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Honey and bee pollen produced by Meliponini (Apidae) in Alagoas, Brazil: multivariate analysis of physicochemical and antioxidant profiles

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Abstract

This study evidenced the physicochemical composition and antioxidant activity (AA) of honeys (n = 31) and pollen (n = 25) of stingless bees species from Alagoas, Brazil. Fifteen parameters were studied under the light of a multivariate analysis. A dendrogram with three groups of honeys was stablished for the different bees, being the group III formed exclusively by samples from genus *Melipona*. *Plebeia* sp. and *Tetragona clavipes* Fabricius (1804) produced honeys with higher pH/acidity, electrical conductivity, phenolic total and AA. Even different bees from the same meliponary, as *M. asilvai* and *T. clavipes*, produced honeys with different AA and chemical profiles, possibly due their different nectar preference. The multivariate analysis of the bee pollen samples also showed three principal components responsible for 74.52% of their variability, clustering nine groups strongly influenced by total phenolics and AA. Honey and pollen produced by *T. clavipes* had the highest phenolic content and AA. Therefore, the chemical characteristics and AA of the Meliponini honey and bee pollen showed here can give support to the market to influence their incorporation in the human diet as sources of potential functional foods.

Keywords: Melipona; polyphenols; flavonoids; stingless bees; *Tetragona clavipes*.

Practical Application: The physicochemical and antioxidant properties of honey and pollen produced by seven Meliponini species.

1 Introduction

Stingless bees (Hymenoptera: Apidae: tribe Meliponini) - also known as native and meliponines, are widely distributed in tropical and some temperate-subtropical regions of the planet. There are more than 600 species of them in different genera, as the most well-known *Melipona* Illiger (1806), and several other of trigoniforms (Kerr et al., 1996; Francoy et al., 2016).

Besides the importance of stingless bees for the pollination, they can be cultivated because of their honey and bee pollen, which are completely different from those produced by bees of the genus *Apis* Linnaeus, 1758 (Rao et al., 2016; Vit et al., 2004). The Brazilian rural population has a strong tradition of using products from stingless bees, especially in the North (Amazonia) and Northeast (dry lands of *Caatinga*) regions (Rebelo et al., 2016; Silva et al., 2013).

Natural honeys contain more than 180 substances (Al et al., 2009), being most of them responsible for the honeys medical properties reported for since ancient times, by aborigines people around the world. Studies on the chemical composition and antioxidant activity (AA) of Melliponini honeys, however, are still scarce (Duarte et al., 2012; Souza et al., 2016). The bee pollen results from the agglutination of the floral nectar and pollen with the salivary substances of bees, being accumulated in the pollen baskets (corbiculae) of bees (structures like small bags), and there are about 250 substances in its chemical composition (Barreto et al., 2006; Nogueira et al., 2012; Silva et al., 2014). Bee pollen also has not been very well studied in Melliponini as in *A. mellifera* (Biluca et al., 2016; Rao et al., 2016) and at despite of its folk therapeutic uses, as anti-allergenic against rhinitis or antiteratogenic (Omarr et al., 2016), the market of natural products has stimulated its consume as food supplement, due to its high content of protein (with all the essentials amino acids), minerals, vitamins, fatty acids and other organic acids, flavonoids, lipids, and carbohydrates (Silva et al., 2009, 2014). The purpose of this study was to investigate the physicochemical and antioxidant profiles of honey and bee pollen produced by stingless bees from Alagoas (Northeast Region of Brazil).

2 Materials and methods

2.1 Samples of honey and bee pollen from meliponini species

Different samples of honey (n = 31) and bee pollen (n = 25), respectively from seven stingless bees species: *Melipona asilvai* Moure (1971); *M. quadrifasciata anthidioides* Lepeletier (1836); *M. scutellaris* Latreille (1811); *M. subnitida* Ducke (1910); *Frieseomelitta varia* Lepeletier (1836); *Tetragona clavipes* Fabricius (1804) and *Plebeia* sp., and six of such species (all the above mentioned less *F. varia*) were collected in four private

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meliponaries from distinct mesoregions of the State of Alagoas (Northeast Brazil), mainly in the *Caatinga* biome, with nests placed in the hollow trunks of trees (as found in nature) or in a standard rational beehive box (Table 1).

Harvesting of honey and bee pollen were carried out during the rainy season (2009/2010) every fortnight, using sterilized syringes (honeys) and sterile glass pots for store all the samples (honey and bee pollen). These pots were deposited on ice inside isothermal boxes to be transported to the laboratory in the Institute of Chemistry and Biotechnology (IQB), Federal University of Alagoas (UFAL), and refrigerated.

2.2 Physicochemical profile of honey

The following physicochemical parameters were evaluated in triplicate of the honey samples, according to the preconized methods: moisture (Association of Official Analytical Chemists, 1997); pH, free acidity, contents of reducing sugar (Vargas, 2006); electrical conductivity, contents of proline and hydroxy-methyl-furfural (HMF) (Bogdanov, 2002); diastase activity (Santos et al., 2003); and color (Bianchi, 1989).

2.3 Total content of phenolics and flavonoids of honey

Each sample of honey (1 g) was diluted in 10.0 mL of sterilized deionized water, filtered (preparation-filter paper), and submitted to the test (Meda et al., 2005). The content of total phenolics in the diluted honeys samples (0.1 g.mL^{-1}) and in aqueous solutions of gallic acid (GA) (0.0-100.0 μg.mL^{-1}, used for the standard calibration curve), was analyzed by spectrophotometry at 760 nm.

On the other hand, the content of total flavonoid in methanolic solutions of honey (100.0 mg.mL^{-1}) and methanolic solutions of quercetin (Q) (5.0 to 120.0 μg.mL^{-1}, used for standard calibration curve) was analyzed by spectrophotometry at 510 nm (Al et al., 2009).

2.4 Antioxidant activity of honey

The antioxidant activity (AA) was determined in triplicate using three methods: (1) *2,2-diphenyl-1-picryl-hidrazil radical* (DPPH) scavenging method (Meda et al., 2005); (2) "*ferric reducing-antioxidant power*" (FRAP) test (Küçük et al., 2007); and (3) inhibition of β-Carotene bleaching, described by Carpes et al. (2008) with modification.

For the first test, each sample of honey was diluted in methanol (40.0 mg mL^{-1}) and 0.75 mL of each dilution was added to 1.5 mL of methanolic DPPH solution (0.02 mg mL^{-1}). Standard curves were prepared using quercetin-Q (0.0-5.0 μg.mL^{-1}) and gallic acid –GA (0.0-5.0 μg.mL^{-1}). For FRAP assay, aliquots of 2.5 mL of aqueous solutions of honey (100.0 mg. mL^{-1}) were used to obtain the reaction mixtures and standard curves were prepared using gallic acid – GA (0.0-100.0 μg.mL^{-1}) and results were expressed as mg of GA equivalents per 100g of honey (mgGAEq.100g^{-1}).

In β-Carotene linoleate model system, a solution of 5.0 mg of β-Carotene in 50.0 mL of chloroform was initially prepared. A volume of 3.0 mL of this solution was added to 40.0 μL of linoleic acid and 400 mg of Tween® 40 in a 100.0 mL round-bottom flask. The chloroform was roto-evaporated at 40.0 °C for 5 min and the residue was homogenized with 100.0 mL of sterilized distilled water (aired for 30 min). Aliquots (3.0 mL) of this emulsion were transferred to different test tubes containing 0.3 mL of aqueous-honey solution (100.0 mg.mL^{-1}) or α-tocopherol solution (90.0 μg. mL^{-1}) as the positive control. The mixtures were incubated (water bath) under shaken (50.0 °C, 150 rpm, 2h) for oxidation, before their absorbance at 470 nm be measured. The antioxidant activity (AA) was estimated according to the equation: AA = [(DRc – DRs)/ DRc] x 100, in which: DRc = degradation of the positive control [In (a/b)/120]; DRs = degradation of the honey sample [In (a/b)/120]; a = absorbance in time zero; b = absorbance after 2 h of reaction.

2.5 Hydroethanolic Pollen Extracts (HPE) and Phosphate Buffer Pollen Extracts (BPS)

The hydroethanolic bee pollen extracts (HPE) and the buffer pollen solutions (BPS) were prepared according to the method described by Carpes et al. (2007), with modifications. To HPE, each sample of bee pollen (1g) was macerated with a solution of 70% ethanol p.a. to a final concentration of 50 mg.mL^{-1} of bee pollen. These suspensions were incubated under shaken (70 °C, 150 rpm, 30 min), centrifuged (1368 *g*, 10 min), and

Table 1. Localization (Alagoas, Northeast region of Brazil) of the meliponaries where the honey and bee pollen studied samples (with codes) were collected (rainy season - 2009/2010).

Municipalities of the Harvest (GPS data)	Samples of Honey (codes)	Samples of Bee Pollen (codes)
Barra de Santo Antônio (North Coast) 09°25'15,11"(S) 35°31'17,64" (W)	FV1	AU2, AU3
Palmeira dos Índios (city between Atlantic Forest and Dryland)- 09°21'25,14" (S) 36°36'06,43" (W)	AU1-AU14	AU4 – AU1
Delmiro Gouveia (Dryland area, near to São Francisco River) - 09°22'36,47" (S) 38°00'00,64" (W)	AM1–AM5, AJ1-AJ2, AP1-AP2	AM1-AM3, AJ1-AJ3, AP1-AP3
Água Branca (Dryland)*	AJ3, PT1-PT3, AG1-AG3	AJ4 – AJ5, PT1, AG1-AG3

Samples of honey and bee pollen: **AG** – Bee "grude" (*Tetragona clavipes* Fabricius, 1804), **AJ** – Jandaíra (*Melipona subnitida* Ducke, 1910), **AP** – Mirim (*Plebeia* sp.), **AM** – Mandaçaia (*Melipona quadrifasciata anthidioides* Lepeletier, 1836), **AU** – Uruçú (*Melipona scutellaris* Latreille, 1811), **FV** - *Frieseomelitta varia* Lepeletier, 1836, **PT** – Papa terra (*Melipona asilvai* Moure, 1971); *GPS Data not evaluated.

the supernatant filtered through a preparation-filter paper. The filtrates were stored in Falcon tubes at 6-8 °C.

On the other hand, to the buffer solution (BPS), each sample of bee pollen (0,5g) was macerated with 10.0 mL of 0.1 M phosphate buffer (pH 6.2) and the suspensions (50.0 $mg.mL^{-1}$) were processed as for the HPE before being stored in Falcon tubes at 6-8 °C.

2.6 Physicochemical profile of the bee pollen

The following physicochemical parameters were evaluated (triplicate) in the bee pollen extracts/solutions (HPE and BPS), according to the specific preconized methods: pH (Vargas, 2006); content of total protein in BPE [as in Lowry et al. (1951), at 660 nm, using a standard curve of bovine serum albumin (0.0 a 100.0 $\mu g.mL^{-1}$)]; total carbohydrates in HPE [as in Yemm & Willis (1954), using sucrose for standard curve (0.0 a 100.0 $\mu g.mL^{-1}$)]; total lipids in HPE (Manirakiza et al., 2001).

2.7 Total content of phenolics and flavonoids in bee pollen

The content of total phenolics in the HPE was determined using the method described by Carpes et al. (2007) and a standard curve of gallic acid (0.5-100.0 $\mu g.mL^{-1}$), expressing the results as mg GA equivalents per g of pollen ($mgGAEq.g^{-1}$).

On the other hand, the content of total flavonoids was determined as described by Al et al. (2009), using a standard curve of quercetin-Q (5.0–150.0 $\mu g.mL^{-1}$). As such, 0.3 mL of 5% $NaNO_3$ was added to each 1.0 mL of diluted HPE (1:100). After 5 min, it was added 0.3 mL of 10% $AlCl_3$ ($v.v^{-1}$) to this reaction mixtures, which were homogenized and after 6 min they were neutralized with 2.0 mL of NaOH 1M before analyzed at 510 nm.

2.8 Antioxidant activity of bee pollen

The AA of stingless bee pollen was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method, as described by Baltrusaityte et al. (2007), with some modifications. To 50 µL of each sample of HPE or control (70% ethanol), 2.0 mL of DPPH solution (6.5. 10^{-5} M) was added. The absorbance of the reaction mixtures was measured at 515 nm, and the inhibition of the AA was given following the formula: $I = [(A_B - A_A) / A_B] \times 100$, in which I = Inhibition percentage; A_A = corresponds to the absorbance of the sample; A_B = corresponds to the absorbance of the blank.

This activity was also analyzed by the FRAP test described by Küçük et al. (2007) with adaptations. To 2.5 mL of each diluted HPE (1:25), it was added 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] and the reaction mixtures were incubated at 50.0 °C for 20 min before the addition of 2.5 mL of trichloro acetic acid (10%). Then, mixtures were centrifuged (1368 *g*, 10 min) and 2.5 mL of each supernatant were added to 2.5 mL of deionized water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the reaction mixtures was measured at 700 nm and the results expressed as $mgGAEq.g^{-1}$ of pollen.

The β-Carotene linoleate model system for AA of the bee pollen samples was determinate as described to the honey samples (item 2.4), but using 0.3 mL of the diluted HPE (1:20). All the analyses were performed in triplicate.

2.9 Statistical and principal component analysis

The relations between the physicochemical parameters and the species of stingless bees were tested by the *software* BioEstat 5.0, with Spearman's rank correlation coefficient. Due to the large number of variables measured in different units, the analyses were conducted using standardized data. The samples were analyzed by principal components *biplot* (Gabriel, 1971), and cluster analyses, using the Euclidean distance and UPGMA method (unweighted pair-group average), considering the general physicochemical characteristics of the honey and bee pollen samples. The highly correlated variables were excluded, using the criterion proposed by Jolliffe (1973), it means, the number of discarded variables must be equal to the components whose variance (eigenvalue) is less than 0.7. Analyses were processed using the *software* SAS/STAT (SAS Institute, 2004) and the *biplots* created in the *software* StatSoft (2001). Such analysis was not performed with honey of *F. varia* since only one sample was available.

3 Results and discussion

3.1 Physicochemical profile of honey

The analysed physicochemical parameters of the 31 samples of honey produced by seven stingless bee species, in different cities from Alagoas (Brazil), mainly at *Caatinga* biome, are showed in Table 2.

Generally, the moisture level of the studied honeys was upper than 25%, being the lowest value observed for honey of *T. clavipes* (19 ± 2%) - the only one that meets the rate defined by National and International legislations for honey of *Apis mellifera* (20.0%). High moisture values for honeys of stingless bees have also been reported before. When Biluca et al. (2016) evaluated 33 samples from 10 Meliponini in Santa Catarina (a State in the South region of Brazil), they found an average moisture ranging from 23.1 to 43.5%, being the highest values detected in honeys of *M. quadrifasciata*. Usually, high values of moisture make easier the honey microbial contamination, and, therefore, its fermentation, spoiling and flavour loosing, leading its loss of quality (Costa et al., 1999). But honeys of *M. scutellaris* and *M. quadrifasciata* harvested in Bahia - a State in the Northeast region of Brazil, did not lost their quality and acceptability after being submitted to a dehumidification process, and their moisture was reduced to approximately 17% (Carvalho et al., 2009).

On the other hand, the pH values of the studied stingless honeys varied from 4.1 (for *Plebeia* sp. samples) to 5.6 (for *T. clavipes* samples), and the average free acidity ranged from 17 $mEq.kg^{-1}$ (for *M. q. anthidioides*) to 125 $mEq.kg^{-1}$ (for *Plebeia* sp.) (Table 2).

In a previous study of Carvalho et al. (2009), with honey of *M. scutellaris* collected in Itaparica Island and Costa do Sauípe (Coastal region of Bahia, Brazil), the free acidity of the samples had a lower variation, ranging from 25.7 to 55.0 $mEq.kg^{-1}$, but in the research carried out more recently by Biluca et al. (2016),

Table 2. Average values (*) of physicochemical parameters of honeys produced by Meliponini species in Alagoas/Brazil (rainy season – 2009/2010).

Stingless bees (number of samples)	Moisture (%)	pH	Acidity free (mEq. kg^{-1})	Electrical Conductivity (mS. cm^{-1})	Proline ($mg.100g^{-1}$)	Reducing Sugars (%)	Diastase (Gothe)	HMF** ($mg.kg^{-1}$)	Colour (mm Pfund)
***M. scutellaris* (n = 14)**	30 ± 2	4.2 ± 0.9	37 ± 14	0.7 ± 0.2	45 ± 27	59 ± 7	2 ± 1	21 ± 1	87 ± 57
***M. q. anthidioides* (n = 5)**	31 ± 2	4.2 ± 0.7	17 ± 10	0.5 ± 0.3	27 ± 15	75 ± 2	3 ± 2	33 ± 27	186 ± 188
***M. subnitida* (n = 3)**	27 ± 3	4.6 ± 0.5	22 ± 11	0.6 ± 0.6	41 ± 4	75 ± 3	3 ± 2	51 ± 6	32 ± 24
***M. asilvai* (n = 3)**	30 ± 4	4.3 ± 0.5	22 ± 11	0.3 ± 0.1	27 ± 9	67 ± 4	2 ± 1	61 ± 27	24 ± 15
***T. clavipes* (n = 3)**	19 ± 2	5.6 ± 0.6	59 ± 12	1.4 ± 0.0	1,477 ± 828	72 ± 5	9 ± 2	18 ± 4	378 ± 12
***Plebeia* sp. (n = 2)**	35 ± 1	4.1 ± 0.9	125 ± 71	1.2 ± 0.1	176 ± 65	72 ± 5	10 ± 8	72 ± 2	403 ± 5
***F. varia* (n = 1)**	30.0	5.2	28.8	1.2	32.48	75.9	19.1	28.9	111.2

*Different parameter (Average values ± standard deviations); **hydroxy-methyl-furfural.

with honeys of ten different stingless bees from Santa Catarina (South of Brazil), the authors found pH variation between 3.3 (*M. q. anthidioides*) and 6.5 (*M. mondury* Smith, 1863), and free acidity oscillating between 16.2 $mEq.kg^{-1}$ (*M. mondury*) and 139.0 $mEq.kg^{-1}$ (*M. bicolor* Lepeletier, 1836). By the same way, Chuttong et al. (2016), investigating 28 samples of honey of 11 stingless bees species from Thailand, found pH ranging from 3.1 to 3.9 (average pH = 3.6), whilst the average free acidity of such honeys was 164.0 $mEq.kg^{-1}$, varying from 25.0 to 592.0 $mEq.kg^{-1}$. Therefore, the low pH value and the high acidity detected in honeys of stingless bees are not favourable conditions for the microbial development, counterbalancing the effect of their high humidity, and they are features that increase the shelf life of such honeys (Lage et al., 2012).

The electrical conductivity ranged from 0.3 $mS.cm^{-1}$ in the *M. asilvai* to 1.4 $mS.cm^{-1}$ in *T. clavipes* (Table 2). Similar result (average of 0.84 $mS.cm^{-1}$) was found for honey of *Plebeia* sp. collected during the dry season of 2008/2009 in the State of Alagoas, Brazil (Duarte et al., 2012). Chuttong et al. (2016) found values for electrical conductivity upper to 2.0 $mS.cm^{-1}$ in honeys from 3 stingless bees species.

Compared to honey of *Apis mellifera* also collected in Alagoas State, previously studied by our group during the dry season (Duarte et al., 2012), the most relevant differences regarding the honeys of Meliponini treated in the present research, besides the higher values of moisture and free acidity already discussed, are the higher values for electrical conductivity and proline in honeys of bees different than *Melipona* (Table 2), as well as lower values for diastase (from 2 Gothe, for honey of *M. asilvai*, to 19.1 Gothe, for honey of *F. varia*) for all the genera of stingless bees (although *Melipona* sp. showed still less diastase activity than other Meliponini). In others researches of stingless bees honeys, the activity of this enzyme was absent or very low detected (Chuttong et al., 2016), but in other (Biluca et al., 2016), such in honey of *T. angustula* (Latreille, 1811), high values such as 49.6 Gothe were reported.

Silva et al. (2014) found proline as the predominant amino acid in bee pollen of *M. subnitida* samples collected in 2009 and 2011 (11.79 and 9.54 $mg.g^{-1}$, respectively), being pollen the main source of the proline of honeys. The proline content of the honeys here studied ranged from 27 mg.100 g^{-1} (*M. q. anthidioides*) to 1,477 mg.100 g^{-1} (*T. clavipes*) (Table 2). Duarte et al. (2012), found contents of proline ranging from 20.16 mg.100 g^{-1} (for *M. scutellaris*) to 94.26 mg.100 g^{-1} (for *Plebeia* sp.) during the dry season, whilst the average concentration of proline in honeys from *A. mellifera* was 74.10 mg.100 g^{-1}. The "Harmonized Methods of the International Honey Commission" preconizes that, for quality assessment, estimating the degree of maturity and detecting tampering by commercial sucrose in *A. mellifera* honeys (Bogdanov, 2002), they should have a content of proline upper than 18.3 mg.100 g^{-1}, and once there is not an official limit for minimum proline in honeys of stingless bees, once the rate for *Apis* honeys is used, all the samples of this study can be appropriate.

Regarding the average percentage of total reducing sugars, the rate defined by the Brazilian legislation as the minimum for consider honey of *A. mellifera* with good quality (65%), was not reached by honeys of *M. scutellaris* (59%) collected during the rainy season in this study (Table 2), although the honeys of the other stingless bees had higher rates of this parameter reaching 75.9% (for *F. varia*). Previous results of Duarte et al. (2012), regarding honeys of stingless bees collected in the same region but during the dry season, showed a variation from 70.28 to 75.3% of reducing sugars. Similar values were observed by Chuttong et al. (2016) for honeys of 11 stingless bees species, in Thailand.

Likewise, the limit of HMF (the product of hexoses dehydration in an acid medium, after prolonged storage under inadequate conditions such as overheating, or adulteration caused by the addition of invert sugar) established by Brazilian legislation (Brasil, 2000) for *Apis* honeys is 60 $mg.kg^{-1}$. The *Codex Alimentarius*, however, recommends a maximum of 80 $mg.kg^{-1}$ of HMF for honeys from tropical countries, since in hot countries the HMF tends to increase more rapidly during long storage of honeys (Bogdanov, 2002). In this case, even honeys of *M. asilvai* and *Plebeia sp.*, which showed respectively average contents of HMF of 61 and 72 $mg.kg^{-1}$, had adequate level of HMF, mainly considering the adverse weather conditions of the region of the harvests, it means, the dried *Caatinga* biome, which has high temperatures even during the small rainy season. However, as aforementioned, particular criteria/limits for the quality control of stingless bees honeys should be proposed considering the differences from meliponine physiology.

Finally, in relation to the colour of the studied honeys, it ranged from white (24 mm Pfund, for *M. subnitida* honey) to dark amber (403 mm Pfund, for *Plebeia* sp. honey) (Table 2). On the other hand, samples from *M. q. anthidioides* also showed

dark color (186 ± 188 mm Pfund). This highest variation of the means between different samples of honey from the same stingless bee specie may be related to environmental factors such as botanical source, geographic origin, and climatic condictions and not analytic error. This organoleptic property is related to floral origin, processing, storage, climatic factors during the nectar flow and the temperature at which the honey is produced within the hive (Bogdanov et al., 2004). However, in global markets, lighter honeys achieve higher prices (Alves et al., 2005).

3.2 Physicochemical profile of bee pollen

The physicochemical characteristics of the twenty-five samples of bee pollen produced by six stingless bee species are showed in Table 3. The bee pollen showed average pH values between 4.9 for *M. subnitida* and 5.9 for *T. clavipes* (Table 3). Rebelo et al. (2016) found lower pH values for bee pollen produced in the Brazilian Amazonia by *M. interrupta* Latreille (1811) and *M. seminigra* Friese (1903), respectively 3.34 and 3.7.

Total reducing carbohydrates in the studied stingless bees pollen ranged from 185 $mg.g^{-1}$ (*M. scutellaris*) to 450 $mg.g^{-1}$ (*T. clavipes*) (Table 3). Carbohydrates commonly found in bee pollen samples include fructose, glucose and sucrose (Silva et al., 2014). The content of total protein (Table 3) in such samples ranged from 45 $mg.g^{-1}$ (*M. scutellaris*) to 99 $mg.g^{-1}$ (*T. clavipes*), while total lipids (Table 3) varied from 2% (*M. subnitida*) to 6% (*T. clavipes*) (Table 3). Although the physicochemical profile of stingless bee pollen is highly related to its floral origin (not evaluated in the present study), it is worth mentioning that samples from *T. clavipes* were collected in the same meliponary of bee pollen samples from *M. asilvai* and *M. subnitida* (Table 1), even though it showed differences in relation to the other samples, with highest value for total protein, carbohydrates and lipids (Table 3).

Bogdanov (2011) showed that bee pollen of *A.mellifera* has total carbohydrates, protein and lipids respectively ranging from 13.0-55.0%,10.0-40.0% and 1.0-13.0%. On the other hand, Vossler (2015) found a content of total protein ranging from 9.78 to 30.41% in bee pollen samples of *Tetragonisca fiebrigi* Schwarz (1938), *M. orbignyi* Guérin (1844), and *Geotrigona argentina* Camargo & Moure (1996), in xeric forests at the Chaco region of Northern Argentina. In turn, studying bee pollen collected from *M. seminigra* and *M. interrupta* in Brazilian Amazonia, Rebelo et al. (2016) detected respectively 25.66% and 44.27% of total carbohydrates, 37.63% and 24.00% of total proteins, and 10.81% and 6.47% of total lipids, showing the same variation already seen in bee pollen of *A. mellifera.*

3.3 Total phenolic, flavonoids and antioxidant activity in honey and bee pollen

For total phenolics in honeys, the highest levels were recorded for *T. clavipes* (136 $mgGAEq.100\ g^{-1}$) and *Plebeia* sp. (104 $mgGAEq.100\ g^{-1}$), whilst the highest values of total flavonoids were found in honeys of *T. clavipes* (55 mg $QEq.100g^{-1}$) and *M. q. anthidioides* (45 $mgQEq.100g^{-1}$). Previous studies of Duarte et al. (2012) have also showed that honeys of *M. q. anthidioides*, especially the samples with a dark amber color, have a highest content of total phenolics than honeys from *A. mellifera* and other *Melipona* spp.

In opposite, the lower value for total phenolics and flavonoids were detected in samples produced by *M. asilvai* (Table 4). A statistically significant high correlation ($p < 0.01$) was seen

Table 3. Average values (*) of some physicochemical parameters of bee pollen samples of Meliponini species in Alagoas/Brazil (rainy season – 2009/2010).

Stingless bees (number of samples)	pH	Electrical Conductivity ($mS.cm^{-1}$)	Total Proteins ($mg.g^{-1}$)	Total Carbohydrates ($mg.g^{-1}$)	Total Lipids (%)
***Melipona scutellaris* (n = 10)**	5.5 ± 0.5	0.2 ± 0.0	45 ± 18	185 ± 98	2 ± 2
***Melipona subnitida* (n = 5)**	4.9 ± 0.3	0.3 ± 0.1	78 ± 8	301 ± 169	2 ± 0
***Melipona q. anthidioides* (n = 3)**	5.0 ± 0.4	0.4 ± 0.0	59 ± 34	215 ± 33	3 ± 1
***Tetragona clavipes* (n = 3)**	5.9 ± 0.2	0.3 ± 0.0	99 ± 9	450 ± 45	6 ± 1
***Plebeia* sp. (n = 3)**	5.0 ± 0.5	0.4 ± 0.0	97 ± 22	264 ± 172	3 ± 3
***Melipona asilvai* (n = 1)**	5.5	0.3	70.8	300.9	3.1

*Average values ± standard deviations.

Table 4. Average contents (*) of **t**otal phenolics and flavonoids in honey and bee pollen samples of Meliponini species in Alagoas/Brazil (rainy season 2009/2010).

Stingless bees (number of samples)	Honey		Stingless bees (number of samples)	Bee pollen	
	Total Phenolics ($mgGAEq.100\ g^{-1}$)	Total Flavonoids ($mgQEq.100\ g^{-1}$)		Total Phenolics ($mgGAEq.g^{-1}$)	Total Flavonoids ($mgQEq.g^{-1}$)
***Melipona scutellaris* (n = 14)**	62 ± 15	29 ± 14	***M. scutellaris* (n = 10)**	12 ± 5	3 ± 2
***Melipona q. anthidioides* (n = 5)**	78 ± 48	45 ± 37	***M. q. anthidioides* (n = 3)**	17 ± 7	5 ± 3
***Melipona subnitida* (n = 3)**	38 ± 11	11 ± 2	***M. subnitida* (n = 5)**	16 ± 6	4 ± 3
***Melipona asilvai* (n = 3)**	32 ± 9	8 ± 2	***M. asilvai* (n = 1)**	6.9	0.3
***Tetragona clavipes* (n = 3)**	136 ± 32	55 ± 20	***T. clavipes* (n = 3)**	21 ± 2	17 ± 5
***Plebeia* sp. (n = 2)**	104 ± 20	35 ± 13	***Plebeia* sp. (n = 3)**	12 ± 1	7 ± 3
***Frieseomelitta varia* (n = 1)**	89.2	29.2	-	-	-

*Average values ± standard deviations.

between total phenolics and flavonoids (0.93) for all the studied honeys (Appendix A: Table S1).

Souza et al. (2016) observed similar results considering the content of total phenolics in honey of *M. scutellaris* (4.2-61.1 mgGAEq.100g^{-1}). However, in honey of *M. subnitida* these authors found a variation between 31.5 mgGAEq.100g^{-1} and 126.6 mgGAEq.100g^{-1}, although both species have pollen from the same floral origin, it means, *Ziziphus joazeiro* Mart. (Juazeiro).

On the other hand, the content of total flavonoid detected in the study of Souza et al. (2016) ranged from 2.4 mgQEq.100g^{-1} to 4.2 mgQEq.100g^{-1} in honeys of *M. subnitida*, and 1.9 mgQEq.100g^{-1} to 4.4 mgQEq.100g^{-1} in honeys of *M. scutellaris* – values lower than the ones found in honeys of stingless bees in the present study.

Different factors influence the profile of total phenolic and flavonoids in honey, depending especially of the floral origin. The nectar is the main source of phenolic compounds present in honey, and it is an auxiliary tool for the analysis of pollen to the identification of the botanical origin of honey (Küçük et al., 2007). Factors such as seasonality and environmental and storage conditions can influence the quality and percentage of these antioxidants in honey (Al-Mamary et al., 2002; Baltrusaityte et al., 2007).

Regarding the bee pollen, the lowest contents of total phenolic and flavonoids were detected in *M. asilvai* samples (6.9 mgGAEq.g^{-1} and 0.3 mgQEq.g^{-1}, respectively), whilst the highest contents were found in pollen of *T. clavipes* (21 mgGAEq g^{-1} and 17 mgEQ.g^{-1}, respectively) (Table 4).

Regarding to the AA, the highest value was observed for honeys of *T. clavipes* according to the FRAP method (110.8 ± 8.8 mgGAEq.100g^{-1} of honey), followed by honey of *F. varia* using different detection methods, such as β-Carotene/linoleic acid system, with 70.46% of discoloration, and DPPH radical scavenging (11.1 mgGAEq.100g^{-1}) (Figure 1A). According to Baltrusaityte et al. (2007) and Küçük et al. (2007), two classes of compounds with AA are present in honeys – the enzymes, including glucose oxidase and catalase, and antioxidants such as ascorbic acid, phenolic acids and flavonoids compounds. Erejuwa et al. (2012) reviewed the results of AA of honey and suggest that when honeys are administered alone or in combination with conventional therapy, they might be usual in the management of chronic diseases commonly associated with oxidative stress.

Therefore, honeys samples of different stingless bees from the same meliponarie, as *T. clavipes* and *M. asilvai*, showed different contents of phenolic, flavonoids and AA. Also, the three methods used to measure the AA showed high correlation which was statistically significant ($p < 0.01$) between the contents of total phenolics and flavonoids. The highest correlation was found between FRAP assay and total phenolics (0.98) and FRAP and total flavonoids (0.91) in honey samples (Appendix A: Table S1).

On the other hand, the highest AA of bee pollen was observed for the samples produced by *T. clavipes* with 123.4 mgGAEq.100g^{-1} using FRAP method. However, with β-Carotene/linoleic acid

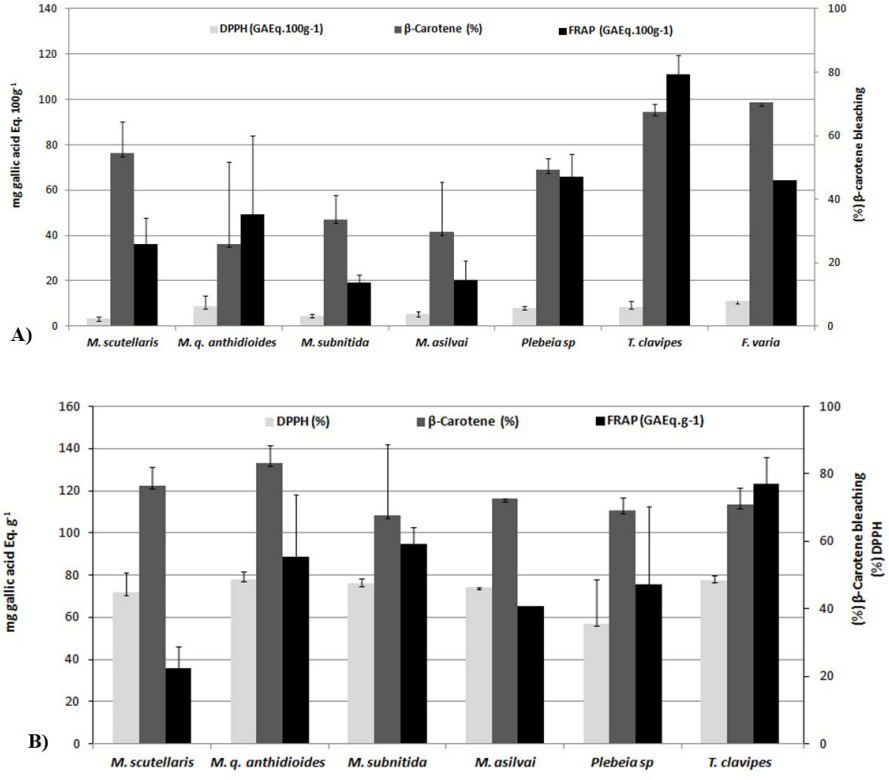

Figure 1. Antioxidant activity of honey **(A)** and bee pollen **(B)** produced by Meliponini species in Alagoas/Brazil (rainy season – 2009/2010), according to three different tests, it means, DPPH radical (*), β-Carotene and FRAP (**). *2-2-diphenyl-1-picrylhydrazyl free radical scavenging; **Ferric Reducing Antioxidant Power.

method the highest value for this parameter was seen with samples of M. *q. anthidioides* (83.3%) followed by the ones produced by *M. scutellaris* (76.5%) (Figure 1B). Yet, in bee pollen only the FRAP test showed correlation statistically significant ($p < 0.01$) between AA and total phenolics (0.55) or with total flavonoids (0.43) (Appendix A: Table S1).

Bee pollen is a product greatly appreciated by the natural medicine because of its potential medical (Komosinska-Vassev et al., 2015). However, wew studies was been reported antioxidant activity from bee pollen from stingless in Brazil. Silva et al. (2009) reported that crude ethanolic extract of bee pollen of *M. rufiventris* (Uruçú amarela) from Ceará State, Brazil, showed high AA, with 0.1 $mg.mL^{-1}$ exhibiting 50% DPPH inhibition. In other study (Harif Fadzilah et al., 2017), bee pollen extracts from *Trigona apicalis, Trigona itama* and *Trigona thoracica* showed total phenolic content ranged 33.4 to 135.9 mg GAE/g and flavonoids 15.2 to 31.8 mg QE/g and highest antioxidant activity of *T. thoracica* gave higher percentage of DPPH inhibition (EC50 was 0.86 mg/mL).

The antioxidant activity of the Meliponini honey and bee pollen showed here can give support to the market to influence their incorporation in the human diet as sources of potential functional foods.

3.4 Multivariate analysis for honey and bee pollen

Tables S2-S5 presents the eigenvalues estimation of variance and accumulative percentage of the total variance (%) obtained by principal component analysis and eigenvectors of all the samples, respectively of honey and bee pollen studied. Since only one sample of honey was produced by *F. varia*, this bee was not considered by the multivariate analysis.

According to the criteria of Jolliffe (1973), from all the variables studied regarding the honey samples of stingless bees, six were eliminated [proline, color, diastase, flavonoids, FRAP, DPPH (GA)], due to their higher correlation for multivariate analyses, and nine were selected. Regarding to the species of stingless bees, from the data showed in Table S2 (Appendix A), three components were needed to explain more than 92.62% of the variability of the honey samples. Among the variables that can be highly correlated with the first component, explaining 51.85% of the total variability of the data, are: a positive combination with moisture and a negative correlation with pH, electrical conductivity, total phenolics, DPPH (Q) and β-Carotene assay. The second component has a positive correlation between free acidity and HMF; and the third component correlated positively with reducing glycids (Appendix A: Table S3). The first two components respond for 77.28% of the variability (Figure 2A).

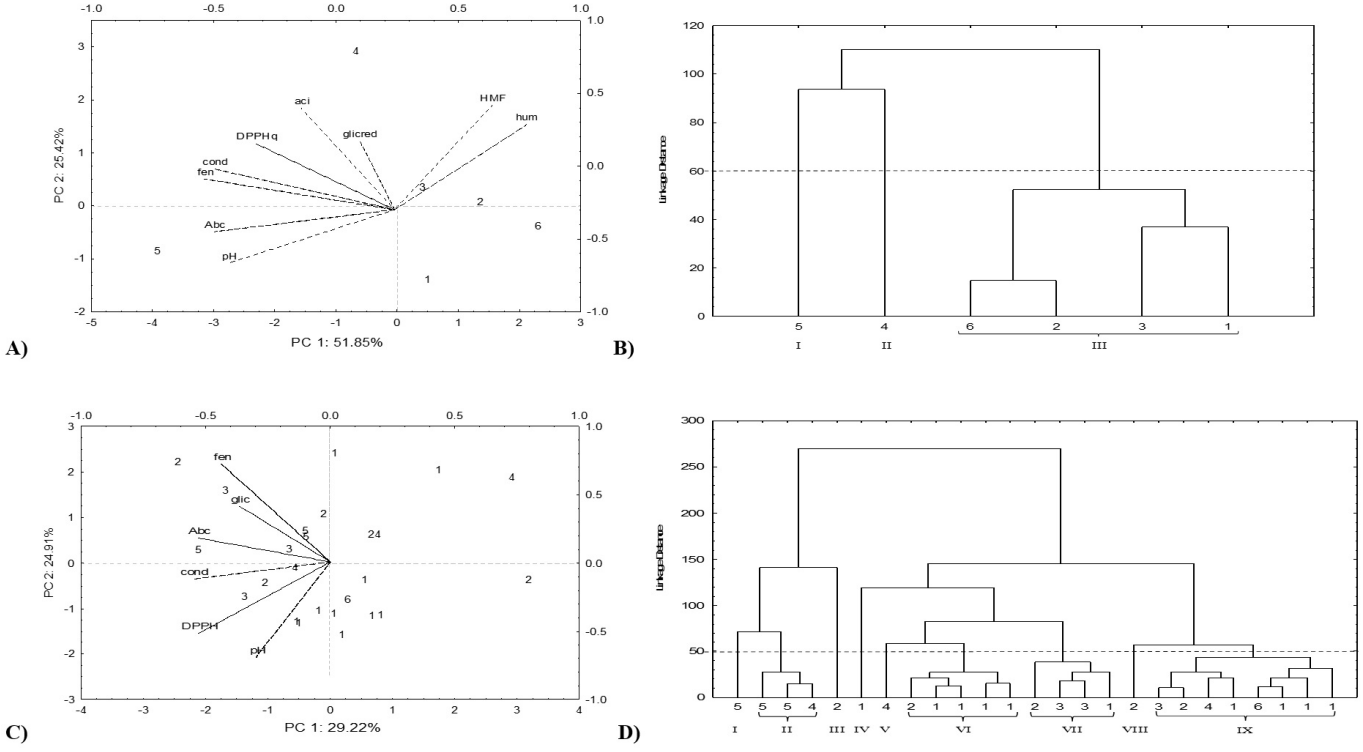

Figure 2. *Biplot* of six stingless bees species in the State of Alagoas/Brazil on the basis of nine evaluated characteristics (pH, aci = acidity, cond = electrical conductivity; hum = moisture; HMF = hydroxymethylfurfural; fen = total phenolics; DPPHq = DPPH mg of quercetina.$100g^{-1}$; glicred=reducing glycides; Abc = antioxidant activity β-Carotene). (**A**) Dendrogram obtained by cluster analysis of six stingless bees, using the average Euclidean distance and the method UPGMA, considering the nine parameters; (**B**) *Biplot* of 25 samples of bee pollen produced by six species Meliponini species in Alagoas/Brazil on the basis of six evaluated characteristics (pH; cond; fen; DPPHq; glic; Abc); (**C**) Clustering using the average Euclidean distance and the method UPGMA of the 25 samples of bee pollen based on the evaluated characteristics; (**D**) (1- *Melipona scutellaris*; 2- *M. subnitida*; 3- *M. quadrifasciata anthidioides* ; 4- *Plebeia* sp.; 5- *Tetragona clavipes*; 6- *M. asilvai*.

The honey samples of *T. clavipes* were characterized by higher values of pH and β-Carotene assay. Samples of *Plebeia* sp. were characterized by higher values of free acidity. This result was quite interesting as well as the evolutionary closeness of different species of bees. The dendrogram for six species of stingless bees according to their honeys chemical composition (Figure 2B) evidenced three groups, being group III formed exclusively by species of the genus *Melipona*, and the other two clusters formed by trigoniforms. Stingless bees collected from the same meliponary and biome (*Caatinga*) such as *T. clavipes* and *M. asilvai* showed distinct antioxidant and chemical composition profiles, probable because they explore different available floral nectar during the small rainy season.

On the other hand, the multivariate analysis of the bee pollen samples (Appendix A: Tables S4 and S5) showed the need of three components to explain more than 74.52% of the variability and their clustering in nine groups. The first component correlated negatively with electrical conductivity, total phenolics, DPPH(Q) and β-Carotene/linoleic. The second component, correlated negatively with pH and DPPH, and positively with total phenolics, whilst the third component correlated positively with reducing sugar and β-Carotene. There is equally strong influence of total phenolics, DPPH(Q) and β-Carotene tests, and the presence of these features in more than one main component (Figures 2C and 2D).

A common botanical origin may explain the similarity found between the examined bee pollen samples. However, the groups one and two, formed by bee pollen samples of *T. clavipes* and *Plebeia* sp. showed a pattern a little more distinct from the others (Figures 2C and 2D), probably due to different floral origin or to the more "humid", which is characteristic of ensiled pollen (samburá) in species of *Melipona* when compared to samples from the trigoniform bee group of "grude" bees.

4 Conclusion

It was seen that honeys samples (n = 31) from six species of stingless bees, even in the same region, season and meliponary from Alagoas, in the Northeast of Brazil showed a statistically significant difference ($p < 0.05$) in relation to moisture, pH/acidity, total phenolics, total flavonoids, reducing sugars and AA. A possible common floral origin may explain the similarity between most of the analyzed pollen samples (n = 25). The results of this study represents a contribution since little is known about their contents of total phenolics and flavonoids, as well antioxidant activity of honey and bee pollen from stingless bees.

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Appendix A. Supplemental information.

Table S1. Spearman correlation coefficient between the total content of phenolics, flavonoids and antioxidant activity of honey and bee pollen produced by Meliponini species in Alagoas/Brazil (rainy season 2009/2010).

Honey Samples						
Parameters	Total Phenolics (mg GAEq.100 g^{-1})	Total Flavonoids (mg QEq.100g^{-1})	FRAP (mg GAEq.100 g^{-1})	DPPH (mg GAEq.100 g^{-1})	DPPH (mg QEq.100g^{-1})	β-Carotene/ linoleic acid system (%)
Total Phenolics (mg GAEq.100g^{-1})	1.00	**0.93**[a]	**0.98**[a]	**0.40**[a]	**0.50**[a]	**0.70**[a]
Total Flavonoids (mg QEq.100g^{-1})		1.00	**0.91**[a]	0.29	**0.39**[a]	**0.66**[a]
FRAP* (mg GAEq.100 g^{-1})			1.00	**0.42**[a]	**0.51**[a]	**0.66**[a]
DPPH** (mg GAEq.100 g^{-1})				1.00	**0.91**[a]	0.05
DPPH (mg QEq.100g^{-1})					1.00	0.11
β-Carotene/ linoleic acid system (%)						1.00
Bee Pollen Samples						
Parameters	Total Phenolics (mg GAEq.g^{-1})	Total Flavonoids (mg QEq.g^{-1})	FRAP (mg GAEq.g^{-1})	DPPH (%)	β-Carotene/ linoleic acid system (%)	
Total Phenolics (mg GAEq.g^{-1})	1.00	**0.66**[a]	**0.55**[a]	0.06	0.36	
Total Flavonoids (mg QEq.g^{-1})		1.00	**0.43**[a]	0.24	-0.07	
FRAP (mg GAEq.g^{-1})			1.00	0.28	0.06	
DPPH (%)				1.00	0.20	
β-Carotene/ linoleic acid system (%)					1.00	

[a]99% of probability; *Ferric Reducing Antioxidant Power; **2-2-diphenyl-1-picrylhydrazyl free radical scavenging.

Table S2. Eigenvalues estimation of variance and accumulative percentage (%) of the total variance obtained by principal component analysis considering six species of stingless bees and nine characteristics evaluated in honey samples.

Principal Components	Eigenvalues	Accumulated %
1	4.67	51.85
2	2.29	77.28
3	1.38	92.62

Table S3. Eigenvectors calculated for six species of stingless bees for honey from State of Alagoas.

Evaluated Characteristic	PC1	PC2	PC3
Moisture	**0.66**	0.61	-0.26
pH	**-0.83**	-0.38	0.24
Acidity	-0.46	**0.73**	-0.46
Electrical Conductivity	**-0.90**	0.29	-0.21
Reducing Glycids	-0.17	0.49	**0.83**
HMF	0.49	**0.75**	0.01
Total Phenolics	**-0.95**	0.22	-0.05
DPPH(Q)	**-0.69**	0.48	0.39
β-Carotene/ linoleic acid system	**-0.90**	-0.16	-0.38

PC1, PC2, and PC3 are principal components.

Table S4. Eigenvalues estimation of variance and accumulative percentage (%) of the total variance obtained by principal component analysis considering six characteristics evaluated for bee pollen of stingless bees.

Principal Components	Eigenvalues	Accumulated %
1	1.75	29.22
2	1.49	54.13
3	1.22	74.52

Table S5. Eigenvectors calculated for 25 samples of bee pollen of six species of stingless bees harvested in the State of Alagoas, Brazil.

Evaluated Characteristic	PC1	PC2	PC3
Electrical Conductivity	**-0.64**	-0.13	0.41
pH	-0.35	**-0.70**	-0.25
Reducing Glycides	-0.43	0.40	**0.71**
Total Phenolics	**-0.52**	**0.72**	-0.14
DPPH	**-0.62**	**-0.52**	-0.30
β-Carotene/ linoleic acid system	**-0.62**	0.17	**0.62**

PC1, PC2, and PC3 are principal components.