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Screening for quality indicators and phenolic compounds of biotechnological interest in honey samples from six species of stingless bees (Hymenoptera: Apidae)

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Abstract

Honey from stingless bees of the genus *Melipona* is a well sought product. Nevertheless lack of legal frameworks for quality assessment complicates the evaluation of food safety and marketing of these products. Seeking to assess the quality of honey from the bees of this genus, physical and chemical analyses, identification of phenolic compounds, and microbiological evaluation from six species of stingless bees was performed. The honey samples showed high reducing sugars, low protein levels and a balanced microbiota. High total phenols and flavonoids and higher antioxidant activity were also recorded. Different phenolic compounds of great biotechnological potential were identified and of these apigenin, kaempferol and luteolin were identified for the first time in honey. To the best of our knowledge, this is one of the few works describing a detail characterization of melipona honey together with identification of the phenolic compounds of significant therapeutic value.

Keywords: honey; chromatography; flavonoids; phenols; antioxidants; stingless bees; pollen types.

Practical Application: This is one of the few works that describe the characterization of honey from seven species of stingless bees together detailing the identification of phenolic compounds of significant therapeutic value, which can serve as an important reference for future studies.

1 Introduction

Despite the lack of standardized legal frameworks for quality assessment, honey produced by eusocial bees of the genus *Melipona* is a product that has shown increasing demand, and even attracted higher prices than the honey produced by bees of the genus *Apis mellifera* (Alves et al., 2005), due to its peculiar flavor, nutritional value, and therapeutic properties (Silva et al., 2013a; Kumul et al., 2015; Kadri et al., 2016).

Honey is reported to prevent the onset of various pathological processes in the human body; such as atherosclerosis, cardiovascular disease and Alzheimer's as well as giardiasis (Mohammed et al., 2015) It has also shown to possess anti-stress, anticarcinogenic (Muhammad et al., 2015) and antibacterial (Nishio et al., 2014) properties. The medicinal properties of honeys are associated with their antioxidant activity which are linked to the phenolic compounds present in the honey samples which may vary depending on the floral source, storage, geography and species of the bees (Liu et al., 2012; Cimpoiu et al., 2013).

Most studies describing the physical and chemical composition of honeys have been conducted for the honey produced by the genus *Apis*, requiring more studies that reference these properties in honey produced by bees of the genus *Melipona* (Alves et al., 2005; Silva et al., 2013a, b). In Brazil, the only two studies published with Melipona honey (*Michmelia seminigra merrillae and Melipona subnitida)* report good antioxidant capacity in the honey produced by this species and relate it to the high concentration of phenolic compounds found in the honey samples (Silva et al., 2013a, b).

To verify if the honey has adequate nutritional quality for consumption and antioxidant capacity, physiochemical, and microbiological analysis, and the identification of phenol compounds are extremely important, since each type of honey has its own physicochemical characteristic and complex chemistry that needs to be assessed mainly in species of stingless bees where the studies are less frequent (Silva et al., 2013b).

The goal of this study was to evaluate the quality of seven honey samples from six different species of the genus *Melipona* through physicochemical, chromatographic, and microbiological analysis. To the best of our knowledge this is one of the few studies detailing the analysis and identification of phenolic compounds from melipona honey.

2 Materials and methods

2.1 Honey samples

The honey samples were collected during the month of May 2013 from six species of *Melipona* stingless bees housed in rational boxes. The total of seven samples were analyzed in triplicate from the species *Melipona quadrifasciata* Lepetellier 1836 (two subspecies *Melipona q. quadrifasciata* and *Melipona q. anthidioides*), *Melipona asilvai* Moure 1971, *Melipona subnitida*

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Ducke,1910 and *Melipona scutellaris* Latreille 1811, from meliponary in São Cristovão city, Sergipe state (11°00'54"S, 37°12'21"W); *Melipona compressipes* Smith 1854 from meliponary in Anajatuda city, Maranhão state (03º15'50"S, 44°37'17"W); and *Melipona mandacaia* Smith 1863, from meliponary in Irecê city, Bahia state (11°18'14"S, 41°51'21"W). The honey samples were collected in sterile 50mL falcon tubes and stored in the freezer at -20 °C until analysis.

2.2 Chemicals and reagents

DPPH (1.1-difhenyl-2-picril-hidrazyl), potassium persulfate, ascorbic acid, ethylene diaminetetra acetic acid (EDTA), and formic acid were acquired from Vetec (Sigma-Aldrich/Brazil), and methanol was bought from Tedia, Brazil.

The standards used for the liquid chromatography were: apigenin, kaempferol, luteolin, quercetin, and narigerin all bought from Sigma-Aldrich. The acids: ferulic, caffeic, p-coumaric, chlorogenic, abscisic, protocatechuic, vanillic, trans-cinnamic, gallic, and Folin-Ciocalteu's phenol reagent were also acquired from Sigma-Aldrich. All chemicals used were of analytical grade.

2.3 Physicochemical, phenols and flavonoids evaluation

The analysis of reducing sugars was carried out as described in AOAC (Association of Official Analytical Chemists, 1995) and the evaluation of the total protein content was performed by the method described in AACC (American Association of Cereal Chemists, 2000).

The total phenolic content was estimated using the spectrometric method of Folin-Ciocalteu' as described by Beretta et al. (2005). The total flavonoid content was determined according to the methodology described by Meda et al. (2005) and Ahn et al. (2007).

2.4 DPPH analysis, phenolic compounds extraction and HPLC-DAD

To analyze the antioxidant activity standard protocol for 2.2-diphenyl-2-picrylhydrazyl (DPPH) by Brand-Williams et al. (1995) and Tominaga et al. (2005) was utilized. Radical DPPH was quantified by spectrophotometry with absorption at 517 nm. The inhibition coefficient (IC_{50}) and antiradical efficiency (EC_{50}) were carried out following the protocol by Kulisic et al. (2006).

The extraction of phenolic compounds was carried out using the methods described by Andrade et al. (1997) and Ferreres et al. (1996) using 10 g of honey dissolved in 50 mL of methanol. Due to the presence of sugars in honey, the samples were subjected to additional Amberlite XAD-2 treatment. The chromatographic separation of the samples with HPLC-DAD was conducted using chromatograph Prominence Shimadzu (Quioto, JAPÃO) equipped with diode array detector (SPDM2O), a reverse phase column Phenomenex Luna C18 (250 mm x 4.6 mm x 5 mm), precolumn Phenomenex C18 (4 mm x 3 mm, with a temperature of 30 °C), and an oven (at 35ºC). The mobile phase consisted of a mixture of 1% aqueous formic acid (A) and methanol (B) with a flow rate of 1 mL/min using the solvent gradient: 0-10 min 10% of B, 10-40 min 55% of B, 40-46 min 55% of B, 46-60 min 75% of B, 60-65 min 75% of B, 65-68 min 10% of B, and 68-70 min 10% of B. The injection volume was 20 µL. The identification of phenolic compounds was based on the retention time and UV/Vis spectra scan (245 nm to 370 nm) which was compared to analytical standards. The gallic, abscisic, and trans-cinnamic acids were evaluated at 270 nm; protocatechuicacid, vanillic acid, luteolin, narigerin, and apigenin at 290 nm; chlorogenic, p-coumaric, and feluric acids at 310 nm; quercetin, caffeic acid, rutin, and kaempferol at 370 nm.

2.5 Botanical origin

For the identification of botanical origin protocols described by Erdtman (1952) and Louveaux et al. (1978) were used. The slides were observed under optical microscope and 1000 pollens were counted from each sample for quantitative analysis. The pollens were grouped in four classes: predominant pollen (> 45%), accessory pollen (≤ 45% to >15%), important pollen (≥3% to ≤15%), and minority pollen (<3%). To identify the families and pollen types, catalagos of taxonomic and morphological reference indicated by several authors were utilized (Barth, 1989, 2004).

2.6 Isolation and identification of microorganisms

Plate count agar (PCA) and Dicloran rose bengal chloramphenicol agar (DRBC) were used for the growth of microorganisms. 100 µL of diluted honey sample (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) was plated on PCA to evaluate Mesophilic aerobic (incubated at 37 ± 1 ºC for 48 ± 2 hours) and psychotropic aerobic microorganisms (incubated at 17 ± 1 ºC for 16 hours and kept refrigerated at 4 °C for five days). DRBC was used to evaluate the yeasts and mold (incubated at 25 ± 3 ºC for five days).

The microorganisms were microscopically and morphologically observed and the total number of microorganisms counted per plate was expressed as colony-forming unit per gram (CFU/g) (Silva et al., 2010).

Identification of the microorganisms was performed by the amplification of their total DNA by PCR, sequencing of the PCR amplified band and *on line* database comparison of the sequenced DNA with other similar DNA sequences present in the GeneBank. For PCR amplification and DNA sequencing, primers corresponding to 16S ribosomal DNA were utilized for bacteria (Lane, 1991). For the amplification and sequencing of the yeast and fungi ITS primers (Bellemain et al., 2010) were utilized.

Overnight cultures of single pure colonies in nutrient broth were used for DNA extraction according to the protocol of Moreira et al. (2010). DNA quantification was performed using Qubit (Invitrogen®). PCR was carried out in a final volume of 20 µL with 2 µL of each 10 µM primer (16S-5'AGAGTTTGATCMTGGCTAG-3'F, 5'TACGGYTACCT TGTTACGACTT-3'R; ITS-5'TCCGTAGGTGAACCTGCG-3'F, 5'GCTGCGTTCTTCATCGATGC-3'R), 8.5 µL Taq Master Mix RED (Amplicon®), 8.5µL ultra-pure water, and 1 µL of 20 ng/µL extracted DNA. PCR was performed using Promega® TC96CG thermocycle using the following conditions: hold of 94 °C for

5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds and extension at 74 °C for 45 seconds. In the end a final extension at 74 °C for 10 minutes was carried out. Electrophoresis was performed on 0.8% agarose gel and stained with Sybr Green (Qiagen®).

Amplified DNA was purified using Wizard® SV Gel Kit and PCR Clean-Up System (Promega®) and sequenced using BigDye® Terminator v.3.1 Cycle Sequencing kit and ABI 3500 sequencer (Applied Biosystem).

The quality of the sequences was verified using the Staden Package Version 2.0 (Staden et al., 2001). Only sequences with phred values above 30 were considered for subsequent analysis. The 16S and ITS1-ITS2 gene sequences were aligned using the ClustalW algorithm embedded in the software MEGA (Molecular Evolutionary Genetics Analysis) version 6.0.5 (Tamura et al., 2007).

After alignment the sequences were inserted into BLAST (Basic Local Alignment Search Tool) and compared to the sequences present in the GenBank.

2.7 Statistical analysis

Analyses of all seven samples were performed in triplicate. The averages were compared by Tukey' test ($p \leq 0.05$) using Assistat software (Statistics Assistance 7.6 beta version).

3 Results and discussion

The results of physico-chemical analysis obtained from the honey samples are presented in (Table 1). The samples showed satisfactory reducing sugar content varying between 34.83% and 78.95%. There is still no legislation regulating the quality of honey produced by stingless bee. Previously reported levels of reducing sugars in *Meliponini* range from 50.60% to 95.60% (Alves et al., 2005). In this study, the lowest level of sugar was observed in honey from *Melipona mandacaia*. Low sugars are indicative of immature honey (Alves et al., 2005) and in the case of *Melipona*, are connected to fermentation by bacteria. The protein content ranged between 0.12 to 0.39 mg/100 g (Table 1).

Honey generally haslow protein content, and the variation in the protein contentis related to the botanical origin of the nectar collected by bees (Noor et al., 2014).

The honey produced by stingless bees has been reported to possess high total phenol content and consequently higher antioxidant capacity compared to other types of honeys (Noor et al., 2014).The total phenols present in the honey samples ranged from 30.71 (*Melipona compressipes*) to 854.02 (*Melipona subnitida*) mg/100 g of Gallic acid equivalent (GAE) (Table 1) which were higher compared to honeys of *Melipona seminigramerrilae*, stingless bees from Amazônia (Northern Brazil), with 17 to 66 mg/g of GAE (Silva et al., 2013a). High total flavonoids content, ranging from 30.24 (*Melipona scutellaris*) to 279.73 (*Melipona subnitida*) mg/100 g of equivalent of quercetin (EQA) (Table 1) were found in all the honey samples analyzed in this study. It is believed that the flavonoids in honey are partially derived from flavonoids in pollens resulting from enzymatic hydrolysis of substances contained in the bee saliva (Anklam, 1998).The levels of flavonoids were also variable according to the species of bees and plants visited by these bees.

The antioxidant activity measured by the DPPH method (EC_{50}) showed values ranging from 25.39 (*Melipona q. quadrifasciata*) to 51.44 (*Melipona scutellaris*) mg/mL (Table 1). The flavonoids which usually exists in larger quantities than phenols and the others compounds are responsible for much of the antioxidant activity of honey (Alvarez-Suarez et al., 2010).

In this study three important flavonoids were identified as major compounds for the first time in the honey samples from stingless bee: kaempferol in honey samples from *Melipona subnitida* (160.85 mg/100 g), *Melipona q.anthidioides* (29.34 mg/100 g), and *Melipona scutellaris* (6.79 mg/100 g), apigenin in *Melipona q. anthidioides* (42.15 mg/100 g) and *M. mandacaia* (29.34 mg/100 g) and luteolin in honeys from *Melipona q. quadrifasciata* (47.87 mg/100 g) and *Melipona asilvai* (41.78 mg/100 g (Table 2).

Both apigenin and kaemferol flavonoids are valuable antioxidants and have relevant anticarcinogenic activity (Kukongviriyapan et al., 2006). The chemoprevcntive action of multiflorais honey has been proven by Moskwa et al. (2014). However further studies are necessary in case of melipona honey. Identification of kaemferol and apigenin, in melipona honey suggests that they might have chemopreventive action.

Luteolin is known to promote cell death by apoptosis and preservation of normal cells and also possesses significant anticancer properties (Chen & Chen, 2013; Dang et al., 2015; Zheng et al., 2014). Its presence in honey produced by *Melipona q. anthidoides* and *Melipona mandacaia* might attribute cell protecting properties.

Table 1. Average values of physico-chemical analysis of honey from six species of stingless bees.

Physicochemical tests	Species						
	Melipona asilvai	*Melipona q. anthidioides*	*Melipona q. quadrifasciata*	*Melipona mandacaia*	*Melipona scutellaris*	*Melipona compressipes*	*Melipona subnitida*
Reducing sugars (g/100 g)	63.39 ± 1^{b}	65.60 ± 0.3^{b}	60.63 ± 0.05^{c}	34.83 ± 1.2^{d}	64.20 ± 1.01^{b}	78.95 ± 0.05^{a}	64.20 ± 0.2^{b}
Protein g/100 g	0.39 ± 0.01^{a}	0.12 ± 0.02^{e}	0.32 ± 0.02^{b}	0.24 ± 0.04^{c}	0.19 ± 0.005^{cd}	0.18 ± 0.01^{de}	0.13 ± 0.011^{ef}
Total phenols mg GAE/g	82.91 ± 1^{d}	161.8 ± 3.4^{c}	82.19 ± 1.2^{e}	61.72 ± 1.1^{f}	192.01 ± 2.8^{b}	30.71 ± 2.01^{g}	854.62 ± 3.8^{a}
Total flavonoid mg QE/g	79.73 ± 1.6^{b}	43.09 ± 2^{f}	75.45 ± 2.71^{c}	45.42 ± 2^{d}	30.24 ± 2^{g}	44.63 ± 2.3^{e}	279.73 ± 4.6^{a}
DPPH (IC_{50}mg/mL)	41.33 ± 0.9^{b}	40.03 ± 0.4^{c}	25.39 ± 0.5^{f}	28.1 ± 0.6^{e}	51.44 ± 0.7^{a}	37.79 ± 1.2^{d}	37.69 ± 1^{d}

Each sample was analysed in triplicate. Values on the same line followed by the same letter are not significantly different ($p \leq 0.05$) by the Tukey's test.

All honey samples analyzed showed the presence of abscisic acid and gallic acid. And these two phenolic compounds were seen as the major compound in honey samples of *Melipona subnitida; Melipona scutellaris and Melipona q. anthidioides,* (Table 2). Abscisic acid is also a plant hormone involved in physiological regulation and according to Kenjeric et al. (2008) can be found in varying amounts in honey samples.

The variation of phenol compounds in the honey samples in this study can be related to the floral preference of each species of bees, as shown in Table 3. In the analysis of pollen types, it was possible to verify the presence of 14 different plant families. Those belonging to Fabaceae (subfamily Mimosaceae) Myrtacea, Euphorbiaceae and Solanaceae, were most frequented by the stingless bees which is consistent with many other surveys carried out in the North and Northeast regions of Brazil (Muniz & Brito, 2007).

The presence of microorganisms in the honey can be explained by what is considered the primary sources of contamination: bee's intestinal flora, soil, water, air, pollen, and nectar. These sources of contamination are practically impossible to avoid because they are naturally occurring. However, hygiene, handling, and packaging of honey are controllable processes which are considered secondary sources of contamination (Różańska & Osek, 2012). Therefore, the aerobic microorganisms are indicators of the degree of deterioration of products and can help determine the useful shelf-life of these products (Franco & Landgraf, 2008). Both yeast and bacteria (Table 4) belonging to different genera were found in all the samples under study, similar to ones described before in honey (Giraldo et al., 2013). It is believed that the amounts of microorganisms in honey are lower than in any other natural food due to their high sugar concentration (Giraldo et al., 2013). In addition to this factor, the presence of phytochemical molecules such as phenols, terpenes, and pinocembrine helps control the growth of microorganisms in honeys (Al-Hind, 2005; Torres-González et al., 2016).

Table 2. Phenolic compounds profile by HPLC-DAD.

Phenolic compounds	Species						
	Melipona asilvai	*Melipona q. anthidioides*	*Melipona q.quadrifasciata*	*Melipona mandacaia*	*Melipona scutellaris*	*Melipona compressipes*	*Melipona subnitida*
Apigenin	-	-	-	**29.34 ± 0.57**	-	-	**42.15 ± 0.47**
Kaempferol	-	**26.28 ± 0.37**	-	-	**6.79 ± 0.25**	-	**160.85 ± 0.93**
Luteolin	**41.78 ± 1.14**	-	**47.87 ± 0.91**	-	-	-	-
Quercetin	-	-	-	-	1.4 ± 0.01	-	-
Naringenin	-	-	-	5.92 ± 0.45	-	-	-
Rutin	-	-	-	-	-	-	-
Gallic acid	1.35 ± 0.2	0.76 ± 0.06	1.06 ± 0.04	1.83 ± 0.37	1.39 ± 0.01	2.43 ± 0.32	1.31 ± 0.02
Feluric acid	-	-	-	-	-		-
Caffeic acid	-	-	122.18 ± 1.9	-	-	25.83 ± 0.45	35.82 ± 0.78
P-cumaric acid	3.8 ± 0.67	7.06 ± 0.89	3.06 ± 0.89	7.91 ± 0.88	-	6.27 ± 0.42	37.03 ± 0.51

Table 3. Frequency and type pollen found in seven samples honey analyzed in this study. 1000 pollen were counted for each sample. Predominant pollen > 45%, accessory pollen ≤ 45% to >15%, important pollen ≥3% to ≤15%, and minority pollen <3%.

		Melipona asilvai	*Melipona q. anthidioides*	*Melipona compressipes*	*Melipona q. quadrifasciata*	*Melipona mandacaia*	*Melipona scutellaris*	*Melipona subnitida*
Plant Family	**Genus**							
Arecaceae	*Cocos* sp	-	-	-	-	0.5% (PM)	-	-
Convolvulaceae	*Jacquemontia* sp	-	-	-	-	-	2.74% (PM)	-
Euphorbiaceae	*Croton* ssp	16.76% (PS)	-	15.4% (PS)	-	15.78% (PS)	-	-
Fabaceae	***Mimosa* ssp**	**70.85% (PD)**	-	**50.88% (PD)**	**92.18% (PD)**	**73.89% (PD)**	**61.01% (PD)**	**22.31% (PS)**
Melastomatoceae	*Clidemia* ssp	3.75% (PI)	-	15.47% (PS)	-	-	-	-
Myrtaceae	***Myrcia* ssp**	-	**22.49% (PS)**	-	-	9.79% (PI)	9.25% (PI)	**62.59% (PD)**
Phytolaccaceae	*Microtea* ssp	-	-	-	-	-	-	0.77% (PM)
Plantaginaceae	*Angelonia* ssp	-	-	-	-	-	-	14.33% (PI)
Rubiaceae	*Mitracarpus* ssp	-	-	12.74% (PI)	-	-	5.74% (PI)	-
Solanaceae	***Solanun* ssp**	-	**71.66% (PD)**	-	-	-	-	-
Tilaceae	*Triumfettas* ssp	3.3% (PI)	-	-	5.6% (PI)	-	-	-
Undetermined 1		2.3% (PM)	2.85% (PM)	2.13% (PM)	1.32% (PM)	-	2.79% (PM)	-
Undetermined 2		3% (PM)	2.9% (PM)	1.03% (PM)	0.24% (PM)	-	2.72% (PM)	-
Total Frequency		100%	100%	100%	100%	100%	100%	100%

Table 4. Microorganisms identified in honey from stingless bees.

Bacteria	Species						
	Melipona asilvai	*Melipona q.anthidioides*	*Melipona compressipes*	*Melipona q. quadrifasciata*	*Melipona mandacaia*	*Melipona scutellaris*	*Melipona subnitida*
Bacillus ssp	+	+	+	+	+	+	-
Klebsiella pneumonia	-	-	-	-	+	-	-
Micrococcus ssp	-	-	-	-	-	-	+
Neisseria ssp	-	-	-	-	-	-	+
Tsukamurella ssp	-	-	-	-	-	-	+
Yeast and Molds							
*Aspergillus s*sp	-	+	+	-	-	+	-
Candida apícola	+	-	-	-	-	-	+
Eurotiales ssp	-	-	-	-	-	+	-
Eutypella ssp	-	+	-	-	-	-	-
Penicillium ssp	-	+	-	+	+	-	-
Starmerella meliponinorum	-	-	-	-	+	-	-
*Trametes s*sp	-	-	-	-	-	-	+

+ Positive in the honey sample / - Negative in the honey sample.

4 Conclusions

The honey samples showed important nutritional and therapeutic properties, and high antioxidant capacity. Good levels of reducing sugar content and low protein levels help to keep the balance of microbiota in honeys, confirming the product quality. Few published articles analyzing above mentioned properties are available for *Melipona* honey. Moreover in Brazil no laws are laid out to asses its quality. This study can thus serve as an important reference for future studies relating to the honey produced by stingless bees.

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