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Red Propolis: Chemical and Mid-Infrared Spectroscopic Characterization and Classification by Geographic Origin

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In this paper, we report the characterization of Brazilian red propolis from two origins. The commercial value of this product is due to its composition and bioactivity, which depend on geographical factors. Total and individual phenolic, antioxidant, and medium infrared spectroscopic (MIR) characterization of 72 red propolis samples from two origins (Bahia (BA) and Rio Grande do Norte (RN), Brazil) were performed. Samples from RN showed higher concentrations of biomarkers. The BA samples showed higher antioxidant capacity despite having lower concentrations of the red propolis biomarker molecules when compared to the RN samples. The MIRS showed differences regarding the origin. The principal component analysis confirmed the differences, generating well-defined groups. Linear discriminant analysis or discriminant analysis and and partial least squares discriminant analysis showed excellent model performance. The models with MIRS data from raw propolis have a high correct classification rate and require no sample preparation. These findings can affect red propolis production, boosting sustainable extractivism, and changing the reality of small communities.

Keywords: bee products, MIRS, HPLC, multivariate analysis, chemometric

Introduction

Propolis is a bee product produced to coat, and reinforce the inner walls of the hive, repair combs, protect from insect invasion, and maintain an antiseptic environment. To do this, bees explore and select plant sources from which they collect the resin that will give rise to propolis.[1,2] The three largest producers of propolis in the world are Russia, China, and Brazil. Brazil produces about 150 tons *per* year and supplies 80% of the demand for propolis in Japan, the country that consumes this product the most. The vast majority of Brazilian production originates from small rural producers. This production is based on sustainable extractivism and joins in associations and cooperatives, which brings an important social and ecological character to this product.[3-5] Propolis is marketed as a functional food, although it has many applications in traditional medicine because of its bioactive properties. Its medicinal use ranges from treating infections in the respiratory and digestive tract, dermatological care, and improving the immune system, to recent studies on counter viral load and cancer cells. Its application ranges from capsules, extracts, and powders, to personal care products such as toothpaste, ointments, and sunscreens, among others, covering the cosmetic, pharmacological, and food industries.[4-6]

The chemical composition and color of propolis may vary according to the botanical source available in the collection region.[7] Its chemical composition varies in quality and quantity of bioactive compounds such as phenolic acids and flavonoids, and its color can vary from dark brown through a greenish hue to reddish-brown.[8] Park *et al.*,[2,9] observing the great variety of types of Brazilian propolis, performed a classification into twelve types according to the botanical origin, chemical constituents, and color. In 2007, the 13th type of Brazilian propolis, the red propolis, was studied. It is considered the rarest type of propolis. Its chemical and pharmacological properties are unique, and its commercial value can reach five times the value of other types of propolis.[5,10,11] This propolis has botanical origins in

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Dalbergia ecastophyllum, a legume that grows abundantly in the mangrove area of the Northeast coast of Brazil.[12] Initial studies on this type of propolis were carried out in the state of Alagoas, where production was consolidated, acquiring a seal of geographical indication. However, other Brazilian states have a well-established production of red propolis such as Bahia. Some other states such as Rio Grande do Norte, Pernambuco, Sergipe, and Paraíba are starting the production.[11,13,14]

Some chemical constituents differentiate it from other types of propolis and are considered biomarkers, such as biochanin A, daidzein, quercetin, and naringenin.[8,13] These and other compounds have been associated with various biological activities such as antibacterial,[15-18] antitumor,[13,16,18,19] anti-inflammatory,[17] antioxidant,[8,13,15,16,19-22] among others. However, geographic location, climatic zones, and seasonality significantly influence the chemical constituents, as well as the various bioactive properties of propolis, thus, there is diversity and uniqueness in each propolis sample.[23,24] Propolis samples can have the same botanical origin, color, and yet be chemically different.[2,9] Since the commercial value of this product is estimated by its constituents and consequently its bioactive properties, it becomes necessary to identify similarities or differences between products of different geographical origins. do Nascimento *et al*.[23] have already found significant differences between Brazilian red propolis samples collected in different seasons using multivariate correlations. While Maldonado *et al*.,[25] using chemometrics, could differentiate Argentinian propolis samples from different origins. However, to the best of our knowledge, no published studies have analyzed the influence of geographical origin on the chemical composition of red propolis, its similarities, differences, and/or uniqueness.

Thus, this study aims to evaluate the influence of the origin on the phenolic composition and antioxidant capacity of red propolis produced in the Brazilian states of Rio Grande do Norte and Bahia. Conventional chemical methods and mid-infrared spectroscopy combined with chemometrics will be used.

Experimental

Samples preparation

Seventy-two samples of red propolis were analyzed. Forty-four samples were collected in the years 2018 and 2019 from an association of beekeepers in Bahia State, Brazil. Twenty-eight samples were collected in the year 2019 from a beekeeper in the city of Natal, Rio Grande do Norte, Brazil. The samples were produced in periods of different rainfall rates, ensuring heterogeneity of samples by different producers, years, and production periods. The samples were stored at −18 °C until the analyses were performed.

Extracts were produced, as proposed by Frozza *et al*.[19] Under the same conditions described by Lima *et al*.[22] 10 g of sample were mixed in a hydroalcoholic ethanol solution 70:30 (v v^{-1}) (Quimidrol, Joinvile, Brazil) to 100 mL of volume. The mixture was stirred on a shaking table (Quimis, São Paulo, Brazil) at 150 rpm at room temperature for 24 h. Then, the mixture was filtered through qualitative filter paper and the solvent was evaporated in a vacuum concentrator (Christ, Diadema, Brazil) until obtaining a powder. The dry extract was kept frozen at −18 °C and prepared in different concentrations with 96% ethanol (v v^{-1}) according to the analytical procedure to be adopted. Results are expressed on a raw propolis dry weight basis.

Total phenolics content

The total phenolics content (TPC) was determined according to Singleton *et al*.[26] and Singleton and Rossi[27] expressed as gallic acid equivalent (GAE) (mg GAE g^{-1} of propolis). The dry extract was resuspended in 1.2 mg of raw propolis mL^{-1}. For the reaction, 0.5 mL of the resuspended extract, 2.5 mL of 10% Folin-Ciocalteau (Êxodo, Sumaré, Brazil) aqueous solution, and 2.0 mL of 7.5% sodium carbonate (Dinâmica, São Paulo, Brazil) were used. The mixture was homogenized and kept at 40 °C for 15 min in a thermoregulated water bath. Then, the mixture was kept at 25 °C for 30 min in the dark. Absorbance was measured in a spectrophotometer (Shimadzu UV 1800, Duisburg, Germany) at 750 nm.

Content of total flavonoids

The total flavonoid content was quantified according to the method described by Sakanaka *et al*.[28] Under the conditions described by Lima *et al*.[22] The dry extract was resuspended at 3.0 mg mL^{-1}. For the analysis, 0.5 mL of the resuspended extract, 2.5 mL of distilled water, and 0.15 mL of 5% sodium nitrite (Êxodo, Sumaré, Brazil) were used. This mixture was homogenized and kept away from light at 25 °C for 6 min. Then, 0.3 mL of 10% aluminum chloride (Dinâmica, São Paulo, Brazil) methanolic solution was added and waited for 5 min. Then, 1 mL of 1 M sodium hydroxide (Dinâmica, São Paulo, Brazil) and 0.55 mL of distilled water were added. The tubes were shaken and read. The amount of TPC was expressed as catechin equivalent (CE) (mg CE g of propolis^{-1}), through a calibration curve of the catechin standard (Sigma-Aldrich, Saint Louis, USA).

High-performance liquid chromatography in reverse phase (HPLC)

The dry extracts were diluted in HPLC-grade methanol at a concentration of 6 mg of raw propolis mL^{-1} and filtered through a syringe filter with 0.22 µm membranes. The chromatographic experiments were carried out with the HPLC (Shimadzu DGU20A5R, Japan) system, equipped with a UV-Vis diode array detector (UV-DAD) and manual injection detector, composed of a C18, 5 µm reverse phase column, 250 mm × 4.6 mm in dimensions (Supelco Analytical, Sigma-Aldrich, Darmstadt, Germany), with an injection volume of 20 µm. The chromatographic separation was based on the method proposed by Park *et al*.[2] and modified by Lima *et al*.[22] The mobile phase used was water/acetic acid (ACS, Rio de Janeiro, Brazil) (19:1, v v^{-1}) (solvent A) and methanol (Êxodo, Sumaré, Brazil) (solvent B), with a constant flow of 1 mL min^{-1}. The gradient elution program was established as follows: 0 to 15 min, 30-40% B; 15 to 30 min, 40-50% B; 30 to 45 min, 50-60% B; 45 to 65 min, 60-75% B; 65 to 95 min, 75-90% B; 95 to 100 min, 90-100% B; 100-110 min, 100-30% B. The column was rebalanced and the total run time was 120 min. The substances were determined by comparing the spectra of the standard in the 200 to 400 nm ultraviolet region obtained by the diode array detector at the maximal absorbance to the individual standard (270 or 310 nm). The retention time and the purity of the peak were considered too. Standards (Sigma-Aldrich, Darmstadt, Germany) of gallic acid, chlorogenic acid, coumaric acid, ferulic acid, catechin, kaempferol, quercetin, naringenin, daidzein, formononetin, and biochanin A were used for identification, and quantification.

Antioxidant capacity by the DPPH method

The primary antioxidant capacity was assessed by the sequestration method of the 2,2-diphenyl-1-picrilhidrazilyl radical (DPPH•) (Sigma-Aldrich, Darmstadt, Germany).[29,30] Dilution and analysis conditions were the same as adopted by Lima *et al*.[22] Five dilutions of the extracts were prepared (0.6, 1.2, 1.8, 2.4, and 3.0 mg of propolis mL^{-1}) in triplicate. A 0.1 mL aliquot of each extract dilution was transferred to test tubes with 3.9 mL of the ethanolic solution (ethanol, 99.8% absolute alcohol) of the DPPH• radical, which had the initial absorbance adjusted to the range of 0.6 to 0.7 in a spectrophotometer at 515 nm. After 30 min of incubation in the dark and at room temperature, the reduction of the DPPH• free radical was measured by reading the absorbance at 515 nm. The result was expressed as EC_{50} (the concentration needed for the extract to sequester 50% of the radical).

Antioxidant capacity by the iron reduction method, reducing power

The reducing power was evaluated according to the procedure described by Oyaizu,[31] with adaptations described by Lima *et al*.[22] The extracts were resuspended at the same concentrations used for DPPH analysis. 1 mL of each extract concentration was used, added 2.5 mL of 0.2 M phosphate buffer solution (pH 6.6) and 2.5 mL of 1% (m v^{-1}) potassium ferricyanide. This mixture was centrifuged (Hettiche®, Universal 320R, Germany) at 1000 rpm for 8 min. 2.5 mL of the supernatant were removed and 2.5 mL of distilled water and 0.5 mL of 0.1% (m v^{-1}) ferric chloride (Synth, Diadema, Brazil) were added. The mixture was homogenized and then absorbance reading was taken at 700 nm. The increase in absorbance indicates greater antioxidant capacity due to the greater reducing power of the ferric ions. The concentration of the extract corresponding to 0.500 absorbances (EC_{50}) was calculated from the graphical representation of the absorbance recorded at 700 nm as a function of the corresponding concentration of the extract.

Antioxidant capacity by the co-oxidation method of the β-carotene: linoleic acid system

The co-oxidation method of the β-carotene:linoleic acid system was described by Miller.[32] Propolis at a concentration of 3 mg mL^{-1} was used. The reactive mixture was prepared with 50 µg linoleic acid (Sigma-Aldrich, Darmstadt, Germany), 200 mg Tween 40 (Dinâmica, São Paulo, Brazil), and 1 mL of β-carotene (Sigma-Aldrich, Darmstadt, Germany) solution at 1 mg mL^{-1} in chloroform. Subsequently, the mixture was subjected to the rotary evaporator (IKA, Staufen, Germany) at 50 °C for 10 min for complete evaporation of the chloroform. This mixture was diluted in 50 mL of water previously saturated with oxygen. This mixture had its absorbance adjusted between 0.6 and 0.7 at 470 nm. 5 mL of the emulsion were transferred to tubes with 0.5 mL of the samples. The tubes were homogenized and immediately the absorbance reading at 470 nm was taken. The tubes were incubated in a water bath at 50 ºC to promote the oxidative process. The reading was repeated every fifteen min until 120 min were completed. The results were expressed as a percentage of oxidation inhibition. The reduction of the absorbance of the system without a sample is considered 100% of the oxidation.

Medium infrared spectroscopy (MIR)

MIR spectroscopic analysis was performed on samples of raw propolis and dry extracts. The spectra were obtained using middle infrared spectroscopy with Fourier transform with total attenuated reflection (FTIR-ATR, Cary 630 FTIR, Agilent Technologies Inc., CA, USA), using the spectral range of 4000 to 600 cm^{-1}, with 4 cm^{-1} resolution, 64 scans, and reading through the diamond crystal. During collection, the temperature was kept constant at 25 °C. About 0.1 g of the samples were placed on the diamond surface for reading. Before each digitization, the white reading was performed, with reading without samples on the surface.

Statistical analysis

The data obtained were analyzed using the statistical program Statistical Analysis System (SAS)® OnDemand for Academics[33] for principal component analysis (PCA) and linear discriminant analysis (LDA); and Chemoface[34,35] software version 1.66 for partial least squares-discriminant analysis (PLS-DA).

Pre-processing of spectroscopic data

The samples were organized into five data sets. The first data set consisted of red propolis classification according to the home state. The second contains the composition of total phenolics, total flavonoids, and antioxidant capacity. The third contains the composition of individual phenolics quantified by HPLC. The fourth and fifth sets contain the absorbance data for the entire MIR spectrum of the raw samples and dry extracts (each set with 14000 variables), respectively. For statistical analysis, spectral data were used with and without mathematical methods of data pre-processing (none, standard normal variate (SNV), multiplicative scatter correction (MSC), first and second derivatives). Exploratory or classificatory models (PCA, LDA, and PLS-DA) for each set of variable individuals were built and the results were compared. For spectral data (full data), the data pre-processing method used to generate the model with the development parameters will be indicated. Only the parameters of the optimized models were presented.

For PCA and LDA the maximum absorbance bands identified in the spectra were used as variables.

Principal component analysis (PCA)

The PCA identifies the interrelationships among the samples. Through this analysis, it is possible to select the appropriate variables for the construction of the model and identify and eliminate outliers.[36] The number of principal components was chosen according to the parameters described by Pereira *et al*.[37] The five sets indicated in the data pre-processing item were used as variables. The variables that showed a low correlation with the principal components of higher variance and a similar correlation among the principal components were discarded.[38]

Linear discriminant analysis (LDA)

The LDA was used to discriminate and classify red propolis samples according to their home state. To carry out the LDA, the data sets indicated in the data pre-processing item were used as variables. The functions were obtained by optimizing the classification parameters described by Chen *et al*.,[39] Santos *et al*.[40] and Lopes *et al*.[41] The Kennard-Stone algorithm was applied to design the sample sets.[42] This algorithm takes a representative portion of the total data set. The samples were divided into a calibration set with 70% of the samples and a prediction set with the remaining 30% of the samples, respectively.

The performance of the models was assessed by the ability to classify red propolis from the states of Rio Grande do Norte and Bahia sensitivity (equation 1) and specificity (equation 2).[41]

$$\text{Sensitivity (\%)} = (TP/(TP + FN)) \times 100 \quad (1)$$

$$\text{Specificity (\%)} = (TN/(TN + FP)) \times 100 \quad (2)$$

where TP: true positive (correctly identified samples); FN: false negative (incorrectly rejected samples); TN: true negative; and FP: false positive.

Partial least squares-discriminant analysis (PLS-DA)

For PLS-DA chemometric analysis the data were divided into two groups as described for LDA. The adjustment parameters followed those proposed by Lopes *et al*.[41] and Pereira *et al*.[37] The classification model was optimized by cross-validation using a set composed of 70% of the samples of each class. The other samples (30%) were used as an external validation set. The number of latent variables in the models was selected according to the method proposed on the software, and the T^2 test was used to determine whether two PRESS values were significantly different. The performance of the models was demonstrated using the same figures of merit described for LDA.

Results and Discussion

Reference analysis results

In this study, 72 samples of red propolis were collected in the states of Rio Grande do Norte (28 samples) and Bahia (44 samples). The maximum and minimum values, mean, and standard deviation of the variables studied for the red propolis samples by the state of production are presented in Table 1.

High variability was observed (Table 1), demonstrating heterogeneity among the samples. However, it is still possible to indicate some compositional patterns according to the state of origin of the red propolis. Individual sample data for the determined parameters are presented in the Supplementary Information (SI) section (Table S1). A figure with a chromatogram of a sample from each state is also presented in the SI section (Figure S1). Samples produced in the state of Rio Grande do Norte (RN) showed higher values for total phenolic constituents and lower values for total flavonoid constituents compared to samples produced in the state of Bahia (BA). For the quantified individual flavonoids (catechin, kaempferol, quercetin, naringenin, daidzein, formononetin, and biochanin A) higher concentrations were found in the samples from RN than in those from BA. The opposite behavior was observed for the individual phenolics (gallic acid, chlorogenic acid, coumaric acid, and ferulic acid), where red propolis samples from BA had higher concentrations. This indicates a direct influence of the production region on the chemical composition of the samples.

Some constituents are considered biomarkers because they are identified exclusively in red-type propolis and confirmed in *Dalbergia ecastophyllum*. Among these constituents are formononetin, daidzein, biochanin A, quercetin, and naringenin.[43-46] Red propolis produced in the RN state shows higher concentrations of the main biomarkers. These constituents are associated with several bioactive properties.[8,13,18,20,21] Sarfraz *et al*.[47] presented several bioactive properties, of biochanin A with emphasis on anticancer, antioxidant, anti-inflammatory, antimicrobial, neuroprotective, as well as antidiabetic and hepatoprotective activity. According to Erlund,[48] quercetin exhibits antioxidant, anticarcinogenic properties, anti-inflammatory, anti-aggregating, and vasodilatory effects. Naringenin has antioxidant, and anti-inflammatory activity and acts in different types of effects on sex hormone metabolism.[48] For Şöhretoğlu and Renda[49] and Sun *et al*.,[50] it has already been well elucidated that daidzein mimics the actions of estrogen improving the cardiovascular system, acts as an anticancer, anti-osteoporosis, antidiabetic, antioxidant, anti-inflammatory, and neuroprotective agent. Formononetin, on the other hand, has immuno-enhancing, antiangiogenesis, antitumor, and hepatoprotective

Table 1. Range of values obtained by the reference methods for red propolis samples from Rio Grande do Norte and Bahia, Brazil

	Rio Grande do Norte (28 samples)			Bahia (44 samples)		
	Range	Mean	SD	Range	Mean	SD
Total phenolics / (mg GAE g^{-1} propolis)	5278.35-16467.89	10414.50	3626.91	18.60-115.62	67.68	19.33
Total flavonoids / (mg CE g^{-1} propolis)	17.38-79.08	38.19	15.73	6.53-152.39	73.41	40.39
Gallic acid / (mg 100 g^{-1})	nd-5.30	1.99	1.85	nd-46.47	8.65	8.17
Chlorogenic acid / (mg 100 g^{-1})	nd-1.30	0.09	0.32	nd-41.60	6.15	8.59
Coumaric acid / (mg 100 g^{-1})	nd-5.59	0.92	1.60	nd-36.93	1.29	5.71
Ferulic acid / (mg 100 g^{-1})	nd-29.56	4.19	7.29	nd-48.62	4.66	9.16
Catechin / (mg 100 g^{-1})	nd-134.00	27.57	47.31	nd-53.58	9.29	14.83
Kaempferol / (mg 100 g^{-1})	3.43-957.92	197.76	199.50	nd-383.67	96.02	89.14
Quercetin / (mg 100 g^{-1})	nd-158.53	31.72	40.77	nd-251.68	26.09	40.25
Naringenin / (mg 100 g^{-1})	nd-702.52	179.38	174.43	nd-442.75	141.05	119.64
Daidzein / (mg 100 g^{-1})	21.24-628.48	267.28	182.80	nd-337.86	76.31	75.58
Formononetin / (mg 100 g^{-1})	1197.52-11544.31	4758.35	2677.23	2.03-11173.64	3737.42	3041.90
Biochanin A / (mg 100 g^{-1})	17.94-2060.40	555.28	559.97	nd-372.09	55.52	67.04
DPPH / (EC_{50} mg propolis mL^{-1})	1.67-4.66	2.83	0.84	0.96-3.59	2.16	0.66
Reducing power / (EC_{50} mg propolis mL^{-1})	0.65-2.95	1.69	0.56	0.32-1.32	0.82	0.23
β-Carotene/linoleic acid co-oxidation (protection) / %	14.28-92.44	59.44	18.37	64.93-97.81	82.57	8.78

The individual phenolic constituents quantified by HPLC are expressed in mg 100 g^{-1} of the sample. SD: standard deviation; nd: not detected; GAE: gallic acid equivalent; CE: catechin equivalent; DPPH: 2,2-diphenyl-1-picrilhidrazilyl radical; EC_{50}: concentration needed for the extract to sequester 50% of the radical.

actions.[51] These surveys emphasize the importance of these constituents in the composition of red propolis samples. In addition, they reinforce the relevance that this apicultural product has in the market and justify its differentiated market value compared to other types of propolis.

Despite having lower concentrations of the biomarkers, the samples from Bahia showed higher antioxidant capacity. This may suggest that: (*i*) some constituents present in red propolis from Bahia may act more efficiently in antioxidant capacity; (*ii*) the constituents from Bahia samples may act synergistically; (*iii*) very high concentrations of some constituents may present pro-oxidant effects. Despite the difference in composition and antioxidant capacity varying according to the origin of the samples, all the propolis analyzed had high concentrations of phenolic constituents and high antioxidant capacity.

MIR spectra interpretation

Mid-infrared spectroscopy was performed on the raw red propolis samples and the dried extracts. In Figure 1 we have the spectral average of the samples from each state, in the region of the spectrum between 4000-600 cm^{-1}.

As described by Lima *et al.*,[22] the main difference observed between the spectra of the raw red propolis samples (Figure 1a) and the dried extract (Figure 1b) is between the bands of 2916, 2849, 1736, and 1467 cm^{-1}, with absorbances of higher intensity in the crude red propolis samples. These same bands were identified in the present study and correspond respectively to the stretching of vibrations of C–H groups of ester carbonyls, and hydrocarbon vibrations.[52] These functional groups are associated with the presence of wax in the samples.[22,53] The region between 1800-600 cm^{-1} showed absorbances of higher intensity in the dry extract compared to the raw propolis samples.

The average spectral behavior of the raw samples and the dried extracts of red propolis were similar for the two states of origin analyzed, varying in absorbance intensities. Some bands showed higher absorbances according to the origin. For the raw red propolis and its dried extracts from BA, higher absorbance intensities were observed in the bands of 1720, 1378, 1289, and 887 cm^{-1} about the samples from RN. These bands correspond to vibrations of C=O and C–O groups, present in aldehydes, ketones, carboxylic acids, esters, and alkanes,[52,54] in addition to vibrations of =C–H and C–H bonds of aromatic compounds. For crude red propolis and its dried RN extracts, higher absorbance intensities were observed at 1620, 1155, 1107, 1081, 1027, 946, 830, 790, 738, and 718 cm^{-1}. The band at 1620 cm^{-1} corresponds to C=C vibrations of alkenes. The region between 1300 and 1000 cm^{-1}, corresponds to vibrations of C–O groups of aldehydes, ketones, carboxylic acids, esters, and alkanes.[52,54] Aromatic rings vibrate strongly in the region of 900 to 600 cm^{-1}, with the bands varying according to the amount and conformation of the benzene ring substitutions.[52] The number of bands in the aromatic ring region suggests the presence of several phenolic compounds with different numbers and locations of substitutions. The concentration of these compounds tends to be higher in the RN propolis samples, which contain higher absorbance intensities. This statement corroborates with the data observed for conventional bench and HPLC analysis, which indicated higher concentrations of total phenolic constituents and individual flavonoids in the RN propolis samples. Furthermore, based on differences in absorbance intensities in specific regions, the MIR infrared spectroscopy data indicate differences in the chemical composition of the red propolis samples according to the state of origin.

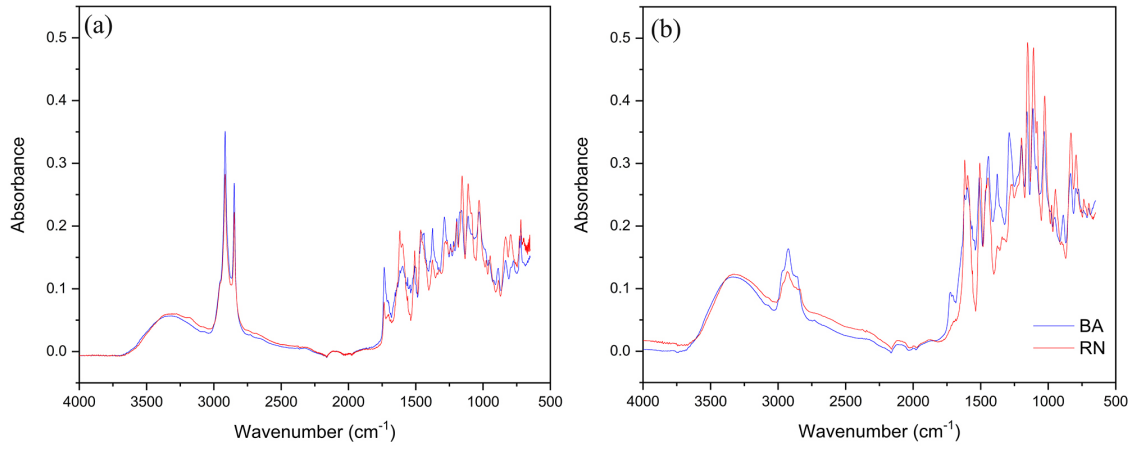

Figure 1. Average mid-infrared spectroscopy with ATR spectra of red propolis samples from the states of Rio Grande do Norte (RN) and Bahia (BA). (a) Spectral averages of the crude samples; (b) spectral averages of dry extracts, obtained in the range between 4000-600 cm^{-1}.

Principal components analysis (PCA)

PCA was performed using four datasets (Figure 2). The first is composed of the results of conventional bench-top analysis of phenolic composition and antioxidant capacity (Figure 2a). The second data set was found by HPLC for individual phenolics (Figure 2b). The third and fourth data sets are composed of the main absorbance bands observed in MIR spectroscopy for the raw red propolis samples (Figure 2c) and their respective dried extracts (Figure 2d).

According to the four sets of data analyzed, it was possible to observe separation among the red propolis samples by the state of origin. For the data of individual phenolics by HPLC, there was the formation of three groups, with the division of the red propolis samples from RN into two groups. These data suggest that the quantified individual phenolics not only indicate differences among the samples according to their origin but may also suggest differences in the composition of samples from the same region. This indicates that there can be expressive variations in constituents, even within the same region of origin. The MIR spectroscopy techniques indicated the separation of red propolis by the state of origin. The same behavior was observed for PCA with the measurements of total phenolics, total flavonoids, and antioxidant capacity.

The PCA thus reinforces the assumption of difference among red propolis samples according to the state of origin and shows the importance of geographic and climatic factors for the composition of these samples.

Linear discriminant analysis (LDA)

An undirected binary model for the classification of red propolis samples from different origins was developed and validated. To perform LDA, the same datasets as described for PCA were used. The samples were separated into two classes, each class being the state of origin of the red propolis. Each class of samples was separated into training sets, used in acquiring the classificatory model, and external validation was used to evaluate the generalization ability of the model. The tuning parameters of the generated models are presented in Table 2.

According to the results presented in Table 2, it is possible to indicate LDA as a highly efficient technique for

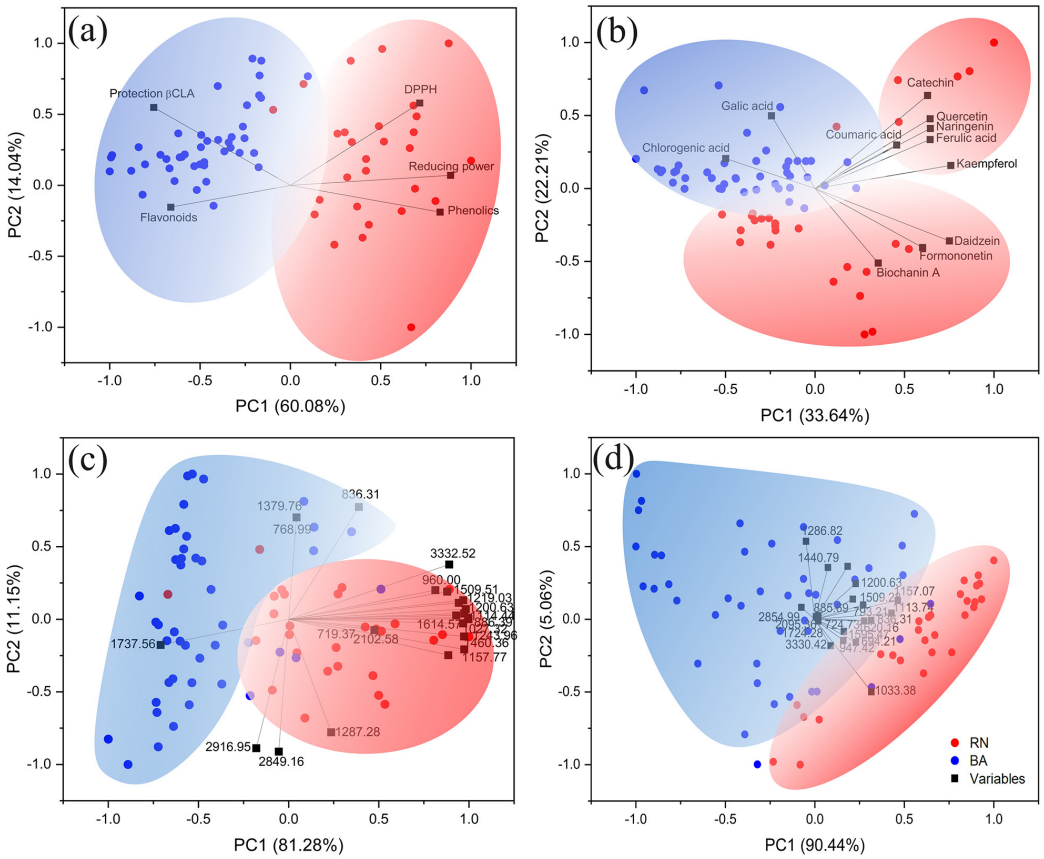

Figure 2. Scores and loads graphs of the unsupervised classification of red propolis produced in the states of Rio Grande do Norte and Bahia in the principal component analysis model. Using data: (a) conventional analysis of composition and antioxidant capacity; (b) individual phenolics by HPLC; (c) MIR spectroscopy of crude samples and; (d) MIR spectroscopy of dry extracts.

Table 2. Classification capacity, sensitivity, and selectivity of binary models to indicate the origin of red propolis using the linear discriminant analysis algorithm

Date used	Calibration			Validation		
	Accuracy / %	Sensitivity / %	Specificity / %	Accuracy / %	Sensitivity / %	Specificity / %
Bench Analysis	100	100	100	100	100	100
HPLC	100	100	100	100	100	100
MIR crude sample	100	100	100	100	100	100
MIR dry extract	100	100	100	96.16	92.31	100

HPLC: high-performance liquid chromatography; MIR: medium infrared spectroscopy.

classifying red propolis samples according to their origin. All data sets generated models with high parameters of correct classification, almost all with 100% correctness. Models calibrated with data from the conventional bench-top, HPLC, and MIR analysis of the samples showed 100% sensitivity and specificity for both training and validation. The analysis with MIR spectroscopy data of the crude samples can be suggested for use in the discrimination of red propolis samples of different origins, as it does not require sample preparation, reagents, and long analysis times.

Partial least squares discriminant analysis (PLS-DA)

For PLS-DA the same dataset as for PCA was used. However, since PLS-DA is generally a non-linear, full-spectrum technique, for the MIR data set, all 14000 absorbance measurements of the raw red propolis samples and the dry extract were used as a variable. The PLS-DA model was evaluated by internal validation analysis followed by external validation analysis where a set of new samples was input and the practical applicability of the model was tested. The model tuning parameters are shown in Table 3.

The models calibrated using the PLS-DA technique showed an excellent rate of correct classification for the red propolis samples according to their origin. Although all data sets showed a high classification rate, the models calibrated with chromatographic phenolic composition data and MIR data of crude propolis highlighted a 100% hit rate in the external validation. These data corroborate what was observed for LDA.

The plots with the regression coefficients as a function of variables for the models calibrated using PLS-DA are presented in Figure 3.

The models obtained with composition and antioxidant capacity data (Figure 3a) and phenolic composition by HPLC (Figure 3b) have categorical variables (independent from each other), while the models obtained with spectral data (Figures 3c and 3d) consist of continuous variables. Because of this, the plot of regression coefficients of the model in Figures 3a and 3b are presented as bars, while the plots in Figures 3c and 3d are presented as continuous lines. In Figures 3a and 3b, a balance between the correlation coefficients of the models is observed, indicating that all variables are important in classifying red propolis samples as to geographical origin. However, it is possible to highlight the relevance of gallic acid in the model using HPLC data. The opposite behavior is observed for coumaric acid, which presented a regression coefficient close to zero, suggesting little relevance in the classification of the samples. For the models calibrated with MIR spectroscopy data, it is observed that the region of the highest significance for the regression coefficients of both models is between 1800 and 600 cm^{-1}. This region is commonly called the "fingerprint" of the sample and is associated with vibrations of functional groups related to bioactive compounds of interest in red propolis such as phenolics and flavonoids.[52]

Table 3. Classification capacity, sensitivity, and selectivity of binary models to indicate the origin of red propolis using the partial least squares discriminant analysis algorithm

Date used	LV	Calibration			Validation		
		Accuracy / %	Sensitivity / %	Specificity / %	Accuracy / %	Sensitivity / %	Specificity / %
Bench analysis	1	100	100	100	90.48	87.50	92.31
HPLC	4	100	100	100	100	100	100
MIR crude sample	3	100	100	100	100	100	100
MIR dry extract	2	100	100	100	95	100	92.31

HPLC: high-performance liquid chromatography; MIR: medium infrared spectroscopy; LV: latent variables.

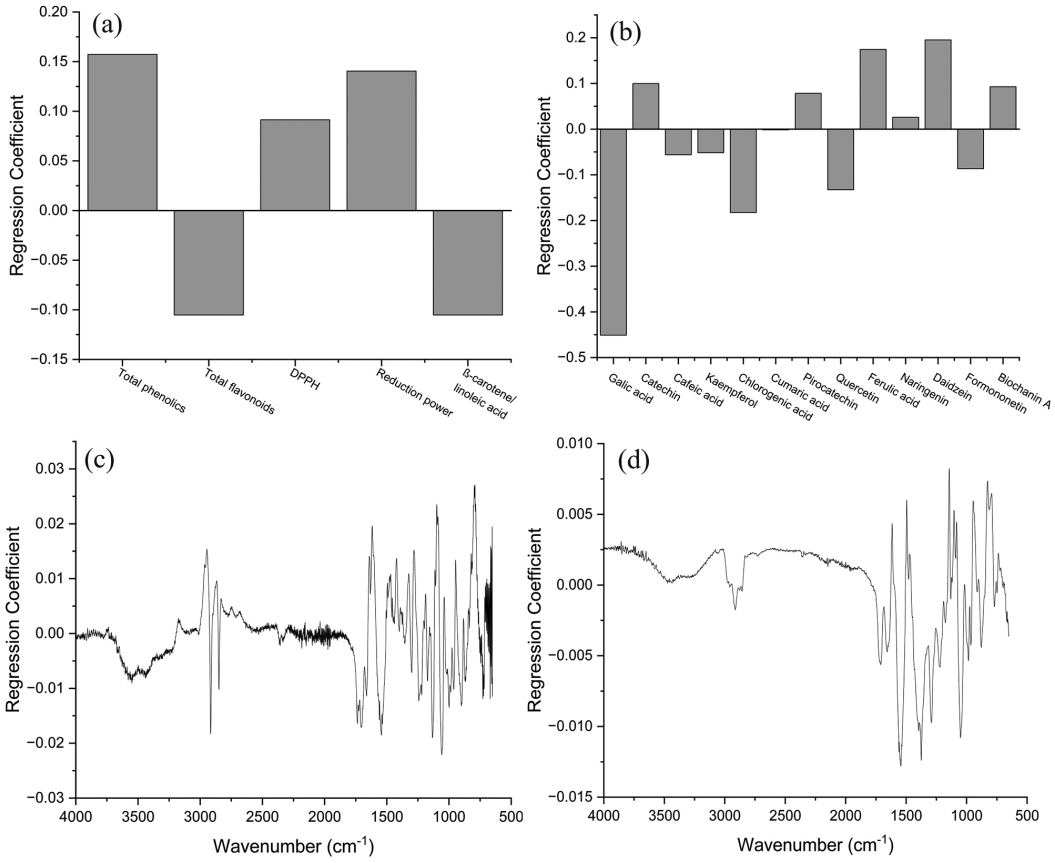

Figure 3. Regression coefficients as a function of the variables of the PLS-DA models to classify red propolis samples from Bahia and Rio Grande do Norte States. (a) Conventional analysis of composition and antioxidant capacity; (b) individual phenolics by HPLC; (c) MIR spectroscopy of crude samples and; (d) MIR spectroscopy of dry extracts.

The models calibrated with MIR data from the dry extracts for LDA and PLS-DA showed a greater error than the models calibrated with MIR data from the raw samples. This is probably due to the extraction process. In general, when samples are subjected to the extraction process, the heterogeneity between them decreases, which can make differentiation more difficult.

The results of this study indicate that despite the presence of specific biomarkers, red propolis samples produced in different Brazilian states have a unique chemical composition in the quality and quantity of constituents. These variations in the composition may imply different bioactive properties for these samples and point to the need for further studies on these properties according to the origin of the propolis. The results also indicate that although red propolis is classified as a single class of Brazilian propolis, it has different compositions and characteristics according to its geographical origin. This points to the possibility of new classifications among these samples regarding their origin, as has been done previously for other types of propolis.

Conclusions

An efficient classification among red propolis samples from different states of origin was achieved through the association of different instrumental measurements and multivariate analysis (PCA, LDA, and PLS-DA). Among the instrumental techniques used in the characterization of red propolis, the models calibrated with MIR spectroscopy data of raw propolis deserve to be highlighted. These models showed a high rate of correct classification and do not require any prior sample preparation, extraction, or solvent use. Regarding statistical analyses, the use of PLS-DA is suggested as it does not require peak selection or any pre-treatment of spectral data, which facilitates practical application.

Based on the observed data, it is possible to point out differences in composition and bioactivity among Brazilian red propolis samples according to their origin. As differences in composition were identified in qualitative and quantitative terms, variations in biological/pharmacological properties can also be identified according to the state of production of red propolis. These findings may enable

studies of new biological active properties that have not yet been elucidated and may also impact the market value of this product. Regions of red propolis production that previously did not have market visibility can be identified as important producers and this can generate income for small producers, boost sustainable extractivism, and change the social reality of these communities.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Amanda Beatriz S. de Lima was responsible for conceptualization, formal analysis, investigation, validation, writing original draft; Thinara F. Oliveira for data curation, investigation; Marcondes V. da Silva for methodology, resources; Sibelli P. B. Ferrão for resources, writing-review and editing; Vítor V. S. de Almeida for conceptualization, resources; Leandro S. Santos for conceptualization, funding acquisition, project administration, resources, supervision, writing review and editing.

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