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RESEARCH ARTICLE

Physicochemical Characteristics of Brazilian Green Propolis evaluated During a Six-Year Period

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Abstract: ***Background:*** Propolis has been used as a natural health product mainly due to the presence of polyphenols, flavonoids, phenolic aldehydes, amino acids, vitamins and others bioactive constituents. To this natural substance are attributed different biological and pharmacological properties which are influenced by its chemical composition and organoleptic properties. The aim of this work was to evaluate the physicochemical properties and parameters of green propolis collected during a period of six years (2008-2013) in the state of Minas Gerais, located at the southeastern region of Brazil.

Methods: The methodology were in accordance with Brazilian legislation on the identity and quality standards of propolis. The evaluated parameters of hydroalcoholic from green propolis were total flavonoids, antioxidant activity - DPPH method, oxidation index, wax content, humidity and insoluble impurities.

Results: Propolis samples collected in different seasons during the years 2008 to 2013 presented mean values of total flavonoids (3.4 ± 0.11 mg/g), antioxidant activity DPPH (4.76 ± 0.16 μg/mL), oxidation index (3, 4 ± 0.33 seconds) and wax (15.14 ± 0.78% m/m), which are in accordance with Brazilian legislation.

Conclusion: Green propolis did not show abrupt seasonal changes during the six years of investigation, and may be considered as an adequate functional ingredient.

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1. INTRODUCTION

Propolis is a resinous substance collected by bees (*Apis mellifera*) from a variety of trees and other vegetable sources. *Baccharis dracunculifolia* represents the main plant source of propolis used by bees in the state of Minas Gerais, in the southeastern region of Brazil [1, 2, 3].

Propolis is a bee product composed primarily of resins, waxes, essential oils, flavonoids and other substances [1-7]. Its composition varies according to the botanical origin, the species of bee that collects it, the season and geographical area of collection pollen and resins [3, 4, 8, 9]. Therefore, it is important to know the profile of physicochemical parameters of propolis, in order to guarantee its biological properties and consequently, quality of the final product.

Propolis is used in beehives for coating parts, sealing cracks and crevices, and protecting against predators [1, 2]. For decades it has been used in traditional medicine due to its biological properties, such as antibacterial, antiviral, antioxidant, antiinflammatory, immunomodulatory, antitumor, antifungal, cytotoxicity, and other pharmacological activities [1-6, 10]. The chemical composition and physicochemical characteristics of propolis depend strongly on the plant source and geographic origin, in addition to climatic conditions, quality of soil, species of bees, among other factors [3-5]. Approximately, 600 substances have been found in propolis, including amino acids, lipids (triglycerides, phospholipids), phenolic compounds (flavonoids), sesquiterpenes and other terpenes, aromatic acids, vitamins (B1, B2, B6, C and E) and minerals (manganese, iron, calcium, aluminum and others) [2, 3-12].

Brazilian propolis is currently classified into thirteen groups, based on its physicochemical characteristics [3-6]. This categorization is explained through the plant biodiversity found in different regions of Brazil [2, 4, 6]. Consequently, the physicochemical properties and chemical composition of propolis could vary, allowing for these different origins [2-10, 15].

The physicochemical pattern of identity and quality of propolis *in natura* and its extracts involves the knowledge about total flavonoids, antioxidant activity, oxidation index, waxes content, humidity level and insoluble impurities. It is important that these parameters be measured because they can influence the biological and pharmacological properties of propolis. Others parameters may be added according to the product being examined and the methodology established in the analytical quality control [13-16].

Considering that propolis presents variations in its composition according to the place of collection and climatic variation, the motivation of the study was to evaluate the seasonal variation of the Hydroalcoholic Extract of Propolis (HEP) produced in Minas Gerais, Brazil, from samples collected in the months of February, March, July, September and October between the years 2008-2013.

2. MATERIALS AND METHODS

2.1. Samples

For a period of six years (2008-2013) Green Propolis samples were collected during the months of February (Summer), March (Summer and Autumn), July (Winter), September (Spring) and October (Spring). Samples of Green Propolis were acquired by Nectar Farmacêutica, which by means of traceability supervises the quality of apiculture facilities located in 15 micro-regions of the state of Minas Gerais. In these regions, there are typical conditions for the growth of *B. dracunculifolia*, by which bees produce Green Propolis [5]. Up to 24 hours, after collection of the samples (≈10.0 g) they were stored in poly-nylon vacuum bags, at a temperature of ≤ -18°C [5].

2.2. Hydroalcoholic Extract of Propolis (HEP)

HEP was prepared based on the procedure described by Park *et al*. [3, 5], with amendments proposed by Melo *et al*. [13]. The sample of propolis was crushed previously and dehydrated in an oven at 60°C. The mixture of 0.3 g of Propolis and 15 mL of 80% ethanol (v/v) was stirred for 10 minutes. Then, the mixture was centrifuged at 3,000 rpm for 3 minutes (Hermle Z 320 centrifuge). The supernatant was filtered on filter paper (Advantec 5A with 0.06 mg ashes/90 mm) and transferred to a 50 mL volumetric flask. The residue was extracted similarly, but using 10 mL of 80% ethanol (v/v). This procedure was repeated three times. All supernatants were transferred to a volumetric flask and the volume completed to 50.0 mL with 80% ethanol (v/v).

2.3. Qualitative Determination of Phenolic Compounds of Hydroalchoholic Extract of Propolis (HEP)

The method used was according to Park *et al*. [17] where the maximum absorption spectrum of the HEP was determined to be in the range of 200 to 800 nm (Beckman Coulter DUTM 640 spectrophotometer). An aliquot of 6.0 μL was carefully diluted in 15.0 mL of ethanol (80% v/v) and maximum absorption spectrum was determined.

2.4. Total Flavonoid

The content of total flavonoid in HEP was determined according to Melo *et al*. [13]. The total flavonoids content was calculated from the calibration curve of quercetin standard (0.01 – 0.2 mg/mL) and expressed as milligrams of quercetin per g of propolis (mg/g). The absorbance of the samples was measured in a spectrophotometer (Shimadzu model UV-1700) at a wavelength of 425 nm in 1 cm optical glass cuvettes.

The total flavonoid content was calculated according to the following formula: **TF (%) = $C_{(mg/mL)}$ x 100/d**, where TF is the total flavonoid contents expressed as quercetin (%, w/w) of dry sample; D = extract density (g/mL) [13**].**

2.5. Determination of Antioxidant Activity

DPPH free radical scavenging activity was measured according to the procedure described by Banskota *et al*. [18] and Hatano *et al*. [8]. In general, HEP was diluted in ethanol or in water (50 mL) and mixed with an equal volume of DPPH solution (60 μmolar). The resulting solution was thoroughly mixed using a Vortex mixer, and the absorbance and absorbance measured after 30 minutes at 520 nm. The scavenging activity was determined through correlated absorbance of sample with DPPH radical and absorbance of blank (100%), so containing only DPPH. The results were expressed in μg per mL DPPH solution [8, 18, 19].

2.6. Oxidation Index

A sample of 0.2 g of green propolis was dissolved in 5 mL of ethanol and allowed to stand for 1 hour at room temperature. Then, distilled water (100 mL) was added and the solution filtered in filter paper n° 3. An aliquot of 1.0 mL of the filtrate was diluted with distilled water (40 mL) and 1 mL of 20% sulfuric acid (v/v) was added. The mixture was stirred for 1 min and then 5 μL of 0.1 M potassium permanganate was added. The time, measured in seconds, was equivalent to the period of the disappearance of pink color and is proportional to the oxidation index [13, 14, 16].

2.7. Wax Content

The wax content was determined from 1.0 g of sample, previously crushed, that was weighed into cellulose cartridge and subjected to exhaustive extraction in a Soxhlet apparatus with 110 mL absolute ethanol, for 6 hours. The extract obtained remained for 24h at 5°C. The extract was then subjected to cold filtration on filter paper n° 3, previously weighed. The filter paper with the sample was dried at 105°C and then placed in desiccators until constant in weight. After filtration, the paper was reweighed. For the calculation, the following formula was used: **Wax (%) = (P3 - P2)/P1**, being P1 = initial weight of sample (g), P2 = weight of filter paper (g), P3 = weight of filter paper + wax (g) [7, 13, 15, 16].

2.8. Humidity

The degree of humidity was defined with samples heated at 105°C for 3 h in a drying oven. Then, the samples were cooled to room temperature in desiccators, and weighed. The process of heating, cooling and weighing of the set was repeated with intervals of 2 h, until constant mass and the difference between two consecutive measurements did not present variation above 0.25% [13, 14]. This process was realized in triplicate and expressed as average.

2.9. Mechanical Mass

To determine the mechanical mass or insoluble particles of Green Propolis, 1.0g of crude sample was treated with 15 mL of chloroform-acetone (2:1 v/v). After stirring for 1 hour, at room temperature, the mixture was filtered in a dried filter paper, previously tared in an analytical balance Tecnal Model 210A. The filter paper containing the insoluble particles was transferred to a watch glass and dried in a Deleo Type 2 stove, at 60ºC initial temperature for 10 minutes, increasing to 80°C for 1 hour. Then, this filter was transferred to a desiccator

and weighed. This procedure was repeated until the sample reached constant weight. The experiment was realized in duplicate and the result expressed as a median [16]. The following formula was used: % mechanical mass = **100 x (A1 – A2) / p**, being **A1** the mass of (filter paper + insoluble particles), **A2** the mass of filter paper, and **p** the mass of Green Propolis sample [16].

2.10. Statistical Analysis

The experiments were conducted in triplicate and the results expressed as mean ± standard deviation of means, were calculated. The results were compared with the limits established by Brazilian legislation [14].

After checking for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test), intergroup variations among the parameters were estimated by means of analysis of variance (ANOVA). The results were complemented with Newman–Keuls multiple range tests to establish possible differences. Qualitative treatments were evaluated by means of the Newman–Keuls test with a confidence interval of 95%, and a 5% significance level ($p \leq 0.05$) for comparison of the samples. A confidence interval of 99% and significance level of 1% ($p \leq 0.01$) were used for comparison among months and groups of samples. The software GraphPad PRISM 5.0 was used for statistical analysis and graphical representation of the data.

3. RESULTS

The qualitative determination of phenolic compounds was conducted for preliminary classification the propolis in accordance with Alencar [20]. The hydroalchoholic extract of propolis (HEP) showed maximum absorption spectrum at 290 nm, similar to Green Propolis.

The physicochemical parameters of Green Propolis collected at different seasons over six years (2008-2013) were shown in (Fig. **1a** to **f**). The samples collected in February, March, May, July, September and October are representative of different seasons of the year. In addition to the results obtained it is possible to visualize the standards established by the Brazilian legislation [14] for propolis quality and identity standard.

General averages were calculated considering the mean and standard deviation of means representative monthly for a period of 6 years. The results were total flavonoids (3.4 ± 0.11 mg/g), antioxidant activity (4.76 ± 0.16 μg/mL), oxidation index (3.4 ± 0.33 seconds) and wax content (15.14 ± 0.78% m/m) no statistically significant variation ($p \geq 0.05$) among the different months during the the six-years of studies was demonstrated. It was observed that parameters humidity (8.09 ± 0.14 %) and insoluble impurities (48.97 ± 1.05 %) showed significant variation ($p \leq 0.05$) among the different months investigated in the six-year period of study.

4. DISCUSSION

The preliminary analysis for the classification of propolis using the maximum absorption spectrum is one of the most widely used physicochemical parameters to evaluate propolis, and is an indicator for type of propolis. The results obtained should be complemented with chromatographic analysis, which was presented according to the study of Figueiredo *et al*. [5].

The Fig. **1a** shows the content of total flavonoids in Green Propolis samples collected over six years. The average found for total flavonoids (3.4 ± 0.11) ($p \geq 0.05$) was above those established by Brazilian legislation [14], and higher than those observed by Loureiro & Galbiati [16] who studied the correlation of the influence of seasonality with the quality of propolis during one year. It was observed that there were no significant changes in the total content of flavonoids and this result suggests that green propolis has a good potential to be used in food products as an ingredient. Castro *et al*. [21] verified the influence of seasonality on the phenolic composition and antibacterial activity of propolis in the southeastern and northeastern regions of Brazil. It was observed that the highest content of total flavonoids was in September.

The results of the means of antioxidant activity of the samples of propolis determined by the DPPH method are presented in Fig. **1b**. The samples of Green Propolis did not present great variations of antioxidant activity according to the months of collection and analysis. The general average was 4.7 μg/mL ($p \geq 0.05$), and these data suggest that independent of season, the variation was minimum along the entire year. Some effects of antioxidant activity of green propolis were the result of synergistic action of all constituents of

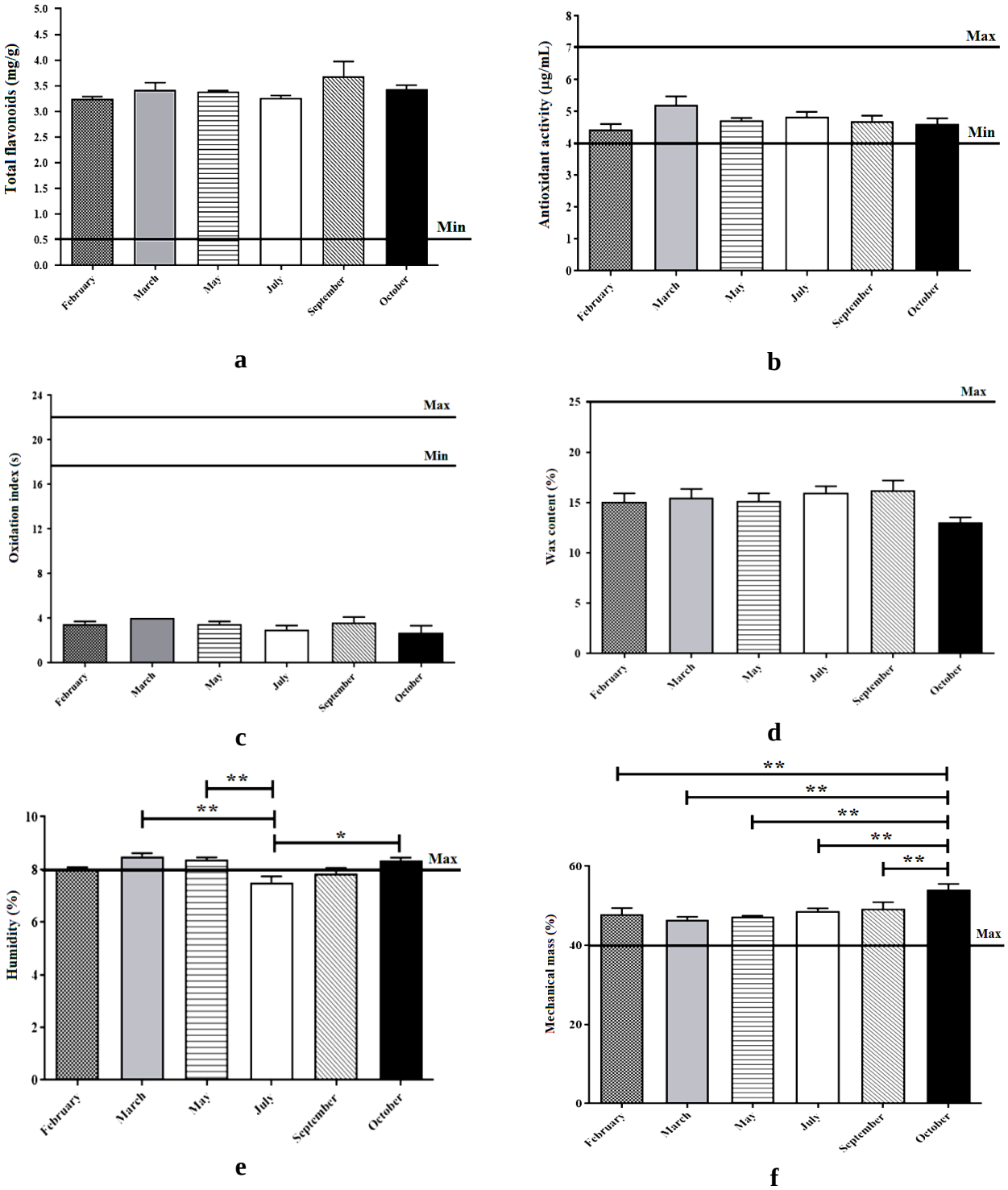

Fig. (1). Physicochemical parameters of Brazilian green propolis presented as average per month during six years of analysis (2008-2013). a = total flavonoids (mg/g), b = antioxidant activity (µg/mL), c = oxidation index (seconds), d = wax content (%), e = humidity (%) and f = mechanical mass (%). ****** = $p \leq 0,05$).

composition [2, 22]. The general averages of antioxidant activity of the samples of propolis collected in the months of March and July were those that presented higher levels, and this effect may be related to the Artepillin C compound, which also presented in higher levels in these months and has antioxidant activity as presented by Figueiredo *et al*. [5].

Teixeira *et al*. [23] studied seasonal variation, the chemical composition and antioxidant activity of Brazilian propolis from the state of Minas Gerais.

Samples were collected monthly for a period of 1 year from three different apiaries. They found that the highest antioxidant activity was observed in samples of propolis collected in the month of March (66.8 ± 2.7 %), being in agreement with results obtained in the present study.

According to studies carried out by Santos [2] and Lustosa *et al*. [24] there is a correlation between the high content of total flavonoids and antioxidant activity in HEP [5, 25].

The oxidation index was evaluated the accordance with the time that propolis retains its oxidation properties. The data of oxidation index in the general average, independent of month, presented in this work was 3.4 seconds and is substantially less than the 22 seconds by Brazilian legislation [14] (Fig. **1c**) and were adequate. Melo *et al*. [13] identified the quality of the propolis from four regions of Brazil. The propolis collected from the southeast region showed an average of 7 seconds for the oxidation index and this result was double as compared to that (3.4 seconds) found in the present study. The oxidant activity here found for green propolis was below that of other studies with other types of propolis [8, 13, 26] and this property demonstrates the good quality of Green Propolis.

Fig. **1d** shows that the average content of wax in the propolis samples collected in the different months was 15%. The differences were not statistically significant ($p \geq 0.05$) among the propolis samples and the values are in accordance with the Brazilian legislation that determines a maximum content of 25% [14].

Bastos [27] studied samples of propolis from Minas Gerais and observed wax contents between 20.86% and 35.53% and these values were higher than those found in the present work. Melo *et al*. [13] verified that propolis samples from the southwestern region of Brazil had average contents of 7.8% of wax. These results are in accordance with Brazilian legislation [14] and were lower than those obtained in the present study. An explanation for the great variation observed in wax content is that the bees can collect more waxes during periods, such as winter when the resins are scarce and collection is difficult so as to the seal the hive [13].

The samples of Green Propolis collected in different seasons over six years (2008-2013) presented general average humidity of 8.3% (Fig. **1e**) and this value is close to that established by Brazilian legislation, which is 8%. This parameter presented a statistically significant difference ($p \leq 0.05$) between the months of collection over the years. The samples of propolis that were collected in the months of March, May and October presented values of humidity above the value recommended by the Brazilian legislation.

Melo *et al*. [13] verified that the propolis samples of the southeastern region of Brazil had average humidity of 6.3%. On the other hand, Funari & Ferro [28] observed that the humidity of propolis collected in the city of Cabreúva, in the state of São Paulo, ranged from 10.86% to 11.00%. The humidity of propolis is related to place where it was deposited inside hive, since the greater contact with the external environment greater will be the influence of water content on propolis, resulting in greater or lesser humidity [13].

Mechanical mass is a component of propolis constituted of insoluble particles which is supposed to exist to contribute to the structure of crude propolis [16]. The mechanical mass determined for Green Propolis samples ranged from 47.33% to 54.10% (Fig. **1f**) and these percentages are higher than the maximum level of Brazilian legislation which establishes maximum of 40% impurities [14]. This parameter refers to the mixture of impurities, such as fragments of wood, leaves, bits of flowers, pollen, insects during processing [13].

Melo [13] found 31.4% of mechanical mass in propolis samples from the southeast region of Brazil, while Bastos [27] obtained the values between 22.25% and 40.73% for propolis samples collected in Minas Gerais, Brazil. Funari & Ferro [28] found an average of 35.22% in propolis samples from Serra do Japi in the state of São Paulo, results different from those found in the present work. These values did not affect the quality of propolis, since the other physicochemical parameters remained adequate, such as total flavonoids, antioxidant activity, oxidation index and wax content [16, 29].

5. CONCLUSION

The results demonstrated that Brazilian Green Propolis samples collected in different seasons

during the years 2008 to 2013, presented a slight seasonal variation in relation to the physiochemical parameters, and can be used as a functional ingredient in food. At the moment, there are no standardized methods to analyze the 13 types of propolis found until now in Brazil, emphasizing the importance of directing more research to investigate and assist in the definition of parameters.

ABBREVIATIONS

DPPH	=	2,2-diphenyl-1-picrylhydrazyl;
HEP	=	hydroalcoholic extract of propolis
B. dracunculifolia	=	*Baccharis dracunculifolia*
≈	=	approximately
≤	=	smaller or equal than
° C	=	degree Celsius
mL	=	milliliter
%	=	percentage
g	=	gram
mg	=	milligram
UV-Vis	=	ultraviolet-visible
Nm	=	nanometer
µg	=	microgram
cm	=	centimeter
mM	=	millimolar
M	=	Molar
h	=	hour
ANOVA	=	analysis of variance
$p \geq 0.05$	=	no demonstrated statistically significant variation
$p \leq 0.05$	=	demonstrated statistically significantly variation

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. Each one has made substantial contribution to the information or materials submitted for publication.

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