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Analysis of Brazilian Propolis by Differential Scanning Calorimetry (DSC) and Thermal Gravimetric Analysis (TGA). Characteristics of Crude Resin, Ethanolic Extracts, Wax and Isolated Compounds

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Introduction

Propolis is a resinous material of different colours ranging from yellow, brown, green and red, composed of resins produced by plants, added to the secretions of bees and mixed with wax. Propolis is used mainly in the construction and preservation of hives (Marcucci et al., 2000). Propolis resin is characterized by naturally occurring chemical compounds, including different phenolic bioactive compounds, waxes, ashes and volatile substances, among others, whose content may widely vary depending on the existing differences in plant ecosystems (Fabris et al., 2013; Fikri et al., 2019; Marcucci et al., 2000). Brazilian green propolis collected from *Baccharis dracunculifolia* (Tomazzoli et al., 2020) in the central Regions is rich in *p*-coumaric acid, prenylated cinnamic acis, such as Artepillin C, caffeic and caffeoylquinnic acids and flavonoids (Carvalho et al., 2019; Veiga et al., 2017). Brown propolis collected from *Araucaria heterophylla* and, sometimes, from *B.dracunculifolia* and *A. heterophylla,* in the southern Regions, contains high levels of vanillin, crysin, pinocembrin and cinnamic acid derivatives, for example, Artepillin C (Marcucci et al., 2008), while red propolis collected from *Daubergia ecastophyllum* and *Symphonia globulifera* (Ccana-Ccapatinta et al., 2020) in the northern Regions are rich in flavonoids, isoflavones and prenylated benzophenones (Vieira de Morais et al., 2021) (Figure 1). All these propolis may present biological activities, including some antimicrobial and antioxidant (Batista et al., 2016; Dantas Silva et al., 2017; do Nascimento et al., 2019a; Ripari et al., 2021; Schnitzler et al., 2010; Touzani et al., 2018; Vieira de Morais et al., 2021), anti-inflammatory (Wang et al., 2015) and antitumoral properties (Nani et al., 2018; Watanabe et al., 2011). Some types of propolis have high market value for their medicinal properties, such as Brazilian green propolis (Berretta et al., 2020). Because there are so many variations in its chemical composition, in particular, regarding bioactive compounds, the characterization of propolis is very important in order to meet quality control standards.

The green color of Brazilian propolis is a consequence of its botanical origin, from the young plant tissues of *Baccharis dracunculifolia* DC, which contains a high concentration of chlorophyll. Brown propolis, on the other hand, is mostly composed of resins of *Pinus* spp., *Eucalyptus* spp. and *Araucaria angustifolia* (Ribeiro et al., 2021). The characteristic red color of the product is due to pigments called retusapurpurins. Some of the red propolis constituents, such as chalcones, neoflavonoids, isoflavones and pterocarpans, are classes of secondary metabolites characteristic of the subfamily Faboideae of the family Fabaceae (Leguminosae) (Frozza et al., 2013). Any change in the characteristic color of these different types of propolis can be associated with changes in their quality.

According to Matsuda et al. (2002) thermal analysis is a chemical tool that gives information about changes on heating of great importance for technological applications. However, there is very limited information on the thermal properties of propolis (Taherzadeh et al., 2021). Only one report has shown the melting interval of propolis to be between 63 and 84 °C (Jobir & Shume, 2020), and another study showed the melting point of a propolis component (caffeic acid) is 195 °C (Majiene et al., 2004). There are many reports about the use of this technique to check the properties of formulations with propolis, such as nanoemulsions (Baygar, 2020; Correa et al., 2019) spray-dryer formulations (do Nascimento et al., 2019b) and films (Hajinezhad et al., 2020; Villalobos et al., 2017).

An integrated thermal analysis can measure transition temperature, weight losses, energies of transition, dimensional changes, modulus change, and viscoelastic properties. Current areas of application, include environmental measurements, composition analysis, product

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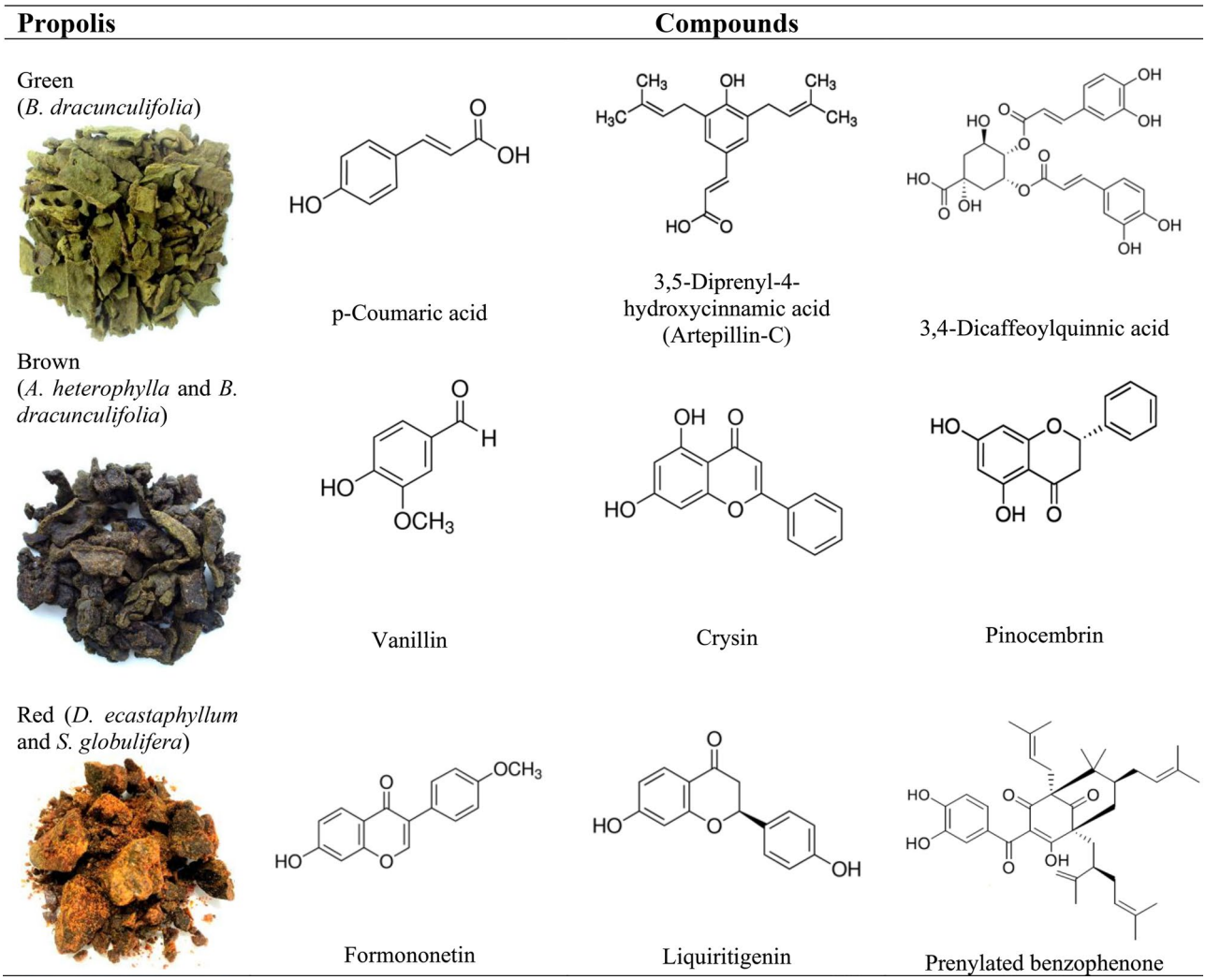

Figure 1. Colours and some compounds identified in Brazilian propolis.

reliability and stability, chemical reactions, and dynamic properties (Matsuda et al., 2002).

Many products with propolis are prepared using heating treatments that may affect their functionalities (Frozza et al., 2013; Park et al., 2002). For this reason, the purpose of this study was to determine the thermal stability of some types of Brazilian propolis and of some selected derivatives (wax and ethanolic extract from green propolis). The study was conducted using Thermal gravimetric analysis (TGA) and Differential scanning calorimetry (DSC), which complements the TGA, to determine the main temperature values, due to the loss of mass of the raw propolis samples, also comparing the results obtained with literature data to ensure an accurate result. The mechanism of thermal degradation of the main components of propolis, previously detected for these samples, was proposed, based on data from the literature.

Results and Discussion

Thermogravimetry provides a quantitative measurement of any weight changes (mass) associated with thermally induced transitions. Table 1 presents thermogravimetric analysis of different propolis samples, i.e., the weight loss of the substances contained in propolis as a function of temperature. Samples 1 to 14 were studied in the crude form. We chose propolis 1 to 3, because they represent the most important commercial samples, and have the chemical markers that we wanted to evaluate thermal stability. Samples 1 to 3 were further subjected to the extraction process in order to compare separately the effects of temperature on EEP and on wax derived from propolis.

It can be seen that the thermal stability of crude propolis and of the EEP is compromised around 100 °C with a relatively low weight loss of 2.5% (mainly volatile substances) followed by a 9% weight loss at 200 °C (mainly volatile substances) whereas an average 75% weight loss at 500 °C can be observed. These differences were statistically significant ($p < 0.001$). The wax contained in propolis resin displayed good thermal stability up to 200 °C. This is to be expected because propolis waxes are composed primarily by monoesters and non-saturated hydrocarbons, which is essentially the composition of propolis wax (Cetin et al., 2021; Rivero et al., 2020) that are thermally stable. TGA results have revealed that beeswax is thermally stable up to 290 °C and completely pyrolyzed at 600 °C (Ebrahim, 2015). Thermal and mechanical properties were determined for the halloysite nanotubes (HNT)/beeswax composites at various compositions. According to Cavallaro et al. (2015) the beeswax degradation temperature was around 220 °C. Some authors reported that there is no considerable weight loss of beeswax up to temperature of around 250 °C (Kabir & Yola, 2020). Other types of natural waxes, such as carnauba wax has thermal stability until 250 °C (ANS, 2014). No studies were found

Table 1. Thermal gravimetric analysis of propolis (crude resin), ethanolic extract and wax.

	Temperature (°C)							
	50	100	200	300	400	500	600	700
Propolis Sample	Weight loss (%)							
Crude resin								
1	–	3	10	32	60	70	73	76
2	–	2	8	30	53	60	64	67
3	–	3	8	36	75	86	89	91
4	–	4	12	36	62	80	96	96
5	–	2	6	30	53	74	87	97
6	–	2	12	35	56	68	72	75
7	–	3	7	30	64	75	95	95
8	–	2	10	34	57	65	70	76
9	–	3	11	33	59	67	78	80
10	–	2	11	33	54	75	86	96
11	–	3	8	36	60	83	89	96
12	–	2	11	33	62	79	90	97
13	–	4	10	32	61	69	90	96
14	–	2	9	35	60	72	77	82
Median		3	10	33	60	73	83	87
	Ethanol extract (EEP)							
Temperature (°C)	50	100	200	300	400	500	600	700
1e	–	2	13	40	68	82	84	86
2e	–	2	18	38	59	75	76	79
3e	–	2	16	38	60	73	75	78
	Wax							
1w	–	–	1.0	7	87	99	99	99
2w		–	–	7	85	96	97	98
3w		–	–	6	87	97	98	98

about thermal analysis using TGA and DSC to analyse propolis wax. Figure 2(A) illustrates weight loss as a function of temperature of a propolis sample in the crude form as well as EEP and wax fractions at temperatures ranging from 50–700 °C. The wax fraction is stable up to 200 °C exhibiting a curve similar to pure substances. The propolis resin exhibited similar behaviour to EEP. The majority of the biologically active components of propolis are in the EEP. At 100 °C the thermal stability of propolis is slightly altered and above 200 °C this process is largely accentuated.

DSC produces a quantitative measure of the transitional temperature and the transitional energy. Table 2 presents the DSC analysis of the propolis resin in the crude form. In all samples we observed two distinct endothermic peaks. One relating to the melting point of propolis wax contained in propolis (endothermic peak 1), with a melting temperature (T_m) ranging between 50–67 °C. Similarly, studies have shown that the beeswax has a T_m ranging from 40 to 70 °C. Other examples of resins and its melting point are, carnauba wax: 82–86 °C, castor wax: 80 °C, candellila wax: 68.5–72.5 °C and beeswax: 64–64 °C (Bucio et al., 2021; Winkler-Moser et al., 2019). The second peak observed was related to the decomposition process of the remaining substances present in propolis. The decomposition temperature (T_d) of propolis ranged from 120 to 200 °C. This variation may be due to differences in the chemical composition of propolis samples coming from different geographical regions. We can compare our findings with known transition temperatures of other resins. The gum benzoin from Sumatra and Siam has a transition temperature above 100 °C (Sankaralingam et al., 2020). Thermal gravimetric results were reported (Pereira et al., 2011) indicating that Dominican, Russian and Colombian resins present relatively high thermal stability under air conditions in the range of 228–300 °C, but the mass loss was observed at 217 °C for Baltic amber. A melting temperature of 127.35 °C was verified for resin samples from almaciga (*Agathis philippinensis* Warb.) from Davao Oriental, Philippines. However, Palawan samples showed average melting points of around 140 °C. A high melting point indicates the presence of polar components that require greater energy to break the intermolecular forces present in the material (Razal et al., 2021).

Figure 2(B) shows the characteristic behavior of the DSC curves of propolis samples. Two endothermic peaks were observed in sample 1:1 wax (T_m = 62 °C) and for the sample 2, the remaining substances contained in propolis (T_d = 145 °C). This was observed in most of the samples. However, in some cases peak 2 unfolded based on the composition and concentration of the different substances present in propolis resin, as observed in sample 10 (T_d = 138, 173 and 198 °C) (Table 2).

Figure 2(C) shows DSC analysis of EEP and wax after extraction of sample 1. It can be seen that the peaks are sharper that those observed with crude resin. Similarly, their T_m and T_d presented with higher values than those found in the crude sample (Figure 2B). In this manner we can confirm that the first endothermic peak in the crude

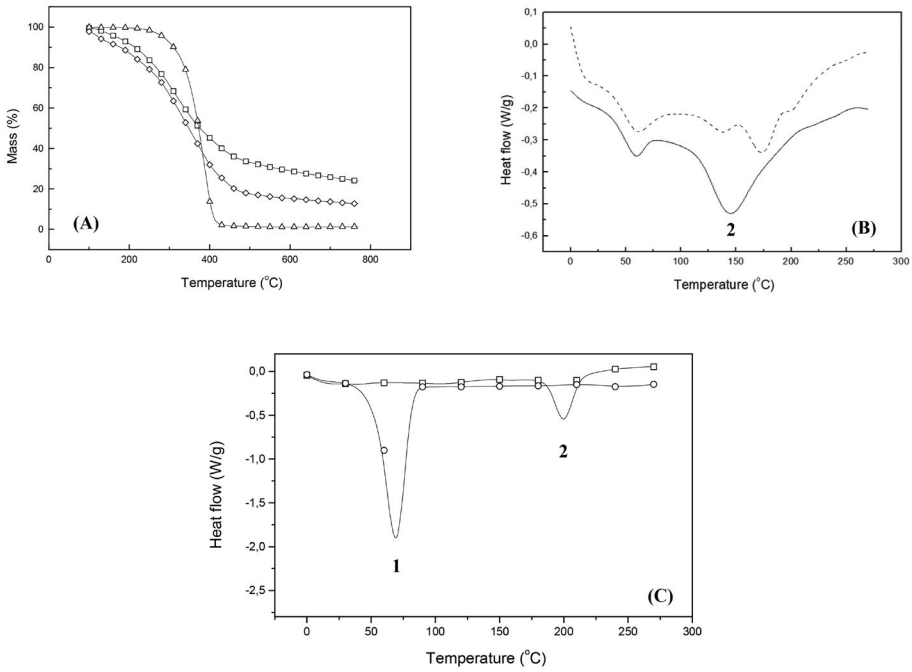

Figure 2. (A) Weight loss as a function of temperature. Crude resin (-□-), ethanolic extract (EEP) (-◊-) and wax (-Δ-) (sample 1).
(B) DSC Analysis for crude resin. Endothermic peak 1 is characteristic of the wax (present in propolis) and endothermic peak 2 is characteristic for the decomposition of the remaining propolis substances. (C) DSC analysis of the EEP (-□-) and wax (-o-) after extraction (sample 1). Legend: sample 1 (- - - -) and sample 8 (—) (see Table 3).

Table 2. DSC Analysis of propolis samples (crude resin).

Propolis Samples	Endothermic peak 1[a]		Endothermic peak 2[a]	
	ΔH (J/g)	Tm (°C)	ΔH (J/g)	Td (°C)
1	10	62	41	145
2	27	64	94	133
3	13	65	44	127
4	17	62	47	137
5	14	63	49	133
6	38	63	31	190
7	29	62	120	130
8	23	67	12; 39	150; 185
9	42	64	77	152
10	58	66	6; 15; 21	138; 173; 198
11	7	51	162	135
12	20	67	79	149
13	5	62	76	130
14	15	67	173	124

[a]The endothermic peak no. 1 refers to the wax contained in the crude resin; the endothermic peak no. 2 denotes other substances present in crude resin. Tm—melting temperature; Td—decomposition temperature.

resin characterizes the presence of wax whereas the second peak represents the remaining substances in the crude resin, which is similar to EEP pattern. The energy spent in these transitions can be utilized to perform semi-quantitative analysis of the concentration of the propolis resin. The peaks observed are, therefore, proportional to the amount of wax or EEP present in propolis. In this manner, thermal analysis could be a semi-quantitative parameter in quality control tests where wax and the EEP can be measured without the need of being extracted.

In order to aid future studies, the degradation mechanism and degradation products of the main propolis components determined previously are presented in Figure 3. The knowledge of these sub products generated from the thermal degradation of propolis is extremely important in the validation and stability indication for the analytical procedures used in the quality studies (Kmiotek et al., 2018). Artepillin C, the major component of green Brazilian propolis, under mild heating conditions and in the presence of air, forms a pyran-oxide derivative, **VII** as the major degradation product, starting from a tertiary hydroperoxide, **IV** which forms an epoxide, **V**, which subsequently undergoes attack by the hydroxyl group on one of the two carbons of the epoxide. Finally, with the loss of one molecule of water the degradation product, **VII** is formed (Arruda et al., 2020) (Figure 3A). Furthermore, Artepillin C when heated to temperatures around 150 °C, originates as degradation product, compound **VIII**, by decarboxylation, i.e., loss of CO_2, as it is proposed for several cinnamic acids (Cheng et al., 2014). Considering the degradation mechanism proposed by Cheng and collaborators, *p*-coumaric acid when subjected to heating at a throne of 150 °C will form the degradation product **IX** (Figure 3B). Flavonoids such as kaempferol are highly temperature sensitive, and their degradation can occur at temperatures of 35 °C. These observations were made by Chaaban and collaborators, when studying the thermal degradation of various flavonoids (Chaaban et al., 2017). Based on these studies, the thermal degradation mechanism of kaempferol is shown in Figure 3(C). The heat treatment at a temperature of 130 °C in the presence of air, results in the degradation products **X**, **XI** and **XII**. It is important to note that product **X** can already form when subjected to much milder temperatures of 35 °C.

Other compounds of great interest present in Brazilian propolis are caffeoylquinic acids, which have several biological and pharmacological effects. Xue et al. (2016) conducted studies, regarding the thermal degradation of these compounds, and were able to identify the degradation products formed, through high-performance liquid chromatography-with photodiode array detection (HPLC-PDA) and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The proposed degradation mechanism for 3,4-dicaffeoylquinic acid is shown in Figure 3(D).

Also, in this study, the authors point out that the main factor of degradation of this class of compounds is temperature, followed by light, highlighting the importance of greater care with the storage of these compounds. Currently, several formulations are developed with propolis, for use in different purposes. Not only the isolated compounds from propolis must be kept under special conditions, as well as the resin itself, must be under stable

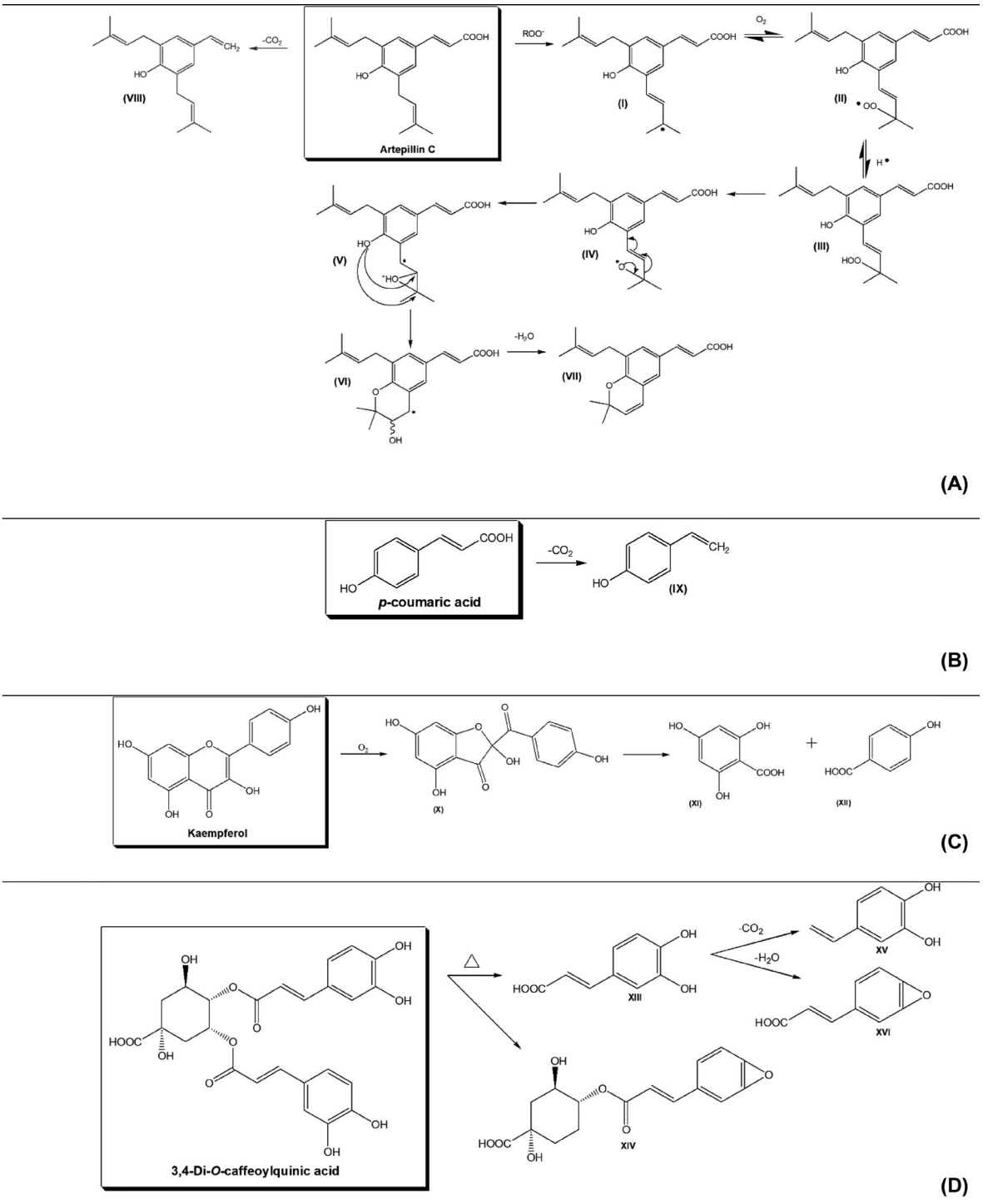

Figure 3. Proposed thermal degradation mechanism for: (A) Artepillin C. (B) *p*-Coumaric acid based on Cheng et al., 2014 (Chaaban et al., 2017). (C) Kaempferol. (D) 3,4-Dicaffeoylquinic acid.

conditions, in order to obtain thermal stability in the final product.

Conclusion

We conclude that it is very important to know the thermal properties of propolis and wax, which is a constituent of it, as well as an ethanolic extract, widely used for medicinal purposes. Propolis-based formulations have been developed, considering the properties of the resin. Hence using a thermally stable raw material is important so that there are no problems with the final formulations, in terms of stability.

Experimental

Samples

Fourteen crude propolis samples of different regions where the predominant propolis-producing species was *Apis mellifera L.* were submitted to thermal analysis. The geographical location and botanic origin of the samples are depicted in Table 3. The location in the country and typification of each sample is showed in Figure 4. Three samples were further extracted with absolute ethanol, yielding an ethanolic extract and wax (Table 3).

Ethanolic Extract of Propolis (EEP) and Wax

Three representative samples of the most commercially important propolis in Brazil were chosen. This type of propolis is BRP (Table 3) whose chemical composition is very similar, regardless of the region of collection; it comes from *Baccharis dracunculifolia*, rich in prenylated compounds. Approximately 20 g of samples 1, 2 and 3 were dissolved in

Table 3. Description of propolis samples.

Propolis samples	Geographic/Botanic origin/Typification	Extract fractions	
		Ethanolic Extract	Wax
1	Atibaia (SP)/*Baccharis dracunculifolia*/BRP	1e	1w
2	Bragança Paulista (SP)/*Baccharis dracunculifolia*/BRP	1e	1w
3	Maringá, PR/Araucaria and *Baccharis dracunculifolia*/BRP(PR)	3e	3w
4	Bambuí, MG/*Baccharis dracunculifolia*/BRP(MG)	–	–
5	Cambará do Sul (RS)/*Araucaria heterophylla*/BRG	–	–
6	Bagé (RS)/*Araucaria heterophylla* /BRG	–	–
7	Bambuí (MG)/*Baccharis dracunculifolial*/BRP(MG)	–	–
8	Nova Petrópolis (RS)/*Araucaria heterophylla*/BRG	–	–
9	Itirapina (SP)/*Baccharis dracunculifolia*/BRP	–	–
10	Caxias do Sul (RS)/*Baccharis dracunculifolia*/BRG	–	–
11	Pinhal (SP)/*Baccharis dracunculifolia*/BRP	–	–
12	Uberaba (MG)/*Baccharis dracunculifolia*/BRP(MG)	–	–
13	São João da Boa Vista (SP)/*Baccharis dracunculifolia*/BRP	–	–
14	Jarinu (SP)/*Baccharis dracunculifolia*/BRP	–	–

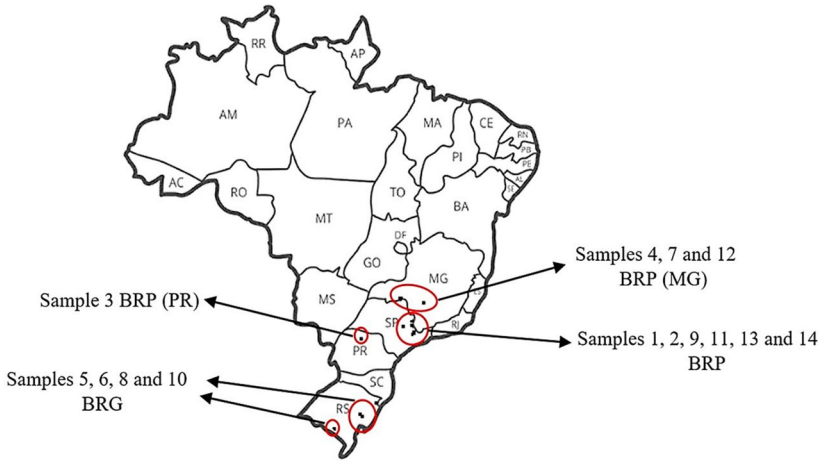

Figure 4. Location of each propolis sample including the typification (Marcucci et al., 2008).

ethanol 70 °GL (Ecibra, Brazil) and refluxed in the Soxhlet apparatus for 24 h at 60 °C. The EEP was evaporated and maintained in a dissecator. The final solution was frozen at −10 °C in order to obtain crystallization of wax, removed by filtration. After processing the samples, three fractions in each sample became apparent which yielded: 40–50% EEP (containing the biological active substances), 10–20% wax and 10–20% insoluble residues.

Thermal Analysis

Thermal gravimetric analysis (TGA) was performed on the Thermal Analyst 2000 (TGA Instruments). TGA data was obtained in the temperature range of 50 to 700 °C under nitrogen atmosphere, at heating rates of 5 °C/min. Samples described in Table 1 were studied in the crude form as a fine powder in order to eliminate the effects of propolis thickness. Differential scanning calorimetry (DSC) analysis was performed on the Thermal Analyst 2000 (TGA Instruments, Waters, Massachusetts, USA). DSC data was obtained in the temperature range of 0 to 300 °C, at a heating rate of 5 °C/min, under nitrogen atmosphere.

Statistical Analysis

Non-parametric tests were employed to measure differences in the parameters measured under different thermal conditions. The data were submitted to the Shapiro-Wilk normality test. Once the normality of the distribution was verified, the data were submitted to analysis of variance (ANOVA) and Tukey test with significance level of 5%. PAST software Version 3.22 was used (Oslo, Norway) (Dasgupta, 2013).

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To beekeepers, for providing samples of propolis.

Disclosure Statement

The authors declare no conflict of interest, financial or otherwise.

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Maria Cristina Marcucci*
ICT-Unesp, Department of Biosciences and Oral Diagnosis, São José dos Campos, São Paulo, Brazil
*Email: cris.marcucci@yahoo.com.br

Ildenize B. S. Cunha
Starret Inf. & Com Ltda., Itu, São Paulo, Brazil
http://orcid.org/0000-0002-2694-286X

Elisabete M. S. Sanchez
Rua Alice Kimaid Farah, São José dos Campos, São Paulo, Brazil

Carolina Passarelli Gonçalves
Pharmacy Department, Anhanguera University of São Paulo - Osasco, São Paulo, São Paulo, Brazil
http://orcid.org/0000-0002-4759-100X

Cristina Cedeño-Pinos and Sancho Bañón
Department of Food Technology and Science and Nutrition, Veterinary Faculty, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Murcia, Spain