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THE EFFECT OF PROPOLIS EXTRACT AND PROPOLIS CANDIES ON THE GROWTH OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* ATCC 43718

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ABSTRACT

Objective: This experiment aimed to analyze the effect of propolis extract and propolis containing candies on the growth of *Aggregatibacter actinomycetemcomitans* using spectrophotometric analysis and colony-forming units (CFU) counts.

Methods: After *A. actinomycetemcomitans* were exposed to propolis extract and candies, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined with spectrophotometry and post-exposure colony counting.

Results: The MIC of propolis extract against *A. actinomycetemcomitans* was determined to be 10%, and the MBC was 20%. A decrease in the total CFU count of *A. actinomycetemcomitans* was observed after propolis extract and candy exposure.

Conclusions: Propolis extract and propolis candies were effective in inhibiting the growth of *A. actinomycetemcomitans* ATCC 43718 *in vitro*.

Keyword: Propolis, Flavonoid, *Aggregatibacter actinomycetemcomitans*, Periodontitis.

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INTRODUCTION

Periodontitis is an oral health problem with a high prevalence. The prevalence of periodontitis is up to 60% [1]. Periodontitis is caused by a bacterial complex. The bacteria that predominantly cause this disease are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [2]. *A. actinomycetemcomitans* is a facultative anaerobic bacterium that causes aggressive and juvenile periodontitis. Based on recent studies, the combination of scaling, root planning, and antibiotic therapy is required to cure periodontitis. However, if the antibiotic is not properly administered, it can cause microorganism resistance. Moreover, systemic antibiotics have a high allergic ratio and are expensive [3]. Therefore, a number of herbal ingredients have been developed as an alternative for the periodontitis therapy. Propolis is an herbal ingredient that has antibacterial activity [4-6].

Propolis has been proven in the medical world to have many benefits. One of them is its antimicrobial effect because of the flavonoid and phenol it contains [7,8]. Propolis that comes from China is proven to have a good antimicrobial effect against periodontal pathogens. Gel that contains propolis, from Brazil, is also proven to lower periodontitis morbidity [2,3]. Another herbal product which has also proven to have antimicrobial activity is honey. Honey contains resin collected by honeybees from various plants. Honey has antimicrobial activity because of the oxidase glucose enzyme that changes glucose to gluconolactone that produces H_2O_2 [9,10].

Propolis is available in various forms, such as mouthwash, gel, and candy. Recently, Universitas Indonesia, represented by Sahlan and Partners, has been developing propolis containing candy as a preventive measure for the dental and oral disease. This experiment aimed to analyze the effect of propolis extract, propolis honey candy with sucrose and palm sugar, and propolis containing candies that are available in the market (propolis containing candy X), on the growth of *A. actinomycetemcomitans*.

METHODS

Sterilization of equipment and materials

All equipment and materials were sterilized using an autoclave at 121°C for 15 minutes.

Making propolis extract concentrations

Propolis extract, 20% concentration provided was diluted with glycerin using the formula: K1V1 = K2V2. K1 = initial propolis extract concentration; V1 = initial propolis extract volume; K2 = result propolis extract concentration; V2 = result propolis extract volume.

Making candy solution/mixture

Propolis candy with honey, honey candy, and propolis candy X were each ground by mortar, and 3 g of each was put into sterilized Erlenmeyer flasks with 10 ml of sterilized brain-heart infusion broth (BHI), according to the formula above. The candy solution/mixture was then filtered with a 0.22 µl diameter Sartorius filter.

Making the agar

BHI (37 g) and 15 g of bacteriological agar powder were diluted in 1 L of aquades in an Erlenmeyer flask. It was then sterilized in an autoclave at 121°C for 15 minutes. After sterilization, 1 ml of Vitamin K was added into the resulting colloid. The Erlenmeyer flask with agar was then put into an orbital shaker. Petri dishes were then filled with 20 ml of the BHI agar.

Bacterial culture

A. actinomycetemcomitans was taken by 20 µL pipette and put into the agar medium under aseptic condition. Then, the bacteria were evened out with a spreader. It was then cultured for 24 hrs at 37°C in an anaerobic environment.

Bacterial dilution

One colony of bacteria was taken from the agar using a loop. It was then put into 7 ml of BHI medium and incubated for 72 hrs at 37 °C in an anaerobic environment. After incubation, the bacteria were standardized into a dilution of 10^5 CFU/ml.

Table 1: Inhibition values of propolis extract against *A. actinomycetemcomitans*

Propolis extract concentration (%)	Inhibition value (%)			Mean value of inhibition (%) ±SD	C
	Sample I	Sample II	Sample III		
0.5	27.44	26.17	25.36	26.32±0.85	0
1	29.51	31.14	27.89	29.51±1.33	0
5	55.32	72.11	75.00	67.48±8.67	0
10	94.31	94.95	94.77	94.68±0.27	0
15	61.55	65.61	60.11	62.42±2.33	0
20	56.23	50.45	45.31	50.66±4.46	0

C: Control without extract exposure, SD: Standard deviation, *A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans*

Table 2: The number of *A. actinomycetemcomitans* colonies after propolis extract exposure

Propolis extract concentration (%)	Number of colonies (10^4 CFU/ml)
0.5	15000
1	13000
5	8100
10	480
15	18
20	0

A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans

Propolis extract minimum inhibitory test and minimum bactericidal test

Propolis extract (100 µl) with 0.5%, 1%, 5%, 10%, 15%, and 20% concentrations were put into a 96-well plate with a triplicate shape. Each 96-well plate was then filled with 100 µL of *A. actinomycetemcomitans* with a dilution of 10^5 CFU/ml [11]. The 96-well plate was put into an enzyme-linked immunosorbent assay (ELISA) reader, and the optical density was checked to measure the bacterial concentration using a wavelength of 450 nm [12]. Then, the 96-well plate was incubated for 48 hrs at 37°C in an anaerobic environment [11]. After incubation, the 96-well plate was put back into the ELISA reader, and the optical density was checked for a second time to measure the bacterial concentration, again, using a wavelength of 450 nm [12]. The inhibition percentage was calculated using the formula:

$$\text{Inhibition} = 1 - \left(\frac{OD_{sample} - OD_{blank}}{OD_{bacterial} - OD_{medium}} \right) \times 100\%$$

Later, the bacterial products from the incubation were planted in BHI broth agar and incubated for 24 hrs at 37°C in an anaerobic environment. The MBC was observed where no bacterial growth had appeared on the agar, with the smallest concentration.

Calculation of bacterial quantity after candy exposure

A. actinomycetemcomitans suspension (100 µl) with a dilution of 10^5 CFU/ml was exposed to 100 µl of each solution of honey sucrose propolis candy, honey palm sugar propolis candy, and propolis candy X [11]. The results of the exposure were then incubated for 48 hrs at 37°C in an anaerobic environment [11]. After incubation, the result of the *A. actinomycete mcomitans* exposure, with each candy solution, was then planted on BHI broth agar and later incubated for 24 hrs at 37°C in an anaerobic environment. The colony size on the agar was later calculated.

RESULTS

The results of the propolis extract inhibition test against *A. actinomycetemcomitans* were as follows: Concentration of propolis extract of 0.5% was 26.32% (±0.85); 1% concentration was 29.51% (±1.33); 5% concentration was 67.48% (±8.67); 10% concentration was 94.68% (±0.27); 15% concentration was 62.42% (±2.33); and 20% concentration was 50.66% (±4.46) (Table 1). The results showed an increasing trend from 0.5% concentration to 10% concentration, but a decreasing trend was shown from 15% concentration to 20% concentration.

The calculated sizes of the *A. actinomycetemcomitans* colonies (10^4 CFU/ml) after extract exposure were as follows: 0.5% concentration was 15000; 1% concentration was 13000; 5% concentration was 8100; 10% concentration was 480; 15% concentration was 18; and for 20% concentration no colony growth was shown. The results showed that the larger the concentration of propolis extract, the less *A. actinomycetemcomitans* colony growth on agar, with a regression equation of: Y = 12985.670-802.272X, with Y = Colony count, and X = Propolis concentration (Table 2).

Based on *A. actinomycetemcomitans* colony calculations, after exposure to propolis containing candies, it was shown that exposure of *A. actinomycetemcomitans* to honey sucrose propolis candy had the lowest mean colony count of 123. This was followed by propolis candy X with a mean colony count of 131. Finally, honey palm sugar propolis candy had a mean colony count of 200 (Table 3). Based on a one-way analysis of variance test, the mean of the *A. actinomycetemcomitans* colony count, after exposure to the three different candies, was significantly different to the control, without propolis extract ($p<0.025$).

DISCUSSION

The minimum inhibitory concentration (MIC) of propolis extract was found at 10% concentration, and the MBC was found at 20% concentration. These data suggest that propolis was effective in inhibiting the growth of *A. actinomycetemcomitans.* The inhibitory ability of propolis is reported to come from the flavonoid it contains. Based on the previous studies, it is a flavonoids proven ability to destroy bacterial cells that causes inhibition and prohibition of bacterial macromolecule synthesis [13]. The results of this study are supported by tests of propolis from China and Brazil that were also effective to inhibit the growth of *A. actinomycetemcomitans* [2,14]. When compared to propolis extract from China, the local propolis extract, tested in this study, was less effective in fighting against *A. actinomycetemcomitans*. The MIC of propolis extract from China was found at 0.25 µg/ml [2]. A difference in propolis content may have been caused by different methods of propolis extraction, the propolis solvent, geographic location of the propolis, and the local flora where the propolis was collected [2].

The local propolis, being tested, was identified to have a flavonoid content of 0.26% while the propolis from China had flavonoid content of 18.792%, being ×72 greater than the local propolis [13]. The difference in flavonoid content, between the types of propolis, produces different inhibitory ability. The inhibitory test of propolis extract, using the optical density method, indicated that the samples showed increasing inhibition up to 10% concentration. However, the inhibition decreased at 15% concentration and 20% concentration. The CFU count of the amount of bacteria continued to decrease until 20% concentration. In this study, the difference, as stated above, might have been caused by

Table 3: *A. actinomycetemcomitans* bacterial colony count after candy exposure

Candy	Colony count (10^6 CFU/ml)		Mean of colony count (10^6 CFU/ml) ± SD	p value of control (K)
	Sample I	Sample II		
PM	135	111	123±12	0.002*
PA	202	198	200±1.5	0.019*
PX	112	150	131±19	0.002*
K	299	260	279±19.5	

*Significantly different (p<0.05), PM: Honey sucrose propolis candy, PA: Honey palm sugar propolis candy, PX: Propolis candy X, CFU: Colony-forming unit, SD: Standard deviation, *A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans*

various factors that influenced the value of the optical density samples because the value of inhibition used the calculated value of optical density. Optical density is influenced by a Specimen's transparency, consistency, color, shape, and size [15]. The samples of propolis extract at 15% and 20% concentrations, which were used in this study, had greater concentrated color and consistency than the propolis extracts at 0.5%, 1%, 5%, and 10% concentration. The color may have influenced the value of the Specimen's optical density.

Optical density not only indicates the amount of living bacteria but also living and dead bacteria. In addition, an antibacterial substance can kill bacterium, but the bacterium remains intact. Therefore, a specimen with high optical density could contain no living bacteria [15]. Based on the regression test of propolis extract and the *A. actinomycetemcomitans* colony count, an equation was derived; Y = 12985, 670-802, 272X (X = propolis concentration, Y = colony count). Based on the equation, we could calculate that in this study every increase of 1% of propolis extract concentration led to a decrease in 802, 272 colonies. In this study, a constant of 12985, 670 showed that the regression equation was initially linear, but after the propolis extract concentration reached Y = 1 (16.16% concentration) the graph no longer remained linear.

Based on the testing of candy exposure against the growth of *A. actinomycetemcomitans*, it was shown that the number of *A. actinomycetemcomitans* colonies after the exposure to honey sucrose propolis candy, honey palm sugar propolis candy, and propolis candy X was less than the control without candy exposure. The smallest colony numbers, in this study, were identified in honey sucrose propolis candy, followed by propolis candy X, and the last was honey palm sugar propolis candy. This may have happened because of the thiamine in palm sugar. Based on the previous studies, thiamine has been identified as having the ability to increase the growth of *A. actinomycetemcomitans* [16,17].

The major difference between the honey sucrose propolis candy and the honey palm sugar propolis candy, in this study, was the sweetener used in each of the candies. Sugar, with a sucrose concentration of 99%, was used as the sweetener for the honey sucrose propolis candy. Palm sugar, with a sucrose concentration of 78%, was used as the sweetener for the honey palm sugar propolis candy. Sucrose is a disaccharide carbohydrate. In bacteria, carbohydrate is used in the process of producing adenosine triphosphate (ATP). ATP is needed in all biosynthetic processes of bacteria in order for the bacteria to live and reproduce. The greater the bacterial growth the bigger the amount of carbohydrate fermented [18]. However, in this study, the honey sucrose propolis candy with a higher sucrose concentration was better at inhibiting the growth of *A. actinomycetemcomitans* than the honey palm sugar propolis candy. This was caused by the limitation of *A. actinomycetemcomitans* growth by carbohydrate fermentation.

Unlike other bacteria in the genus *Streptococcus* that are able to ferment various kinds of carbohydrates starting from monosaccharide carbohydrate, such as glucose, to disaccharide carbohydrate, such as sucrose, *A. actinomycetemcomitans* is only able to ferment a limited number of carbohydrates, such as glucose, fructose, and mannose. The limited ability of *A. actinomycetemcomitans* is caused by its inability to produce an enzyme that can ferment sucrose. In the process of carbohydrate fermentation, bacteria need to produce enzymes to degrade and oxidize carbohydrates. Glucose is a simple carbohydrate (monosaccharide) so that almost all bacteria are able to ferment glucose, whereas sucrose is a disaccharide carbohydrate, which has a glycosidic bond between the glucose and the fructose, which cannot be dissolved by *A. actinomycetemcomitans*. Because of the limited ability of *A. actinomycetemcomitans* to ferment the carbohydrate, the high level of sucrose in the honey sucrose propolis candy did not increase the growth of *A. actinomycetemcomitans.*

The concentration of propolis extract in the honey propolis candies, in this study, was 5%. The growth of the bacterial colony on 5% propolis extract was 81×10^5, whereas on honey sucrose propolis candy and honey palm sugar propolis, it was, respectively, 123×10^6 and 200×10^6. The results showed that propolis extract of 5% had a higher inhibitory ability against *A. actinomycetemcomitans* than either honey sucrose propolis candy or honey palm sugar propolis candy. This outcome may have been caused by the glucose in the honey propolis candy that lowered the effectiveness of the candy [18]. In this study, the effect of each component: Palm sugar, sucrose, honey, and glucose syrup were not individually tested against the growth of *A. actinomycetemcomitans* because the compounds available were in the form of candy. The results of this study are parallel to the hypothesis which stated that propolis extract was effective in inhibiting the growth of *A. actinomycetemcomitans*, and honey sucrose propolis candy, honey palm sugar propolis candy, and propolis candy X were able to decrease the number of colonies of *A. actinomycetemcomitans* ATCC 43718.

CONCLUSIONS

Propolis extract has been proven to be able to inhibit the growth of *A. actinomycetemcomitans* ATCC 43718, which was shown by the value of the MIC of 10% and the MBC of 20%. Every increase in propolis extract, in this study, was able to decrease the number of *A. actinomycetemcomitans* colonies based on the equation Y = 12985, 670-802, 272X (X = propolis concentration, Y = colony count). Honey sucrose propolis candy, honey palm sugar propolis candy, and propolis candy X have been proven to be able to significantly decrease the number of *A. actinomycetemcomitans* ATC43718 colonies compared to the control without exposure to propolis candies (p<0.025). The writers suggest that propolis extract and propolis candies need to be tested with other methods both *in vitro* and *in vivo*. Furthermore, studies about the characteristics of palm sugar and sucrose, in terms of bacterial growth, should also be conducted.

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