Influence of pH variation during propolis extraction with the use of water as solvent
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ABSTRACT
Propolis is a substance that shows a variable and complex chemical composition with antimicrobial, antioxidant and antiviral properties, associated to its high concentration of flavonoids and phenolic compounds. The objective of this work was evaluating the effect of pH variation on propolis extraction, prepared with water as solvent. In this regard, six different pHs were tested and compared with samples without variation. In order to verify the quality of the obtained extracts a determination of flavonoids, phenolic contents, antimicrobial and antioxidant activities was carried out. Propolis extracts were prepared using 1g crude crushed propolis for each 15mL of solvent. Six samples were prepared at pH 2 up to 10. The flavonoids were measured by colorimetric method of aluminium – flavonoid complexation. Phenolic contents were calculated by the Folin-Ciocalteau method. The antioxidant activity was analysed by two different methods: DPPH and FRAP, and the antimicrobial activity against Staphylococcus aureus was measured by the disc diffusion method and the formation of a zone of growth inhibition. In aqueous extracts the results were better in basic pHs, reaching an increase of 160% in flavonoids and 25% in phenolics when pH 8.0 was used. The antioxidant activity was higher when higher pH was used, reaching an increase in the activity of almost 100% in both methods when compared with the sample without pH variation, which represents a value similar to that obtained by ethanolic extract. The use of water as propolis solvent in basic pHs produced an extract with higher concentration of flavonoids and polyphenols against normally aqueous extract which provides a better product in relation to antimicrobial and antioxidant activities. Thus, this work showed that the use of alkaline water as solvent could be an alternative to ethanolic propolis extracts.

Keywords: Propolis; flavonoids; phenolic compounds; pH; antimicrobial activity.

INTRODUCTION
Propolis is a resinous substance prepared by honeybees from buds, leaves and exudates of trees and plants mixed with pollen, wax and enzymes secreted from the bees. Some important characteristics have been reported concerning this substance, such as antimicrobial and antioxidant effects, anesthetic properties and others. Due to these characteristics, which can bring health benefits, propolis is considered a functional ingredient and has attracted much attention in recent years as an important substance that can be used in foodstuffs, beverages, cosmetics and medicine to improve health and prevent diseases. As propolis is a hard resinous substance, it cannot be consumed in its natural form. Its usual way of preparation is by powdering the resinous, followed by an extraction in alcoholic or aqueous medium. Flavonoids are one of the most important constituents and can represent around 50% of the propolis contents, depending on the region where it is collected, since its characteristics are influenced by botanical, geographical factors and weather. The antioxidant activity of propolis from various geographic origins was studied and different activities were found for each region. Its properties have been widely investigated confirming its antibacterial, antiviral, antifungal and antiprotozoan activities among others. Due to its antimicrobial activity, ethanolic propolis extracts inhibited the growth of Streptococcus and is more active against Gram positive bacteria than against Gram-negative ones.

The effect of the pH variation during the extraction of flavonoids and polyphenols has been studied by many authors, working with different vegetal species. The pH variation could have a positive or a negative effect on extraction, depending on the interaction of the polyphenols with other constituents of each plant. For propolis extraction, no reports for pH variation could be found.

The most common propolis in Brazil is usually collected in the southeast region, characterized as group 12 (Brazil has 12 different groups of propolis, with distinct characteristics) and presents a great amount of soluble substances, anti-microbial activity against Staphylococcus aureus and Streptococcus mutans and...
greater anti-inflammatory activity than samples from other parts of the country, which can be associated with the higher concentrations of flavonoids and phenolic compounds found in this group [6].

Considering the widespread use of propolis, the objective of this work was to evaluate the effect of pH variation on propolis extraction, prepared with water as solvent. Six different pHs were tested to each solvent and were compared with samples without pH variation. Final extracts were quantified regarding flavonoids and phenolic contents to verify the relation between the pH and solvent in the extraction efficiency. Moreover, analyses of antimicrobial and antioxidant activities were carried out for all the extracts.

MATERIALS & METHODS

Propolis

Raw propolis was obtained from Apis mellifera beehives in the State of São Paulo, Brazil, and was acquired in a single batch, in order to minimize the variability. It was stored under refrigeration (4°C) until the extracts preparation.

The aqueous propolis solution was prepared from crude propolis previously comminuted in a bench blender, homogenized, weighed on a semi-analytical balance and mixed to deionized water. Each extract of 100mL was prepared in a proportion of 20% propolis and 80% solvent. The mixture was kept at room temperature in a closed recipient for five days and the recipient was manually stirred once a day. After this period, the sample was centrifuged (Beckman - Allegra 25-R, Beckman Coulter, Munich, German) at 8800g for 20 min. Finally, the resulting extract was stored under refrigeration (4°C) in a closed recipient in the dark.

Acid samples were prepared with the addition of HCl 1M and basic samples with the addition of NaOH 1M. The addition of acid or base occurred every day during the extraction period because they react with the flavonoids and phenolic compounds present in the raw propolis, being consumed when these compounds are extracted. In each extraction day, the maximum of 0.20 mL of base or acid was added.

Determination of total flavonoids

The total flavonoid content of the propolis solutions was determined by the aluminium complexation method [7]. In this procedure, the extracted solutions were diluted in the respective solvents in the proportion of 1:10 (0.5 mL) and mixed with 0.1 mL of 10% aluminium nitrate, 0.1 mL of 1mol/L potassium acetate and 4.3 mL of 80% ethyl alcohol. The samples were kept at room temperature for 40 min and the absorbance was read at 415 nm in a spectrophotometer (UNICO-2800, UNICO Sci., New Jersey, USA). Quercetin was used as the standard for the calibration curve. The mean of three readings was used and the total flavonoid content expressed in mg of quercetin equivalents (mg/g).

Determination of the phenolic compounds

The polyphenols in the propolis solutions were determined by the Folin-Ciocalteau colorimetric method [8]. According to this procedure, the extracted solution was previously diluted in the proportion of 1:10 (0.5 mL) and then mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na2CO3. The absorbance was read at 760 nm in spectrophotometer (UNICO-SQ-2800, UNICO Sci., New Jersey, USA) after 1 hour of incubation at room temperature. Gallic acid was used as the standard for the calibration curve. The mean of three readings was used and the total phenolic content expressed in mg of gallic acid equivalents (mg/g).

Antimicrobial activity

Antimicrobial activity of the propolis samples was investigated by the disc diffusion method, using Staphylococcus aureus [9].

Sterile paper discs of 5mm diameter were impregnated with 10μL of the propolis extracts and were let to dry under vacuum at room temperature overnight and after that, they were incubated at 60°C for 4 hours to remove any residual solvent which might interfere with the result. The papers were placed on agar medium, uniformly seeded with a standardized inoculum of the test organism, and the plates were incubated at 37°C for 24 hours. After the incubation, the zones of growth inhibition around the discs were measured to evaluate the propolis antimicrobial activity. Plates were made by using only the solvent control (water and alcohol), to show the effect of the solvent on the antimicrobial activity.
Antioxidant activity
FRAP assay
Propolis extracts were redissolved at a concentration of 50 mg/mL and diluted to 1000 and 2000 μg/mL. Aliquots (100μL) of each diluted extract were mixed with 3 mL of freshly prepared FRAP reagent. This solution was kept at 37°C for 30 minutes. The FRAP values are obtained by comparing the absorbance change of blue coloured complex at 593 nm. Aqueous solutions of known ferrous sulphate concentrations in the range of 100 – 1000 μM were used for calibration. In order to make comparison, Trolox was also tested under the same conditions as a standard antioxidant compound.

DPPH assay
Propolis extracts were redissolved at a concentration of 10 mg/mL. Aliquots of 0.3mL of sample solution was rapidly mixed with 0.3 mL of 1.0 mM DPPH radical solution and 2.4 mL of 99% ethanol. The scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid (0.1 and 1.0 mM) was used as positive control.

Statistical analysis
The results were statistically analysed by the analysis of variance to determine significant differences between the samples. The analysis of the means was performed through the Tukey procedure at p<0.05 using the software STATISTICA 5.0 (StatSoft, Inc., Tulsa, USA).

RESULTS & DISCUSSION
Table 1 shows the quantification of the extracted compounds for each sample. For the aqueous extracts, the results for flavonoid concentration showed that all samples presented statistical differences compared to the initial solution (extracted without pH variation) at 95% significance. However, only the samples with a more basic pH (6.0 and 8.0) had an increase on concentration of the main functional compounds. This behaviour was the same for phenolic compounds in the samples at pH 6.0 and 8.0, which had statistical difference from the initial solution, at pH 4.3.

Table 1. Flavonoids and phenolic compounds in extracts prepared with different pHs

<table>
<thead>
<tr>
<th>Solution</th>
<th>Flavonoids (mg/g)*</th>
<th>% variation</th>
<th>Total polyphenols (mg/g)**</th>
<th>% variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.0</td>
<td>12.08 ± 0.54 (a)</td>
<td>-48.96</td>
<td>36.96 ± 0.04 (a)</td>
<td>1.07</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>11.41 ± 1.56 (a)</td>
<td>-51.80</td>
<td>35.48 ± 0.44 (b)</td>
<td>-2.98</td>
</tr>
<tr>
<td>pH 4.3</td>
<td>23.67 ± 2.14 (d)</td>
<td>-</td>
<td>36.57 ± 0.35 (a.b)</td>
<td>-</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>33.21 ± 2.62 (b)</td>
<td>-40.30</td>
<td>40.37 ± 0.27 (c)</td>
<td>10.39</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>61.42 ± 1.51 (c)</td>
<td>159.48</td>
<td>45.41 ± 0.40 (d)</td>
<td>24.17</td>
</tr>
</tbody>
</table>

Values are represented by mean ± S.D of three experiments
* quercetin equivalents
** gallic acid equivalents
Means with different superscript letters within a column are significantly different at p< 0.05

The results showed that the aqueous samples at pH 6.0 and 8.0 extracted more flavonoids and phenolic compounds than the extraction without pH variation. Lowering the pH for both solvents did not have significant effect on functional compounds extracted. The effect of pH in the extraction of total phenols from meadowsweet (Filipendula ulmaria L.) was studied using water as solvent and it was verified that increasing the pH from 3.9 to 6.4 the total phenols content was 33% higher [10]. On the other hand, the efficiency of catechins extraction from green tea was tested and observed a decrease on the extraction efficiency when pH was increased, reaching a 30% less extraction at pH 6.0 and 15% at pH 7.6 [11]. Analysing these results, the effect of pH on phenolic compounds extraction can be positive or negative, as it depends on the interaction of these compounds with raw material.

Flavonoids and phenolic quantitative results were compared in order to choose which combination of solvent and pH is the best one to produce a novel propolis extract.

Antimicrobial activity of the aqueous extracts without pH variation and the basic aqueous samples was tested, due to their flavonoids and phenolic highest content. A control sample using only ethanol and an ethanolic extract was also evaluated, to measure the influence of the solvent on the antimicrobial activity.
Table 2 shows the mean diameter of inhibitory zone obtained for each sample, in mm. Samples were statistically different at 95% significance in Tukey test. However, when the aqueous extract was at pH 8.0, it did not present a significant statistical difference from the alcohol used as a standard.

Table 2. Antimicrobial activity of the propolis extracts against *Staphylococcus aureus*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition zone of microbial growing (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (control)</td>
<td>1.32 ± 0.97a</td>
</tr>
<tr>
<td>Ethanolic extract (pH 4.3)</td>
<td>4.85 ± 0.85b</td>
</tr>
<tr>
<td>Aqueous extract (pH 4.3)</td>
<td>0.00 ± 0.00c</td>
</tr>
<tr>
<td>Aqueous extract (pH 6.0)</td>
<td>0.00 ± 0.00c</td>
</tr>
<tr>
<td>Aqueous extract (pH 8.0)</td>
<td>2.40 ± 0.39e</td>
</tr>
</tbody>
</table>

*measured from the ending of the disk and the growing ending, by the mean of five repetitions

Means with different superscript letters are significantly different at p<0.05.

Despite of the increase on flavonoids and phenolic compounds in the basic aqueous samples, they did not show the same activity against *Staphylococcus aureus* when compared to the ethanolic extract. The sample without base addition and the sample at pH 6.0 had no activity against the microorganism, while the sample at pH 8.0 showed a little activity, 62% lower than the activity obtained for the ethanolic sample.

It is reported that the antimicrobial activity of propolis reflected its constituents, which may differ from each producing area and season, depending on its chemical composition [12]. The pH effect on antimicrobial activity of ethanolic extracts at pH 5.0 was studied and found to be the most effective against *Staphylococcus aureus*, which is the normal pH of the extract [13]. When the inhibition zone obtained by extracts with different ethanol concentration was compared, aqueous extract and extracts prepared with only 20% of ethanol had no activity against *Staphylococcus aureus*, achieving the better activity against this microorganism when 80% of ethanol was used in the extract [1]. Moreover, an inhibition zone of microbial growing similar to the one obtained in this work at pH 8.0 was reported, when testing extracts using as solvent a combination of water, alcohol and one of the following surfactants: polissorbate 80 or sodium lauryl sulfate [14]. The surfactants facilitate binding between molecules that were not naturally attracted by water. This comparison reinforced the idea that alkaline pH acts as a tensoactive during propolis extraction.

The antioxidant activity of aqueous propolis extracts was tested by two different methods, to explore the different mechanisms involved in each evaluation. In the FRAP assay the antioxidant capacity is measured as the ability to reduce 2,4,6-trypyridyl-s-triazine-Fe(III) complex to 2,4,6-trypyridyl-s-triazine-Fe(II) complex and may be related to the presence of iron-chelating compounds in the samples. In contrast, the DPPH assay involves a fast electron transfer process from phenolic compounds to the DPPH radical. DPPH is more correlated to the antioxidant capacity of compounds in situ, than the FRAP assay [15]. The results obtained to propolis extracts are shown in Table 3.

Table 3. Antioxidant activity of propolis extracts with different pHs.

<table>
<thead>
<tr>
<th>Solution</th>
<th>FRAP value *</th>
<th>DPPH scavenging b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.0</td>
<td>42,44 ± 0,78</td>
<td>304,76 ± 7,41</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>45,48 ± 1,33</td>
<td>385,71 ± 22,22</td>
</tr>
<tr>
<td>pH 4.3</td>
<td>24,17 ± 1,66</td>
<td>180,95 ± 7,41</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>44,17 ± 0,57</td>
<td>190,48 ± 19,60</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>44,44 ± 1,01</td>
<td>414,29 ± 46,26</td>
</tr>
</tbody>
</table>

*In μmol Fe²⁺/mg dry weight of extract

The extracts prepared with pH modification had higher activity than the extract without the addition of acid or base, what could be a consequence of the major effect of the polyphenols in the antioxidant power. The results obtained for both methods had the same correlation among the samples tested. Because propolis is rich in flavonoids and phenolic acids, the increase in antioxidant capacity with pH is consistent with the effect of pH in the antioxidant activity in red wine [16]. Likewise, the antioxidant activity of carrot in different pHs (4 and 9) was greater at higher pH [17].
CONCLUSION

According to the results overview, the studied propolis extracts represent an important functional product, rich in flavonoids and polyphenols. By using alkaline water as the extraction solvent, the amount of extracted compounds increased as so their antimicrobial activity comparing with the extract prepared without pH modification. In conclusion, water could be used as an alternative solvent for propolis extraction, with a similar behaviour than the ethanolic one and without its disadvantages.

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REFERENCES