Antioxidant Activity of Brazilian Organic Propolis and Its Relation to Seasonal Variation

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Abstract: Propolis is a non-toxic, brownish resinous mixture of organic constituents and volatiles compounds collected from the plant buds and exudates by Apismellifera bees. It has a variety of biological effects, such as antiviral, antimicrobial, anti-inflammatory, anticancer, and antioxidant activities. Propolis is a rich source of phenolic compounds, as well as environmental influences its chemical composition. In this present study, the evaluation of propolis was performed and produced collected from south Brazil. The aim of the research was to analyze the antioxidant activity of the propolis samples collected in different times during summer, autumn, and spring. The organic propolis was analyzed by CG-MS. For flavonoid content, summer was the season that presented the most varied values. For total phenolic content as well as for the antioxidant activity, autumn was the season which showed the highest values. The volatiles found in this type of propolis brought glimpses about its biological activities. In conclusion, certified organic propolis shows a high potential of use by the pharmaceutical and food industry, as well as on functional food manufacturing.

Keywords: Organic propolis, Antioxidant activity, Phenolic compounds, Flavonoids, Season.

I. Introduction

Apismellifera bees produce propolis while they visit and collect exudates from plant buds and sprouts, and bark of trees. The biodiversity found in the site of hives affects the propolis chemical composition (Aguero et al., 2011) that contains mainly, antioxidant compounds such as phenolics, flavonoids, phenolic acids, esters, aldehydes, and ketones. All these chemical substances present not only antioxidant activity, but also antimicrobial, anti-inflammatory, antiviral, and antifungal activities (Duarte et al., 2008; Oldoni et al., 2011; Bezerra et al., 2012; Silva et al., 2013). Geographical variations as well as the botanical origin influence the chemical composition and the color of propolis (Bonvehí and Gutiérrez, 2012). These variations influence the chemical characteristics of propolis occur due to the flora, climate, and seasonal variation found in the site of production (Barlak et al., 2011). The assessment of the chemical profile of phenolic and flavonoid compounds in propolis is a relevant procedure not only for characterizing the origin of the samples, but also for guaranteeing its commercial quality (Piccinelli et al., 2011). Brazilian propolis has gained popularity as a healthy food supplement and has been extensively used in foods and beverages, where it is claimed to improve health and prevent ailments such as inflammation, heart diseases, diabetes, and even cancer (Oldoni et al., 2011). Previous data state that propolis from Brazil and Venezuela is poor in flavonoids (Bonvehí and Gutiérrez, 2012). In Brazil, propolis has been classified in 13 botanically types. However, only four types out of them have been botanically identified; type 4 (Southern region) Populus spp., type 6 (Northeast region) Hyptis didiaricata, type 12 (green propolis, Southeast region) Baccharis dracunculifolia, and type 13 (red propolis, Northeast region) Dalbergia castellaweyi (L.) Tau (Park et al., 2002; Silva et al., 2008).

Seasonality is also a predominant factor that influences the propolis chemical composition since, the different seasons influence the phenology of the plants, the biosynthesis, and the accumulation of diverse secondary metabolites such as coumarins, flavonoids, lignins, tannins, terpenes, carotenoids, essential oils, saponins, cinnamogenic glycosides, glicosinolates, and alkaloids (Cartea et al., 2011). These metabolites are considered a natural defense strategy of the plants against insects and parasites (Bastos et al., 2011). The metabolites extracted from the plants vary due to environmental clues such as temperature, humidity, drought,
soil type, as well as the duration and the intensity of sunlight (Aguero et al., 2011). Considering that, the highest propolis production is assumed to occur during the summer and rainy seasons when plants are on flowering time and sprouting phase. The propolis collected in Minas Gerais in March, May, and November presented a high antioxidant activity, in which the derivatives of cinamic acid were the most abundant (Teixeira et al., 2010). Thus, seasonality and the time of propolis collection were responsible for the chemical profile of the compound (Sulaiman et al., 2011), and the climate, altitude, latitude, and rain precipitation differences corroborated with the concentration and variability of the active substances in a propolis produced in apicole seasons (Bastos et al., 2011). All these factors influence the antimicrobial, antioxidant, and antiviral activities of propolis (Antunes, 2011).

Organic propolis is produced in natural environments found in native forests, in reforestation areas, where it is believed to be a contamination and pollution free-zone. The production of organic propolis is certified, whereby it is audited periodically by the organic certifying agencies, and it can be traded if it presents, for example, the National Organic Program (NOP) from the United States Department of Agriculture, Kiwa BCS Öko-Garantie GmbH (Kiwa, 2016), and CEE (European) (Kiwa, 2016) as well as to Brazilian regulations (IMO, 2016). The main aim of this study was to evaluate the antioxidant activity of organic propolis samples collected during the summer, autumn, and spring seasons, as well characterize the volatile compounds.

II. Materials and Methods

A number of 78 propolis samples certified as organic propolis were collected in a period between February 21, 2011 and January 05, 2012 by 11 beekeepers responsible for bee apiary located in 14 municipalities, in which 10 are located in southern Paraná state and 4 in the northern part of Santa Catarina state. The municipalities were Mato Rico (24° 42' S, 52° 8' W), Turvo (28° 55' S, 49° 41' W), Prudentópolis (25° 12' S, 50° 58' W), Campo Magro (25° 22' S, 49° 27' W), Campo Largo (25° 27' S, 49° 31' W), Irati (25° 28' S, 50° 39' W), Pinhão (41° 11' S, 7° 32' W), Bituruna (26° 9' S, 51° 33' W), União da Vitória (26° 13' S, 51° 5' W), General Carneiro (26° 25' S, 51° 19' W), Canoinhas (26° 10' S, 50° 23' W), Três Barras (26° 7' S, 50° 18' W), Papanduva (26° 22' S, 50° 8' W) and Santa Terezinha (25° 26' S, 54° 24' W).

Sample collection was performed according to the rules of international certification of organic production and handling operations, namely National Organic Program (NOP) from the United States Department of Agriculture (USDA, processes no. 22422 and 23511), Kiwa BCS Öko-Garantie GmbH 2016 (processes no. A-2016-00005/2016-01341 and A-2016-00005/2016-01342), and CEE (European) (processes no. 22422 and 23511) [12], as well as to Brazilian regulations (OrgânicosBrasil, processes no. PR 106 and 12-0030).

Propolis Extraction

The samples were cleaned and grounded using liquid nitrogen and kept at -18°C. The organic propolis extracts (OPEE) were prepared using 2 grams of each sample with 25 mL of ethanol (80%, v/v). The extraction was made in ultrasound bath for 15 minutes. Then, the extracts were refrigerated at 4°C for 24 hours, and filtered. Total phenolic content (TPC)

The total phenolic content (TPC) was analyzed according to the Folin-Ciocalteau method developed by Singleton et al. (1999). The TPC was measured as gallic acid equivalent. Folin-Ciocalteau reagent is a complex solution of polymeric ions formed by phosphomolybdic and phosphotungsticheteropolyacids. This reagent oxidizes phenolates, reducing the acids to a MoW (blue) complex.

The OPEs were diluted in a proportion of 1:50 or 1:100. An amount of 0.5 mL of the diluted sample was transferred to a tube, in which 2.5 mL of Folin-Ciocalteau was added. The reagent was diluted in water in a proportion of 1:10 and the mixture rested for about 8 minutes. After that, 2 mL of 4% Sodium carbonate were added to the tubes and they were left to stand for 2 hours in a dark room. The absorbance was measured by Shimadzu UVmini 1240 spectrophotometer in λ=740 nm. A blank sample was treated in the same conditions and the results of total phenolic content were shown as gallic acid equivalent (GAE).

Flavonoid content

The analyses of flavonoids content were performed according to the method developed by Park et al. (1995) using quercetin as standard. This reaction is based on formation of chelates between aluminum and flavonoids, mainly flavonols (3-hydroxyflavone) as quercetin, for example. In alcoholic solutions, flavonols and the aluminum chelate have a bathochromic shift of spectral band position in the absorption, reflectance, transmittance or emission spectrum, resulting in color alteration (Jurd and Geissman, 1956).

The colorimetric reaction was performed by mixing 0.5 mL of OPEE (1:2 or 1:5), 4.3 mL of ethanol: water 80% (v/v), 0.1 mL Aluminum chloride 10%, and 0.1 mL of 1M Potassium acetate. It was used 0.1 mL of potassium acetate and 4.9 mL of ethanol 80% (v/v) as control. After 40 minutes in a dark room, the absorbance of the samples was measured in the Shimadzu spectrophotometer UVmini 1240 in λ= 415 nm. The results of the flavonoid content were shown in quercetin based on its standard curve.
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Scavenging of synthetic free radical DPPH free radical scavenging assay

The determination of the radical scavenging activity of the OPEEs was carried out using DPPH\(^*\) (1, 1-diphenyl-2-picrylhydrazyl) assay as described by Mensor et al (2001). Since DPPH\(^*\) is a stable radical presenting a violet color, it accepts an electron or one hydrogen radical in order to become a stable molecule, which is reduced in the presence of antioxidants acquiring a yellow color. In the radical form, DPPH\(^*\) is absorbed in a \(\lambda=517\) nm, disappearing once it gets reduced by hydrogen donated by an antioxidant compound.

In a dark room, the reaction was formed by 0.5 mL of OPEs diluted in a proportion of 1:50 or 1:100, 3.0 mL of absolute ethanol 100%, and 0.3 mL of DPPH\(^*\) in a 0.5 mMethanolic solution. After that, the mixture was vortexed for 1 minute and incubated in a dark room for 20 minutes in room temperature. The activity of the anti-radical was determined in antioxidant activity (AA). As standard, 0.5 µM Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used. As control sample, it was used 0.5 mL of ethanol 80%, 3 mL of absolute ethanol 100%, and 300 µL of DPPH\(^*\). A blank sample was prepared with ethanol 100% to zero out the spectrophotometer.

ABTS free radical scavenging assay

For antioxidant activity, it was used the ABTS\(^{2,2'}\)-azinobis (3-ethyl-benzothiazoline-6-sulphonate). The radical ABTS\(^{·}\) was formed by the reaction between 7 mMABTS and 140 mM Potassium persulphate. This mixture was incubated in a temperature of 25° C, in a dark room for 12 hours. Once the radical was formed, it was diluted with ethanol until an absorbance of 0.700 nm ± 0.200 nm in \(\lambda=734\) nm was obtained. In a dark room, an aliquot of 3.0 mL of the ABTS solution was added to 30 µL of each OPE sample (1:5 or 1:10). The mixture was vortexed for 1 minute, left to stand at room temperature for 6 minutes. The absorbance of the solutions were measured in \(\lambda=734\) nm. As standard, 0 - 15 µM Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analog of vitamin E, was used. The antioxidant activity results were presented in TEAC (antioxidant activity equivalent to Trolox) (Rufino et al., 2007).

Analysis of volatile compounds

The volatile analysis was performed as described in the literature by Fu et al. (2009). The extracts were analyzed in headspace with 1 g of ground propolis in a temperature of 100°C in a GC-MS, Shimadzu modelQP 2010 Plus, by using a fused-silica capillary column (5% phenyl-95% polydimethylsiloxane, 30 x 0.25 mm; 0.25 µm film thickness) installed in a GC-MS (QP 2010 Plus) to separate the compounds. The injector temperature was 220°C. The employed column temperature was 40°C (2 min) - 200°C (15 min) at a rate of 4°C/min. The injection was done in a splitless mode and the Carrier gas (He) was 1.0 ml/min. The CG-MS operated in an ionization mode. The electrons were accelerated to 70 eV and scanned at 4-60 Da. The temperatures of the ions source as well as the GC-MS interface were 200 and 230°C, respectively. The GC-MS peaks were identified by comparison with data from the profiles from Wiley® 8 and 1,2 FFNSC.

Statistical Analysis

For the statistical analyses it was used the R Development Core Team software (2011). The Shapiro-Wilk test verified the normality of data; Kruskal-Wallis for comparing the medium content among the seasons, and Levene for comparing the variances among the seasons as well.

III. Results and Discussion

The organic propolis (OP) collected during summer time presented great variability in flavonoid content (Levene test, p=6%) in regard to the other seasons (Fig. 1). Through statistical analysis, major and minor variabilities in the content were detected. The OP with maximum flavonoid content was found in Irati city, 4.76 mg Q.E.g\(^{-1}\) (quercetin equivalent). Flavonoid content varied from 0.26 mg Q.E.g\(^{-1}\) to 0.76 mg Q.E.g\(^{-1}\) in spring, in which 25% of the samples showed 0.29 mg Q.E.g\(^{-1}\), 50% showed 0.34 mg Q.E.g\(^{-1}\), and 75% showed 0.76 mg Q.E.g\(^{-1}\). In autumn, the found results ranged from 0.0 mg Q.E.g\(^{-1}\) to 0.92 mg Q.E.g\(^{-1}\), in which 25% showed 0.09 mg Q.E.g\(^{-1}\), 50% showed 0.36 mg Q.E.g\(^{-1}\), and 75% showed 0.92 mg Q.E.g\(^{-1}\). Therefore, in summer, the results varied from 0.0 mg Q.E.g\(^{-1}\) to 2.31 mg Q.E.g\(^{-1}\), with a maximum of 4.76 mg Q.E.g\(^{-1}\). It is worth noting that 25% of the samples showed 0.11 mg Q.E.g\(^{-1}\), 50% 0.68 mg Q.E.g\(^{-1}\), and 75% showed 2.31 mg Q.E.g\(^{-1}\). These findings demonstrated that the organic propolis that was tested is poor in flavonoid content, corroborating with other analyses originated from Brazil and Venezuela (Souza et al., 2010; Bonvehíaudi Gutiérrez, 2012).
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Figure 1. Flavonoid content (mg QE.g⁻¹) in organic propolis (OP) samples collected in different seasons.

It is possible that the reason for having flavonoid content higher in summer, mainly in Irati city, is that temperature was higher (15° C - 27° C) than in autumn and spring. Regarding rain precipitation, it was similar to spring (179 mm to 180 mm). Besides that, the predominant vegetation found in that region is araucaria (Araucariaceae family) and pinus trees (Pinaceae family), which have in their chemical composition limonene (α-limonene and α-limonene, respectively), a major flavonoid (Hirota et al., 2012). It is likely that, since temperature was high in summer, the number of visits to the araucaria trees as well as to the pinus trees by the bees could have increased. In response to that, an increase of the amount of secondary metabolite was produced resulting in high flavonoid content.

For phenolics, statistical significance was found on their content among diverse seasons (Levene, p=0.04%). Phenolic content varied much more during autumn than in spring (Fig. 2). Thus, it can be claimed that phenolic content was more heterogeneous in autumn. Also, it was observed that phenolic content varied from 9.34 mg GAE.g⁻¹ (gallic acid equivalent) to 23.82 mg GAE.g⁻¹ in spring, 6.80 mg GAE.g⁻¹ to 38.54 mg GAE.g⁻¹ in autumn and 8.60 mg GAE.g⁻¹ to 23.60 mg GAE.g⁻¹ in summer. In autumn, it can be verified that General Carneiro city was the one that presented a maximum value of phenolic content (72.55 mg GAE.g⁻¹).

Nevertheless, other authors (CASTRO; CURY; ROSALEN, 2007) found in Green propolis (type 12) the highest phenolics content in spring and summer (77.15 +/- 0.98 mg GAE.g⁻¹ and 59.98 +/- 0.98 mg GAE.g⁻¹ to 75.15 +/- 2.25 mg GAE.g⁻¹, respectively) in regard to organic propolis.

Figure 2. Total phenolic content (mg GAE.g⁻¹) in organic propolis (OP) samples collected in different seasons.

Also, there was a significance difference among the seasons considering the mean values for ABTS⁺. In other words, the mean values for ABTS⁺ was significantly higher in spring than the other seasons, (p=0.3%, Kruskal-Wallis) (Fig. 3). Moreover, discrepant values were found in terms of antioxidant activity in
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Bituruna city during spring (127.40 mg Trolox.g\(^{-1}\)), 116.335 mg Trolox.g\(^{-1}\), 128.40 mg Trolox.g\(^{-1}\), and 78.38 mg Trolox.g\(^{-1}\), respectively for Santa Terezinha, Bituruna, and General Carneiro in summer. Discrepant values for autumn showed 99.78 mg Trolox.g\(^{-1}\) and 212.08 mg Trolox.g\(^{-1}\) for General Carneiro, 198.95 mg Trolox.g\(^{-1}\) and 384.60 mg Trolox.g\(^{-1}\) for Santa Terezinha, and 104.6 mg Trolox.g\(^{-1}\) and 158.84 mg Trolox.g\(^{-1}\) for Bituruna city.

In regard of antioxidant activity by the DPPH\(^{•}\) method, autumn was the season in which there was higher heterogeneity in antioxidant activity and content (Fig. 4). It was observed that the antioxidant activity varied from 5.1 µmol Trolox.g\(^{-1}\) to 84.30 µmol Trolox.g\(^{-1}\) in autumn, where its maximum showed a value of 148.10 µmol Trolox.g\(^{-1}\). For summer, values varied from 4.50 µmol Trolox.g\(^{-1}\) to 50.30 µmol Trolox.g\(^{-1}\), and 18.40 µmol Trolox.g\(^{-1}\) to 53.22 µmol Trolox.g\(^{-1}\) for spring (Fig. 4). These differences among the obtained values for the seasons were statically significant, which means that DPPH\(^{•}\) varied a lot more in autumn than in the other seasons (Levene, \(p=7\%\)). Therefore, an increase in the DPPH\(^{•}\) and phenolic values tend to be observed in autumn, once in summer, these content demonstrated low values. These values were found in propolis from the southern region of Brazil by other authors (Souza et al., 2010; Teixeira et al., 2010).

**Figure 3.** Antioxidant activity of OPEE assessed by ABTS\(^{•}\) scavenging essay method (mg Trolox.g\(^{-1}\)).

As flavonoid and phenolic contents, seasonality had a drastic effect on the antioxidant activity by the DPPH\(^{•}\) as well (Levene, \(p=7\%\)) and ABTS\(^{•}\) (Kruskal-Wallis, \(p=0.3\%\)). Therefore, it was noticed that the values for DPPH\(^{•}\) and phenolics were higher in autumn. Nevertheless, DPPH\(^{•}\) and phenolic contents tended to increase in autumn and had higher values than summer (Fig. 4). These results were also found in propolis from the southern region of Brazil (Souza et al., 2010; Teixeira et al., 2010). Moreover, it was possible to observe that the highest phenolic compound content demonstrated the highest antioxidant activity.

**Figure 4.** Antioxidant activity of OPEE assessed by DPPH\(^{•}\) scavenging essay method (µmol Trolox.g\(^{-1}\)).
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A possible explanation for having results showing high phenolic content (72.55 mg GAE.g⁻¹) and high antioxidant activity by DPPH (148.10 µmol Trolox.g⁻¹) and ABTS (384.60 mg Trolox.g⁻¹) essay methods is that the chemical compounds found in the organic propolis collected in autumn may have high activity due to the stress suffered by the plants. It is known that plants synthesize secondary metabolites when they are visited by insects and face hydric and temperature stresses so that they can defend themselves. These metabolites include flavonoids, chalcones, dihydroflavanoids, anthocyanins, and condensed tannins (Garcia and Carril, 2009; Cartea et al., 2011). Considering this matter in the region where organic propolis was collected, rain precipitation during autumn was scarce compared to spring and summer seasons. In an average, rain precipitation varied from 94 mm to 145 mm against 160 mm to 179 mm in summer. In terms of temperature, it varied from 9°C to 25°C in autumn, in which it was much lower than in spring and summer (11°C - 24°C and 16°C - 33°C, respectively) (Inmet, 2012).

Volatiles compounds in Brazilian organic propolis

Volatiles are chemical compounds that are found in propolis in low concentration, in which their aroma helps to characterize its botanical origin (Miguel and Antunes, 2011). Through GC-MS headspace analysis, the Brazilian organic propolis was tested in our laboratory to analyze its chemical profile. Various chemical compounds such as alpha-pinene(144.05%), beta-pinene (53.38%), alpha-limonene (8.45%), beta-myrcen (12.4%), delta-cadinene (10.1%), gama-muurolene (7.5%), beta-phellandrene (7.15%), alpha-selinene (5.85%) and many others, which some of them were never described by the literature (Tab. 1), were found in the volatile composition of OP collected in autumn and summer. Since compounds such as alpha-pinene, beta-pinene, alpha-limonene, beta-myrcen, delta-cadinene, gama-muurolene, beta-phellandrene, and alpha-selinene were founded in organic propolis, it is likely to say that organic propolis has similarities in regard of green propolis. It is known that the botanical origin of green propolis is Baccharis dracunculifolia (Marióstica Junior, 2008; Alencar et al., 2005), commonly known in Brazil as “alecrim-do-campo” (wild rosemary) or “vassourinha” (little broom). Moreover, these findings suggest that one of the plant sources for organic propolis could possibly be B. dracunculifolia. This specie is the source for green propolis and, presents in its chemical composition artepellin C, which is known due its high antioxidant activity (Szliszka et al., 2013). According to the literature, the volatile limonene, for example, reduces oxidative stress, platelet aggregation, and allergic airway inflammation as well (Hirota et al., 2012) and, α-pinene, β-pinene present antimicrobial activity against C. albicans, C. neoformans, R. oryzae, and MARS (S. aureus resistant to metacillin) (Silva et al., 2012). Thus, these results suggest that the biological activity of OP can also present similar results in these aspects.

Table 1 – Average in percentage (%) of major compounds detected in OP volatiles by GC-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>(x) Compound</th>
<th>(x) %</th>
<th>RI</th>
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<tr>
<td>Alpha-pinene</td>
<td>144.05</td>
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<td>Beta-pinene</td>
<td>53.38</td>
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<td>Beta-myrcen</td>
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<td>Beta-phellandrene</td>
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<td>Alpha-limonene</td>
<td>8.45</td>
<td>2571.5</td>
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<td>Gama-muurolene</td>
<td>7.5</td>
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<td>Delta-cadinene</td>
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<td>Alpha-thujene</td>
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<td>Camphene</td>
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<td>Myrcen</td>
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</table>

IV. Conclusion

In summary, seasonality demonstrated to influence the Brazilian certified organic propolis in terms of total phenolic content and antioxidant activity. In autumn, certified organic propolis presented high phenolic content and antioxidant activity possibly due to low temperature and rain precipitation. For flavonoids, summer was the season in which showed higher flavonoid content also probably due to high rain precipitation. The volatiles found in this type of propolis brought glimpses about its botanical origin and biological activities. Certainly, certified organic propolis shows a potential of application by the pharmaceutical and food industry, as well as on functional food manufacturing.
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