at pH 3.6 to (1:1:10) parts by volume, respectively. The extracts were dissolved in ethanol at a concentration of 1 mg mL^{-1}. An aliquot of 20 μL of solution test was mixed with 180 μL of FRAP solution. Absorption of the reaction mixture was measured at 590 nm using the Thermo Fisher Scientific microplate reader (Waltham, MA, USA). Ethanol concentrations (50–500 mM) of trolox were used to construct a calibration curve.

Total phenols

Total polyphenols were determined using the Folin–Ciocalteu Colorimetric Method, (Singleton, Orthofer, & Lamuela-Raventós, 1999) based on the 96-well microplate as described by Bobo-García et al. (2015). A total of 20 μL of the hydroethanolic pollen extract were mixed with 100 μL of Folin–Ciocalteu diluted at 1:4 and stirred for 60 sec on a 96-well microplate. The mixture was left for 240 s and then 75 μL of sodium carbonate solution (100 g L^{-1}) was added. The mixture was stirred at medium-continuous speed for 1 min. After 2 h at room temperature, the absorbance at 750 nm was measured using the Thermo Fisher Scientific microplate reader (Waltham, MA, USA). Gallic acid dilutions (10–200 g L^{-1}) were used to construct a standard curve.

Total flavonoids

The analysis was done following the methodology proposed by Herald, Gadgil, and Tilley (2012). In brief, to each of the 96 wells was added 100 μL of distilled water, followed by 10 μL $NaNO_2$ (50 g L^{-1}) and 25 μL of sample extract or standard solution. After 5 min, 15 μL of $ALCl_3$ (100 g L^{-1}) was added to the mixture, after 6 min 50 μL of NaOH (40 g L^{-1}) and 50 μL of distilled water were added. The plate was shaken for 30 sec on the Thermo Fisher Scientific microplate reader (Waltham, MA, USA) prior to measuring the absorbance at 515 nm. Quercetin (5–100 g L^{-1}) was used to generate a standard curve.

Analysis of phenolic compounds by HPLC-PDA

The identification and quantification of phenolic compounds was performed according to the method previously described by Moreira et al. (2017). The pollen extracts (20 μL) were injected into an HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an LC-20AD pump, a DGU-20AS degasser, a CTO-10AS VPN column oven, a SIL automatic multimeter-20 A HT, and a SPD-M20A photodiode array detector (PDA). The separation of phenolic compounds was performed using a Phenomenex Gemini C_{18} column (250 mm × 4.6 mm, 5 μm) at 25 °C and the chromatograms were recorded at 280, 320, and 360 nm depending on the maximum wavelength of the phenolic compound. The mobile phase is composed of methanol (solvent A) and water (solvent B) both acidified with 0.1% formic acid. The phenolic compounds were analyzed using a gradient elution at 1.0 mL min^{-1} with the following schedule: 0–13 min: 20–26.5% A; 13–18 min: 26.5% A; 18–25 min: 26.5–30% A; 25–50 min: 30–45% A; 50–60 min: 45–50% A; 60–70 min: 50–55% A; 70–90 min: 55–70% A; 90–100 min: 70–100% A, followed by 100% A for 5 min and back to 20% A in 10 min and 5 min reconditioning before the next injection. The identification of phenolic compounds was performed by comparing the UV absorption spectra and the retention time of each compound with those of pure standards injected under the same conditions. For the quantification of phenolic compounds, different concentrations of each standard were prepared from the respective stock solution in methanol at concentrations ranging from 1 to 5 g L^{-1} and stored at –20 °C, and the results were expressed in milligrams of compound per liter (mg L^{-1}).

Antibacterial activity

The microorganisms used for the antimicrobial assays were strains of *Staphylococcus aureus* (ATCC 43300)™, *S. aureus* (ESA 83138150), *S. aureus* (ESA 32), *Pseudomonas aeruginosa* (ATCC 15442)™, *Pseudomonas* spp. (MRC.4), and *Pseudomonas* spp. (MRC.10). These were used as reference microorganisms and obtained from the authorized distributor (LGC Standards SLU, Barcelona) ATCC (American Type Culture Collection) as well as the same microorganisms obtained from clinical isolates (biological fluids from the Local Hospital) that were identified in the Laboratory of Microbiology of the Agricultural School of Bragança, Portugal.

The inoculum of the bacteria were suspended in liquid medium, Brain Heart Infusion (BHI), and incubated in an orbital bacteriological oven (model SI50), at 37 °C for 24 h. A bacterial suspension of 1×10^8 CFU in a 0.85% sterile saline solution with optical density of 0.3 on the McFarland scale, read at 540 nm in UV–visible spectrophotometer (Unicam Hekios Alpha), was prepared following three successive dilutions (1:10) in liquid medium (BHI) to obtain a suspension equivalent

Table 1. Total phenolic content and total flavonoids (mg g^{-1}) and antioxidant activity of *Apis mellifera and Trigona spinipes* pollen samples dried in stove, lyophilized, and fresh.

		Pollen			
Variables	Species	Lyophilized	Dry	Fresh	CV (%)
Total phenols (mg GAE g^{-1})	*A. mellifera*	9.9Bc ± 0.42	13.84b ± 0.78	20.45Aa ± 1.40	3.68
	T. spinipes	12.72Ab ± 0.50	14.06a ± 0.19	14.18Ba ± 0.46	5.82
Total Flav (mg QE g^{-1})	*A. mellifera*	3.82 ± 0.33	3.41 ± 0.04	3.53 ± 0.03	5.34
	T. spinipes	4.63 ± 0.16	4.73 ± 0.44	4.82 ± 0.04	5.80
FRAP (mmol TE g^{-1})	*A. mellifera*	36.95Bb ± 2.38	36.71Bb ± 1.68	49.10Ba ± 2.38	6.92
	T. spinipes.	50.40Ab ± 2.91	64.21Aa ± 1.71	60.24Aa ± 4.36	4.62
DPPH (μmol TE g^{-1})	*A. mellifera*	10.87Bb ± 0.05	11.21Bb ± 1.21	15.41Ba ± 1.17	14.58
	T. spinipes	17.68Ab ± 1.84	28.72Aa ± 1.85	26.36Aa ± 3.03	5.78
BCB (%)	*A. mellifera*	91.86Ba ± 1.56	87.87Bb ± 1.07	91.27Ba ± 0.58	1.36
	T. spinipes	96.40Aab ± 0.04	95.62Ab ± 0.24	96.71Aa ± 0.06	0.46

Different letters (lower case) in the same row represent statistical differences ($p < 0.05$) by the Tukey test. Different letters (upper case) in the same column of the results represent statistical differences ($p < 0.05$) by the F test. DPPH, Flavonoids (Flav) and FRAP. Coefficient of variation (CV).

to 1×10^5 CFU mL^{-1}. The antimicrobial activity was determined by the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (CMB) based on the 96-well microplate microdilution method, with the intention of defining the lowest concentration capable of inhibiting the growth of microorganisms (Morais, Moreira, Feás, & Estevinho, 2011).

Statistical procedures

The experimental design was completely randomized, in a subdivided plot scheme, with six treatments and three replications. The treatments consisted of a combination of two bee species (*A. mellifera* and *T. spinipes*), which were allocated to the plots, and three methods of pollen conservation (dry, lyophilized, and fresh) in the subplots. The effect of species versus method interaction on the analyzed variables was verified through analysis of variance. The effect of species on each method was compared using the *F* test and the effect of method on each bee species was compared by means of the Tukey test. In addition, the analysis of principal components was used to check the relationship between the variables and samples. All analyses were performed using software R version 3.0.2 (Team, 2017).

Results

Contents of total phenols and flavonoids

There was an interaction ($p < 0.0001$) between bees' species and the conservation method for the parameter "total phenols." The highest value of total phenols (20.45 ± 1.40 mg GAE g^{-1}) was found on fresh *A. mellifera* pollen samples. The lyophilized pollen presented the lowest total phenol content for both species studied, although they differed from each other with *A. mellifera* pollen presenting higher value for total phenols than *T. spinipes*. There was no interaction ($p > 0.05$) amongst bees' species and the conservation method for the parameter "total flavonoids." The values found for the total flavonoid content were between 3.41 ± 0.04 mg QE g^{-1} (dry pollen of *A. mellifera*) and 4.82 ± 0.04 mg QE g^{-1} (fresh samples of *T. spinipes*) (Table 1).

Antioxidant activity

Concerning the antioxidant activity it was observed the interaction between bees' species and the storage methodology regardless of the technique used to determine the antioxidant activity [FRAP ($p = 0.0005$), DPPH ($p < 0.0001$), and BCB ($p = 0.0006$); these results are expressed in Table 1. The antioxidant activity assessed by the FRAP method of *A. mellifera* pollen was similar amongst dried and lyophilized samples. On the other hand, the pollen produced by *T. spinipes* presented higher antioxidant activity when the drying methodology was applied. The antioxidant activity results obtained using the DPPH methodology has a very similar pattern for both species. A significant correlation ($p < 0.05$) was observed between the total flavonoid content and the antioxidant activity determined by both techniques: [DPPH ($r = 0.85$) and FRAP ($r = 0.82$)].

The antioxidant activity ascertained by the BCB technique for fresh and lyophilized samples did not differ ($p > 0.05$) among each bee pollen type. Yet, lower antioxidant activity values were obtained for *T. spinipes* dried pollen.

The values obtained using DPPH, FRAP, and BCB differed among species, being the highest values found in *T. spinipes* pollen.

Analysis of phenolic compounds – HPLC

There was a significant interaction between species of bees and conservation method for the variables gallic acid ($p < 0.0001$), rutin ($p < 0.0001$), resveratrol ($p = 0.0005$), kaempferol-3-*O*-glucoside ($p < 0.0001$), kaempferol-3-*O*-rutinoside <0.0001), and quercetin ($p < 0.0001$). The kaempferol-3-*O*-glucoside was the most abundant compound in *A. mellifera* lyophilized pollen (65.50 ± 2.76) and its content was higher than that of *T. spinipes* pollen, regardless of the storage

Table 2. Phenolic composition (mg.100 g^{-1}) of *Apis mellifera* and *Trigona spinipes* pollen samples submitted to different methods of preservation (dried in stove, lyophilized and fresh) determined by HPLC-PDA (mean ± SD).

		Pollen			
Variables	Species	Lyophilized	Dry	Fresh	CV (%)
Gallic acid	*A. mellifera*	10.07Bb ± 1.01	33.63Ba ± 1.75	10.74Bb ± 0.71	4.47
	T. spinipes	46.20Ab ± 1.65	81.77Aa ± 1.67	34.07Ac ± 1.65	3.82
4-Hydroxyphenylacetic acid	*A. mellifera*	nd	nd	5.55 ± 0.39	
	T. spinipes	nd	nd	nd	
Epicatechin + Acid resorcilic	*A. mellifera*	nd	nd	nd	
	T.spinipes	82.70b ± 0.80	101.90a ± 1.78	nd	
Synaptic acid	*A. mellifera*	nd	nd	nd	
	T. spinipes	3.51 ± 0.56	3.57 ± 0.73	3.61 ± 0.75	
Rutin	*A. mellifera*	5.49Aa ± 0.43	4.28b ± 0.23	1.65Bc ± 0.19	10.20
	T. spinipes	4.45B ± 0.54	4.52 ± 0.47	4.09A ± 0.26	8.67
Resveratrol	*A. mellifera*	7.28Ba ± 0.26	5.52Ba ± 0.35	2.64Bb ± 0.34	12.94
	T. spinipes	12.33A ± 1.95	12.67A ± 0.75	10.69A ± 0.83	10.05
Phloridzine	*A. mellifera*	nd	nd	nd	
	T. spinipes	4.19 ± 0.39	4.58 ± 0.75	3.68 ± 0.41	
Myricetin	*A. mellifera*	4.82a ± 0.30	4.37ab ± 0.32	3.70b ± 0.46	8.01
	T. spinipes	5.23 ± 0.34	5.22 ± 0.50	4.70 ± 0.54	9.87
Quercetin-3-*O*-glucopyranoside	*A. mellifera*	3.63a ± 0.45	2.55b ± 0.21	nd	
	T. spinipes	4.36 ± 0.36	3.24 ± 0.73	nd	
kaempferol-3-*O*-glucoside	*A. mellifera*	65.50Aa ± 2.76	52.63Ab ± 2.84	18.66Bc ± 2.09	4.03
	T. spinipes	39.48Ba ± 0.71	41.53Ba ± 0.80	30.53Ab ± 0.70	4.85
Kaempferol-3-*O*-rutinoside	*A. mellifera*	37.47Aa ± 2.05	30.80Ab ± 0.46	11.58Bc ± 0.54	5.34
	T. spinipes	22.87Ba ± 1.75	24.37Ba ± 0.70	17.80Ab ± 1.57	5.64
Naringenin	*A. mellifera*	nd	nd	nd	
	T. spinipes	3.30 ± 0.41	3.40 ± 0.56	2.69 ± 0.51	
Quercetin	*A. mellifera*	6.48Aa ± 0.45	7.23Aa ± 0.41	2.26Bb ± 0.39	10.94
	T. spinipes	3.65B ± 0.68	3.82B ± 0.70	3.67A ± 0.41	11.98
Phloretine	*A. mellifera*	nd	nd	9.55 ± 0.53	
	T. spinipes	nd	nd	nd	
Tiliroside	*A. mellifera*	Nd	nd	nd	
	T. spinipes	23.10a ± 1.31	nd	19.57b ± 0.78	
Kaempferol	*A. mellifera*	3.28 ± 0.34	3.62 ± 0.37	2.88 ± 0.23	16.04
	T. spinipes	4.42 ± 0.54	5.34 ± 0.48	4.26 ± 0.50	6.34

Different letters (lower case) in the same row represent statistical differences ($p < 0.05$) by the Tukey test. Lack of letter (lower case) in the same row indicates no significant difference ($p > 0.05$). Different letters (upper case) in the same column of the results represent statistical differences ($p < 0.05$) by the *F* test. nd – Not detected.

methodology. On *T. spinipes* samples, the compounds epicatechin + resorcylic acid were predominant for the lyophilized (82.70 ± 0.80) and dried pollen (101.90 ± 1.78). The conservation methods influenced the concentration of the phenolic compounds quantified for the *A. mellifera* pollen and it was verified that lyophilization is the most efficient dehydration method, reducing processing losses. For the *T. spinipes* pollen, the conservation methods did not show significant differences for the majority of the quantified compounds and it was verified that the pollen submitted to drying and lyophilization processes had the same behavior as the fresh pollen. Pollens of both species had a higher amount of flavonoids among the quantified compounds (Table 2).

Antibacterial activity

All extracts presented antibacterial activity against *S. aureus* (ATCC 43300)^{TM}, *S. aureus* (ESA 83138150), *S. aureus* (ESA 32), *P. aeruginosa* (ATCC 15442)^{TM}, *P. aeroginosa* (MRC.4), and *P. aeroginosa* (MRC.10) (Table 3). The lyophilized *A. mellifera* pollen sample (PLA) did not present minimal bactericidal activity at the concentrations tested. The *S. aureus* bacteria showed MIC of 10 (mg mL^{-1}) – the lowest among the microorganisms tested. The fresh *A. mellifera* pollen sample (PFA) presented lower MIC and CMB.

Multivariate analysis

The first two components explained 83% total variation of the data, suggesting that they are sufficient to explain the most important information. The group composed by *A. mellifera* (dry and lyophilized) pollen samples were characterized by having higher values of KpfGsd, KpfRsd, and Qct. On the other hand, the fresh *A. mellifera* pollen formed an isolated group with higher quantification of TPC, Pht, AcREp and lower amount of QctGsd, Rvt, Myt, and Rtn, evidencing the differences between fresh and lyophilized pollen dried in stove. However, *T. spinipes* pollen presented on its composition compounds that were not observed for *A. mellifera* pollen (Achac, AcREp, AcSp, Pld, Ngn, Trd). The dried and lyophilized *T. spinipes* pollen contained higher contents of AcGl, Myt, Kpf, and Rvt. Nevertheless, the

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (CMB) in (mg g^{-1}) of *Apis mellifera* and *Trigona spinipes* pollen samples against the tested microorganisms.

		Apis mellifera			*Trigona spinipes*		
Microorganisms		lyophilized	Dry	Fresh	lyophilized	Dry	Fresh
(ATCC 43300)	CMI	100	50	10	30	50	50
	CMB	NA	100	50	75	75	75
(ESA 83138150)	CMI	30	10	30	20	50	50
	CMB	150	150	50	100	100	100
(ESA 32)	CMI	100	75	20	30	30	30
	CMB	150	100	30	50	50	50
(ATCC 15442)	CMI	75	50	30	30	30	30
	CMB	NA	100	75	100	75	100
(MRC4)	CMI	75	50	30	30	30	30
	CMB	150	100	50	75	75	50
(MCR10)	CMI	75	50	30	50	50	50
	CMB	150	100	50	75	75	75

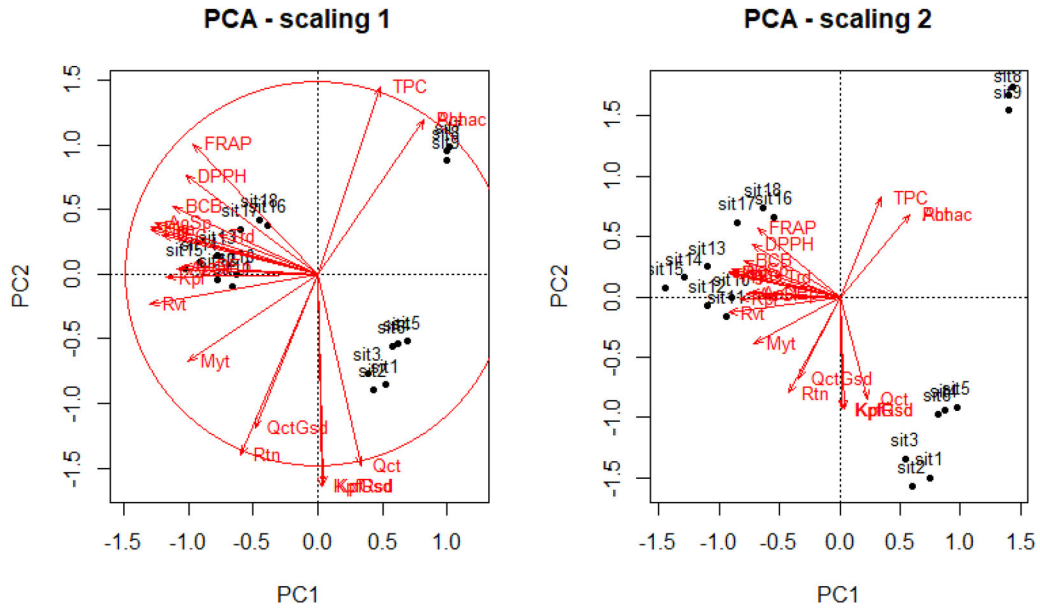

Figure 1. PCA biplots of total phenolic and flavonoid compounds and antioxidant activity assessed by DPPH, FRAP, and BCB. AcGl_Gallic acid; Achac_4-hydroxyphenilacetic acid; AcREp_Epicatechin + b-resorcylic acid; AcSp_Sinapic acid; Rtn_Rutin; Rvt_Resveratrol; Pld_Phloridzin; Myt_Myrecitin; QctGsd_Quercetin-3-*O*-glucopyranoside; KpfGsd_Kaempferol-3-O-glucoside; KpfRsd_Kaempferol-3-rutinoside; Ngn_Naringenin; Qct_Quercetin; Pht_Phloretin and Trd_Tiliroside. The Sit 1, Sit 2, Sit 3_Lyophilized *A. mellifera* pollen; Sit 4, Sit 5, Sit 6_dry *A.mellifera* pollen; Sit 7, Sit 8, Sit 9_fresh *A. mellifera* pollen; Sit 10, Sit 11, Sit 12_lyophilized *T. spinipes* pollen; Sit 13, Sit 14, Sit 312 15_dry *T. spinipes* pollen; Sit 16, Sit 17, Sit 18_fresh *T. spinipes* pollen.

fresh samples of *T. spinipes* pollen showed higher values of antioxidant activity.

Figure 1 shows the PCA biplots of total phenolic and flavonoid, phenolic compounds, and antioxidant activity (assessed by DPPH, FRAP, and BCB). PC1 and PC2: 50.26% and 32.42%, respectively, of proportion explained.

Discussion

The results obtained in this study for the content of total phenols confirm those found by De-Melo et al. (2016) with values between 9.2 and 21.4 mg GAE g^{-1} for lyophilized and oven-dried bee pollen. Zuluaga et al. (2016) presented higher values (24.79 ± 3.3 to 33.69 ± 1.37 mg GAE g^{-1}) for *A. mellifera* pollen processed at high pressure and stated that the storage method employed influenced the quantification of phenols. The higher total phenol content of fresh *A. mellifera* pollen samples may be due to chemical reactions (Maillard reaction) that caused the sample to darken. Similar fact was reported by Barene, Daberte, and Siksna (2015) where the bee bread stored at temperatures of 30–40 °C became darker and with high moisture content. The method used to analyze the total phenol content in pollen is generally Folin–Ciocalteu, in this procedure the color change is measured spectrophotometrically (UV–vis). According to Ares, Valverde, Bernal, Nozal, and Bernal (2018), this is not a specific test because this reagent does not only react with

phenols, but with any reducing substance of the sample, emphasizing the importance of the chromatographic analysis.

Several methods with different detection mechanisms were used to analyze antioxidant activity due to differences in the composition and content of antioxidant agents. The results obtained in this study for antioxidant activity for the *T. spinipes* pollen are similar to the results presented by de Florio Almeida et al. (2017). The results reported in this study were slightly lower than those. Using the DPPH method to assess antioxidant activity, De-Melo et al. (2016) found values between 31.7 ± 0.7 and 81.1 ± 3.7 mmol Tg g^{-1} for dried and lyophilized *A. mellifera* pollen samples, highlighting the differences between the processing methods. Studies indicate that the phenolic compounds present in the bee pollen are responsible for their antioxidant activity (Kim et al., 2015). However, compounds such as polyphenols (anthocyanins, flavonoids, phenolic acids, ketones, stilbenes, and tannins), carotenoids, ascorbic acid, fatty acids, proteins, and other substances may also influence this activity (Huang et al., 2017). Natural antioxidants can be used as natural additives, helping to preserve some nutrients, sensorial characteristics, or some biologically active ingredients of foods submitted to storage or processing (Li et al., 2014).

Freire et al. (2012) performed the quantification of the phenolic compounds of *A. mellifera* bee pollen from the region of Canavieiras, Bahia, and detected the flavonoids isoquercetin, myricetin, tricetine, quercetin, luteolin, selagin, kaempferol, and isorhamnetin. Flavonoids, such as rutin and myricetin, were reported in *A. mellifera* pollen from the southern Brazil by Carpes et al. (2013) and was also reported by de Florio Almeida et al. (2017). The presence of other flavonoids, such as quercetin and kaempferol, was also identified in pollen from the same region of Brazil. In pollen from *A. mellifera* from Greece nine polyphenols were identified namely *o*–*p*-cocmaric acid, ferulic acid, myricetin, cinnamic acid, quercetin, naringenin, hesperitin, and kaempferol (Fanali, Dugo, & Rocco, 2013). Rzepecka-Stojko et al. (2015) evaluated bee pollen from Poland and found the following compounds: gallic acid, caffeic acid, ferric acid, 4-hydroxycinnamic acid, *trans*-*p*-coumaric acid and *trans*-cinnamic acid, five flavonoids: rutin, myrmycin, quercetin, kaempferol, and isorhamnetin. According to Sun, Guo, Zhang, and Zhuang (2017) the presence of flavonoids such as quercetin and rutin in bee pollen increases the nutritional and biological quality of the product due to their high antioxidant and anti-melanogenesis function. Flavonoid composition has also been considered an important factor to distinguish the floral origin of the pollen, which may be an important parameter for markers of denomination of origin (Zhou et al., 2015). Therefore, the floral and geographical origin and the species of bees are key elements influencing the qualitative and quantitative composition of phenolic compounds of bee pollen. In the same context, the conservation method is also known to influence the phenolic composition of *A. mellifera* pollen. Regarding pollen produced by *T. spinipes*, as far as the authors know, there is no information available in the literature.

The antimicrobial activity against Gram-positive bacteria is in agreement with that found by Pascoal et al. (2014) where good efficiency of the *A. mellifera* pollen extract was reported for the antimicrobial activity in Gram-positive strains. According to Morais et al. (2011), Gram-negative bacteria are more resistant because the chemical structure of their cell wall is more complex. One of the compounds on the wall, the polysaccharide, determines the antigenicity, toxicity, and pathogenicity of the microorganisms. In addition, this bacterial group has a greater amount of lipids that act as an extra protective coating around the cell membrane, reducing its exposure to the pollen extract. Studies like this are important due to the harmful emergence of antibiotic resistance and the growing interest in natural therapy as effective coadjutant and source of compounds with pharmacological potentialities.

Conclusions

The studied conservation methods influenced the phenolic composition and the biological properties of the pollen produced by both bee species (*A. mellifera* and *T. spinipes*). Our results suggest that lyophilization is the best way to preserve the content of phenolic compounds in the *A. mellifera* pollen. However, on the other hand, for bee pollen produced by *T. spinipes* the storage method did not significantly influence the phenolic composition. Therefore, having into account the costs inherent to each conservation process and the hereby reported results it is suggested that bee pollen from *T. spinipes* (a fermented product with little susceptibility to contamination) must be stored fresh. The profile of phenolic composition of pollen produced by *T. spinipes* differs quantitatively and qualitatively in relation to the pollen from *A. mellifera*. The fresh and dried samples presented higher antioxidant activity due to their higher content in phenolic compounds.

Disclosure statement

The authors declare that there are no conflicts of interest. Also are indebted to the careful and constructive criticisms of the reviewers.

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