Propolis protects against oxidative stress in human saliva

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Summary
Oxidative stress plays an important role in periodontal health and disease and it is important to find natural compounds to control the oxidative stress in the periodontal area. Therefore, the aim of this study was to determine the effect of Apis mellifera propolis (AmP) and Melipona favosa propolis (MfP) on the Fenton reagent-induced oxidative stress of human saliva. Human saliva was incubated for 10 min at 37°C in the presence of Fenton reagent, to induce the oxidative stress, and propolis ethanolic extract dilutions. After the incubation, the salivary total antioxidant activity was assayed by the ABTS method. It was found that the Fenton reagent caused a decrease in salivary antioxidant activity. AmP dilution protected and even increased the salivary total antioxidant activity after the Fenton reagent-induced oxidative stress. MfP only protected the salivary total antioxidant activity against oxidative stress. In conclusion, propolis could be used to maintain and even increase the antioxidant capacity of saliva during an oxidative stress.

Keywords: antioxidant activity, Apis mellifera, Melipona favosa, nitrites, periodontitis, polyphenolics, propolis, saliva.

Introduction
The involvement of reactive oxygen species (ROS) in periodontal pathology has been studied in the last decades (Battino et al., 1999; Chapple, 1997). Several studies have demonstrated that early-onset forms of periodontitis are associated with functionally activated polymorphonuclear leukocytes (PMN) exhibiting increased ROS production (Guarnieri et al., 1991; Shapira et al., 1991; Kimura et al., 1993). The detection of ROS oxidation products, the elevation of iron and copper ions that in the presence of H2O2 can catalyze the production of the most reactive-free radical species by the Fenton reaction (Equation 1), and the identification of an imbalance in the oxidant/antioxidant activity within periodontal pockets, suggests a significant role for ROS in periodontal tissue destruction (Liskmann et al., 2006). Recently, it has been reported that the antioxidant activity is reduced in chronic periodontitis patients (Canakci et al., 2009). Sculley and Langley-Evans (2003) have demonstrated that subjects with advanced periodontitis tended to have greater oxidative injury and the lowest total antioxidant status.

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH} \ (\text{Equation 1}) \]

Propolis is a complex mixture of chemical constituents and its composition is determined by the constituents of native vegetation and by the season of collection (Ghisalberti, 1979; Bankova et al., 2000). Propolis has been used in traditional medicine since the primordial times of humanity. Propolis has several biological activities including antibacterial, antifungal, antiprotozoan, antiviral, curative, anesthetical, antitumour, immunomodulation and anti-inflammatory activities, among others (Ghisalberti, 1979; Marcucci, 1995). These actions have been attributed to propolis phenolic composition (Lahouel et al., 2004).
Despite increasing use of propolis worldwide (Marcucci, 1995), few studies have been carried out to determine the effect of propolis on saliva antioxidant activity and of Melipona species, which are distributed in the Neotropics (Camargo and Pedro, 2007). Saliva is the first biological fluid that encounters the possible free radicals found in the consumed food. The salivary antioxidant system includes various molecules, the most important of which are uric acid and the peroxidase systems (Greabu et al., 2007). Uric acid contributes to approximately 70% of the salivary total antioxidant capacity (Negler et al., 2004).

Oxidative stress, occurring as a consequence of imbalance between free radicals and inactivation of these species by antioxidant defence system, is capable of causing damage to various cellular and extracellular constituents in the oral cavity. Therefore, the development of new therapies for the treatment of the oral cavity diseases is of great relevance (Walker, 1996). In dentistry, the use of propolis has been proposed in different areas including cariology (Park et al., 1998), oral surgery (Magro-Filho and Carvalho, 1990, 1994), endodontics (Al-Shaher et al., 2004; Silva et al., 2004), oral pathology (Silva et al., 2000), periodontology (Gebara et al., 1996; Murray et al., 1997) and dental traumatology (Martin and Pileggi, 2004). However, it is not clear the effect of propolis on the antioxidant status of human saliva. Therefore, the aim of this study was to determine the effect of both Apis mellifera propolis and Melipona favosa propolis on the Fenton reagent-induced oxidative stress of human saliva.

Materials and methods

Chemicals

All reagents and solvents used were of analytical grade. Ultrapure water (Millipore, Milli-Q system) was used to prepare standard solutions, dilutions and blanks. Folin-Ciocalteu phenol reagent was obtained from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride and sodium carbonate from Merck.

Ethanolic propolis extract

Commercial Apis mellifera ethanolic propolis extract (20%, w/v) was provided by La Casita de la Miel (Maya, Mérida, Venezuela). The Melipona favosa ethanolic propolis extract was obtained by the extraction of 30 g of propolis collected in Peninsula de Paraguaná, Falcón State, Venezuela up to 100 g with 95% ethanol, during 24 h at room temperature. The 1/10 dilutions of both extracts were used in the treatment of human saliva.

Saliva sample

Whole saliva was collected in a quiet room between 9 am and noon to avoid circadian changes, and was obtained by expectorating into disposable tubes. About 1 mL of whole saliva was collected in tubes and centrifuged immediately to remove any cell debris (5,000 rpm for 5 min). The supernatant was removed and used for the determination of total antioxidant activity.

Saliva treatment

Saliva sample was incubated for 10 min at 37°C in the presence of Fenton reagent (combination of 35% hydrogen peroxide and 1 mM iron sulfate), to induce the oxidative stress of human saliva sample. The saliva control contained 10 µL of distilled water and 10 µL of 95% ethanol. Briefly, 90 µL of saliva was incubated with 10 µL of Fenton reagent (5 µL of 35% H2O2 + 5 µL of 1 mM iron sulfate) in the presence of 10 µL of either Apis mellifera propolis (AmP) extract or Melipona favosa propolis (MfP) extract. After the incubation, the salivary TAA was assayed by the ABTS method (Re et al., 1999).

Total antioxidant activity

Total antioxidant activity was assayed by the Trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999). TEAC was measured by decolorization of the ABTS** radical cation. To prepare the ABTS reagent, 7 mM ABTS and 4.9 mM ammonium persulfate were mixed 1:1, covered with foil for 16 h, and diluted up to an absorbance of 0.6-0.7 at 734 nm and equilibrated at 37°C. For the salivary TEAC, 10 µL of saliva sample was added to diluted ABTS solution made up with 40 µL of ABTS reagent plus 960 µL of 20% (v/v) ethanol. Absorbance at 0 and 6 min were recorded and the decolorization percentage was calculated. A calibration curve of Trolox concentrations vs decolorization percentage was used to estimate mM TEAC.

Total polyphenol content

Total polyphenol content in propolis was determined by the Folin-Ciocalteu colorimetric method (Singleton et al., 1999). Diluted propolis sample (0.1 mL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent (1:10). The mixture was vortexed and 0.4 mL of 7.5% (w/v) Na2CO3 was added. Absorbance was measured at 765 nm after 10 min at 37°C. Total polyphenol content was expressed as mg of gallic acid equivalents (GAE)/g of propolis.

Total flavonoid content

Total flavonoid content in propolis sample was determined by the method of Woisy and Salatino (1998) with minor modifications. To 0.5 mL of the propolis extract, 0.5 mL of 20 mg AlCl3 per mL of 95% ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as mg of quercetin equivalents (QE)/g of propolis from a calibration curve.
Nitrite determination

The concentration of nitrite was determined by a colorimetric assay based on the Griess reaction (Green et al., 1982). Briefly, to 0.05 mL of propolis sample, 0.1 mL of 14 mM sulfanilamide in 2 N HCl, 100 mL of 4 mM N-(1-naphthyl)-ethylenediamine (NED) in water, and 0.75 mL of 0.2 M KCl-HCl (pH 1.5) were added. The samples were incubated at 37ºC for 10 min and then were centrifuged at 5,000 rpm for 10 min. Absorbance was measured at 540 nm and sodium nitrite was used as a standard.

Statistical analysis

The results are reported as the means ± SD of six independent replicates. Statistical analysis of data was carried out by computer using SPSS 12.0 software. One-way ANOVA and Bonferroni post hoc multiple comparison tests were used to analyze data. P-values less than 0.05 were considered significant.

Results

As is shown in Table 1, saliva in the presence of ethanol 95% had 0.45 ± 0.09 mM TEAC. Fenton reagent decreased the salivary TEAC (0.22 ± 0.13 mM). However, when saliva sample was oxidized with Fenton reagent in the presence of (1/10) diluted AmP extract, the decrease in salivary TEAC not only was prevented, but increased to a lower value (1.14 ± 0.18 mM) than that displayed by saliva with AmP extract (1.47 ± 0.09 mM). MfP extract provided protection against the saliva oxidation (0.22 ± 0.13 mM) reaching a TEAC value of 0.43 ± 0.07 mM, similar to that of saliva without oxidation.

The ethanolic propolis extracts (AmP and MfP) are characterized in Table 2. The AmP used in this work had a higher content of polyphenols (184.81 ± 4.18 mg GAE/g propolis), flavonoids (91.27 ± 5.23 mg QE/g propolis) and nitrites (3.43 ± 0.13 mM), compared to the MfP (8.36 ± 0.50 mg GAE/g propolis, 0.06 ± 0.004 mg QE/g propolis, 0.35 ± 0.02 mM nitrites).

Discussions

Propolis extracts were reported to have antioxidant activity (Jasprica et al., 2007; Sheng et al., 2007; Moreira et al., 2008). To our best knowledge this is the first report that has shown that propolis can increase salivary antioxidant capacity in vitro. This observation could be explained by the fact that the AmP used in this work had a relatively high content of polyphenols (184.81 ± 4.18 mg GAE/g of propolis) and flavonoids (91.27 ± 5.23 mg QE/g propolis); whereas MfP only protected saliva and this could be due to a lower content of polyphenols and flavonoids, as shown in Table 2. These compounds are known as potent antioxidants, and the total polyphenol content has been considered the main responsible agent for the antioxidant activity of different propolis extracts (Kumazawa et al., 2004).

In both extracts (AmP and MfP) there was nitrite, but the nitrite concentration was higher in AmP than in MfP. Nowadays, it is proposed that nitrite could be transformed into nitric oxide (NO) in the oral environment (Lundberg et al., 2008), and NO could act at a low concentration as an antioxidant in the saliva (Hummel et al., 2006). In that way the nitrite content might affect the total antioxidant activity of saliva.

In conclusion, AmP and MfP extracts could be used both to protect against oral oxidative stress and to improve the antioxidant status of oral environment. This effect could be mediated by the content of polyphenols, flavonoids and nitrite in both extracts.

### Table 1. Effect of ethanolic extracts of propolis from *Apis mellifera* (AmP) and *Melipona favosa* (MfP) on the Trolox equivalent antioxidant capacity (TEAC) of human saliva treated by Fenton reagent.

<table>
<thead>
<tr>
<th>Samples</th>
<th>TEAC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva + water + 95% ethanol</td>
<td>0.45 ± 0.09a</td>
</tr>
<tr>
<td>Saliva + Fenton reagent + 95% ethanol</td>
<td>0.22 ± 0.13b</td>
</tr>
<tr>
<td>Saliva + water + AmP</td>
<td>1.47 ± 0.09c</td>
</tr>
<tr>
<td>Saliva + Fenton reagent + AmP</td>
<td>1.14 ± 0.18c</td>
</tr>
<tr>
<td>Saliva + water + MfP</td>
<td>0.52 ± 0.07c</td>
</tr>
<tr>
<td>Saliva + Fenton reagent + MfP</td>
<td>0.43 ± 0.07c</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 6). Means sharing the same letter are not significantly different by Bonferroni multiple comparison test (p < 0.05).

### Table 2. Concentrations of polyphenols, flavonoids and nitrite in the ethanolic extracts of propolis from *Apis mellifera* (AmP) and *Melipona favosa* (MfP).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Polyphenols (mg GAE/g propolis)</th>
<th>Flavonoids (mg QE/g propolis)</th>
<th>Nitrite (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmP</td>
<td>184.81 ± 4.18†</td>
<td>91.27 ± 5.23*</td>
<td>3.43 ± 0.13*</td>
</tr>
<tr>
<td>MfP</td>
<td>8.36 ± 0.50</td>
<td>0.06 ± 0.004</td>
<td>0.35 ± 0.02</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 3). Means are significantly different by unpaired t test (*, p < 0.0001). GAE means gallic acid equivalents and QE means quercetin equivalents.
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