In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development

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Abstract

Recently, two chemically different types of Brazilian propolis (type-3 and -12) were shown to have cariostatic properties. This study aimed to evaluate the influence of their isolated fractions on mutans streptococci viability, glucosyltransferases (GTFs) activity and caries development in rats. The ethanolic extracts of propolis (EEPs) were serially fractionated into hexane (H-fr), chloroform, ethyl acetate, and ethanol. The ability of the four fractions and EEP to inhibit Streptococcus mutans and Streptococcus sobrinus growth and adherence to a glass surface was examined. The effect on GTFs B and C activity was also determined. For the caries study, 60 Wistar rats infected with Streptococcus sobrinus were treated topically twice daily as follows: (1) EEP type-3, (2) H-fr type-3, (3) EEP type-12, (4) H-fr type-12, and (5) control. In general, the H-fr from both types of propolis showed the highest antibacterial activity and GTFs inhibition. Furthermore, the EEP and H-fr type-3 and -12 were equally effective in reducing dental caries in rats. The data suggest that the putative cariostatic compounds of propolis type-3 and -12 are mostly non-polar; and H-fr should be the fraction of choice for identifying further potentially novel anti-caries agents.

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1. Introduction

Over the last few decades, worldwide increase in the use of natural products for pharmacological purposes has been observed (Cragg et al., 1997). Propolis is a natural resinous hive product collected by Apis mellifera bees from tree buds and mixed with secreted beeswax (Ghisalberti, 1979; Burdock, 1998). Many biological activities, such as antimicrobial, cytostatic, anti-inflammatory properties (Ghisalberti, 1979; Burdock, 1998), have been attributed to the ethanolic extract of propolis. The chemical composition of propolis is complex; flavonoids and (hydroxyl) cinnamic acid derivatives are considered to be the primary biologically active compounds in propolis (Burdock, 1998). Furthermore, its composition is highly variable, depending on its geographical origin (Greenaway et al., 1990; Bankova et al., 1992; Park et al., 1997). To date, 12 distinct types of Brazilian propolis have been chemically characterized and classified from type-1 to -12 (Park et al., 2000).

Recent studies have shown the anti-caries potential of propolis from the Southern (type-3) and Southeastern (type-12) regions of Brazil (Park et al., 1998; Koo et al., 1999, 2000a,b,c). Both samples reduced the incidence of caries and dental plaque accumulation in vivo (Koo et al., 1999, 2002). Two action mechanisms have been associated with the anti-caries/anti-plaque properties of propolis: (1) antimicrobial activity against cariogenic bacteria, and (2) inhibition of glucosyltransferase enzymes (GTFs) activity (Koo et al., 2000a). However, all of these studies were conducted with...
crude ethanolic extract of propolis and little is known about the putative anti-caries compounds in these samples. Considering that propolis fractionation is the first step in identifying the active compound(s) of this natural product, this study aimed to evaluate the influence of isolated fractions of propolis type-3 and -12 on mutants streptococci and GTFs activity in vitro, and on caries development in vivo.

2. Materials and methods

2.1. Propolis samples and fractionation

Crude samples of *Apis mellifera* propolis were obtained from two different regions of Brazil: southern and southeastern, classified as type-3 and -12, respectively (Park et al., 2000). The ethanolic extract of propolis (EEP) at 20% (w/v) in aqueous ethanol (80%, v/v) was prepared as described elsewhere (Park et al., 1997). The EEP was further fractionated according to a polarity gradient, using standard protocols as described by Duarte et al. (2003). The EEP was serially fractioned with hexane, chloroform, ethyl acetate and ethanol. Each of the four fractions was monitored by paper chromatography and developed by UV light (λ = 254 and 366 nm) (Duarte et al., 2003). The isolated fractions (1 g) were dissolved in 10 ml of 80% (v/v) ethanol.

2.2. Susceptibility testing

The antimicrobial activity of propolis extracts was examined by determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) guidelines and Koo et al. (2000b). The bacterial strains used were: *Streptococcus mutans* Ingbritt 1600 and *Streptococcus sobrinus* 6715, which are proven cariogenic pathogens (Bowen et al., 1988). To determine MIC, the starting inoculum was 5 × 10^5 CFU/ml, and the test extract concentrations ranged from 12.5 to 1600 μg/ml (for EEP) and 6.25 to 800 μg/ml (for propolis fractions). The control vehicle was ethanol (final ethanol concentration: 0.6%, v/v). For determining MBC, an aliquot (50 μl) of all incubated tubes with concentrations higher than MIC was sub-cultured on BHI agar, supplemented with 5% of defibrinated sheep blood with a Spiral plater (Whitley Automatic Spiral Plate®). MBC was defined as the lowest concentration that allows no visible growth on the agar (Koo et al., 2000b). Three separate experiments were conducted for each concentration of the extracts tested.

2.3. Inhibition of growing cell adherence to glass surface

To assess bacterial adherence to a glass surface, microorganisms were grown at 37°C, 10% CO₂ at an angle of 30° for 18 h in test tubes as detailed by Hamada and Torii (1978) and Koo et al. (2000c). *Streptococcus mutans* Ingbritt 1600 or *Streptococcus sobrinus* 6715 was grown in BHI broth plus 1% (w/v) sucrose containing sub-inhibitory concentrations (sub-MIC) of the test extracts or control (final ethanol concentration: 0.6%, v/v). After incubation, the adherent cells were washed and re-suspended in an ultrasonic bath (Sonics and Material Inc., VibraCell®). The number of adherent cells was measured spectrophotometrically at 550 nm (Duarte et al., 2003). Three separate experiments were carried out for each concentration of the extracts.

2.4. GTFs activity determination

The GTF B and C enzymes were obtained from the culture supernatants of *Streptococcus milleri* KSB8 (which harbors the gffB gene of *Streptococcus mutans* GS-5) and *Streptococcus mutans* WHB 410 (whose gffB, gfc and gff genes were deleted), and purified to near homogeneity by hydroxyapatite column chromatography (Venkitaraman et al., 1995; Wunder and Bowen, 1999). GTF activity was measured by incorporating 14C-glucose from labeled sucrose (NEN Research Products, Boston, MA, USA) to glucans (Venkitaraman et al., 1995; Koo et al., 2000a). The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 μmol of glucose during the 4 h reaction period. GTF B or C was mixed with a series of the EEP or the fractions at two-fold dilution (concentration ranging from 50.0 to 400.0 μg/ml) and incubated with 14C-(glucosyl)-sucrose substrate (0.2 μCi/ml; 200.0 mmol/l sucrose, 40 μmol/l dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to a final concentration of 100 mmol/l sucrose (200 μl final volume). Ethanol (final concentration: 8%, v/v) was used as control. The radio-labeled glucan was collected and determined by scintillation counting (Venkitaraman et al., 1995; Koo et al., 2000a).

2.5. Caries study

The animal experiment was performed as previously described by Bowen et al. (1988) and Koo et al. (2002) and was approved by the Ethical Committee on Animal Research at the State University of Campinas (UNICAMP), which is in accordance with the internationally accepted principles for laboratory animal use and care. Sixty Wistar female rat pups, aged 19 days, specific pathogen and mutans-free, were purchased from CEMIB-UNICAMP (Brazil). On three consecutive days, the pups were infected with an actively growing culture of *Streptococcus sobrinus* 6715 and checked to confirm the infection as described elsewhere (Bowen et al., 1988). Pups aged 24 days were randomly divided into five groups of 12 animals and had their teeth treated topically, using a camel hair brush for 30 s, twice daily as follows: (1) propolis type-3; (2) H-fr type-3; (3) propolis type-12; (4) H-fr type-12 and (5) ethanol 80% (v/v). The concentration of tested EEPs and H-frs was 2.5%. Each group of 12 animals was provided with diet 2000 (Keyes,
1959), containing 50% sucrose, ad libitum. The experiment proceeded for 5 weeks, at the end of which the animals were killed by CO2 asphyxiation. The lower left jaw was aseptically dissected, suspended in 5.0 ml of sterile saline solution and sonicated (three 10 s-pulses at 5 s-intervals, at 30 W (Sonic and Materials Inc., Vibracell®). The suspension was plated on mitis salivarius agar plus streptomycin to estimate Streptococcus sobrinus populations and on blood agar to determine total cultivable flora. The jaws were de-fleshed and the teeth prepared for caries scoring. Caries on the smooth and sulcal surfaces and its severity (Ds, dentin exposed; Dm, 3/4 of the dentin affected) was evaluated by means of Larson (1981) modification of the Keyes system.

The data were subjected to ANOVA in the Tukey–Kramer honest standard deviation (HSD) test for all pairs using JMP version 3.1, software for statistical visualization (Fitzgerald and Keyes, 1960). The level of significance was 5%.

3. Results

3.1. In vitro study

The MIC, MBC and the values at which bacterial adherence was inhibited are shown in Table 1. The MIC values of EEPs and their hexane (H-fr) and chloroform (Chlo-fr) fractions from both types of propolis ranged from 25 to 400 μg/ml. In contrast, ethyl acetate and ethanol fractions did not show inhibition at the concentrations tested in this study. The MBC values of propolis type-3 extracts were two–eight times higher than the MIC values, whereas most of the propolis type-12 extracts did not show any bactericidal effect (except H-fr). In general, the order of potency against Streptococcus mutans and Streptococcus sobrinus viability was: H-fr > EEP > Chlo-fr. Streptococcus sobrinus appears to be more susceptible to propolis type-3 than Streptococcus mutans, but no difference was observed with propolis type-12.

Furthermore, this study investigated whether propolis fractions affect the sucrose-dependent adherence of growing mutans streptococci cells to a glass surface. The adherence assays were conducted using sub-MIC levels, since false positive results could be generated due to antimicrobial activity. Growing bacterial cultures (S. mutans and S. sobrinus) were exposed to sub-inhibitory concentrations (test 1) and to concentrations twofold higher than the MIC values (test 2). In test 1, the EEPs and their antibacterial fractions (H-fr and Chlo-fr) did not inhibit growing cells from adhering to a glass surface. It is interesting to note that the EtAc-fr of both types of propolis, which were devoid of antibacterial activity, inhibited bacterial adherence at concentrations between 50 and 800 μg/ml. In contrast, the EtOH-fr did not show inhibition at the concentrations tested in this study.

The effects of propolis on GTF B and C activity are shown in Figs. 1 and 2. The EEPs extracts and their four fractions reduced GTF B and C activity to a remarkable extent in solution (50–80% of enzymatic activity inhibited at a concentration of 200 μg/ml).

Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Propolis type-3</th>
<th>Propolis type-12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Streptococcus mutans</td>
<td>Streptococcus sobrinus</td>
</tr>
<tr>
<td></td>
<td>H.1600</td>
<td>S.6715</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude propolis (EEP)</td>
<td>25–50</td>
<td>200–400</td>
</tr>
<tr>
<td>H-fr</td>
<td>25–50</td>
<td>50–100</td>
</tr>
<tr>
<td>Chlo-fr</td>
<td>25–50</td>
<td>200–400</td>
</tr>
<tr>
<td>EtAc-fr</td>
<td>25–50</td>
<td>200–400</td>
</tr>
<tr>
<td>EtOH-fr</td>
<td>25–50</td>
<td>200–400</td>
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<td></td>
<td>200–400</td>
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<td>200–400</td>
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<tr>
<td></td>
<td>400–800</td>
<td>400–800</td>
</tr>
</tbody>
</table>

The values are expressed in concentration range (μg/ml). The MIC, MBC and Adh. values are between the concentrations above.

a The cell adherence inhibition value is for sub-inhibitory concentrations.
Fig. 1. Effect of ethanolic extract of propolis (EEP) type-3 and -12 and their fractions on glucosyltransferases (GTF B) activity. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

Fig. 2. Effect of ethanolic extract of propolis (EEP) type-3 and -12 and their fractions on GTF C activity. For the control, ethanol (80%, final concentration) was used. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

3.2. Caries study

The rats remained in apparent good health during the 5-week experiment. Weight gains were not significantly different among the treatment groups ($P > 0.05$). The percentage of Streptococcus sobrinus 6715 recovered from the rat jaws was calculated from the total cultivable flora and the Streptococcus sobrinus population. The groups treated with the propolis extracts did not show any significant effects on the level of infection by Streptococcus sobrinus compared to that of the control group ($P > 0.05$) (data not shown), although lower counts for both total and Streptococcus sobrinus population were observed in the groups treated with the H-fr of type-3 and -12 propolis.

The effects of the treatments on the incidence and severity of smooth-surface and sulcal caries are shown in Table 2. The animals treated with crude extracts and their H-frs showed significant cariostatic properties on smooth-surface caries, especially on the severity of smooth-surface lesions. In general, the animals treated with crude extracts showed no statistical difference in either incidence or severity of smooth-surface caries when compared to their respective H-frs ($P > 0.05$). The incidence and severity of sulcal caries were unaffected by the propolis extracts compared to control group ($P > 0.05$).

4. Discussion

Dental caries development involves a series of events in the biofilm on the tooth surface, where bacterial interactions with diet occur. There is a general consensus that the frequent consumption of carbohydrates, mainly sucrose, can result in the emergence of cariogenic microorganisms, such as mutans streptococci (Fitzgerald and Keyes, 1960; Hamada et al., 1984; Loesche, 1986). The ability of mutans streptococci to produce extracellular polysaccharides, mainly glucans, has been recognized as a critical factor in the pathogenesis of

Table 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total smooth-surface</th>
<th>Smooth-surface severity</th>
<th>Total sulcal</th>
<th>Sulcal Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_s$</td>
<td>$D_m$</td>
<td>$D_s$</td>
<td>$D_m$</td>
</tr>
<tr>
<td>Propolis type-3</td>
<td>10.4 (2.0) b</td>
<td>1.9 (1.0) b</td>
<td>0.3 (0.3) b</td>
<td>35.7 (2.4) a</td>
</tr>
<tr>
<td>H-fr type-3</td>
<td>13.6 (2.3) ab</td>
<td>0.7 (0.4) b</td>
<td>0.1 (0.1) b</td>
<td>31.1 (1.1) a</td>
</tr>
<tr>
<td>Propolis type-12</td>
<td>13.8 (2.3) ab</td>
<td>2.5 (1.5) b</td>
<td>n.d. b</td>
<td>30.0 (1.9) a</td>
</tr>
<tr>
<td>H-fr type-12</td>
<td>19.4 (2.7) ab</td>
<td>4.6 (0.9) ab</td>
<td>0.6 (0.6) ab</td>
<td>30.9 (2.4) a</td>
</tr>
<tr>
<td>Ethanol 80% (control)</td>
<td>22.0 (3.4) a</td>
<td>8.3 (2.1) a</td>
<td>3.0 (1.5) a</td>
<td>35.4 (2.3) a</td>
</tr>
</tbody>
</table>

Values followed by the same lower case letters are not significantly different from each other ($P > 0.05$). ANOVA, comparison for all pairs using Tukey–Kramer HSD (SAS, 1995). n.d., not detectable.
inhibited bacterial adherence solely by inhibiting glucan synthesis. It is noteworthy that EtAc-frs to have any influence on bacterial cell adherence to a glass surface, since a large proportion of the insoluble glucans adhere and to accumulate on the tooth surface, leading to the formation of cariogenic biofilm communities (Schilling and Bowen, 1992; Marsh and Bradshaw, 1995). Therefore, mutans streptococci and/or the GTFs should be prime targets for any therapeutic agent aimed at preventing dental biofilm-related diseases, such as dental caries.

Brazilian propolis is known for its complex and variable chemical composition; so far, 12 chemically distinct types of propolis have been identified (Koo et al., 1999; Park et al., 2000, 2002). Furthermore, it has been found that specific types of Brazilian propolis (type-3 and -12) exhibit anti-caries/anti-plaque properties in vitro and in vivo (Koo et al., 1999; 2000a,b,c). The composition of propolis type-3 and propolis type-12 is quite complex, as recently reported by Park et al. (2002). Propolis type-3 is a rich source of flavonoids, such as pinobanksin, kaempferol, apigenin, pinocembrin, chrysin and galangin. In contrast, only a few flavonoids were identified in propolis type-12. Namely kaempferide, kaempferol, isosakuranetin and pinobanksin in addition to p-coumaric, cinnamic acid derivatives and terpenes (Park et al., 2002). However, there is scant information about the potentially anti-caries compounds in these propolis samples. Therefore, this study aimed to identify the most active fraction of Brazilian propolis type-3 and -12, as a first step toward identifying the active constituents of this promising natural anti-caries/anti-plaque product.

The findings of this study confirm and extend previous observations on the anti-caries properties of propolis type-3 and -12 (Koo et al., 1999; 2000a,b,c). Here, it was shown that Chlo-fr and, especially H-fr of both propolis types were the most effective extracts, indicating that the putative antibacterial and anti-GTFs action in vitro. GTF B and C inhibition has many implications with regard to bacterial adherence to and further accumulation on the tooth surface, since a large proportion of the insoluble glucans synthesized by these enzymes is retained by the pellicle and provides binding sites for Streptococcus mutans and other oral cariogenic bacteria, thus contributing to the in situ formation of dental biofilm (Schilling and Bowen, 1992). Furthermore, GTF B and C are proven virulence factors associated with the pathogenesis of dental caries (Yamashita et al., 1993). Glucans also contribute to the bulk and structural integrity of dental biofilm (Hotz et al., 1972; Cury et al., 2000). However, sub-MIC levels of H-fr were not sufficient to have any influence on bacterial cell adherence to a glass surface; it is likely that sub-MIC levels (<25 µg/ml) are too low to inhibit GTFs activity. It is noteworthy that EtAc-frs inhibited bacterial adherence solely by inhibiting glucan synthesis.

The in vivo experiment in this study showed that topical application of either crude extracts of their H-frs showed cariostatic effect on smooth-surface caries without showing reduction of the percentage of Streptococcus sobrinus 6715 infection; their effect on sulcal caries was negligible. Considering the reduction of smooth-surface caries and lack of effect on Streptococcus sobrinus levels in the animals’ plaque, the in vivo data in this study suggest that the cariostatic properties of propolis extracts are related to glucosyltransferases activity inhibition rather than to antimicrobial action. The results of the present study are not comparable with those of a previous study using the same type of crude propolis, because a different experimental model was used in the former, since the animals were not desalivated. In this model, the presence of saliva is an important factor to consider because it could have interacted with propolis and its fractions. The extract concentrations used in this study were also considerably lower than that of the previous study (2.5% versus 5.0%, w/v). In general, the propolis samples showed a similar effect on smooth-surface caries development compared to that of their respective hexane fractions. Propolis type-3 and its hexane fraction were the most effective treatments for preventing both the incidence and severity of smooth-surface caries. It would appear that hexane fractions harbor most of the active compounds of propolis type-3 and -12, and should be the fractions of choice for further chemical characterization and isolation.

Acknowledgments

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