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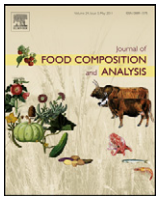

Original Research Article

Phenolic compounds, melissopalynological, physicochemical analysis and antioxidant activity of jandaíra (*Melipona subnitida*) honey

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ABSTRACT

Profile of phenolic compounds, melissopalynological, physicochemical analysis and antioxidant activity of *Melipona subnitida* honeys from Brazil are presented. The constituents in the EtOAc fraction were identified by HPLC-DAD. The melissopalynological analysis showed 19 pollen types from 9 families. *Mimosa caesalpiniifolia* was the predominant pollen type in 8 of the 9 honey samples. The physicochemical analysis revealed that the samples showed a similar profile. All jandaíra honey samples had similar characteristic profile of phenolic compounds, strong antioxidant activity accompanied by high total polyphenol contents. The flavonoids naringenin, quercetin, and isorhamnetin along with gallic, vanillic, 3,4-dihydroxybenzoic, and cumaric acids are common to eight samples and were adequately quantified. The two isomers of abscisic acid (*trans–trans* and *cis–trans*) present in major quantity in the sample 9 were isolated and quantified in all samples. The antioxidant activity of the honey samples strongly correlated with their phenolic content.

1. Introduction

Meliponina honey is a valuable bee product with a long history as a substantial part of the diet of the Northeastern Brazilian population. The stingless bee, *Melipona subnitida* Ducke "jandaíra", is native to Northeastern Brazil. This species is commercially valued by the local population because of the medicinal properties attributed to its honey and pollen loads. However, the primary importance of this species is associated with environmental conservation and fruit production because they pollinate wild plants and cultivated crops in the semi-arid Caatinga (shrub vegetation) and humid pre-Amazonian forest regions (Silva et al., 2006). Meliponina honey is not included in the international standards for honey (Codex, 2001) and is not controlled by the food control authorities because there is very little knowledge about this product. Furthermore, there are no quality control regulations for this honey and therefore there are no safety assurances for consumers.

A few colonies of the total 80 colonies of *M. subnitida* produce as much as 6.0 L of honey per year during the rainy season in the Caatinga regions of Brazil (Cortopassi-Laurino et al., 2006). The tradition of selling stingless bee honey in the Brazilian markets has only recently become profitable. Small jars that indicate the origin of the stingless bee species can be found in local markets in the production areas. The composition of this honey is rather variable and primarily depends on the floral source. However, certain

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external factors also play a role, such as seasonal and environmental factors and processing methods.

Honey is a supersaturated solution of sugars, of which the main constituents are fructose (38%) and glucose (31%). A wide range of minor constituents is also present in honey, many of which are known to have antioxidant properties. These minor constituents include phenolic acids and flavonoids (Martos et al., 2000), certain enzymes (glucose oxidase, catalase), ascorbic acid, carotenoid-like substances (White, 1975), amino acids and proteins (White, 1978). The phenolic compounds in the honey under study are the phenolic acids and flavonoids, which are considered potential markers for the botanical origin of honeys (Alvarez-Suarez et al., 2012a). These phenolic acids are divided into two subclasses: the substituted benzoic acids and cinnamic acids. The flavonoids present in honey are divided into three classes with similar structures: flavonols, flavones and flavanones. These flavonoids are important because of their contribution to the color, taste and flavor of the honey and also because of their beneficial health effects (Estevinho et al., 2008).

Antioxidant activity, or simply antioxidant capacity, is the ability and potential of the honey to reduce oxidative reactions within the food systems and human health (Frankel et al., 1998). The antioxidants that naturally occur in honey contribute to its antioxidant capacity (Erejuwa et al., 2012). Furthermore, the phenolic profile of honeys and consequently their antioxidant capacity depend on the floral sources used to collect honey. The predominance of a particular floral source in honey is primarily influenced by geographical, seasonal and environmental factors (Andrade et al., 1997). Therefore, different properties of the honeys are expected because the composition of the active compounds in honey from different locations is likely to be different.

In this study, we conducted both melissopalynological and physicochemical analyses of jandaíra honey. In addition, the major phenolic constituents of the honey samples were extracted and analyzed using high performance liquid chromatography-diode array detector (HPLC-DAD). The identified phenolics were quantified. The total phenolic contents were determined using the Folin–Ciocalteu test. The antioxidant properties of the honey and the extracts were also studied by testing their scavenging effect on the DPPH (1,1-diphenyl-2-picryl hydrazyl) and ABTS (2,2 azinobis 3-ethylbenzothiozoline-6-sulfonic acid) radical activities and linoleic acid oxidation.

2. Materials and methods

2.1. Honey samples

Nine samples of *M. subnitida* "jandaíra" honey were collected from two semi-arid regions within the state of Paraiba, Brazil. Samples 1–8 were obtained in February 2009 from experienced producers in the municipality of Soledade, and sample 9 was collected in March 2009 at Sítio Riacho, which is in the municipality of Vieirópolis (Fig. 1). The honey was collected into small storage bottles from the hives and was refrigerated at 4 °C until it was analyzed six months later.

2.2. Reagents and standards

Apigenin (4′,5,7-trihydroxyflavone), isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), 8-methoxykaempferol (3,4′,5,7-tetrahydroxy-8-methoxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone),

Fig. 1. Map of Paraiba showing the distribution of honey jandaíra samples studied. Municipality of Soledade (◆, samples 1–8) and municipality of Vieirópolis (●, sample 9).

myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), quercetin (3,3′,4′, 5,7-pentahydroxyflavone), tricetin (3′,4′, 5,5′,7-pentahydroxyflavone), dihydromyricetin (3,3′,4′,5,5′,7-hexahydroxydihydroflavonol), taxifolin (5,7,3′,4′-tetrahydroxydihydroflavonol) and naringenin (3′,4′,5,7,-tetrahydroxydihydroflavanone) had been previously isolated and identified from the pollen loads (Silva et al., 2006, 2009). Ferulic acid, 3-hydroxy-4-methoxycinnamic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, sinapic acid, 4-methoxycinnamic acid, chlorogenic acid, 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and syringic acid were obtained from Sigma–Aldrich (Hamburg, Germany); gallic and vanillic acids were obtained from Fluka Chemie AG (Buchs, Switzerland). The abscisic acids, which were used as standards, were isolated and identified using ^{1}H NMR, ^{13}C NMR and HPLC–MS in this work. All reagents used were of analytical grade. Folin–Ciocalteu's phenol Reagent, DPPH (1,1-diphenyl-2-picryl hydrazyl), potassium persulfate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), β-carotene, linoleic acid and ferrozine were obtained from Sigma–Aldrich (Sternheim, Germany) and butylated hydroxytoluene (BHT) was supplied by Acros Organics (Belgium). ABTS (2,2 azinobis 3-ethylbenzothiozoline-6-sulfonic acid) was purchased from Fluka Chemie GmbH (Switzerland). Ascorbic acid was purchased from Vetec (Brazil). EDTA (ethylenediamine tetraacetic acid) was obtained from Nuclear (Brazil). Formic acid (Vetec, Brazil) and methanol (TEDIA) were analytical grade.

2.3. Melissopalynological analysis

The honey samples were treated using the typical melissopalynological methods (Louveaux et al., 1978). The pollen sediment was acetolyzed (Erdtman, 1960), mounted on glycerin jelly and sealed with paraffin. To determine the frequency classes, 500 pollen grains were counted from each sample. The pollen types were placed into four percentage classes, as determined by Louveaux et al. (1978): predominant pollen (>45%); secondary pollen (≤45% to >15%); important minor pollen (≤15% to ≥3%); and minor pollen (<3%). The pollen slides from the Palynothecae of Universidade Estadual de Feira de Santana (Bahia, Brazil) and pollen catalogs were used to identify the botanical affinity of the pollen types.

2.4. Extraction of the phenolic compounds from honey and the isolation of **1** and **2** from sample 9

The extraction was performed using previously described methods (Andrade et al., 1997; Ferreres et al., 1994) with the following modification: 50 g of honey was dissolved into 250 mL of distilled water and the solution was adjusted to pH 2.0 by adding concentrated HCl. The use of acidic water (pH 2 with HCl) for diluting the honey before passing it through the Amberlite XAD-2 column is highly recommended because the flavonoid aglycone recovery is >95% and there are also some other non-flavonoid phenolic compounds that are detected (Ferreres et al., 1994); however, polyphenolic compounds, such as gallic and *p*-hydroxybenzoic acids, exhibit a lower affinity toward Amberlite XAD-2 (Michalkiewicz et al., 2008). The fluid samples were then filtered through cotton wool to remove the solid particles. The filtrate was mixed with 100 g of Amberlite XAD-2 resin (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3–1.2 mm) and stirred on a magnetic stirrer for 30 min. The Amberlite resin particles were then packed on a glass column (42 cm × 3.2 cm), washed with acidified water (pH 2 with HCl, 250 mL) and subsequently rinsed with distilled water (300 mL) to remove all sugars and other polar constituents of the honey (Ferreres et al., 1996). The phenolic compounds were eluted with 300 mL of methanol. The methanol extracts were concentrated under vacuum at 40 °C in a rotary evaporator.

The presence of sugars gives the MeOH extract a syrupy texture, which causes difficulties for injecting the sample into the HPLC. Therefore, the additional treatment of the sample was necessary. After the additional treatment, the residue was dissolved in 5 mL of distilled water and extracted three times using 5 mL of ethyl acetate. The extracts were combined and the solvent was removed *in vacuo*. Four replicate extractions were performed for each sample. The average yield of the MeOH extracts and the ethyl acetate fraction (n = 4) ranged between 80–350.0 mg/50 g and 15–30 mg/50 g product, respectively. The dried extracts were stored in a refrigerator at 4 °C until further analysis. To isolate the two principal compounds present in sample 9 (Fig. 2), the MeOH extract (310.0 mg) was chromatographed on a Sephadex LH-20 column using methanol as the mobile phase. Compounds **1** and **2** were then purified by

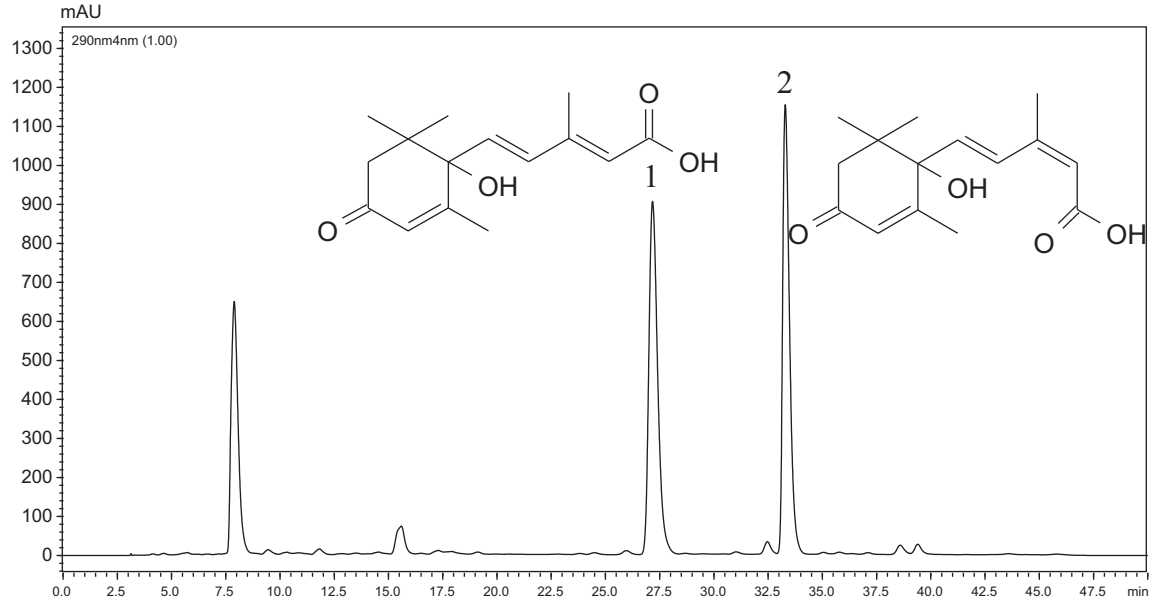

Fig. 2. Chromatogram of the jandaíra (*Melipona subnitida*) honey sample 9. **1**: *trans*, *trans*-abcisic acid and **2**: *cis*, *trans*-abcisic acid.

semi-preparative HPLC on a Shimpack CLC-ODS column (10 mm × 250 mm × 5 μm) at a flow rate of 3 mL/min using a mobile phase of H_2O (A) and methanol (B): 0 min 42% B, 20 min 47% B, and 23 min 90% B at 290 nm. The purity of the compounds was examined using analytical HPLC with diode array detection. The pure, isolated compounds (25.0 and 35.0 mg of **1** and **2**, respectively) were analyzed in a Bruker ARX500 instrument: ^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz. The samples were dissolved in DMSO-d_6. LC–ESI-MS analyses were performed using an Esquire 3000 Plus (Bruker Daltonics). The chromatographic conditions were the same as those used for the analytical method.

2.5. HPLC analysis

Chromatographic analyses were performed using a Shimadzu Prominence LG2OAT HPLC equipped with a photodiode array detector (SPDM2O) and a reversed-phase column (Shimpack CLC-ODS, 4.6 mm × 250 mm × 5 μm). For the benzoic and cinnamic acid derivatives, the mobile phase consisted of a mixture of 5% aqueous formic acid (A) and methanol (B) at a flow rate of 1 mL/min. A gradient elution was used, starting with 20% B up to 15 min, 30% B at 20 min, 40% B at 30 min and isocratic at 40% B up to 45 min. The flavonoids were separated using a mobile phase consisting of 1% aqueous formic acid (A) and methanol (B) at a flow rate of 1 mL/min. The mobile phase was delivered using the following solvent gradient: 0–3 min 40% B, 5–15 min 45% B, 17–25 min 50% B, 27–35 min 55% B, and 35–40 min 70% B. The injection volume was 10 μL. Chromatograms were recorded at 290 nm and 340 nm. The identification of phenolics was based on the retention times, UV-spectra and chromatographic comparison (co-injection) with authentic markers.

2.6. Quantification of the phenolics and abscisic acids *1* and *2*

Because of the importance of natural polyphenols, interest in their identification and quantification in honey samples has significantly increased in recent years (Pyrzynska and Biesaga, 2009). The flavonoids isorhamnetin, naringenin and quercetin were quantified using the external standard method based on peak area. Analyses were performed by plotting a calibration curve. To construct the calibration curve for each flavonoid, working solutions with concentrations between 0.5 and 40 μg/mL were prepared from each stock solution by diluting appropriate volumes with methanol, which were then correlated with the measured area. The area of these peaks was plotted and the corresponding concentration of flavonoids was calculated from the calibration curve. For each sample, the quantitative analyses were performed in triplicate at 290 nm for naringenin (flavanone) and 320 nm for isorhamnetin and quercetin (flavonol). The phenolic acids, such as cinnamic, 3,4-dihidroxy, gallic and vanillic acids, were quantified against their standards (0.5–5.0 μg/mL) at 290 nm. The abscisic acid, both of *trans*, *trans*- and *cis*, *trans*- (250–700 μg/mL) isomers, were determined against the standard (250–1200 μg/mL) at 290 nm.

2.7. Physicochemical analysis

The physicochemical analysis of the honey samples consisted of the following basic determinations, which were performed in duplicate: pH, free acidity, hydroxymethylfurfural, water content, ash content, nitrogen, reducing sugars and moisture.

2.8. Determination of the total phenolic content

The total soluble phenolic content of the samples was determined with the Folin–Ciocalteu reagent, according to the method of Slinkard and Singleton (1977) that was modified by using gallic acid as a standard phenolic compound. Appropriate aliquots of the solutions prepared from pure honey (300 μL, 30 mg/mL), MeOH (methanol) extract (300 μL, 1 mg/mL) and EtOAc (ethyl acetate) fraction (60 μL, 1 mg/mL) were transferred to a 3 mL volumetric flask and diluted with distilled water. Folin–Ciocalteu reagent (300 μL) was added and the contents of the flask were mixed thoroughly. After 3 min, 180 μL of Na_2CO_3 (sodium carbonate, 2%) was added and then the mixture was completed diluted to 3 mL and distilled water and then allowed to stand for 2 h in an ultrasonic bath. The absorbance was measured at 760 nm in a spectrophotometer. The amount of total phenolic compounds was determined in micrograms of gallic acid equivalent using the equation obtained from the standard gallic acid graph.

2.9. DPPH$^\bullet$ radical scavenging assay

The free radical-scavenger activity was determined using the DPPH assay, as described previously (Silva et al., 2006). The antiradical activity was evaluated using a dilution series to obtain five concentrations. This process involved mixing the DPPH solution (60 μM in ethanol) with an appropriate amount of pure honey, MeOH extract and EtOAc fraction followed by homogenization. After 30 min, the remaining DPPH radicals were quantified by measuring the absorption at 517 nm using a spectrophotometer. The percentage of inhibition was given by the formula: percent inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control solution and A_1 was the absorbance in the presence of the sample and standards.

2.10. ABTS$^{\bullet+}$ radical cation decolorization assay

The radical cation decolorization assay was based on the method described by Re et al. (1999). ABTS was dissolved in water to yield a final concentration of 7 mM. The ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS$^{\bullet+}$ solution was diluted to give an absorbance of 0.700 ± 0.025 at 734 nm with ethanol before use. Then, appropriate amounts of the ABTS$^{\bullet+}$ solution were added into 3 mL of the sample solutions in ethanol at five concentrations: 1–15 mg/mL (pure honey), 10–150 μg/mL (EtOAc fr.) and 25–300 μg/mL (MeOH ext.). After 10 min, the percentage inhibition of absorbance at 734 nm was calculated for each concentration, which was relative to the blank absorbance (ethanol). The capability to scavenge the ABTS$^{\bullet+}$ radical was calculated using the following equation: ABTS$^{\bullet+}$ scavenging effect (%) = $[(A_0 - A_1/A_0) \times 100]$, where A_0 is the initial concentration of the ABTS$^{\bullet+}$ and A_1 is absorbance of the remaining concentration of ABTS$^{\bullet+}$ in the presence of sample.

2.11. Antioxidant activity in linoleic acid oxidation

This experiment was conducted using the method of Emmons et al. (1999) with some modifications. β-Carotene (10 mg) was dissolved in 1 mL of chloroform and 50 μL was added to 530 μL of linoleic acid and 40 μL of Tween 20. Oxygenated deionized water (100 mL) was added and the solution was thoroughly mixed. Aliquots of 3 mL of the carotene/linoleic acid emulsion were mixed with samples of the bee honey (10 mg/mL honey pure, 100 μg/mL MeOH extract and 50 μg/mL EtOAc fraction) and incubated in a water bath at 50 °C. The emulsion oxidation was monitored spectrometrically by measuring the absorbance at 470 nm over a period of 60 min. The control sample contained solvent in place of the extract. The antioxidant activity was expressed as the percentage of inhibition relative to the control after a 60 min

incubation period using the following equation: $AA = 100(DR_C - DR_S)/DR_C$. Where AA is the antioxidant activity, DR_C is the degradation rate in the presence of the control ($=Abs_i - Abs_f$), DR_S is the degradation rate in the presence of the sample ($=Abs_i - Abs_f$), Abs_i is the initial absorbance at time 0 and Ab_f is the absorbance at 20, 40, 60 and 80 min. Trolox (a water-soluble Vitamin E analog) at a concentration of 200 μg/mL was used as the reference antioxidant.

2.12. Statistical analysis

All samples were analyzed in triplicate unless stated otherwise and the results were expressed as average ± standard deviation. All statistical analyses were performed using the Microsoft Excel 2007 software package (Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Physicochemical analysis

The honey standards of Brazil have been established only for the *Apis mellifera* honey following international standards guidelines. It was for this reason that the physico-chemical tests were necessary. With the exception of sample 9, which was a yellow-brownish color, all of the honey samples studied had a bright yellow color. The results from the physicochemical analysis of the honey samples are presented in Table 1.

The moisture values ranged from 22.2 to 24.4%, which are above the limit allowed for honey from Apis. Knowledge of the moisture content in honey is useful for improving its conservation and storage by preventing the growth of molds on its surface (Gomes et al., 2011; Estevinho et al., 2012). High levels of humidity can be considered important for the Meliponina honey because they affect other characteristics such as viscosity, fluidity and conservation. In general, some of the physicochemical parameters of the honey produced by stingless bee species differ compared to the honey of *A. mellifera*, especially in moisture, which is very high and causes the honey to be more fluid. This honey is less sweet and has a higher moisture content, which favors fermentation. Furthermore, this honey requires greater levels of care during harvesting and needs to be refrigerated. Discrimination between the honeys (stingless bee and *Apis*) based on sensory attributes was performed using descriptive analysis and employing multivariate statistics, and it was verified that honey from the stingless bee, *M. subnitida*, was perceived as the most similar in relation to flavor and aroma acceptance and sensory characteristics to the conventional *Apis* product (Ferreira et al., 2009).

Hydroxymethylfurfural (HMF) is a widely recognized parameter for evaluating the honey freshness and/or overheating. The HMF content of the honeys analyzed was not more than 60.0 mg/kg and because the HMF content is indicative of the freshness of the honey, this result suggests that the organic honeys are fresher (Gomes et al., 2011).

The pH values and the free acidity varied from 2.9 to 3.7 and 24.5 to 93.5 mequiv./kg, respectively. The low pH of the honey inhibits the presence and growth of microorganisms and causes the honey to be compatible with many food products in terms of pH and acidity. This parameter is of great importance during the extraction and storage of honey because it influences its texture, stability and shelf life (Gomes et al., 2011). The values observed for the ash content varied from 0.01 to 0.27%. The ash content represents the richness of the minerals in the honey and is a parameter that is often used for quality control purposes.

The percentage of reducing sugars in the samples ranged from 50.5 to 72.5% (Table 1). Six samples presented values below the limits stipulated by legislation, which recommends minimum values of 60 g/100 g. Our results for the reducing sugars suggest a different sugar profile for jandaíra honey that deserves further investigation to determine the sugars that are present. The percentage of proteins in the samples ranged between 0.09 and 0.26%. The water activity for the honey samples ranged between 0.650 and 0.720, which is important for determining the stability of certain processed foods.

3.2. Melissopalynological analysis

Results from the qualitative pollen analysis for the 1–9 jandaíra honey samples are summarized in Table 2. All results are listed as percentages of the total pollen content in each sample. Overall, 19 pollen types from 9 families were identified from the 9 honey samples analyzed.

Mimosa caesalpiniifolia (Fabaceae/Mimosoideae) was the predominant pollen type in 8 of the 9 honeys. This pollen type was present in a total of 8 samples, which represents a minimum of 34.62% to a maximum of 95.69% of total pollen. *M. caesalpiniifolia* Benth. (sabiá) is a very common plant species in the Caatinga region and its presence in jandaíra honey in large amounts is expected. Samples 1, 6, 7 and 8 contained a large percentage of pollen (>90%) from this single species. These samples also displayed similar physicochemical data (Table 1).

A significant amount of *Mimosa tenuiflora* (Fabaceae/Mimosoideae) was present in all samples; 33.24% of the total pollen in sample no. 3. *Chamaecrista* sp. (Fabaceae/Caesalpinioideae) was the second most abundant pollen type identified and was the predominant pollen in sample 9. This pollen type was also observed in two additional samples (3 and 5), but with slightly lower amounts, with values of 0.72 and 1.21%, respectively. Pollen grains from a number of other species were present in a large number of the honey samples, although at generally lower levels. A number of specific plant varieties similarly represented were also present in the 9 honeys, at levels ranging from 1.0% to 22.5% of total pollen grains. All of these are relatively common plants in Caatinga.

Table 1
Physicochemical composition of jandaíra (*Melipona subnitida*) honey samples.

Sample	Physicochemical parameters							
	pH	Free acidity (mequiv./kg honey)	Ash (g/100 g honey)	HMF (mg/kg honey)	Nitrogen (mg/100 g)	Reducing sugars (g/100 g honey)	Moisture (g/100 g honey)	Aw
1	3.40 ± 0.0	24.66 ± 0.16	0.08 ± 0.0	15.71 ± 0.2	0.26 ± 0.00	66.77 ± 0.12	22.30 ± 0.00	0.720 ± 0.00
2	2.90 ± 0.0	58.33 ± 0.16	0.09 ± 0.0	10.80 ± 0.1	0.18 ± 0.00	72.77 ± 0.14	23.40 ± 0.00	0.720 ± 0.00
3	3.10 ± 0.1	52.73 ± 0.14	0.06 ± 0.0	13.06 ± 0.1	0.26 ± 0.00	60.10 ± 0.10	22.20 ± 0.00	0.700 ± 0.00
4	3.30 ± 0.0	57.00 ± 0.00	0.05 ± 0.0	14.70 ± 0.1	0.18 ± 0.00	55.27 ± 0.15	23.40 ± 0.00	0.720 ± 0.00
5	3.06 ± 0.0	59.66 ± 0.16	0.05 ± 0.0	13.36 ± 0.1	0.18 ± 0.00	55.50 ± 0.28	24.40 ± 0.00	0.700 ± 0.00
6	3.46 ± 0.0	28.16 ± 0.16	0.03 ± 0.0	13.26 ± 0.1	0.18 ± 0.00	55.67 ± 0.16	23.40 ± 0.00	0.680 ± 0.00
7	3.50 ± 0.0	33.16 ± 0.16	0.05 ± 0.0	11.70 ± 0.2	0.26 ± 0.00	50.50 ± 0.28	23.40 ± 0.00	0.650 ± 0.00
8	3.50 ± 0.0	35.50 ± 0.50	0.04 ± 0.0	15.76 ± 0.1	0.18 ± 0.00	50.67 ± 0.16	23.00 ± 0.00	0.690 ± 0.00
9	3.83 ± 0.1	25.00 ± 1.80	0.20 ± 0.2	14.70 ± 0.2	0.09 ± 0.00	51.83 ± 0.16	23.00 ± 0.00	0.700 ± 0.00

Table 2
Melissopalynological analysis of bee jandaíra (*Melipona subnitida*) honey samples.

Family	Botanical origin	Honey 01 % (frequency)[a]	Honey 02 % (frequency)[a]	Honey 03 % (frequency)[a]	Honey 04 % (frequency)[a]	Honey 05 % (frequency)[a]	Honey 06 % (frequency)[a]	Honey 07 % (frequency)[a]	Honey 08 % (frequency)[a]	Honey 09 % (frequency)[a]
Asteraceae	*Gomphrena*	–	0.15 (MP)	–	0.15 (MP)	0.15 (MP)	–	–	0.16 (MP)	0.17 (MP)
Burseraceae	*Commiphora leptophloeos*	–	–	1.29 (MP)	1.08 (MP)	0.60 (MP)				
Euphorbiaceae	*Croton*						–	–	0.16 (MP)	0.17 (MP)
Fabaceae	*Anadenanthera colubrina*	0.45 (MP)	6.01 (IP)	4.87 (IP)	1.85 (MP)	1.06 (MP)	–	0.31 (MP)	0.48 (MP)	2.00 (MP)
	Chamaecrista	–	–	0.72 (MP)	–	1.21 (MP)				
	Chamaecrista ramosa						–	–		5.00 (IP)
	Chamaecrista sp.									35.17 (SP)
	Mimosa caesalpiniifolia	95.61 (PP)	55.62 (PP)	34.67 (SP)	74.19 (PP)	60.73 (PP)	95.69 (PP)	92.02 (PP)	93.27 (PP)	1.17 (MP)
	Mimosa lepidophora	1.36 (MP)	–	0.57 (MP)	–	–				5.00 (IP)
	Mimosa tenuiflora	2.42 (MP)	11.86 (IP)	33.24 (SP)	11.44 (IP)	18.13 (SP)	4.31 (IP)	7.36 (IP)	4.81 (IP)	20.50 (SP)
	Piptadenia moniliformis									6.17 (IP)
	Prosopis juliflora	–	20.49 (SP)	11.60 (IP)	3.55 (IP)	6.34 (IP)			0.96 (MP)	
	Senna	0.15 (MP)	–	1.29 (MP)	0.77 (MP)	1.96 (MP)	–	0.31 (MP)	0.32 (MP)	10.67 (IP)
	Zornia	–	1.54 (MP)	–	0.15 (MP)	–				
Myrtaceae	*Eucalyptus*	–	0.31 (MP)	0.57 (MP)	0.31 (MP)	0.91 (MP)				
	Myrcia	–	–	0.29 (MP)	0.31 (MP)	–				
	Psidium	–	3.70 (IP)	8.45 (IP)	6.18 (IP)	8.16 (IP)	–	–	–	9.83 (IP)
Phytolaccaceae	*Microtea*	–	0.15 (MP)	0.14 (MP)	–	–				
Scrophulariaceae	*Scoparia dulcis*	–	–	–	–	0.15 (MP)	–	–	–	2.33 (MP)
Solanaceae	*Solanum*	–	–	0.86 (MP)	–	0.30 (MP)				
Sterculiaceae	*Waltheria*	–	–	0.14 (MP)	–	0.15 (MP)				
Not identified[b]		–	0.15 (MP)	1.29 (MP)	–	0.15 (MP)	–	–	–	0.17 (MP)
Total (%) pollen		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100

[a] PP: predominant pollen (>45%). SP: secondary pollen (16–45%). IP: important minor pollen (3–15%). MP: minor pollen (<3%).
[b] A single pollen type.

Table 3
Quantitative analysis of compounds identified in the jandaira honey (μg/50 g honey).

Honey	Phenolics content (μg/50 g honey)							Abscisic acid (μg/50 g honey)	
	Benzoic acids			Cinnamic acids	Flavonoids			*Trans, trans* (**1**)	*Cis, trans* (**2**)
	3,4-Dihidroxy	Gallic	Vanillic	Cinnamic	Isorhamnetin	Naringenin	Quercetin		
1	6.6	5.4	14.7	12.9	6.0	60.9	16.5	63.3	85.5
2	16.8	10.8	12.6	6.6	11.1	65.1	89.1	33.3	214.2
3	21.9	12.9	42.0	8.4	8.1	103.5	66.6	39.3	177.0
4	13.5	10.5	13.5	10.8	8.4	48.3	63.0	22.0	61.2
5	19.5	2.7	18.8	14.7	6.0	73.5	49.5	333.0	182.4
6	8.4	7.8	7.8	4.2	2.0	53.1	37.2	36.0	32.4
7	9.3	8.4	13.8	5.1	5.1	56.1	36.3	73.5	52.5
8	11.1	13.5	9.9	5.1	33.0	28.2	36.3	39.0	59.1
9	–	–	–	–	–	–	–	6498.6	4170.0

Based on this small sample set, therefore, it would appear that the *M. subnitida* bees in the Northeastern semi-arid region primarily feed on nectar from plants of the Fabaceae family. In a previous study concerning the pollen loads from jandaíra bees, it was observed that grains were the predominant pollen type (Silva et al., 2006). There are many endemic plant species with important beekeeping potential that are good geographical markers and the presence of their pollen grains in the bee honey and stingless bee products from the semi-arid regions has been confirmed by pollen analysis of honeys (Oliveira et al., 2010). Although this potential is recognized throughout the Northeastern flora, particularly in the Caatinga, little is known about the potential beekeeping species. The Caatinga is perhaps the only Brazilian phytogeographic region whose flora has not been subjected to continuous and systematic pollen studies (Oliveira et al., 2010).

3.3. HPLC analysis and identification of **1** and **2**

Other than flavonoids (Martos et al., 2000; Alvarez-Suarez et al., 2012b), the most common phenolic acids generally found in honeys are benzoic and cinnamic acids and their esters (Sabatier et al., 1992). To evaluate the presence of these compounds in jandaíra honey, we selected 24 phenolic compounds that are commonly found in honey samples, which comprise five benzoic and eight cinnamic acid derivatives, ten flavonoids and chlorogenic acid.

The HPLC-DAD analysis of the EtOAc fraction of jandaíra honey revealed the presence of phenolic compounds. Cinnamic, 3,4-dihydroxybenzoic, gallic and vanillic acids were detected in samples 1–8. These compounds have been identified based on their retention time and the UV spectral characteristics, which were compared to those of standards. The chromatograms recorded at 320 nm confirmed the presence of quercetin, naringenin and isorhamnetin in samples 1–8. The HPLC chromatograms of the phenolic fractions indicated that the honeys tested had similar phenolic profiles, except for sample 9. The identified compounds were quantified (Table 3).

The presence of naringenin in eight out of nine samples may be related to the botanical origin of the honey, because in all of these samples it is present as the main component of the *M. caesalpiniifolia* pollen type (Fabaceae/Mimosoideae). A previous study concerning the pollen loads collected from jandaíra (*M. subnitida*) with 98.95% of the *Mimosa gemmulata* pollen type also indicated the presence of naringenin, suggesting that it may be specific for these species (Silva et al., 2006).

The flavonoid patterns of honeys from two species of *Melipona* were analyzed by Vit et al. (1997), and it was verified that the number of flavonoid types present in individual samples varied to some extent, but the diversity of flavonoids in the tropical honeys is lower than that previously measured in honeys from temperate zones. A subsequent study by Vit and Tomás-Barberán (1998) on the flavonoids in stingless bee honey showed that honeys from the same geographical origin had very similar flavonoid profiles. Therefore, it was proposed that the flavonoid profiles of a therapeutic product called honey eye drops, which is made of droplets of stingless bee honey and used for eye treatment, could be used for honey authentication and quality control and for determining the geographical origin of the honey.

This observation is consistent with our results because samples 1–8 (Fig. 1) were collected in the same geographical region in the semi-arid region. Sample 9, which contains pollen that is predominantly from a species of the genus *Chamaecrista* (35.1%), showed the presence of two principal components (wavelength 290 nm) (Fig. 2). These two compounds were isolated and purified using a combination of Sephadex LH-20 chromatography and semi-preparative HPLC. The ^{1}H NMR and ^{13}C NMR analysis indicated that compound 1 corresponds to *trans*, *trans*-abcisic acid and compound **2** corresponds to the *cis*, *trans*-abcisic acid isomer (Fig. 2). The LC–ESI-MS of **1** and **2** presented a peak at *m*/*z* 265 that is relative to the molecular weight [M+H].

The other relevant fragments were 208, 190 and 162. After isolating and identifying the abscisic acids **1** and **2**, these acids were identified and quantified in the honey samples 1–9. This isoprenoid, (±)-abscisic acid, appeared in all of the analyzed samples as a major component. In samples 1–8, the amount was 22.0–89.1 μg/50 g honey and in sample 9, the amount was 6498.6 and 4170.0 μg/50 g honey to *trans*–*trans*- and *cis*–*trans*-abscisic acid, respectively. The presence of this plant hormone may be due to the hydric stress that the plant species experiences in the semi-arid region where the honey was collected. The role of abscisic acid as a universal plant stress hormone is well established. Under mild stress conditions when the soil begins to dry and the water potential of the leaves is not or only slightly affected, abscisic acid is accumulated in root tissues, released to the xylem vessels and transported to the shoot where the stomatal and meristematic activities are regulated to help the plant cope with the stress situation (Jiang and Hartung, 2008). This plant hormone has been previously recognized as a phytochemical constituent for the authentication of honey and as a floral marker (Ferreres et al., 1996; Bertoncelj et al., 2011). The nectar of the *Erica* flower was analyzed and both isomers were also observed as the main constituents, which confirmed the floral origin of the compounds found in Portuguese honey.

3.4. Total phenolic content

The amount of total phenolics estimated using the Folin–Ciocalteu reagent in the different samples ranged from 80.2 to 166.1 mg GAE/g (gallic acid equivalent by gram of extract) in the MeOH extract, 125.9 to 391.9 mg GAE/g in the EtOAc fraction and 1.2 to 1.3 mg GAE/g in the pure honey. The highest total phenolic levels were detected in the EtOAc fractions and the lowest in pure honey (Table 4). This colorimetric assay presents a low specificity

Table 4
Total phenolics and antioxidant activity of jandaíra honey samples.

Sample	Total phenolic content (mg GAE/g ± SD)			DPPH (EC_{50})[a]			ABTS (EC_{50})			β-Carotene bleaching (% O.I.)[b]		
	Pure	MeOH	AcOEt	Pure (mg/mL ± SD)	MeOH (μg/mL ± SD)	AcOEt (μg/mL ± SD)	Pure (mg/mL ± SD)	MeOH (μg/mL ± SD)	AcOEt (μg/mL ± SD)	Pure (% ± SD)	MeOH (% ± SD)	AcOEt (% ± SD)
1	1.2 ± 0.0	95.7 ± 5.5	130.2 ± 0.2	11.1 ± 0.1	208.6 ± 2.1	87.8 ± 0.9	7.1 ± 0.0	42.9 ± 0.4	33.9 ± 1.5	54.6 ± 3.2	50.3 ± 2.1	54.0 ± 0.6
2	1.1 ± 0.0	102.6 ± 0.8	242.3 ± 0.4	11.2 ± 0.3	138.1 ± 3.1	64.2 ± 0.7	6.1 ± 0.0	37.0 ± 0.8	23.2 ± 0.7	51.5 ± 5.1	53.6 ± 2.6	72.1 ± 1.8
3	1.1 ± 0.1	109.9 ± 1.6	240.8 ± 2.3	12.9 ± 0.3	157.4 ± 1.0	62.0 ± 0.1	7.0 ± 0.1	42.7 ± 1.5	18.2 ± 0.2	58.3 ± 0.5	55.4 ± 2.2	64.3 ± 1.7
4	1.1 ± 0.1	81.0 ± 0.3	166.7 ± 7.3	12.5 ± 0.1	171.9 ± 2.9	75.9 ± 0.7	6.7 ± 0.3	49.8 ± 0.4	28.3 ± 0.2	48.8 ± 2.8	45.4 ± 0.2	47.9 ± 2.7
5	1.3 ± 0.3	81.5 ± 3.1	195.8 ± 3.0	10.6 ± 0.6	144.3 ± 3.9	64.5 ± 0.6	8.1 ± 0.1	42.1 ± 2.4	20.4 ± 0.1	55.2 ± 1.5	29.7 ± 4.1	50.6 ± 6.1
6	1.3 ± 0.2	101.1 ± 1.1	125.9 ± 3.7	10.6 ± 0.1	184.9 ± 0.3	75.3 ± 0.7	8.3 ± 0.3	27.9 ± 0.5	29.5 ± 0.7	53.7 ± 4.3	34.8 ± 2.0	66.3 ± 0.4
7	1.3 ± 0.1	80.2 ± 0.0	131.3 ± 3.3	10.7 ± 0.3	201.2 ± 5.0	81.3 ± 0.9	9.7 ± 0.1	53.1 ± 0.8	32.7 ± 0.1	55.3 ± 2.5	42.2 ± 3.5	68.2 ± 1.2
8	1.3 ± 0.1	106.5 ± 0.9	125.9 ± 3.1	10.9 ± 0.0	202.0 ± 5.7	84.23 ± 0.8	9.4 ± 0.4	37.8 ± 0.6	23.6 ± 1.4	58.4 ± 1.8	36.4 ± 0.4	58.0 ± 2.6
9	1.2 ± 0.1	166.1 ± 1.4	391.9 ± 3.2	12.1 ± 0.0	108.5 ± 1.7	43.5 ± 0.9	8.3 ± 0.0	21.2 ± 0.3	13.2 ± 0.3	74.6 ± 4.3	63.2 ± 2.0	87.1 ± 2.7

Mean value ± standard deviation: $n = 3$.
[a] Concentration of antioxidant required to reduce the original amount of the radicals by 50%.
[b] Oxidation inhibition.

because it not only measures total phenols but it also reacts with any reducing substance; therefore, the color reaction can occur with any oxidizable phenolic hydroxy group and many non-phenolic compounds (sugars) present in honey (Alvarez-Suarez et al., 2009). Even with these interfering compounds present in honey, the content of phenolic compounds in pure honey was more than one hundred times lower than that obtained in the EtOAc fraction of honey that had a greater concentration of phenolic. These samples contained more than 90% of *M. caesalpiniifolia* pollen.

3.5. Antioxidant activities

Three different methods were used to determine the antioxidant properties of the jandaíra honeybee, which allows us to obtain information about the activity of these extracts during the different stages of the oxidation reaction (Prior et al., 2005). The methods used included the inhibition of β-carotene, co-oxidation in a linoleic acid model system and the scavenging of DPPH and $ABTS^{\bullet+}$. Based on antioxidant tests, different varieties of honey from various countries and geographical regions have been shown to exhibit high antioxidant properties (Alvarez-Suarez et al., 2010, 2012b). All of the extracts assayed prevented the bleaching of β-carotene in the carotene/linoleic acid mixtures (Table 2). However, different samples exhibited varying degrees of antioxidant capacity. All honey samples exhibited free radical scavenging activity. As shown in Table 2, the EC_{50} values ranged from 10.6 to 12.9 mg/mL for pure honey, 108.5 to 208.6 μg/mL for the MeOH extract and 43.5 to 87.8 μg/mL for the EtOAc fraction in the DPPH radical-scavenging assay. The EC_{50} results for the ABTS test varied from 6.1 to 9.7 mg/mL, 21.2 to 53.1 μg/mL and 13.2 to 33.9 μg/mL, for pure honey, the MeOH extract and the EtOAc fraction, respectively. The lowest inhibition activity was observed in the pure honey (Table 2). The antioxidant activity (AA) was determined based on the inhibition of the coupled oxidation of β-carotene and linoleic acid at $t = 60$ min (Table 2). The antioxidant activity ranged from 23.8% to 70.3% ($t = 60$ min), with 51.5–74.6%, 29.7–63.2%, 47.9–87.1% in the pure honey, the MeOH extract and the EtOAc fraction, respectively. Sample 9 presented the highest activity during all three antioxidant assays and the oxidation rate ratios for this sample were either significantly better or equal to that of Trolox. The antioxidant activities of the jandaíra honeybee samples are shown in Table 2.

4. Conclusions

The melissopalynological analysis of *M. subnitida* honeys from Brazil indicated the presence 19 pollen types from 9 families. *M. caesalpiniifolia* (sabiá) was the predominant pollen type in eight of the nine honey samples analyzed. The physicochemical analysis revealed that all samples had a similar profile regarding pH, free acidity and hydroxymethylfurfural, water, ash, nitrogen, reducing sugars and moisture content. The moisture values ranged from 22.2 to 24.4%. The high levels of humidity can be considered important in the *Meliponina* honey because they affect other characteristics such as viscosity, fluidity and conservation. All of the jandaíra honey samples shared a similar phenolic profile, except for sample 9, which was collected from a different geographical location. The flavonoids naringenin, quercetin and isorhamnetin were common to samples 1–8, as the gallic, vanillic, 3,4-dihydroxybenzoic and coumaric acids. The two isomers of abscisic acid (*trans*, *trans* and *cis*–*trans*) that were present in major quantities in the sample 9 were isolated and quantified. All of the identified compounds were adequately quantified. All of the honey samples exhibited high antioxidant activity. The present study demonstrated that the phenolic content of the jandaíra honey

samples is partially responsible for their antioxidant activity, which supports the relevance of this type of honey being an important dietary source of antioxidant compounds and its traditional use as a medicinal product.

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