

Full Length Research Paper

Synergic effect of associated green, red and brown Brazilian propolis extract onto *Streptococcus mutans* and *Streptococcus sanguinis*

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Propolis is an organotherapeutic product collected by honeybees and has relevant pharmacological properties, highlighting the high antimicrobial activity. This study aimed to evaluate *in vitro* the synergistic effect between three ethanol extract of different Brazilian propolis samples: green (*Baccharis dracunculifolia*) (A), red (*Dalbergia ecastophyllum*) (B) and brown (*Copaifera* sp) (C) propolis by antimicrobial sensitivity of *Streptococcus mutans* (ATCC 25175) and *Streptococcus sanguinis* (ATCC 10557), through the agar diffusion method. Aliquots of each microorganism containing 1.0×10^6 CFU / mL were inoculated on Mueller- Hinton agar supplemented with 5% dextrose and sterile blank discs containing 20 μ L of each propolis sample (A, B, C) and combined (A + B, A + C, B + C, and A + B + C) were planted on the agar. Tetracycline 30 mg discs and blank discs containing 70% alcohol served as controls. After incubation at 37°C in bacteriological incubator in a 5% CO₂ atmosphere for 24 and 48 h the inhibition zones were measured. The results showed that all extracts inhibited the growth of both microorganisms, while the samples (B) and (A + B), were significantly more effective than the others. For samples B and C similar results were observed.

Key words: *Streptococcus mutans*, *Streptococcus sanguinis*, Brazilian propolis, antimicrobial activity, synergism.

INTRODUCTION

Dental caries is an infectious disease of worldwide public health concern, especially in developing countries. It is characterized by the colonization and accumulation of oral microorganisms on dental surfaces, resulting in the formation of dental plaque (or bacterial biofilm) and demineralization of the tooth structure (Shelwits et al.,

2007; Farsi, 2008; Asokan et al., 2008; Libério et al., 2009). Many bacteria have been described in association with the cariogenic process, especially large populations of acidogenic and aciduric bacteria, such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and *Lactobacillus*, which are capable of

demineralizing enamel by producing an acidic environment (Loesche, 1986; Mikuls, 2010; Parisotto et al., 2010; van Gemert-Schriks and van Amerongen, 2010). The investigation of natural products with antimicrobial activity has attracted the attention of many researchers, motivated mainly by the increasing bacterial resistance to traditional antimicrobial agents (Cragg et al., 1997; Sheldon, 2003; Rates, 2001; Salatino et al., 2005) and the side effects are frequently observed after the use of antibiotics (Auerbach et al., 2010). In this context, propolis has been extensively studied regarding their antimicrobial property (Cunha, 2001; Dziedzic et al., 2013; Bueno Silva et al., 2013; Dualibe et al., 2007; McLean and Sheikh, 2010).

Propolis is a resinous mixture of substances collected by honey bees (*Apis mellifera*) from various plant sources. It is used by the bees for example, to seal holes in their honeycombs and protect the hive entrance (Björnsson et al., 2010; Marcucci, 1995). It is considered responsible for the low incidence of bacteria and moulds within the hive. The action against microorganisms is an essential characteristic of propolis, and humans have used it for centuries for its pharmaceutical properties. Besides its antibacterial, antifungal and antiviral properties, propolis presents many other beneficial biological activities such as antioxidant, antiinflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory, antimutagenic (Ali et al., 2010; Santos, 2012; Ferreira et al., 2007; Silva-Filho et al., 2008). For these reasons, propolis has been used as a popular remedy in folk medicine, in apitherapy, as a constituent of biocosmetics, health foods and in numerous other purposes (Vervelle et al., 2010; Stepanovic et al., 2003; Siqueira et al., 2009). Brazilian samples present striking differences in their chemical composition when compared with samples from temperate zones. *Brazilian red propolis is derived from Dalbergia ecastophylum* (Kalogeropoulos et al., 2009; Daughsh et al., 2008), while the green propolis is derived from *Baccharis dracunculifolia* (Libério et al., 2009; Park et al., 2002; Alves et al., 2000), and brown propolis is derived from *Copaifera sp.* (Anthony et al., 2005; Veiga Jr., 2007; Santos et al., 2008; Stupp et al., 2008; Salomão et al., 2008). Besides, differences are also found among tropical samples depending on the local flora at the site of collection (CLSI, 2011). This paper aims to study the susceptibility of *S. mutans* and *S. sanguinis* for associated samples of red, green and brown Brazilian propolis ethanolic extracts.

MATERIALS AND METHODS

Preparation of propolis extracts

Crude samples from red (*D. ecastophylum*), green (*B. dracunculifolia*) and brown (*Copaifera sp.*) propolis were

acquired at PharmaNéctar[®] (Belo Horizonte, Brazil). The samples were grinded and then weighed 30 g each to be added to the liquid extractor (alcohol 70%). After percolating for three days, the extracts were filtered and stored at 70°C for seven days for this to evaporate the solvent. After the samples were dried, each was weighed, yielding the following weights: 5.553, 14.123 and 4.261 g, respectively. Each sample was added to alcohol 80% resulting in propolis extracts at 10%. In 1.5 mL polypropylene tube with snap-on caps were placed mixtures of ethanolic extracts to be tested as follows: 100 µL of green propolis extract (A) + 100 µL of red propolis extract (B), 100 µL of green propolis extract (A) + 100 µL of brown propolis extract (C), 100 µL of brown propolis extract (C) + 100 µL of red propolis extract (B) and the addition of three propolis extracts in the ratio of 100 µL each.

Evaluation of antimicrobial activity

Cariogenic bacteria *S. mutans* (ATCC 25175) and *S. sanguinis* (ATCC 10557) were tested in this study. Sterile blank discs (CECON-Sao Paulo-Brazil) were soaked with 20 µL of each extract alone (A, B, C) and combined (A + B, A + C, B + C, A + B + C) and grown on Mueller-Hinton agar (Difco, USA) previously seeded with 1.0×10^8 CFU/mL of each microorganism. The plates were incubated at 37°C in bacteriological incubator in 5% CO₂ atmosphere, for 24 and 48 h and after that, the inhibition zones were measured (Sforzin, 2000).

Statistical analysis

The results were calculated with mean (M) and standard deviation (SD). The nonparametric Kruskal-Wallis test was used for statistical analysis. A p-value less than $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

All isolates (A, B, C), as well as the associated samples extracts propolis (A + B, A + C, B + C), inhibited the tested microorganisms growth *in vitro* with greater effectiveness than control antibiotic tetracycline (Table 1). However, the red propolis (B) showed inhibition zones larger and significantly different from A and C. The inhibition zones observed for samples A and C were similar. All propolis samples were effective in inhibiting *S. mutans*. The antimicrobial activity from the combined extracts is more effective in sample A + C, while the sample B had the same results when tested alone and combined. Furthermore, combination A + B + C was not significant when comparing with samples A, B, or C, nor with combination A + B.

Table 1. Susceptibility of cariogenic biofilm microorganisms to green (A), red (B) and brown (C) Brazilian propolis extract.

Propolis extract	<i>Streptococcus mutans</i>		<i>Streptococcus sanguinis</i>	
	24 h	48 h	24 h	48 h
	Inhibition zones (M ± SD) (mm)			
A	21.50 ± 0.50	21.50 ± 0.50	16.67 ± 1.09	16.67 ± 1.00
B	*27.25 ± 0.25	*27.25 ± 0.25	19.33 ± 0.94	19.33 ± 0.94
C	21.00 ± 0.00	21.00 ± 0.00	15.67 ± 0.19	15.67 ± 0.19
A + B	*27.50 ± 0.82	*27.50 ± 0.82	18.67 ± 0.25	18.67 ± 0.25
A + C	20.33 ± 0.94	20.33 ± 0.94	17.00 ± 1.10	17.00 ± 0.10
C + B	*22.67 ± 0.43	*22.67 ± 0.43	18.00 ± 1.08	18.00 ± 1.08
A + B + C	*25.50 ± 0.41	*25.50 ± 0.41	15.67 ± 1.00	15.67 ± 1.00
Ethanol 70%	No	No	No	No
Tetracyclin 30 µg	18.33 ± 1.79	18.33 ± 1.79	17.00 ± 1.41	17.00 ± 1.41

Inhibition zones (mm) media (M) ± standard deviation (SD) in diffusion agar plates of three experiments. *, Significant $p < 0.05$.

Brazilian green propolis is originated from *B. dracunculifolia* of southeastern Brazil and is attracting many researches in the world (Libério et al., 2009; Sforzin et al., 2007; Pereira et al., 2011), because of its antimicrobial, anti-inflammatory, healing and anaesthetic properties. These results were expected and have been richly studied in literature (Awale et al., 2008; Trusheva et al., 2006). Brazilian red propolis originated from *D. ecastophyllum*, native plant in the wetlands of the Atlantic coast of northeastern Brazil and has been studied for their physicochemical characteristics and their antimicrobial, antitumor and anti-inflammatory properties (Lio et al., 2010). However, brown propolis which originated from *Copaiba* sp, is little studied and was effective against *S. mutans* and *S. sanguinis* like green propolis. When these two types of propolis were associated, they showed greater effectiveness demonstrating that there was a synergism between them. The association of the three propolis samples showed no greater efficiency in inhibiting the microorganism's growth. The results observed for red propolis in this study were expected and showed that it has better antimicrobial properties than green propolis and brown propolis against cariogenic microorganisms.

This work focused on the synergistic effect that can occur when different types of propolis are associated. However, the synergism was observed only when green and brown propolis were associated. Other studies should be made to better clarify the effect of the combination, especially with respect to chemical compounds that may inhibit or activate other unknown factors responsible for the results observed here. Furthermore, the quantitative and qualitative differences in the composition of propolis samples from different regions worldwide affect the intensity and range of its antimicrobial activity (Bonhevi et al., 1994). The mechanism of the antibacterial action of propolis has not been completely elucidated. Apparently, it is associated

with a synergistic action of compounds (Takaisi-kikuni and Schilcher, 1994; Tao et al., 2013). In this regard, studies with *Streptococcus agalactiae* have revealed that the mechanism of action might involve the formation of pseudomulticellular streptococci, disorganization of the bacterial cytoplasm and membranes, partial bacteriolysis, and inhibition of protein synthesis (Cushnie and Lamb, 2005). Since propolis samples may possess high contents of distinct flavonoids, the antibacterial mechanisms associated with these substances may be the same as those of crude propolis: inhibitions of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism (Kujumgiev et al., 1999). Copaiba propolis was recently discovered in Brazil and has still been little studied, and this is the first study of its activity against cariogenic microorganisms. Also in this study, we evaluated the synergistic effect of brown propolis when associated with red and green propolis. Plants of the *Copaifera* L. genus (Fabaceae-Caesalpinioideae), popularly known as "copaiba" has its main constituents oils as sesquiterpenes and diterpenes (Gomes et al., 2007; Attia et al., 2013). The most common sesquiterpenes are caryophyllene, copaene, zingiberene, bisabolene, and bergamotene. The main diterpenes are kaurenic, hardwichiic, kovalenic, polyalthic, and copalic acid (this last is considered a characteristic diterpene of the genus *Copaifera*). Copaiba oil has been used in folk medicine as an anti-inflammatory and antimicrobial (Stupp et al., 2008). Propolis of copaiba can contain these same components to a lesser or greater amount. The chemical profile of propolis has similarity with the profiles of chemical components found in plants from which the bees collect resins. The diversity of propolis is directly related to the season of the year it was collected, light intensity, type of terrain, bee species and plant species. Within this universe of microbial diversity profile of the plants (Malik et al., 2013), can be extrapolated to understand the antimicrobial activity of various types of

propolis worldwide.

Conclusion

The sensitivity test against the microorganisms *S. mutans* and *S. sanguinis* showed inhibitory action of ethanol extracts of propolis alone and their combinations. It was observed that the sample of red propolis (B), alone and combined with Propolis green (A) = (A + B) was significantly more effective than the others. Samples B and C showed similar synergistic effects.

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