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Original Article

PHYSICOCHEMICAL CHARACTERIZATION AND FLAVONOID CONTENTS OF ARTISANAL BRAZILIAN GREEN PROPOLIS

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ABSTRACT

Objective: Green propolis (*Baccharis dracunculifolia*) is widely used in Brazilian folk medicine and elsewhere for treating varied illness. To address the need to meet its chemical composition and pharmacological effects, this study aimed to assess the physicochemical properties of green propolis from the metropolitan region of Vale do Aço, Minas Gerais, Brazil.

Methods: Besides wax and ash contents analysis, flavonoids were analyzed by UV-visible scan spectrophotometry and gas chromatography coupled to mass spectra (GC-MS).

Results: Samples were under the limits of ashes adopted in Brazil, and ranged from 4.84% to 12.78% regarding loss on drying. Wax content of propolis from the winter sample (10.84%) was higher than summer (8.94%) and autumn samples (7.22%). Summer samples, however, presented the highest concentration of flavonoids (1.697 mg/mL), and followed by autumn and winter samples, respectively.

Conclusion: Our findings raised the possibility that this source of propolis may be useful an adjuvant phytotherapeutic product, and contributes to investigations that seek to verify the biological activities of green propolis.

Keywords: Green propolis, Flavonoids, Folk medicine, Chemical analysis.

INTRODUCTION

Propolis (or royal jelly) is a general name for the resinous substance collected by bees from various plant sources and taken to hives, where it is combined to glandular secretions, enzymes and waxes. In beehives, propolis is used for coating parts and to seal cracks and crevices, protecting it against invaders, and also to mummify animal carcasses. The composition of raw propolis varies with the source, therefore, in order to understand the causes of the differences in its chemical composition, it is necessary to investigate its botanical origin and conditions such as the weather, topography, vegetation, and the period in which the resin was collected [1-3].

The wide diversity of substances present in propolis has been taken as its quality indicator. The chemical composition of propolis is complex and includes aromatic acids, esters, aldehydes, ketones, terpenoids, steroids, polysaccharides, amino acids and other organic and inorganic compounds. Propolis is composed of about 55% resinous compounds and balsam, 30% beeswax, 10% ethereal and aromatic oils, 5% bee pollen and different organic compounds. Approximately 300 substances were identified in propolis from different sources, and antitumor, antiviral, anti-inflammatory, antimicrobial, cytostatic, antifungal, wound healing and hepatoprotective activities have been described or this natural product. Propolis flavonoids can inhibit lipid peroxidation, aggregation of platelets and improve capillary fragility. Propolis has also an important activity against reactive oxygen species (ROS). The antioxidant activity of flavonoids present in propolis was described as elimination of ROS and protection of the cell membrane [4-6].

The Brazilian propolis produced in cerrado (savannas zones at Brazil southeastern and mainly in the State of Minas Gerais) is known as "green propolis" and the botanical species *Baccharis dracunculifolia* is the main source. Most of the known biological activities of propolis are assigned to phenolic compounds such as flavonoids. Hydroethanolic preparations of propolis are commonly prescribed by Brazilian physicians in drops for dilution in water prior to administration mainly for the treatment of infectious diseases. Other forms of propolis are also used as dietary supplements in varied countries, like the United States of America, Japan and Europe, to prevent diseases and improve health as a whole [7-11].

Conversely, few studies provide reports regarding the quality of the plant material from such a widely-used phytotherapeutic product. Considering the wide use of green propolis in Brazilian folk medicine and the use of its other forms elsewhere, and the need to meet its characteristics concerning the pharmacological effects, this study aimed to evaluate the physicochemical features of green propolis from the metropolitan region of Vale do Aço, Minas Gerais, Brazil, which is used for the preparation of different end-products that are sold throughout Brazil. Besides traditional assays like wax and ash contents, flavonoids were analyzed by UV-visible scan spectrophotometry and gas chromatography coupled to mass spectra (GC-MS). The scarcity of data regarding green Brazilian propolis makes our study even more relevant.

MATERIALS AND METHODS

Samples

Propolis samples used in this study were provided by Apismel Indústria e Comércio de Mel Ltda. This samples were collected on autumn (month: june), winter (august) and summer (march), at an Apiary in the metropolitan region of Vale do Aço (Minas Gerais state) by the scraping method, with chisels from the inner parts of the hives. After separation of impurities, each sample was packaged in plastic containers, protected from light and kept at -20° C, until analyzed.

Sensory analysis

Sensory analyses were performed at 25 °C as described by the Brazilian Ministry of Agriculture standards [12] for color, taste, aroma, consistency and granulometry, using aliquots of 80 g.

Ash content

The ash content of the samples was determined at 600 °C with a conventional laboratory furnace after drying an aliquot of 2 g at 350 °C. The sample was maintained for 1 h at 350 °C to reduce the organic carbon content and smoke production, and for 2 h at 600 °C.

This analysis was performed in triplicate [14].

Determination of loss on drying (Moisture)

Drying was performed in triplicate at the temperature of 105 °C for 3 h in a drying chamber, and samples were then cooled in a dessicator and weighted. The process of heating, cooling and weighing of the set was repeated with intervals of 2 h, until constant mass (when the difference between two consecutive weightings did not exceed 5 mg) was achieved [14].

Preparation of methanolic extract

The methanolic extract of Propolis was obtained by Soxhlet. An aliquot of approximately 10 g of powdered samples was dried in hot-air oven at 105 °C for 1 h. Porcelain beads and approximately 150 ml of methanol were added to the system, which was maintained under reflux for approximately 8 h. After cooling, the extract was kept in a sealed container protected from light exposure until analysis. This procedure was carried out in triplicate.

Methanol-insoluble residues

The cartridge used in Soxhlet extraction containing substances that were not dissolved in methanol, was placed in a chapel of exhaustion for 1 h for evaporation of solvent. Then, it was taken to a preheated oven at 105° C for 2 h. The system was cold and the cartridge (containing the residue) was weighted. The process of heating, cooling and weighing the cartridge was repeated hourly, until constant mass (when the difference between two consecutive weightings did not exceed 5 mg) was achieved. This analysis was carried out in triplicate [14].

Dry weight (solids soluble in methanol)

A 5 ml aliquot of the wax free methanolic extract of propolis was transferred to dry porcelain capsule, heated in an oven at 105 °C for 2 h, cooled in desiccator and weighted. The process of heating, cooling and weighing of the set was repeated with intervals of 1 h until constant mass was achieved (when the difference between two consecutive weightings did not exceed 5 mg). This analysis was performed in triplicate and the dry residue content (soluble solids in methanol) was calculated by the ratio between the mass of waste deposited in the cartridge and the initial mass of raw propolis extracted, corresponding to the rate of 5 mL, in percentage [14].

Wax content

The methanolic extract of propolis was kept at 4 °C for 24 h and subsequently at -20 °C for 30 minutes. The solution was then filtered in Buchner funnel (with filter paper previously dried and weighed) under vacuum (400 mmHg), and the wax deposited on the filter paper was washed with cold methanol until clarification. The volume of wax-free extract was measured and packaged in sealed glass container, taken to a chapel of exhaustion for 1 h to eliminate the excess of solvent, and then deposited in a preheated oven at 105° C for 2 h, cooled in a desiccator and weighted. The process of heating, cooling and weighing was repeated with intervals of 1 h until constant mass (when the difference between two consecutive weightings did not exceed 5 mg) was achieved. This analysis was performed in triplicate.

Total flavonoids content

Raw samples were crushed for obtaining a powdery form, and a hydroethanolic extract (70% v/v) was obtained as previously described. The analysis of flavonoids in this extract was performed using the colorimetric reaction. An aliquot of the extract (2 ml) was transferred to a 25 ml volumetric flask, containing 1 ml of aluminum chloride solution (2.5% w/v). The final volume was adjusted with ethanol and after 30 minutes the absorbance of the solution were determined at 425 nm by ordinary spectrophotometric method. Calibration curves for this method were achieved with different concentrations of standard quercetin [15].

Scan on UV-visible region

The UV-visible spectra for the hydroethanolic extract previously prepared was obtained through the addition of a small aliquot of the extract in a quartz cuvette and analyzed in a scanning spectrophotometer. The extract was analyzed with the same concentration of propolis (0.150 mg/g). Absorbance data were recorded against a blank reagent, and the spectra were determined in scan range from 200 to 600 nm, averages were calculated, and the means were recorded.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

A dichloromethane extract (95% v/v) was prepared for GC-MS analysis by the adding 10 ml of dichloromethane to 3 g of propolis samples. The mixture was stirred overnight in darkness and then filtered using cellulose membranes. The extract was kept at 4 °C until analyzed. The GC-MS analysis of the dichloromethane extract was performed in a Shimadzu GC-17A equipped with a DB-5 capillary column (30 m x 0.25 mm ID, 0.25 µm film), using He as the carrier gas. The column temperature was programmed to increase from 60 °C to 240 °C at a rate of 3°C/min. The column was maintained at 240 °C for 20 minutes [9, 10].

Statistics

Differences regarding the assessed parameters were analyzed using ANOVA followed by Tukey test. The significance level was set at $p<0.05$, and highly significant, values were set as $p<0.001$.

RESULTS AND DISCUSSION

Sensory analysis

The Green propolis samples had resinous and balsamic aroma, greenish color, soft, strong and acid flavors, rigid consistency at room temperature and heterogeneous fragments. The results obtained from the examination of sensory characteristics analysis of propolis are presented in table 1. The rigidness of the samples in this study indicated high resin content, what was appropriate to our samples, given that the biological activities of propolis have been attributed to substances contained in this fraction. Samples of propolis with pronounced characteristic aroma and flavor indicate whether the sample is new or not. In the present study, the samples sensory characteristics indicated that propolis was recently produced [8, 16].

Table 1: Sensory characteristics analysis of samples in each season

Parameter	Classification parameter	Summer samples	Autumn samples	Winter samples
Aroma	Resinous	X	X	X
	Balsamic	X	X	X
Color	Amber			
	Reddish			
	Greenish	X	X	X
Flavor	Soft		X	
	Strong	X		X
	Bitter	X	X	X
	Spicy			
Aspect	Clear	X	X	
	Turbid			X
	Homogenous	X	X	
	Heterogenous			X

All samples analyzed had sensory characteristics within the standards proposed by the Brazilian Ministry of Agriculture through the normative instruction N° 3. Our data have some overlapping features with previous studies that explored the characteristics of propolis from the city of Maringá, Brazil [16], differing only regarding the taste, what was not analyzed in this study. We observed that even the samples being collected in different periods of the year, the analyzed sensory characteristics were not significantly different; this is probably due to the similar botanical source.

Ash content

The ash content of samples was determined to establish the amount of residual non-volatile substances in the incineration process. This parameter is particularly important for propolis available at common markets, once this analysis may indicate possible tampering by impurities such as sand. In comparison with the Brazilian Ministry of Agriculture legislation, the samples results were under the limits of ashes (>5%); however, the samples collected during the summer had a highest ash content ($p<0.05$).

The physicochemical properties of propolis from Franca and Passos cities (southeastern region of Brazil) were previously analyzed, and it was seen that ash contents ranged from 1.34% and 10.92% [8]. Our data suggest that our samples had no addition of any contaminant, which shows the quality of this material to be used as medicine (Table 2). The ash levels found indicate that even being from different periods, propolis was in accordance to quality standards. However, the difference in values between the samples was statistically significant, indicating that the composition of propolis is not constant throughout the year.

Loss on drying

Our samples ranged from 4.84% to 12.78% regarding loss on drying (Table 2). The propolis sample from the autumn season had the highest value (12.78%), and differed statistically from those collected during summer (4.84%) and winter (7.36%). The legislation sets maximum moisture values of 8%. Therefore, autumn samples had higher levels than the legislation limit, what can affect the shelf life when stored for a longer period of time. High moisture content is critical to the preservation of propolis samples, since excessive water can lead to hydrolysis reactions of active molecules, and favor microbiological growth.

Wax content

Propolis is described as a blend of resins, balsams, waxes and other components. Determining waxes content is important because the phenolic compounds and other active molecules may be not present in this fraction; therefore, high wax content may indicate a low concentration of resins and of biologically active molecules. We observed that the wax content of propolis from the winter sample (10.84%) was higher than the summer (8.94%) and autumn samples (7.22%) (Table 2). All samples were in accordance with the legislation, which stipulates maximum values of 25% propolis wax *in natura*.

Values of 14.53% waxes were found when parsing the propolis from the city of Franca, and values of 2.44% from samples of the city of Crabeúva, both in the State of São Paulo [8, 17]. This suggests that wax levels can vary in different regions of Brazil. There are evidences that propolis is mixed with beeswax in varied concentrations, and also the biotransformation of plant resin/exudates by bees to form propolis have been described [18].

Table 2: Summarization of physicochemical parameters, Data is expressed as means ± standard deviation. Means followed by the same letter had no statistical difference (Tuckey test)

Parameter	Summer samples	Autumn samples	Winter samples
Ash content	3.84 ± 0.13 b	2.44 ± 0.27 a	2.43 ± 0.28 a
Loss on drying	4. 84 ± 0.52 a	12.78 ± 0.20 b	7.36 ± 1.00 a
Wax content	8.94 ± 0.16 a	7.22 ± 0.02 a	10.84 ± 0.05 b
Dry weight (solids soluble in methanol)	53.92 ± 0.24 b	40.42 ± 1.1 a	43.24 ± 0.16 a
Methanol-insoluble residues	39.01 ± 0.37 a	42.44 ± 0.20 a	52.31 ± 1.36 b

Flavonoids analysis

Winter propolis samples presented the highest value of methanol-insoluble residues (52.31%). The difference in values between the samples collected may have been influenced by the period in which each was produced. Values of 31.08% of waste were found in Brazilian samples using grain alcohol [19]. Thus, in addition to the seasonal factor, the choice of solvent can influence directly on insoluble residues of samples. As for the dry weight, the sample collected in summer showed the highest content (53.92%), when compared to autumn (40.42%) and winter samples (43.24%), what is consistent with the observation of others that the summer sample has large number of substances soluble in methanol, such as flavonoids, what was demonstrated by GC-MS analysis (Fig. 3).

Previous studies showed that propolis produced in summer presented high concentration of flavonoids [8, 16-18]. For the propolis samples collected in the region of Vale do Aço, this result was also observed. According to our data, summer samples presented the highest concentration of flavonoids (1.697 mg/mL), followed by autumn and winter samples, with concentrations of 1.275 g/mL and 0.543 mg/mL respectively. Highly significant differences ($p<0.01$) were detected when comparing summer samples to winter and autumn samples.

Seasonality is considered a primordial factor for this variation, since the nature and quantity of active molecules, such as flavonoids, are not constant throughout the year. Therefore, geographic location and local vegetation may affect the composition of propolis. Concentrations lower than 3% were found using this same method of quantification for flavonoid content from Brazilian propolis extracts from the States of São Paulo, Santa Catarina and Rio Grande do Sul [20]. Propolis samples produced in the State of Paraná presented 5.05% of flavonoids when extractions were performed with the same solvent system of this work [21].

The UV absorption spectrum has been widely used for evaluation of propolis, what is justified by the presence of phenolic compounds, especially the class of flavonoids, which absorb in the UV wavelength, ranging from 240-285 nm and 300-355 nm (Agrawal, 1989). The scanning of Spectra Green propolis extracts of summer, autumn and winter obtained showed absorptions features of flavonoids (Fig. 2). Summer samples had higher absorbance in fixed wavelength of flavonoids (UV region) when compared to the other extracts. The maximum absorbance of propolis extract of summer was about 4 and 2 times higher when compared, respectively, with extracts of winter and autumn. Varied absorption profiles for samples collected at different times have been described previously [22-25].

GC-MS is a powerful technique which has been increasingly used in natural products research. The chromatogram in fig. 3 from summer samples shows the presence of Ethyl 3-prenilcinamate at the peak 44 (retention time 55). Six compounds isolated from propolis were analyzed for the first time through GC-MS in 2011 [23]. An investigation of ether extracts of propolises from 11 countries of Europe and Asia together with extracts of the buds of their principal plant precursors were performed by GC-MS, and chemical compositions of the exudates of aspen, white birch and silver birch buds were determined [18].

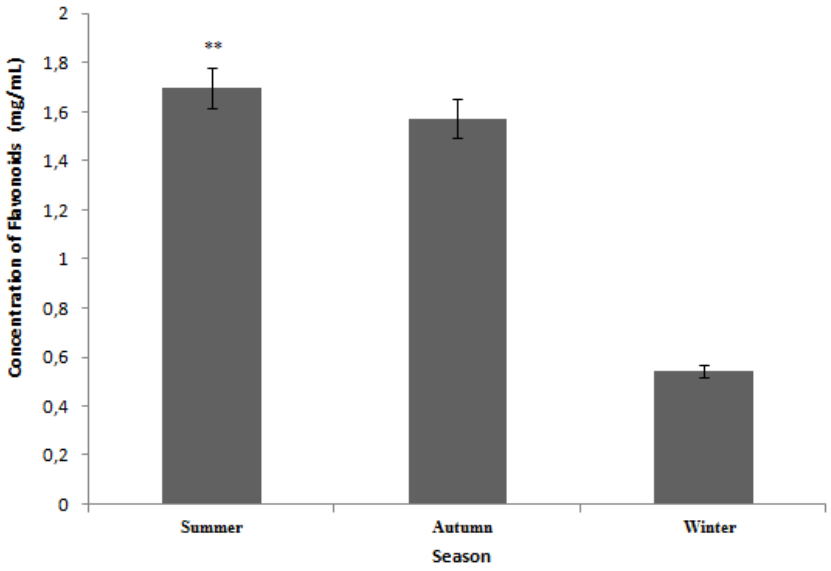

Fig. 1: Total flavonoid content in each season. ** Symbol indicates p<0.001

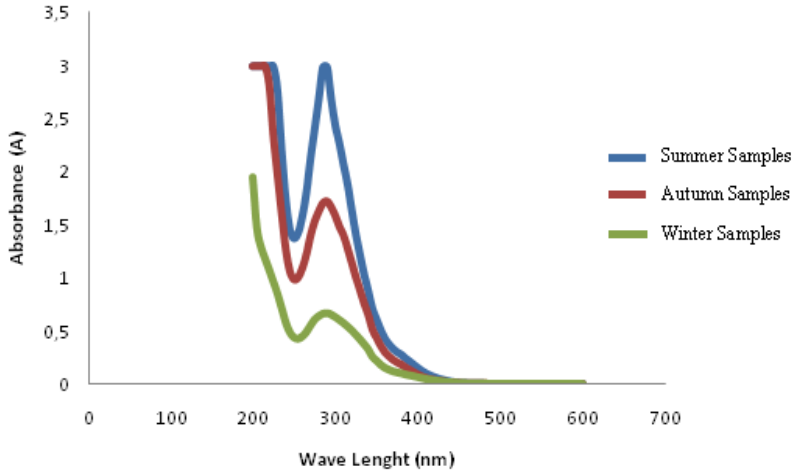

Fig. 2: Spectrophotometric scanning of green propolis extracts produced in summer, autumn and winter

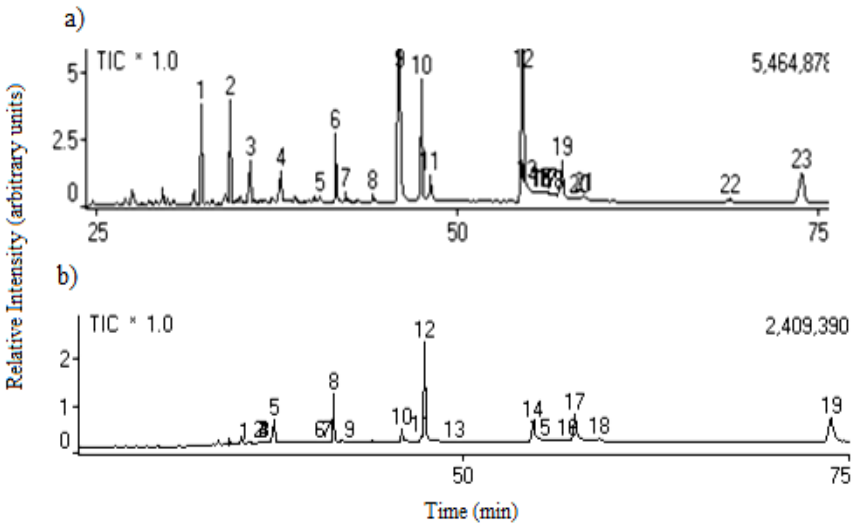

Fig. 3: GC-MS Chromatograms from the standard (a) and the dichloromethane extract of green propolis collected in the summer (b)

CONCLUSION

The present study provided evidence that the characteristics assessed in the samples of propolis are within the limits set by the Brazilian Ministry of Agriculture, although the data obtained showed that there was statistical difference in the results of the analyzed samples collected during the year, suggesting that the season and period of production and collecting of propolis interferes with the variety and concentration of phytomolecules present in this product. These findings raised the possibility that this source of propolis may be useful an adjuvant phytotherapeutic product, and contributes to investigations that seek to verify the biological activities of green propolis, what may also support future pharmaceutical and alimentary exploration of propolis from this region.

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CONFLICTS OF INTEREST

Authors have no conflicts of interest

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