Antibacterial Effect of Propolis Oral Spray against *Streptococcus mutans*

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Abstract

Propolis extract is known to be an effective antibacterial agent against cariogenic bacteria, however, the antibacterial activity of commercial products have never been demonstrated. The aim of this study was to investigate the antibacterial activity of marketed propolis oral spray against *Streptococcus mutans* by using a direct contact test *in vitro*. Chlorhexidine mouthrinse (0.2%) and phosphate buffer saline (PBS) was included as positive and negative control, respectively. After 1-min direct contact with antibacterial agent, or the oral spray, and washed, *Streptococcus mutans* growth was observed in culture medium for up to 10 hr. The optical density measurement at 600 nm (OD_{600}) and pH in *Streptococcus mutans* cultures were recorded every 2 hr to demonstrate bacterial growth and acid production. At 0 hr, each culture was adjusted to 0.1 OD_{600} containing 3×10^7 colony forming units (CFUs/mL) of *Streptococcus mutans*. After *Streptococcus mutans* was exposed to PBS, cell growth was 0.94±0.01 (mean±SD) OD_{600}, or approximately 3×10^8 CFUs/mL in 10 hr. In contrast, 0.08±0.01 and 0.07±0.01 (mean±SD) OD_{600} or approximately 2×10^7 CFUs/mL were measured in cultures of *Streptococcus mutans* which had direct contact to propolis oral spray, and chlorhexidine mouthrinse, respectively. This result showed that propolis oral spray significantly inhibited growth of *Streptococcus mutans* ($p \leq 0.01$) analysed by one-way ANOVA test. Consistently, *Streptococcus mutans* cultured after PBS treatment produced acid more than the *Streptococcus mutans* contacted to propolis oral spray, or chlorhexidine mouthrinse. Culture pH of PBS group reduced from 7 to 5 in 10 hr, while culture pH of propolis oral spray, or chlorhexidine mouthrinse group, remained at 7. Culture pH after a direct contact to propolis oral spray and chlorhexidine mouthrinse were significantly higher than PBS group ($p \leq 0.01$) analysed by one-way ANOVA test. In conclusion, one-minute contact of propolis oral spray, similar to chlorhexidine mouthrinse, is adequate for inhibition of growth and acid production of *Streptococcus mutans in vitro*. This finding suggested that oral application of propolis oral spray may prevent the colonization of cariogenic bacteria.

Keywords: antibacterial effect, direct contact test, propolis extract, *Streptococcus mutans*

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Introduction

Dental caries is a multifactorial disease. The ecological plaque hypothesis suggests that environmental changes may cause an imbalance in the dental biofilm that support the growth of cariogenic bacteria. Several factors, such as the host factors; saliva, tooth anatomy, immune response, genetic factor; and environmental factors such as high sugar diets, can affect the microbial ecological balance in the dental plaque [1, 2]. Epidemiological studies have implicated *Streptococcus mutans* as the primary pathogen of enamel caries in children and young adults [2, 3]. There are positive correlations of percent mutans streptococci, including *Streptococcus mutans* and *Streptococcus sobrinus,* in saliva and plaque with a higher risk for early childhood caries [4]. Thus, an elevation of mutans streptococci, as the caries risk factor, is one of the criteria for caries risk assessment in young children released by American Academy of Pediatric Dentistry [5], and other caries activity assessment [4].

Streptococcus mutans efficiently ferment a range of sugars and generate acids including lactic, formic and acetic acid as metabolic end product. Lactic acid, the strongest acid, produced by *Streptococcus mutans* can cause the plaque pH changes to below the critical pH for enamel demineralization. Increased number of *Streptococcus mutans* in plaque results in more acid production at faster rate, thereby enhancing tooth demineralization. When this acidic environment is continued, it promotes the growth of acidogenic and acid-tolerant bacteria and disturb the balance of other microorganisms in the plaque community to an advantage of *Streptococcus mutans* colonization [1, 4, 6, 7].

The standard care for dental caries is to eliminate bacterial biofilm, by using mechanical methods, such as brushing and interdental flossing, or chemical method such as antibacterial mouthrinse [8]. Chlorhexidine mouthrinse is considered to be the gold standard antiplaque agent and commonly used, when necessary, for the latter method. Long term use of this agent may be beneficial for patients with mental disability, physical disability, motor function disturbance and muscle coordination disturbance. However, side effects of chlorhexidine include brown discoloration of teeth, oral mucosa erosion and a bitter taste reduce patient compliance, especially in young patients. Moreover, the development of chlorhexidine-resistant microbial strains may possibly occur when chlorhexidine are frequently used [9, 10]. Recently, alternative antibacterial agents including natural extracts that can be used orally are being studied for a novel approach in caries prevention purposes [11]. In dentistry, propolis extract has been reported to be an effective antibacterial agents. Several compounds in propolis inhibit glucosyltransferase activities from *Streptococcus mutans* [12, 13]. At present, there are propolis oral spray purchasable in consumer market for indication of bad breath and sore throat reducing. To use oral spray is feasible and beneficial in young children or disability patients to maintain their oral health. However, anti-oral bacterial activity of the propolis extract after being processed during manufacturing remains unknown. The aim of this study was to investigate the antibacterial activity of propolis oral spray against *Streptococcus mutans in vitro*.

Materials and methods

Streptococcus mutans (ATCC 25175) were cultured on brain-heart infusion (BHI) agar (HIMEDIA®) at 37ºC in 5% CO_2 incubator (Forma Steri-Cycle CO_2 Incubator, Thermo Scientific) for 48 hr. A single colony was transferred to BHI broth (HIMEDIA®), incubated overnight in the same condition as above, and continuously shaken at 240 rpm (IKA KS 130 basic Shaker, USA). To assess antibacterial effect of propolis oral spray *in vitro*, direct contact test adapted from Ehsani et al.[14] & Herrera et al.[15] was used. Briefly, the overnight cultures (14 ml) were centrifuged at 5000 rpm for 8 min, resuspended in 9 ml of phosphate-buffered saline (PBS), and then pooled in 50 ml erlenmeyer flask. Then, bacterial suspension was adjusted with PBS to optical density of 1.0 at 600 nm wavelength (OD_{600}), using a spectrophotometer (Thermo Scientific™ GENESYS 20), and 1 ml of bacterial suspension was added into 1.5 ml tubes. Bacterial cells were centrifuged at 8000 rpm for 5 min, PBS was discarded, and 100 µl of propolis oral

spray, 0.2% chlorhexidine mouthrinse (positive control) or PBS (negative control) were added to cell pellet, and briefly vortexed, and direct contact was allowed for 1 min. Then, 1 ml of PBS was added immediately to dilute the test solution and pellets were washed 2 times in 1 ml PBS. After washed and PBS was discarded, 1 ml of BHI broth was added, and bacterial cells were transferred into 15 ml plastic test tube containing 9 ml of BHI broth. Each culture was adjusted again to 0.1 OD_{600} containing approximately 3 x 10^7 CFUs/mL of *Streptococcus mutans*, then cultures were incubated at 37ºC, 5% CO_2 and shaken as above. Bacterial cultures were monitored every 2 hr (at 0, 2, 4, 6, 8 and 10 hr), and optical density measurement (OD_{600}) and pH were recorded. The pH measurement of the bacterial broth was taken using a pH indicator strip (Panreac, pH range 4.5-10). A strip of filter paper was immersed in each bacterial broth until the color change remains stable, and the color on the strip was compared standard chart provided by the manufacturer to determine the pH value of each sample by only one examiner throughout the study. The measurement was performed in triplicates and the means of optical density were plotted to show exponential growth of *Streptococcus mutans* for up to 10 hr. The means of optical density and pH at 10 hr were analyzed by one-way ANOVA to compare the treatments. The statistical analyses was performed using SPSS (version 22.0) and the significance level was set at 0.05.

Results and Discussion

After 1-min direct contact, the bacterial growth and pH of culture media were evaluated for up to 10 hr. The optical density of *Streptococcus mutans* cultures was changed from 0.1±0.01 to 0.94±0.01 (mean±SD) OD_{600}, or approximately 3 x 10^7 CFUs/mL to 3 x 10^8 CFUs/mL, in 10 hr after exposure to PBS. In contrast, the growth of cells were affected after having a direct contact with propolis oral spray and chlorhexidine mouthrinse. *Streptococcus mutans* cultures showed 0.08±0.01, and 0.07±0.01, OD_{600} or approximately 2 x 10^7 CFUs/mL at 10 hr after direct contact to propolis oral spray, and chlorhexidine mouthrinse, respectively (figure 1). This result showed that propolis oral spray and chlorhexidine mouthrinse significantly inhibited growth of *Streptococcus mutans* ($p \leq 0.01$) when compared with PBS group analysed by one-way ANOVA test.

Consistently, pH measured from *Streptococcus mutans* cultures in PBS group was reduced from 7 to 5 within 10 hr as shown in figure 2. On the other hand, pH of *Streptococcus mutans* cultures contacted to propolis oral spray, or chlorhexidine mouthrinse, was significantly higher than pH of PBS group ($p \leq 0.01$) analysed by one-way ANOVA test. Culture pH of both groups were above the critical pH of demineralization and maintained at pH 7 throughout the experiment. These findings suggested that propolis oral spray and chlorhexidine mouthrinse not only affected the growth but also inhibited acid production of *Streptococcus mutans*.

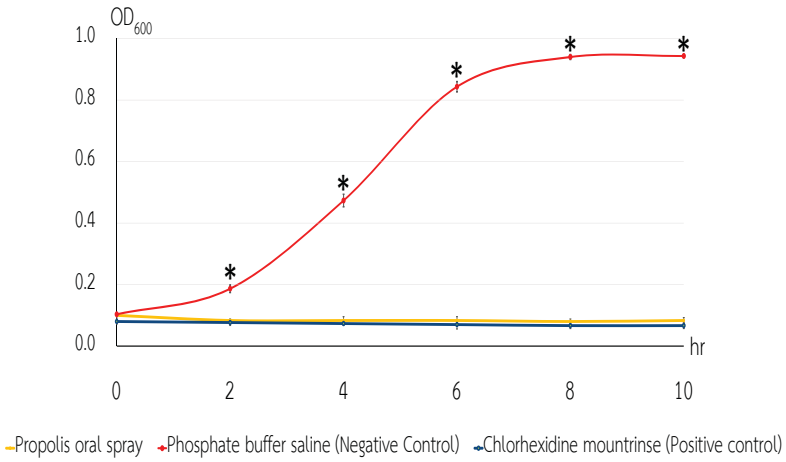

Figure 1. Growth curve of *Streptococcus mutans* after treatment. At 0 hr, each culture was adjusted to 0.1 OD_{600} after a 1-min direct contact to propolis oral spray (purple), PBS (red) and chlorhexidine mouthrinse (green). Optical density was recorded every 2 hr for up to 10 hr, and the means of 3 replicates are shown. Error bars demonstrate standard deviation. Three independent experiments were performed, and one experiment representing all data is shown. This result show that propolis oral spray and chlorhexidine mouthrinse significantly inhibit growth of *Streptococcus mutans* ($p \leq 0.01$) when compared with PBS group analysed by one-way ANOVA test.

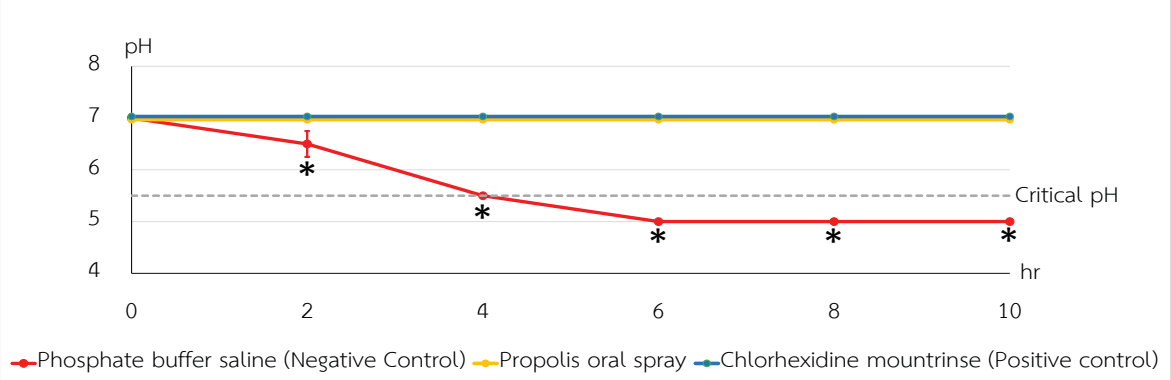

Figure 2. pH of *Streptococcus mutans* cultures after treatment. At 0 hr, each culture was adjusted to 0.1 OD_{600} after a 1-min direct contact to propolis oral spray and chlorhexidine mouthrinse (purple) and PBS (red). Culture pH was recorded every 2 hr for up to 10 hr by using pH indicator strip, and the means of 3 replicates are shown. Error bars demonstrate standard deviation. Three independent experiments were performed, and one experiment representing all data is shown. The result show that pH of *Streptococcus mutans* cultures contacted to propolis oral spray, or chlorhexidine mouthrinse, is significantly higher than pH of PBS group ($p \leq 0.01$) analysed by one-way ANOVA test.

Several compound in propolis i.e. apigenin, kaempferol, pinocembrin and pinobanksin-3-acetate could inhibit streptococcal glucosyltransferase activities which is the virulence factors in the pathogenesis of dental caries. With these compounds, apigenin displayed the most potent inhibition of glucosyltransferase activities. Apigenin inhibited 90.5 to 95% of the activity of all of the glucosyltransferases tested in solution and 30 to 60% on surface-adsorbed enzymes [12]. Propolis extract showed antibacterial effect against relevant bacteria in dentistry i.e. *Streptococcus mutans, Streptococcus salivarius, Streptococcus sorbrinus*, *Escherichia faecalis* and *Lactobacillus casei* [13, 14]. Among these pathogens, *Streptococcus mutans* is considered as a potent initiator of caries which formed a significantly greater proportion of the carious lesion [2, 3]. In the previous *in vitro* study, propolis extracted with 80% ethanol and propolis extract with water showed significant antibacterial effect against *Streptococcus mutans* in agar diffusion method [13]. Different method was used in this study because propolis oral sprays containing peppermint oil and spearmint oils affected the result of agar or disk diffusion method, so direct contact test had been used instead. However, the result demonstrated that propolis oral spray which being processed during manufacturing had similar antibacterial effect against *Streptococcus mutans* to propolis crude extract. Besides propolis oral spray, propolis mouthrinse had already been evaluated the antimicrobial activity on *Streptococcus mutans* by volunteers performed 21 rinses divided into 3 rinses per day for 7 day. The result showed that propolis mouthrinse could reduce the concentration of *Streptococcus mutans* about 81% of all saliva samples collected in young individuals [16]. Thus, propolis oral spray should be further studied whether its antimicrobial activity remains potent on *Streptococcus mutans* in dental biofilm. Microorganisms remaining in the biofilm structure are more resistant to antibiotics than those in the planktonic. Because biofilms present in the oral cavity are complex community comprising more than 700 strains of microbial anchored to solid surfaces such as tooth enamel, thus biofilms express properties not exhibited by the same organisms growing in culture [17, 18].

Conclusion

In conclusion, one-minute direct contact of propolis oral spray is adequate for inhibition of growth and acid production of *Streptococcus mutans in vitro*, similar to chlorhexidine mouthrinse. This finding suggested that propolis oral spray demonstrates antibacterial properties and oral application of propolis oral spray may prevent the growth of cariogenic bacteria.

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