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Physicochemical characterization, bioactive compounds and antioxidant activity of *apis mellifera* honey from western paraná state, brazil

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Abstract
The objective of this work was to evaluate the physicochemical characteristics, the bioactive compounds and the antioxidant potential of honey produced by Africanized bees (Apis mellifera L.) from municipalities in western Paraná state. Sixty-six samples of honey were analyzed from October 2018 to August 2019. The physicochemical parameters evaluated were: color, moisture, hydroxymethylfurfural, pH and free acidity, with mean values of 0.36±0.24nm, 18 .80±1.01%, 10.34±3.24mg.kg^{-1}, 4.01±0.18 and 17.36±4.11 meq.kg^{-1}, respectively. The bioactive compounds (phenolic compounds and flavonoids) and the antioxidant activity were also analyzed, and the mean values of 16.34±7.41mg.GAE 100 g-1 were observed for Total Phenols, 21.35±9.63mg.QE 100 g^{-1} for flavonoids and 0.09±0.03µmol.TE g^{-1} for antioxidant activity (DPPH). The honey samples were influenced by their phytogeographic origin and have antioxidant potential.

Keywords: flavonoids, phenolic compounds, DPPH, phytogeographic origin.

Caracterização físico-química, compostos bioativos e atividade antioxidante do mel de *apis mellifera* do oeste paranaense

Resumo
O objetivo do trabalho foi avaliar as características físico-químicas, os compostos bioativos e o potencial antioxidante do mel produzido por abelhas africanizadas (*Apis mellifera* L.) de municípios do Oeste paranaense. Foram analisadas 66 amostras de mel, no período de outubro de 2018 a agosto de 2019. Os parâmetros físico-químicos avaliados foram: cor, umidade, hidroximetilfurfural, pH e acidez livre, com valores médios de 0,36±0,24nm, 18,80±1,01%, 10,34±3,24mg.kg^{-1}, 4,01±0,18 e 17,36±4,11 meq.kg^{-1}, respectivamente. Também foram analisados os compostos bioativos (compostos fenólicos e flavonoides) e atividade antioxidante, sendo observados valores médios de 16,34±7,41mg.EAG 100 g^{-1} para Fenois Totais, 21,35±9,63mg.EQ 100 g^{-1} para flavonoides e 0,09±0,03μmol.ET g^{-1} para atividade antioxidante (DPPH). As amostras de mel sofreram influência quanto a sua origem fitogeográfica e possuem potencial antioxidante.

Palavras-chave: flavonoides, compostos fenólicos, DPPH, origem fitogeográfica.

Introduction

Honey, in addition to being a natural food, has high nutritional quality and valuable therapeutic properties (DAS *et al*., 2015), mainly due to its biological activity, chemical composition and physical properties (SILVA *et al*., 2016).

Bioactive compounds, quantitatively, in smaller proportions in honey, are responsible for the therapeutic properties, among which the phenolic compounds (flavonoids and phenolic acids) are responsible for the antioxidant activity (DI MARCO *et al*., 2016). There are factors intrinsic to honey that give this product its own antioxidant activity, such as glucose-oxidase enzymes, catalase and compounds such as carotenoids, organic acids, ascorbic acid, amino acids and proteins (MACHADO DE-MELO *et al*., 2018).

However, the antioxidant capacity is related and determined according to its presence in honey, influenced by several factors like floral and geographical (phytogeographic) origin, processing, handling and storage and the bee species (SILVA *et al*., 2020).

Scientific research has investigated the chemical and biological properties of honey, with interest in their applications as an antioxidant in medical treatments for oxidative stress (SPILIOTI *et al*., 2014), in their use as antiviral, antifungal, antitumor (AHMED *et al*., 2018), anti-inflammatory and immune stimulant (ORYAN *et al*., 2016). As a result of this interest, the objective of this work was to evaluate the physicochemical characteristics, bioactive compounds and antioxidant potential of honey produced by Africanized bees (*Apis mellifera* L.) from municipalities in western Paraná state.

Materials and methods

Location and collection of samples

The research was carried out with samples of honey from municipalities in the western region of Paraná state. The climate of the region is classified as Cfa, according to Köppen (1948), mesothermal humid subtropical, with hot summers with a tendency to rain concentration, winter with infrequent frosts (average temperature below 18°C), with no defined dry season. The average annual temperature is approximately 22°C, with the highest monthly average values being concentrated in the months of January and February, around 28°C, and the coldest periods being concentrated in the months of June, July and August, with monthly minimum average temperatures around 18°C.

The soil of the region is composed of eutrophic purple Latosol (45%), eutrophic structured purple earth (45%) and eutrophic litholic soils (10%) (EMBRAPA, 2006). The cities' activities are based on the agricultural sector, highlighting the production of milk, soy, corn, chicken, swine and industries.

In the municipalities of western Paraná, the coverage designated by Veloso et al. (1991) of Semideciduous Seasonal Forest, also called Subtropical Rain Forest, Interior Plateau Forest or Tropical Forest, covering large portions of the state of Paraná, such as the West and North (MAACK, 2002).

As a relevant phytogeographic feature, the Artificial Lake of Itaipu is located in the western region of Paraná, and this stretch of impoundment of the Paraná River is known as the Paraná III basin. The vegetation that borders the lake is formed by the reforestation of areas of anthropized riparian forest, carried out from 1979, with the construction of the Itaipu Binacional Hydroelectric Power Plant, called the green belt. This protection strip has an average width of 217 meters on both banks, and an extension of around 2,900 km, in addition to areas of riparian forest in the river basins that form the Paraná III Basin (BP III - dammed area) and Legal Reserve (OSTROVSKI, 2014).

However, land use is intense in this region and agriculture is highly mechanized, predominantly grain crops: soy, corn and wheat, making the landscape components contrasting in some municipalities.

For this research, 66 samples of honey produced by Africanized bees (*Apis mellifera* L.), during the period from October 2018 to March 2019, from family beekeepers from four municipalities in the western region of Paraná, were analyzed, distributed as follows: Santa Helena (17 samples) (Latitude 24º51'37"S and Longitude 59º19'58"W), Entre Rios do Oeste (17 samples) (Latitude 24º42'16"S and Longitude 54º14'03"W), Terra Roxa (16 samples) (Latitude 24º09'25"S and Longitude 54º06'02"W) and Marechal Candido Rondon (16 samples) (Latitude 24º33'22"S and Longitude 54º03'24"W).

These municipalities are considered *Lindeiros* (lakeside municipalities), as they have all or part of their territory on the shores of Lake Itaipu. However, due to the diversity of landscapes found in the region, for the purpose of comparing the physicochemical characteristics of the honey samples, they were classified as Lakeside ones (n=34 samples) and *Afastados do Lago* (distant from the lake) (n=32 samples).

Thus, according to the geographic position of the apiaries of the suppliers of the evaluated samples, the municipalities of Entre Rios do Oeste and Santa Helena were considered as Lakeside municipalities, due to the arrangement of the beekeepers' hives, closer to the reforestation areas that border the lake. The municipalities of Marechal Cândido Rondon and Terra Roxa were considered distant from the lake, since the producers who donated the samples have their hives allocated in regions further away from the lake.

Analysis and characterization of honey

Color, moisture, pH and free acidity

The methodology used to determine the color of the honey samples was proposed by Vidal and Fregosi (1984) using the Shimadzu UV-1800 UV-VIS spectrophotometer, using glycerin as a blank and reading at a wave of 560 nm. The honey moisture was determined following the Chataway refractometer method (AOAC, 1990), for which a light wave is emitted falling on the honey, being converted into percentage of moisture, as described by Chataway (INSTITUTO ADOLFO LUTZ, 2008). The pH was determined with a benchtop pHmeter Simpla PH140 previously calibrated with buffer solutions of 4 and 7. To carry out this procedure, the method of Moraes and Teixeira (1998) was used, using 10g of each sample of honey, later mixed in 25mL of distilled water in a 50mL beaker, shaken and left to rest for 10 minutes until the moment of reading. To determine the free acidity, the method described by Zenebom (2008) was used, in which the free acidity is the measure obtained by the titration of sodium hydroxide until its equivalence.

Hidroximetilfurfural (HMF)

The determination of HMF content was performed according to AOAC (1990), based on UV absorbance reading, after clarification of the samples with Carrez reagents (I and II) and the addition of sodium bisulfite.

The solutions used in the analysis of hydroxymethylfurfural were: a) Carrez solution (I), where

15g of potassium ferrocyanide dissolved in 100mL of distilled water and Carrez solution (II) were used for the preparation of 30g zinc acetate dissolved in 100mL of distilled water and finally the 0.2% sodium bisulfite solution in distilled water. This solution was prepared at the time of analysis. Absorbances were determined at wavelengths 284nm and 336nm in a Shimadzu UV-1800 UV-VIS spectrophotometer.

Phenolic compounds, flavonoids and antioxidant activity

Extract preparation

The extracts were obtained by the cold hydroalcoholic extraction method proposed by Vedana *et al*. (2008), 1g of honey was weighed and diluted in 10mL of 80% methanol, followed by vortex homogenization. The mixture was then subjected to an ultrasonic bath for 20 minutes, then passed through a centrifugation process at 20,000rpm, at 4ºC for 20 minutes, filtered through qualitative paper and stored at 18ºC.

Total phenolics

The Folin-Ciocalteau spectrophotometric method was used with adaptations by Rotili *et al*. (2018). For this procedure, 0.5mL of honey extract, prepared initially, was used, mixed with 2.5mL of Folin-Ciocalteau water solution (1:10), remaining at rest for 3 minutes. 2.0mL of sodium carbonate solution and water were added sequentially, standing in the dark and incubated at a temperature of 50ºC. After this follow-up, the samples were placed in an ice bath following readings at 760nm in a spectrophotometer.

A calibration curve was constructed for gallic acid as a standard ($y= 0.1632x+0.0041$; $R^2 = 0.9917$), and the results were expressed in gallic acid equivalent (mg.GAE mL^{-1} sample).

Antioxidant activity

The antioxidant activity of honey extract was measured through its capacity to sequester DPPH (2,2-diphenyl-1-picrylhydrazyl), according to Rufino *et al*. (2007). 0.5mL of honey extract and 0.3mL of DPPH radical (0.5mmol L^{-1}) in 3.0mL of 80% methanol were added and left to stand in the dark for 60 minutes. After this period, the samples were read in the Shimadzu UV-1800 UV-VIS spectrum at an absorbance of 517nm. 3.5mL of 80% methanol added to 0.3mL of DPPH solution was used as a blank. Results were expressed in µmol g^{-1} of dry weight, in Trolox equivalent (TE), calculated by adjusting the calibration curve for Trolox at concentrations ranging from 20.0 µmol to 200.0 µmol.TE g^{-1}.

Determination of total flavonoids

For the determination of total flavonoids, the method described by Meda (2005) was used, with some modifications. For this, 0.5mL of honey extract were used, to which 4.3mL of a 2% solution of aluminum trichloride ($AlCl_3$) were added, initially prepared, diluted in 80% ethanol, being homogenized in the Vortex Mixer Kasvi. After this procedure, the samples were stored in the dark at room temperature for 40 minutes, the time necessary for the solutions to react with the extract. After this time, absorbance readings were taken at 415nm, where a mixture of 2.5mL of the extract with 2.5mL of 80% ethanol was used. Total flavonoids were determined using the quercetin standard at concentrations ranging from 10-70mg L^{-1} for calibration of the calibration line (R^2: 0.9972), through the average of three readings. The total flavonoid content was expressed in mg of quercetin equivalents/100g of honey (mg.QE $100g^{-1}$).

Statistical analysis

The parameters color, moisture, HMF, total phenol, flavonoids and DPPH were carried out in triplicate, while conductivity, pH and acidity were carried out in duplicate. The highest and lowest values, mean and standard deviations (SD) were calculated for the analyzed physicochemical parameters of honey. The t test for independent samples was used to verify the possible statistical differences between the municipalities close to the lake (Santa Helena and Entre Rios do Oeste) and those further away (Marechal Cândido Rondon and Terra Roxa). The t<5% test for independent samples was performed after verifying the homogeneity of variances by means of ANOVA. In addition, principal component analysis (PCA) was used to verify the association between variables and sample elements. All statistical analyzes were performed using R software version 3.0.2 (R Foundation, Vienna, Austria).

Results and Discussion

The data of the physical-chemical parameters evaluated in the honey samples, considering together the four municipalities belonging to the western region of Paraná state, are presented in table 1.

The analyzed honeys had an average moisture content of 18.8±1.01%, with pH ranging from 2.56 to 4.60 (4.01±0.18) and acidity from 8.30 to 28.30 (17.36±4.11 meq.kg^{-1}).

These results were similar to those reported by other studies for humidity (CAMARGO *et al*., 2014; GALHARDO *et al*., 2020). A research in previous years showed similar pH values to those found for honey samples from the same region. Moraes *et al*. (2014) found mean values of 3.96 and Galhardo *et al*. (2020) of 3.10. As for the acidity, Galhardo *et al*. (2020) found mean values of 34.54 meq.kg-1.

The mean value observed for HMF was 10.34±3.24, with electrical conductivity ranging from 298.90 to 666.60 (442.6±94.87 µS.cm-1). For color, the average value found was 0.36±0.24, with values ranging from 0.16nm to 1.02nm, and the most predominant color for these samples was light amber.

Other studies reported similar results for HMF in which Galhardo *et al*. (2020) found values lower than 13.67 mg kg^{-1}. The same authors found a mean of 349.00 µS cm^{-1} for electrical conductivity. For color, the research by Camargo *et al*. (2014), Moraes *et al*. (2014) and Galhardo *et al*. (2020) found similar values, ranging from 0.12 to 0.945nm.

Of the values found for humidity, 4.60% of the samples presented values above the values allowed by the Brazilian

legislation (maximum of 20%).

Table 1. Physicochemical parameters in honey samples from western Paraná state.

Parameter	Max.	Min.	Average
Humidity (%)	20,80	15,80	18,80±1,01
HMF (mg kg^{-1})	16,02	3,77	10,34±3,24
pH	4,60	2,56	4,01±0,18
Acidity (meq kg^{-1})	28,30	8,30	17,36±4,11
Conductivity (µS cm^{-1})	666,60	298,90	442,6±94,87
Color (nm)	1,02	0,16	0,36±0,24
Total phenol (mg.GAE $100g^{-1}$)	30,50	1,56	16,34±7,41
Flavonoids (mg.QE 100 g^{-1})	52,80	9,35	21,35±9,63
DPPH (μmol.TE g^{-1})	0,22	0,01	0,09±0,03

HMF: Hidroximetilfurfural. Max.: Maximum found value. Min.: Minimum found value.

The values observed for HMF showed results below 60 mg kg^{-1}, the maximum value determined by the Brazilian legislation, indicating that the honey was stored for a short time and in an adequate way. Considering that honey is naturally acidic according to the Brazilian legislation and that the values found for pH and acidity were within the permitted range, indicating the absence of fermentation by microorganisms. For color, the Brazilian legislation recommends amber as a parameter (BRASIL, 2000).

The determination of electrical conductivity is not required by the Brazilian legislation (BRASIL, 2000).

The mean amount of total phenols ranged from 1.56 to 30.50 mg.GAE 100 g^{-1}, with a mean of 16.34±7.41 mgGAE 100 g^{-1}. The amount of total flavonoids observed ranged from 9.35 to 52.80 mg.QE 100 g^{-1}, with a mean of 21.35±9.63 mg.QE 100 g^{-1}. The mean value found for antioxidant activity, with the capture of the DPPH radical, was 0.09±0.03 µmol.TE g^{-1}. Results that are in accordance with the Brazilian legislation, conferring antioxidant potential to honey from the regions studied.

In a similar study Galhardo (2018) found an average value of 36.3 mg.GAE 100 g^{-1} with an amplitude of 11.39 to 61.27 mg.GAE 100 g^{-1} for the amount of phenols in the western region of Paraná, values that are closely associated the colors of honey. For flavonoids Bueno Costa (2016) evaluated honey samples from different regions of Rio Grande do Sul state, obtaining results from 2.98 to 10.46 mg.QE 100 g^{-1}, results lower than those found in this work. As for the antioxidant activity with DPPH radical capture Galhardo *et al*. (2020) found similar values, justifying that the increase in the values found may be correlated with the degradation of honey, through the enzymatic reactions undergone during the storage period, or by Maillarde reaction. The antioxidant activity, as well as the bioactive compounds, may vary, according to the time of honey harvest, due to the type of flowering, since this directly interferes with its color.

When comparing the municipalities far from the lake with the municipalities on the lakeside (Table 2), the value found for HMF had a significant difference, since the results for the honey samples far from the lake were lower than those for the samples from the lakeside municipalities, however, both are in accordance with the Brazilian legislation.

Significant differences were observed for honey acidity for the two distinct regions, and the honeys from the region far from the lake obtained higher values than those from the lakeside (Table 2). The difference is justified because the two distinct regions have different floral sources and tree species, and the nectar source could have differentiated the acidity between the regions.

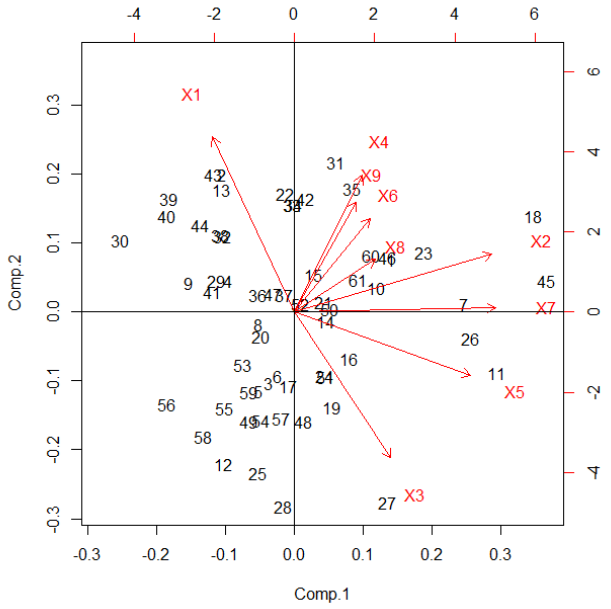
Table 2. Comparison between groups of samples distant from the lake and lakeside for physicochemical parameters of honey.

Parameter	Distant (n = 32)	Lakeside (n = 34)	t<5%	*p-value*
Humidity (%)	18,75± 0,93	18,83±1,15	-0,30	0,764
HMF (mg kg^{-1})	9,04±3,04	11,67±2,96	-3,41	0,002
pH	3,99±0,23	4,03±0,46	-0,49	0,626
Acidity (meq kg^{-1})	18,95± 3,80	15,72± 4,15	3,17	0,002
Conductivity (µS cm^{-1})	436,24±97,90	449,18±97,07	-0,51	0,606
Color (nm)	0,35±0,21	0,38±0,28	-0,47	0,637
Total phenols (mg.GAE 100 g^{-1})	14,11±7,40	18,14±6,66	-2,23	0,030
Flavonoids (mg.QE 100 g^{-1})	20,04±7,36	22,69±11,72	-1,05	0,296
DPPH (μmol.ET g^{-1})	0,09±0,04	0,09±0,01	-0,48	0,630

The diversities of flora and temperature of both regions gave the honeys variation in the composition of total phenols, explaining the difference between them. It was evident that higher temperatures in the lakeside region caused greater accumulation of total phenols in honey.

In PCA, the proportion of variance explained by the first two factors was 44% (Figure 1). Axis I explains 26% of the data variance, it is positively associated with all variables except pH. The flavonoids variable best explains the physicochemical parameters on axis I (0.524), followed by

color (0.515) and acidity (0.459). Axis II explains 18% of the total variation, it is formed by the positive contribution of the variable colors, HMF, conductivity, DPPH, phenolics and pH, the latter being the one that best explains the variance of the data on axis II (0.549). Axis II is also negatively associated with the variable moisture (-0.457) and acidity (-0.198). Thus, we can say that flavonoids, color, acidity and pH are the variables that best explain the differences between the honey samples.



X1 = pH, X2 = color, X3 = humidity, X4 = HMF, X5 = acidity, X6 = conductivity, X7 = flavonoids, X8 = DPPH, X9 = phenol components

Figure 1. Scores of the first two principal components of a PCA of physicochemical variables of A. mellifera honey.

Along with honey studies carried out in previous years, the western region of Paraná received the Indication of Origin seal, a point of great importance for producers. The parameters that were evaluated in this work confirm the quality of the product in terms of absence of tampering with physicochemical characteristics, justifying the use of this seal by the evaluated beekeepers. In addition, this characterization validates the information that relates these characteristics of the product to its phytogeographic origin, providing important elements for the application of Denomination of Origin to honey in the region.

Conclusion

The content of total phenolic compounds, total flavonoids, antioxidant activity (DPPH) and physicochemical characteristics were influenced by the phytogeographic origin of the samples and have antioxidant potential.

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