Botanical source investigation and evaluation of the effect of seasonality on Brazilian propolis from *Apis mellifera* L.

Maira Maciel Tomazzoli*1, Amélia Regina Somensi Zeggio2, Remi Dal Pai Neto2, Leandro Specht3, Christopher Costa4, Miguel Rocha4, Rosendo Augusto Yunes5, Marcelo Maraschin6

1Universidade Federal do Paraná – Depto. de Fitotecnia e Fitossanidade – Lab. de Ecolfisiologia Vegetal, C.P. 19061 – 81531-990 – Curitiba, PR – Brasil.
2Universidade Federal de Santa Catarina – Depto. de Fitotecnia – Lab. de Morfogênese e Bioquímica Vegetal – Núcleo de Produtos Naturais, C.P. 476 – 88040-900 – Florianópolis, SC – Brasil.
3Universidade Federal de Santa Catarina/Centro de Ciências Biológicas – Dept. de Microbiologia, Imunologia e Parasitologia, C.P. 476 – 88040-900 – Florianópolis, SC – Brasil.
4Universidade do Minho/ Centro de Engenharia Biológica, Campus de Guimarães – 4710-057 – Braga – Portugal.
5Corresponding author <mairatomazzoli@gmail.com>

ABSTRACT: Bees generally use different botanical sources of resins for the production of propolis. The elucidation of botanical sources of propolis and identification of the effects of seasonality on the chemical profile of propolis are recognized as two important aspects in the development of a high quality product. Thus, our objective was to elucidate the botanical source and identify the effect of the seasons on the chemical profile of propolis produced in southern Brazil. The chemical profile of the samples was analysed by spectrophotometric and chromatographic techniques and the results were coupled to multivariate analysis. Field observation of the foraging behaviour of *Apis mellifera* L. revealed its preference for the *Baccharis dracunculifolia* DC species. *p*-Coumaric acid, 3, 4-dicaffeoylquinic acid, 3, 5-dicaffeoylquinic acid, drupanin, and artepillin C which were identified in both plant and propolis samples. Moreover, higher artepillin C amounts have been detected in samples collected in the summer and autumn, while the main compounds of *p*-coumaric acid and quercetin were available in spring and winter sampled propolis, respectively. *Baccharis dracunculifolia* has been identified as a plant species preferred by *A. mellifera* in foraging resin for the production of propolis in southern Brazil. The contents of balsam, total phenolic compounds, and flavonoids varied significantly over the seasons, with values above the minimum required by the legislation, thus assuring a quality pattern for this biomass.

Keywords: *Baccharis dracunculifolia*, honeybees, phenolics, seasons

Introduction

Propolis is a substance collected by honeybees from plant sources which is added to salivary enzymes, beeswax, and pollen (Bankova et al., 2000). Bees use propolis to seal openings in the hive, deter the entrance of intruders and maintain the hive temperature close to 35 °C (Salatino et al., 2005). The chemical heterogeneity of propolis is widely known since it has numerous biological properties (Marcucci, 1995) such as antibacterial (Sforcin et al., 2000), antifungal (Sforcin et al., 2001), antiviral (Búfalo et al., 2009), anti-tumoral (Bassani-Silva et al., 2007), immunomodulatory (Orsatti et al., 2010), anti-diabetic (Zamami et al., 2007) and anti-ulcer (Barros et al., 2007) attributes.

Bees use different botanical sources for the production of propolis, and may even use more than one botanical source. Numerous studies have confirmed that different species of the *Populus* spp. are resin donors for the production of propolis *P. nigra* known to be a major resin donor in Europe, North America, and non-tropical regions of Asia and New Zealand (Greenaway et al., 1988; Markham et al., 1996; Bankova et al., 2000). Phytochemical analysis was an aid in proving that the *Populus alba* species had been correctly identified as the main botanical source of propolis in southern Brazil, Argentina and Uruguay (Park et al., 2002). In Venezuela, the *Clusia* spp. had been identified as the botanical source for the production of this propolis (Cuesta-Rubio et al., 2002).

In Brazil, several types of propolis with different chemical compositions have been identified due to the higher plant diversity (Paganotti et al., 2014). The two best known and studied botanical sources of Brazilian propolis are *Dalbergia* spp. and *Baccharis dracunculifolia* DC (Salatino et al., 2005; Daugsch et al., 2008). The species *B. dracunculifolia* is the main botanical source of the green propolis found in southeastern Brazil (Bastos et al., 2011; Oliveira and Bastos, 1999).

*Baccharis dracunculifolia* is a perennial, dioecious shrub native to Brazil. Its leaves have glandular trichomes which act as a barrier to predatory insects and assist in the interaction of the species with bees to collect the plant resin (Oliveira and Bastos, 1999). The species has great importance due to its secondary metabolite composition, which, for the most part, include the compound known commercially as Artepillin C®, a fingerprint marker for green propolis. The compound has a high market value with several properties beneficial to human health such as tumor treatments (Ahn et al., 2007).

Knowledge of plant sources used by bees for the production of propolis and identification of the effects of seasonal changes on the chemical profile of the propolis are extremely important in standardizing such biomass. Therefore, field observations of the bees’ foraging behavior together with chromatographic techniques were applied to the elucidation of the botanical sources, chemical profile, the identification of the seasonal effect on the chemical profile and the evaluation of the quality standard of propolis of *Apis mellifera*. 
Materials and Methods

Characterization of the study site

The foraging behavior of *A. mellifera* was monitored by visual observation in relation to the collection of resin by bees. These observations were performed from the field site in the city of São Joaquim, in the state of Santa Catarina, southern Brazil during all four seasons in 2014 and the summer and autumn of 2015. The field work was carried out on four apiaries in the region: Apiary 1 - 24° 28' 22.9" S, 49°56'14.4" W; Apiary 2 - 24° 28' 6.7" S, 49°56'14.1" W; Apiary 3 - 24° 27' 51.7" S, 49°56'22.3" W; Apiary 4 - 24° 28' 14.9" S, 49°56'26.4" W (average altitude = 1,360 m). The minimum distance between these apiaries was 420.96 m and the maximum 1,650.06 m. The area under study is a vegetation zone known as “Mix Ombrophile Forest”.

The foraging behavior was defined by a consideration of the botanical species that were close to the four apiaries producing propolis. The behavior of the insects was recorded with a digital camera just after the arrival of the bees at the plant for the collection of resin on the branch apex. In order to determine the botanical origin, resin samples from the donor plants foraged by the bees were first collected, and then compared with propolis samples harvested from a beehive in a nearby area through chemical analysis. The plant resin was sampled in the spring of 2014 and the summer and autumn of 2015 (n = 3). The plants collected were taxonomically identified and a voucher specimen was deposited under the reference number FLOR0067646 [Florianópolis, in the state of Santa Catarina, Brazil].

Propolis and plant resin extraction

Propolis samples (n = 19) were harvested from the previously described four apiaries in all seasons of 2014 and the summer and autumn of 2015. The preparation of hydroalcoholic extract (HE) of propolis and plant resins followed the protocol described by Popova et al. (2004), with a number of modifications. Propolis and plant resin samples (500 mg) were added to 25 mL 70 % ethanol (v/v) and incubated for 24 h. The extracts were filtered through a cellulose support under vacuum, and the filtrate collected to which EtOH 70 % (v/v) was added making a final volume of 25 mL. Waxes present in the propolis extract were eliminated by freezing and filtering the extract. The HE of propolis was used for the determination of the total phenolic, flavonoid, and balsam contents.

Reversed-phase high-performance liquid chromatography (RP-HPLC-UV)

The phenolic compounds were analyzed by RP-HPLC-UV using a Thermo Scientific Dionex UltiMate 3,000 liquid chromatograph equipped with a C18 reversed phase column [BioBasic-18, 150 mm x 4.6 mm Ø, 5 µm], thermostated at 40 °C and a UV detector. Samples were eluted at 0.8 mL min⁻¹, using a linear gradient of 0.5 % formic acid [v/v] [solvent A] and methanol [Solvent B], starting with 15 % B [0-10 min], increasing to 70 % B [10-55 min], then decreasing to 15 % B [55-60 min]. Chromatograms were recorded at 280 nm. For the quantification of compounds, an external standard curve of artepillin C (y = 0.2461x, r² = 0.99) at concentrations of 56.25, 112.5, 225, 450, and 900 µg mL⁻¹ was used. The metabolite contents were expressed in equivalent [µg mL⁻¹] artepillin C, considering the average of three consecutive injections/samples. The phenolic compounds were identified by comparing the retention times of authentic standard samples which were injected into a mass spectrometer to confirm the compound identity as described below.

Mass spectrometry - ESI (+)-MS/MS

An aliquot (10 µL) of methanolic extract was diluted in 990 µL of methanol containing 0.1 % NH₃·OH (w/v) to obtain the mass spectra in a Bruker MicroTOF QII spectrometer using a quadrupole/TOF hybrid mass analyzer. Spectra were recorded in negative mode, setting up the ion source and detector configurations as follows: electrospray ionization source [IES], 2.5 kV capillary voltage, desolvation temperature at 200 °C, and scanning window at mass acquisition between 80 and 1,000 m/z. The total time taken to obtain the mass spectra was one min. The spectra in MS/MS were obtained using collision energy of 35 eV.

Quality analysis of propolis

The quality of the southern Brazilian propolis was evaluated in accordance with Norm n° 3, issued by the Ministry of Agriculture, Livestock and Food Supply, Brazil, in January 2001 [MAPA, 2001]. The minimum and maximum values required by legislation for the alcoholic extract of propolis were not less than 0.50 % (w/w) for Phenolic compounds, 0.25 % (w/w) for Flavonoids and 11 % (w/v) for Balsam.

Total phenolic content

Total phenolic contents were determined by the Folin-Ciocalteau reagent (FCR) method. An aliquot (40 µL) of propolis HE was added to 3.16 mL distilled water, 200 µL Folin-Ciocalteau reagent, 600 µL 10 % Na₂CO₃ in water [w/v] and incubated for 2 h. Next, the solution was transferred into a quartz cuvette (3 mL) and the absorbance measured at 760 nm in a UV-Vis spectrophotometer. Calibration was achieved by using an external standard curve of gallic acid (y = 0.0013x, r² = 0.99) at concentrations of 100, 300, 500, 700, 1,100, 1,300, 1,500, 2,000, and 2,500 µg mL⁻¹ [Folin and Ciocalteau, 1927].
Total flavonoid content
The flavonoid content was determined by the aluminum chloride colorimetric method (Popova et al., 2004). An aliquot (200 µL) of propolis HE was added to 4.7 mL methanol and 100 µL 5 % AlCl₃ in methanol (w/v) and left to stand for 30 min. The solution was then transferred to a quartz cuvette (3 mL) and the absorbance recorded at 425 nm. Calibration was performed by means of an external standard curve of quercetin at concentrations of 5, 25, 50, 100, 300, 400, and 500 µg of quercetin mL⁻¹ (\(y = 0.0028x, r^2 = 0.98\)).

Balsam content
The balsam content was determined by the gravimetric method (Popova et al., 2004). In order to perform this, three HEs were concomitantly prepared as described above. Aliquots (5 mL) of propolis HEs were transferred to a crucible porcelain pot and evaporated in an oven (60 °C) to constant mass. The percentage of balsam in the extracts was calculated by mass difference before and after the evaporation of the solvent. This procedure was carried out in triplicate.

Exploratory data analysis
The chromatographically profiled data of plant and propolis samples were processed using multivariate statistical techniques, i.e., principal component analysis (PCA) and hierarchical clustering analysis (HCA). For the HCA, the Euclidean distance between two samples was used as the similarity parameter; while the unweighted arithmetic average (UPGMA) method was used for the hierarchical clustering process. For this purpose, scripts were written in the R (v. 3.1.1) language using the packages specmine, ChemoSpec, and hyperSpec packages. Data were presented in terms of mean ± standard deviation of three repetitions (n = 3). The F-test of analysis of variance (ANOVA) was applied and, where significant, the data were submitted to the Tukey and Scott-Knott tests at 5 % probability (\(p < 0.05\)).

Results and Discussion

Observation of the bee’s foraging behavior
The field observations allowed identifying several honeybees (Apis mellifera) foraging the native species Baccharis dracunculifolia, in different populations close to the apiaries. The honeybees were recorded fragmenting the vegetative apex for resin exudation and using their first pair of legs to collect and transfer the resin to the opposite corbicular, as described in Figure 1. Importantly, Teixeira et al. (2005) and Park et al. (2004) have related the preference of bees foraging B. dracunculifolia resin as they collected resins from leaf buds and both unexpanded and expanded leaves. Other plant species present in the field were also monitored, such as Araucaria angustifolia (Bertol.) Kuntze, but no honeybees were recorded foraging this plant species. Thus, the field observations of the behavior of Apis mellifera allowed for identifying a predilection for B. dracunculifolia.

The chemical profile of propolis HE determined by RP-HPLC-UV was compared with the profile of B. dracunculifolia to determine the botanical source.

Identification of botanical source of propolis – chemical analysis
The chromatographic profiles of B. dracunculifolia were similar in all seasons and the highest contents of artepillin C were found in the plant samples collected in the autumn, \(p < 0.05\) (Table 1). Interestingly, a remarkable likeness was detected between the HEs of B. dracunculifolia and summer-collected propolis samples (Figure 2), 3-Coumaric acid, 3, 4-dicaffeoylquinic acid, 3, 5-dicaffeoylquinic acid, drupanic, and artepillin C were identified in both B. dracunculifolia and propolis (Table 1). The compounds identified by RP-HPLC-UV were confirmed by direct injection into ESI-MS (Figure 1) by the fragmentation of the compounds as described in Table 2.

A similar result was found by Simões-Ambrosio et al. (2010) that detected increases in artepillin C amounts in B. dracunculifolia samples in the period from Feb (summer) to Apr (autumn). Furthermore, it has been reported that due to the interaction between B. dracunculifolia and A. mellifera, the best time to produce propolis rich in artepillin C in southeastern Brazil, is from Dec (summer) to Apr (autumn) (Sforcin et al., 2012). Moreover, Bastos et al. (2011) noted that bees do not collect the resinous material in the blooming stage from B. dracunculifolia, but rather in the growth stage, a period when secondary metabolites important to the interaction with insect and predators seem to be produced.

In a follow-up experiment, hierarchical clustering (HCA) and principal components (PCA) analyses were applied to the chromatographic profile dataset to gain insights into the botanical source (Figure 3 and 4). The

Figure 1 – Apis mellifera collecting B. dracunculifolia resin for the production of propolis.
resulting classification tree revealed samples discriminated into three main groups (Figure 3). The first one had two samples collected in the summer "sum3" and "sum8". These samples, when analyzed by RP-HPLC-UV showed identical chromatographic profiles. The second group consisted of B. dracunculifolia samples ("bac_sum", "bac_spr" and, "bac_aut"), three samples of propolis from the summer ("sum1", "sum2", and "sum6") and one from the autumn ("aut3"). These samples had similar chromatographic profiles, especially the following compounds; acid ρ-coumaric, 3, 4-dicaffeoylquinic acid, 3, 5-dicaffeoylquinic acid, drupanin, and artepillin C. Finally, the third group contained samples from all four seasons, and within this group, each smaller cluster consisted of samples from the same season. For the PCA, the first three components PC1 (30 %), PC2 (24 %), and PC3 (13 %) explained 67 % of the total variance in the dataset (Figure 4). In addition, PCA identified a cluster of two samples summer, "sum3" and "sum8" grouped in PC1 and PC2 negative.
of its typical green color (Instituto Mineiro de Agropecuária, 2011; Hata et al., 2012). Green propolis is characterized by the presence of artepillin C as a biochemical marker at high concentrations. For comparative purposes, a green propolis sample from southeastern Brazil was also analyzed by PCA and RP-HPLC-UV. The chromatographic profiles suggest significant similarity between the green propolis, the summer-collected propolis in São Joaquim county, and *B. dracunculifolia* resin, particularly in relation to the acid compounds ρ-coumaric, 3, 4-dicaffeoylquinic acid, 3, 5-dicaffeoylquinic acid, drupanin, and artepillin C (Table 1).

**Figure 2** – Chromatographic profiles (RP-HPLC-UV, λ = 280 nm) of hydroalcoholic extracts of branch apexes of *B. dracunculifolia* and propolis. In each spectrum the peak numbers represent the same compounds, i.e., peaks (2) ρ-coumaric acid, (3) 3, 4-dicaffeoylquinic acid, (4) 3, 5-dicaffeoylquinic acid, (7) drupanin, and (10) artepillin C.

**Figure 3** – Hierarchical clustering dendrogram (UPGMA method) of hydroalcoholic extracts (HE) of propolis samples collected during the summer (sum), spring (spr), autumn (aut), and winter (win) in 2014 and 2015 of branch apexes of *B. dracunculifolia* (bac).

*Baccharis dracunculifolia* has been described as the most important botanical source of southeastern Brazilian propolis, which is known as green propolis because of its typical green color (Instituto Mineiro de Agropecuária, 2011; Hata et al., 2012). Green propolis is characterized by the presence of artepillin C as a biochemical marker at high concentrations. For comparative purposes, a green propolis sample from southeastern Brazil was also analyzed by PCA and RP-HPLC-UV. The chromatographic profiles suggest significant similarity between the green propolis, the summer-collected propolis in São Joaquim county, and *B. dracunculifolia* resin, particularly in relation to the acid compounds ρ-coumaric, 3, 4-dicaffeoylquinic acid, 3, 5-dicaffeoylquinic acid, drupanin, and artepillin C (Table 1).

**Determination of chemical composition of propolis – seasoning effect**

The various propolis studied differed in their chromatographic profiles over the seasons (Table 1). In this study, propolis samples collected in the spring and winter showed very low levels or absence of artepillin C. The highest content of quercetin, 19.12 ± 0.05 to 34.58 ± 0.39 mg g⁻¹ (*p* < 0.05), was found in the win-
of propolis and seasonality was also studied by Nunes et al. [2018]. Samples produced in the south and southeastern regions of Brazil were identified as the major constituents in propolis, and cinnamic acid derivatives were detected in the summer-harvested propolis. In general, the highest levels of artepillin C were found in almost all collecting seasons, with quantitative and qualitative differences between them. Artepillin C was detected at higher levels in samples collected between Nov (spring) and Dec (summer), similar to the findings herein described, where the highest levels of that compound were detected in the summer-harvested propolis. In general, prenylated compounds and cinnamic acid derivatives were identified as the major constituents in propolis samples produced in the south and southeastern regions of Brazil [Marcucci et al., 2000].

The relationship between the chemical profile of propolis and seasonality was also studied by Nunes and Guerreiro [2012], through multivariate statistical techniques. The PCA of the compounds identified allowed for the separation of the samples into three main groups according to their respective collection seasons, i.e., summer, spring, and autumn. The main compounds identified by those authors varied greatly in the samples throughout the seasons of the year. This result was expected because it is well known that the chemical composition of propolis is directly related to the metabolism of the plant source resin, which undergoes seasonal variation caused by one or more biotic factors.

In Brazil, due to its huge plant diversity, a large number of plant species sources of resin for the production of propolis is found, which complicates the elucidation of the preference criteria used by bees for the selection of resin sources. Thus, at certain times over the year bees have more than one plant donor source of resin for the production of propolis, as noted in southern Brazil. In this study, it is worth mentioning that other plant species not as yet identified taxonomically were also found to donate resin for the production of propolis, especially during the spring and winter. However, multivariate analysis applied to the RP-HPLC-UV data set suggest that for certain propolis samples such as "sum1", "sum2", "sum6", and "aut3" the main botanical source of resin is B. dracunculifolia. Moreover, it is important to note that 68 % of the analyzed propolis samples presented artepelin C in their composition, a well-known chemical signature of the botanical species B. dracunculifolia.

Determination of physicochemical parameters of propolis

In order to ensure the quality of apiarian products in Brazil, the Ministry of Agriculture, Livestock and Food Supply published Norm No. 3 in Jan 2001 [MAPA, 2001], which established physicochemical parameters to regulate the quality of propolis and its extracts in the market. Among the physicochemical characteristics the minimum and maximum amounts of phenolics, flavonoids, and dry extract [balsam] stand out.

The results from Table 3 reveal the highest content of phenolics (7 % ± 0.51) [p < 0.05] in the summer/2014-collected samples, while propolis harvested in the autumn/2014 contained the lowest flavonoid amount (0.37 % ± 0.04). As regards the balsam numbers, the autumn/2014 samples revealed a low content, i.e., 19 % ± 4.99 [p < 0.05], as the remaining samples were similar, ranging from 34 % [summer/2015] to 39 % [spring/2014], p < 0.05.

All the propolis samples met the minimum quality parameters required by the Ministry of Agriculture, Livestock and Food Supply of Brazil phenolic (0.5 % w/w), flavonoid (0.25 % w/w), and balsam (11 % w/w) contents. These findings are in agreement with the work of Castro et al. [2007], identifying similar amounts of total phenolics and flavonoids in propolis samples from southeastern Brazil. In addition, in this study, a positive correlation between the flavonoid and phenolic contents...
Table 3 – Total phenolic, flavonoid, and balsam (%) contents of the hydroalcoholic extract of propolis samples from southern Brazil.

| Seasons       | Total phenolics* | Total flavonoids** | Balsam    |
|---------------|------------------|--------------------|-----------|
| Summer/2014   | 7.00 ± 0.51 a    | 1.00 ± 0.12 a      | 38.00 ± 12.12 a |
| Autumn/2014   | 4.00 ± 0.28 b    | 0.37 ± 0.04 b      | 19.00 ± 4.99 b  |
| Winter/2014   | 5.00 ± 0.42 b    | 0.91 ± 0.07 a      | 35.00 ± 8.47 a  |
| Spring/2014   | 5.00 ± 0.43 b    | 1.00 ± 0.21 a      | 39.00 ± 8.69 ab |
| Summer/2015   | 4.00 ± 0.37 b    | 1.00 ± 0.06 a      | 34.00 ± 7.34 ab |
| Autumn/2015   | 5.00 ± 0.37 b    | 1.00 ± 0.07 a      | 37.00 ± 4.04 a  |

Values are reported as means ± SD (n = 3). Letters indicate significant differences (Tukey’s test, p < 0.05). *Gallic acid equivalent. **Quercetin equivalent.

Acknowledgments

The financial support by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) is acknowledged [Process no 490383/2013-0]. This study was partially funded by Project PropMine, funded by the agreement between Portuguese Fundação para a Ciência e a Tecnologia and Brazilian CNPq [Process no 73: 627-648]. The owners of the Apiário Real® Company are also acknowledged for their logistical support and the supply of propolis samples. The authors also wish to thank the “Financiamento do Plano Estratégico de Unidades de I&D” of UID/BIO/04469/2013 unit. The researcher fellowship from CNPq on behalf of M. Maraschin is also acknowledged.

Authors’ Contributions

Conceptualization: Tomazzoli, M.M.; Somensi-Zeggeo, A.R.; Specht, L.; Maraschin, M. Data acquisition: Tomazzoli, M.M.; Specht, L.; Pai-Neto, R.D. Data analysis: Tomazzoli, M.M.; Costa, C.; Rocha, M.; Maraschin, M. Design of methodology: Tomazzoli, M.M.; Costa, C.; Rocha, M.; Maraschin, M. Software development: Costa, C.; Rocha, M.; Maraschin, M. Writing and editing: Tomazzoli, M.M.; Somensi-Zeggeo, A.R.; Specht, L.; Pai-Neto, R.D.; Yunes, R.A.; Rocha, M.; Maraschin, M.

References

Ahn, M.; Kunimasa, K.; Ohta, T.; Kumazawa, S.; Khamihira, M.; Kaji, K.; Uto, Y.; Hori, H.; Nagasawa, H.; Nakayama, T. 2007. Suppression of tumor-induced angiogenesis by Brazilian propolis: major component artepillin C inhibits in vitro tube formation and endothelial cell proliferation. Cancer Letters 252: 235-243.

Bankova, V.S.; Castro, S.L.; Marucci, M.C. 2000. Propolis: recent advances in chemistry and plant origin. Apidologie 31: 3-15.

Barros, M.P.; Sousa, J.P.B.; Bastos, J.K.; Andrade, S.F. 2007. Effect of Brazilian green propolis on experimental gastric ulcers in rats. Journal of Ethnopharmacology 110: 567-571.

Bastos, E.M.A.E.; Santana, R.; Calaça-Costa, A.G.F.; Thiago, P.S. 2011. Interaction between Apis mellifera L. and Baccharis dracunculifolia DC, that favours green propolis production in Minas Gerais. Brazilian Journal of Biology 71: 727-734.

Búfalo, M.C.; Figueiredo, A.S.; Sousa, J.P.B.; Candeias, J.M.G.; Bastos, J.K.; Sforcin, J.M. 2009. Anti-poliovirus activity of Baccharis dracunculifolia and propolis by cell viability determination and real-time PCR. Journal of Applied Microbiology 107: 1669-1680.

Castro, M.L.; Cury, J.A.; Rosalen, P.L. 2007. Propolis from southeastern and northeastern of brazil: the influence of seasonality in antibacterial activity and phenolic composition. Quimica Nova 30: 1512-1516 (in Portuguese, with abstract in English).

Cuesta-Rubio, O.; Frontana-Urbe, B.A.; Ramirez-Apan, T.; Cardenas, J. 2002. Polyisoprenylated benzenophenones in Cuban propolis: biological activity of nemorosone. Zeitschrift fur Naturforschung C 57: 372-378.

Daugsch, A.; Moraes, C.S.; Fort, P.; Park, Y.K. 2008. Brazilian red propolis: chemical composition and botanical origin. Evidence-based Complementary and Alternative Medicine 5: 435-441.

Folin, O.; Ciocalteau, V. 1927. On tyrosine and tryptophane determinations in proteins. The Journal of Biological Chemistry 73: 627-648.
Greenaway, W.; Scaysbrook, T.; Whatley, F.R. 1988. Composition of propolis of Oxfordshire, UK, and its relation to poplar bud exudate. Zeitschrift für Naturforschung 43: 301-304.

Hata, T.; Tazawa, S.; Ohita, S.; Rhyu, M.R.; Misaka, T.; Ichihara, K. 2012. Artepillin C, a major ingredient of Brazilian propolis, induces a pungent taste by activating TRPA1 channels. PLoS ONE 7: e48072.

Instituto Mineiro de Agropecuária. 2011. Designation of Origin in the State of Minas Gerais within the Scope of the Production of Green Propolis, Ordinance n° 1138 of 13 May 2011 = Instituto Denominação de Origem no Estado de Minas Gerais no Âmbito da Produção de Própolis Verde, Portaria n° 1138 de 13 de maio de 2011. IMAP, Belo Horizonte, MG, Brazil [in Portuguese].

Kujumgiev, A.; Tsvetkova, I.; Serkedjieva, Yu.; Bankova, V.; Christov, R.; Popov, S. 1999. Antibacterial, antifungal and antiviral activity of propolis from different geographic origin. Journal of Ethnopharmacology 64: 235-240.

Markham, K.R.; Mitchell, K.A.; Wilkins, A.L.; Daldy, J.A.; Lu, Y. 1996. HPLC and GC-MS identification of the major organic constituents in New Zealand propolis. Phytochemistry 42: 205-211.

Mencighelli, C.; Joaquim, L.S.D.; Félix, G.L.Q.; Somensi, A.; Tomazzoli, M.M.; Silva, D.A.; Berti, F.V.; Veleirinho, M.B.R.; Recouvreux, D.O.S.; Zeri, A.C.M.; Dias, P.F.; Maraschin, M. 2013. Southern Brazilian autumnal propolis shows anti-angiogenic activity: an in vitro and in vivo study. Microvascular Research 88: 1-11.

Ministério da Agricultura, Pecuária e Abastecimento [MAPA]. 2001. Norm Instruction n° 3, 19 Jan 2001 = Instrução Normativa n° 3, de 19 de Janeiro de 2001. MAPA, Brasília, DF, Brazil [in Portuguese].

Nunes, C.A.; Guerreiro, M.C. 2012. Characterization of Brazilian green propolis throughout the seasons by headspace GC/MS and ESI-MS. Journal of the Science of Food and Agriculture 92: 433-438.

Oliveira, V.C.; Bastos, E.M. 1999. Morphological and anatomical aspects of the leaf of Baccharis dracunculifolia DC. (Asteraceae) as regards to the identification of the botanical origin of propolis. Acta Botanica Brasilia 12: 431-439 [in Portuguese, with abstract in English].

Orsatti, C.L.; Missima, F.; Pagliaroni, A.C.; Bachiega, T.F.; Búfalo, M.C.; Araújo Jr, J.P.; Araújo Jr, J.M. 2010. Propolis immunomodulatory action in vivo on toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. Phytotherapy Research 24: 1141-1146.

Paganiotti, R.S.N.; Rezende, J.C.; Barbeira, P.J.S. 2014. Discrimination between producing regions of Brazilian propolis by UV-VIS spectroscopy and partial least squares discriminant analysis. Current Analytical Chemistry 10: 537-544.

Park, Y.K.; Alencar, M.S.; Scamparini, A.R.P.; Aguiar, C.L. 2002. Propolis produced in south Brazil, Argentine and Uruguay: phytochemical evidence for the plant origin. Ciência Rural 32: 997-1003 [in Portuguese, with abstract in English].

Park, Y.K.; Paredes-Guzman, J.F.; Aguiar, C.L.; Alencar, S.M.; Fujiwara, F.Y. 2004. Chemical constituents in Baccharis dracunculifolia as the main botanical origin of Southeastern Brazilian propolis. Journal of Agricultural and Food Chemistry 52: 1100-1103.

Popova, M.; Bankova, V.; Butovska, D.; Petkov, V.; Nikolova-Damyanova, B.; Sabatini, A.G.; Marazzan, G.L.; Bogdanov, S. 2004. Validated methods for the quantification of biologically active constituents of propolis-type propolis. Phytochemical Analysis 15: 235-340.

Salatino, A.; Teixeira, É.W.; Negrì, G.; Message, D. 2005. Origin and chemical variation of Brazilian propolis. Evidence-Based Complementary and Alternative Medicine 2: 33-38.

Sforcin, J.M.; Fernandes, A.; Lopes, C.A.M.; Bankova, V.; Funari, S.R.C. 2000. Seasonal effect on Brazilian propolis antibacterial activity. Journal of Ethnopharmacology 73: 243-249.

Sforcin, J.M.; Fernandes, A.; Lopes, C.A.M.; Funari, S.R.C.; Bankova, V. 2001. Seasonal effect of Brazilian propolis on Candida albicans and Candida tropicalis. Journal of Venomous Animals and Toxins 7: 139-144.

Sforcin, J.M.; Souza, J.P.B.; Filho, A.A.D.S.; Bastos, J.K.; Búfalo, M.C.; Tonuci, L.R.D.S. 2012. Baccharis dracunculifolia, One of the Main Plant Sources of Brazilian propolis = Baccharis dracunculifolia, uma das Principais Fontes Vegetais da Própolis Brasileira. UNESP, São Paulo, SP, Brazil [in Portuguese].

Simões-Ambrosio, L.M.C.; Gregório, L.E.; Sousa, J.P.B.; Figueiredo-Rinhel, A.S.G.; Azzolini, A.E.C.S.; Bastos, J.K.; Lucisano-Valim, Y.M. 2010. The role of seasonality on the inhibitory effect of Brazilian green propolis on the oxidative metabolism of neutrophils. Fitoterapia 81: 1102-1108.

Teixeira, É.W.; Negrì, G.; Meira, R.M.S.A.; Message, D.; Salatino, A. 2005. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. Evidence Evidence-based Complementary and Alternative Medicine 2: 85-92.

Thomé, V.M.R.; Zampieri, S.; Braga, H.J.; Pandolfo, C.; Silva Júnior, V.P.; Bacic, I.; Laus Neto, J.; Soldatelli, D.; Gebler, E.; Ore, J.D.; Echeverria, L.; Mattos, M.; Suski, P.P. 1999. Agroecological and Socioeconomic zoning of Santa Catarina state = Zoneamento Agroecológico e Socioeconômico de Santa Catarina. EPAGRI, Florianópolis, SC, Brazil [in Portuguese].

Vibrans, A.C.; Sevegnani, L.; Gasper, A.L.; Língner, D.V. 2012. Floristic and Forest Inventory of Santa Catarina: diversity and conservation of forest remnants = Inventário Florístico Florestal de Santa Catarina: Diversidade e Conservação dos Remanescentes Florestais. Edifurb, Blumenau, SC, Brazil [in Portuguese].

Zamami, Y.; Takatori, S.; Koyama, T.; Goda, M.; Iwatani, Y.; Doi, S.; Kawasaki, H. 2007. Effect of propolis on insulin resistance in fructose-drinking rats. Yakugaku Zasshi 127: 9085-2073 [in Japanese, with abstract in English].

Sci. Agric. v.77, n.6, e20180258, 2020