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Research Article

DIFFERENCES IN THE EFFECTS OF 0.05% AND 0.1% PROPOLIS FLAVONOIDS ON *IN VITRO* BIOFILM FORMATION BY *STREPTOCOCCUS MUTANS* FROM CHILDREN'S DENTAL PLAQUE

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ABSTRACT

Objective: This study was conducted to analyze the effects obtained with different concentrations (0.5 and 0.1%) of propolis flavonoids on *in vitro* biofilm formation by clinical *Streptococcus mutans* strains isolated from children's dental plaque.

Materials and Methods: *S. mutans* isolated from children's dental plaque was assayed for biofilm formation in 96-microwell plates using crystal violet.

Results: The effects on *S. mutans* biofilm formation were the same for propolis flavonoids administered at concentrations of 0.05 and 0.1% (p>0.01).
Conclusion: A 0.05% propolis flavonoids concentration was deemed as effective as a 0.1% concentration at inhibiting *S. mutans* biofilm formation.

Keywords: Flavonoid, Propolis, *Streptococcus mutans*, Biofilm, Crystal violet.

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INTRODUCTION

Dental caries is a common oral health problem in children in different parts of the world. In Latin–American countries, the prevalence of child dental caries reached 60% in 2016 [1]. The caries prevalence of children aged 5–6 years in Southeast Asian countries in 2015 was even higher, ranging from 25% to 95%, with an average of 75% [2]. In Indonesia, the prevalence of untreated active caries, based on Basic Health Research (RISKESDA) in 2013, was 53.2% [3]. The prevalence of caries in children under 5 years of age in Indonesia is reported as 90% [4].

Dental caries is a dynamic process that causes progressive damage to dental tissue (enamel, dentine, and cementum) and involves demineralization of inorganic parts of teeth and dissolution of the organic parts. The onset and progression of a carious lesion depend on factors such as hosts, microorganisms, and substrates that interact in recurrent cycles. Microorganisms form dental plaque, and the amount of plaque increases with time. Various strategies have been implemented to reduce caries rates, including strengthening the host, eliminating microorganisms, and changing dietary patterns [5].

Caries can be prevented by eliminating microorganisms through the administration of antibacterial agents. The antibacterial effect is intended to inhibit the bacterium *Streptococcus mutans*, which is the bacterial species that causes caries. A commonly used antibacterial agent is chlorhexidine, considered the gold standard because of its broad antibacterial spectrum [6]. However, the use of antibacterial agents derived from natural ingredients is increasing in popularity as an alternative. One source of these ingredients is propolis, a resin produced by bees.

Propolis has been widely used to treat a number of diseases. The composition of propolis depends on the ecosystem, climate, plant species, and bee species [7-9]. In Indonesia, the *Trigona* bee species are found in Sulawesi, and the main component of propolis from these bees is flavonoids [7,8]. Flavonoids derived from plants and fruits have known antibacterial, anti-inflammatory, and antioxidant effects and can prevent caries by antibacterial action [10,11]. Consequently, the antibacterial potency of flavonoids against various types of bacteria is now being widely studied. For example, studies on bacteria such as *E. coli* indicate that flavonoids can inhibit *E. coli* by influencing bacterial cell membrane interactions [10]. Several studies have revealed that flavonoids from various sources can also inhibit the growth of *S. mutans*. Flavonoids are known to inhibit the caries process by inhibiting the glucosyltransferase (GTF) enzyme, which in turn inhibits dental plaque formation and biofilm maturation, as well as tooth demineralization [12-14].

Clinical strains of *S. mutans* live under different environmental conditions that are experienced by *S. mutans* laboratory strains. *S. mutans* clinical strains live in the highly dynamic environment of the oral cavity, which increases their ability to survive and adapt. Therefore, these strains differ in physiological and biochemical properties and are typically more virulent than *S. mutans* laboratory strains [15,16]. The clinical strains also have the ability to grow 2–4 times faster, produce more acid, have higher caries activity, and form more biofilms when compared to the laboratory strains [15,17,18]. However, the study of the effectiveness of propolis flavonoids against *S. mutans* bacteria is still limited to laboratory strains.

The previous studies of minimal inhibitory levels indicated that propolis flavonoids inhibit the growth of laboratory strains of *S. mutans*, with a 0.1% concentration being the most effective at inhibiting *S. mutans* after 24 h of incubation [8]. Propolis flavonoids at various concentrations are known to inhibit the ATPase enzyme of *S. mutans* bacteria *in vitro*. Concentrations of 0.5, 0.25, 0.1, 0.075, and 0.05% propolis flavonoids gave the same antibacterial effect as 10% ethanol [19]. The aim of the present study was, therefore, to determine whether 0.05 and 0.1% concentrations of propolis flavonoids would inhibit the *in vitro* biofilm formation by clinical *S. mutans* strains isolated from children's dental plaque.

MATERIALS AND METHODS

Ethical statement

This study was approved by the Ethical Committee of the Faculty of Dentistry Universitas Indonesia (Number: 42/ethical approval/FKGUI/VII/2017; protocol number: 050390517).

Flavonoid extraction from propolis

Flavonoids from raw propolis obtained from *Trigona* sp. Beehives in Luwu, North Sulawesi, were extracted using a maceration method [8]. 1 kg of *Trigona* sp. propolis was transferred to an electric macerator, and 5 L of 95% ethanol was added as a solvent. Maceration was conducted by stirring 12 times for 15 min, with a grace period of 5 min between stirring, followed by immersion for 120 h. The material was then filtered by gravity through filter paper to separate the filtrate from the dregs. The ethanol in the filtrate was evaporated on a porcelain plate, leaving a thick extract. The extract was put into an Erlenmeyer flask, 500 mL of toluene was added, and the mixture was stirred until dissolved. A 1.5 L volume of a water: ethanol solution (2:1, v/v) was added, and the mixture was again stirred until homogeneous. After standing for 24 h, the solution was transferred to a separatory funnel and allowed to form 2 layers. The bottom solution was removed and evaporated to obtain a viscous extract.

The 0.05 and 0.1% propolis flavonoid solutions were prepared by diluting the extract with distilled water (i.e., 0.05 and 0.1% propolis flavonoid preparations were obtained by dissolving 5 and 10 mg of the extract, respectively, in 10 mL sterile distilled water).

Subjects

The participants were healthy children 6–9 years of age. Each subject had fully erupted first permanent molars, and no caries was visible on the buccal surface of upper first permanent molars and/or on the lingual surface of the lower first permanent molars.

Sample collection

Plaque samples were taken using sterile cotton swabs from the buccal surfaces of the upper first permanent molars and the lingual surfaces of the lower first permanent molars, using two sweeps on each surface. The plaque was then inserted into Eppendorf tubes containing 1 mL of phosphate-buffered saline (PBS) and placed in a cooler box.

Bacterial culture and identification

A 25 µL volume of solution was taken from the Eppendorf tubes with a pipette and cultured on TYS20B medium in a Petri dish. The Petri dish was inserted into an anaerobic jar containing an atmosphere of 5% CO_2, 10%H_2, and 85% N_2 gas and incubated at 37°C for 24 h. Successful culture of *S. mutans* culture on TYS20B agar was confirmed by a visualization method and by microscopy following gram staining. From each culture of *S. mutans* on TYS20B agar, one colony was taken and transferred to a test tube containing 5 mL TYS broth solution for culture on liquid medium. After 24 h, the optical density (OD) was measured with a microplate reader at a 450 nm wavelength. The obtained OD value was converted to calculate the number of colonies (colony-forming units/mL). The culture was then diluted to obtain a concentration of 1×10^6 bacteria/mL, which was then used for the biofilm assay.

Biofilm assay

A 100µL volume of bacterial culture in diluted TYS Broth medium and 100µL of prepared propolis flavonoids were then added to each well of a 96-microwell plate to give a total volume of 200 µL per well. Negative control wells of TYS Broth medium alone and positive control wells containing the *S. mutans* ATCC 25175 laboratory strain in TYS broth medium were also included. Duplicate wells were prepared for all treatments. The 96-microwell plate was incubated at 37°C in an anaerobic jar containing 5% CO_2, 10% H_2, and 85% N_2 gas. After 24 h of incubation, the bacterial cells that did not form biofilms on the well base (i.e., the planktonic portion) were removed, and each well was washed twice with 200 µL PBS. A 200µL volume of 0.5% crystal violet was then added and incubated at 37°C for 15 min. The remaining crystal violet solution was discarded, the biofilm was washed once with 200 µL PBS, and 200 µL of 96% ethanol solution was added to each well. The absorbance OD of the formed biofilm was then measured at 490 nm using a microplate reader.

Statistical analysis

Data were analyzed by an unpaired t-test, with significance set at $p < 0.01$, to determine the difference in the effects of 0.5 and 0.1% propolis flavonoids on biofilm formation by clinical *S. mutans* strains isolated from children's dental plaque.

RESULTS

The OD values in the flavonoids-treated wells were larger than the OD values for the negative control (mean OD =0.054), which was run as a blank. The OD of this blank was set to OD =0, and all OD values in the treatment groups and the positive control were measured against that blank.

The mean OD values for the 0.5% propolis flavonoids (OD =0.047) and 0.1% propolis flavonoids (OD =0.049) treatments were smaller than the mean OD value for the positive control (OD =0.061), as shown in Table 1. The statistical analysis shown in Table 2 indicates no significant difference ($p=0.810$) between the effects of 0.5% and 0.1% propolis flavonoids on *in vitro* biofilm formation by *S. mutans* strains isolated from children's dental plaque.

DISCUSSION

The aim of this research was to analyze the effects of 0.5 and 0.1% concentrations of propolis flavonoids on *in vitro* biofilm formation by clinical *S. mutans* strains isolated from children's dental plaque. This study is a follow-up to a similar previous study that investigated propolis flavonoids effects on *S. mutans* laboratory stocks, but the aim of the present study was to more closely approximate the organisms that actually populate the oral cavity. The *S. mutans* laboratory strains have been repeatedly subcultured for years under optimal conditions with many organic and inorganic growth factors [15]. Research using laboratory strains should be confirmed using clinical strains to ensure that *in vitro* results validly represent those obtained with *in vivo* strains [15].

In this study, we used *S. mutans* clinical strain samples derived from dental plaques taken from healthy subjects. The objective was to isolate *S. mutans* strains that had grown in culture conditions that were always different and to obtain a situation close to the actual oral environment. The oral environment is always changing, so *S. mutans* strains in the oral cavity will show an improved ability to adapt and survive. Previous work has shown that *S. mutans* clinical strains can grow 2–4 times faster and produce more acids when compared to laboratory strains [15]. Other studies have also suggested that *S. mutans* clinical strains have higher caries activity when compared with *S. mutans* laboratory strains [17].

Plaque was sampled using a sterile cotton swab with slightly bent ends. In accordance with previous literature, the use of a cotton bud or cotton swab is an effective method for plaque sampling and for evaluating *S. mutans* in the plaque [20]. The plaque was taken from the buccal surface of the maxillary first permanent molars and the lingual surfaces of the mandibular first permanent molars, as the posterior molar teeth are areas where the *S. mutans* bacterial colonies are most commonly formed. The more anterior teeth tend to have fewer *S. mutans* colonies [21]. In addition, *S. mutans* colonies are also more available on the buccal/lingual surfaces than on the occlusal and proximal surfaces [22]. The plaque taken on each of the surfaces represented two separate swabs, as a larger number of swabs per surface increased the amounts of *S. mutans* collected [22].

The accumulation of *S. mutans* on tooth surfaces is the main etiology of caries. Therefore, efforts continue to prevent caries by eliminating *S. mutans* bacteria from the teeth. One way to do this is by treatment with antibacterial agents. The gold standard antibacterial is chlorhexidine [6]. However, antibacterial ingredients that are sourced from natural materials are increasingly being developed and in demand. The aim of the present study was to investigate a propolis flavonoids extract as a natural antibacterial agent [7].

Propolis is a material collected by bees from various plants and mixed with secretions from the bee hypopharyngeal gland. Propolis has known antibacterial properties and has been widely used for various purposes in clinical trials in the dentistry field. The propolis flavonoids are important compounds and active biologic components, so flavonoids are believed to play an important role in the observed antibacterial properties of propolis [23,24]. The flavonoids used in the present study were derived from raw propolis obtained from the honeycombs of *Trigona* sp. in the Luwu area of North Sulawesi. This propolis was extracted by a maceration method, followed by chromatography, to yield 10.61 g of flavonoids from 100 g of crude propolis. This yield is consistent with other reports in the literature of flavonoid percentages ranging from 5% to 26% in crude propolis [25].

Flavonoids are found in a variety of plants, including fruits and vegetables, and they are compounds with known antibacterial properties. Flavonoids are natural compounds that have a low toxicity, are safe to eat, and rarely elicit allergic reactions [26]. The propolis flavonoids extract used in this study was administered at concentrations of 0.1 and 0.05%. The 0.1% concentration was chosen because previous studies had reported this concentration as the most effective at inhibiting the growth of *S. mutans* bacteria [8]. The 0.05% concentration was chosen because it was the smallest concentration that previously gave an equally good antibacterial effect when compared to four higher concentrations [19].

In this study, samples of children's plaque containing *S. mutans* were cultured on TYS20B agar medium. This medium is one of the most selective media for *S. mutans*. Culturing was done on a selective medium to obtain only *S. mutans* bacteria that could grow on that agar medium. Of the selective media for *S. mutans*, TYS20B medium has the best sensitivity, so it was used in this study [27].

The growth of *S. mutans* was confirmed by visualization and gram staining after culture in TYS20B medium. The visualization identified small yellowish-white colonies characteristic of *S. mutans*. Microscopy examination confirmed a morphology of chains of purple, round microorganisms, consistent with *S. mutans* [28].

The *S. mutans* strains were cultured in the selective TYS broth liquid medium to avoid contamination with other bacteria during incubation. The amounts of bacteria were determined indirectly by turbidity measurements made after incubation in TYS broth media for 24 h. Measurements were also made using by culturing bacteria in 96-microwell plates, followed by OD determinations. After 24 h, the bacteria were already in the stationary phase, so the bacterial count remains relatively unchanged [29]. Therefore, the bacterial counts were conducted after 24 h.

Biofilm assays were performed when the biofilm formation process had reached the maturation stage. The 96-microwell plates used for the biofilm test were incubated for 24 h, as this is the ideal time for biofilm incubation according to the available literature, as it improves the maturation and biofilm adhesion [30]. Other literature also indicates that biofilm maturation is achieved after 24 h and that bacterial growth in the biofilm has ceased and stabilized by that time [31,32].

In the biofilm assays, all the treatment groups showed larger OD values compared to the negative control blank, confirming the growth and biofilm formation of the *S. mutans* clinical strains isolated here. Bacterial growth and biofilm formation will increase the turbidity of the solution within the well, while crystal violet will stain the biofilm. A larger biofilm formation will intensify the purple color and increase the OD [29,30]. The biofilm assay results, as shown in Table 1, indicate that the mean OD was smaller for the propolis flavonoids treatment groups (*S. mutans* clinical strains+medium+propolis flavonoids) than for the positive control (*S. mutans* ATCC 25175+medium). The formation of biofilms by the *S. mutans* clinical strains was clearly inhibited by propolis flavonoids, whereas the formation of biofilms in the positive control laboratory strain was not affected.

The *S. mutans* clinical strains have a higher virulence, are able to grow 2–4 times faster, and form more biofilms when compared with laboratory strains [15, 18] Therefore, in the treatment groups, the formation of biofilms will be faster and more extensive for the *S. mutans* clinical strains than for the laboratory strains. The biofilm formation was smaller in the treatment groups with the *S. mutans* clinical strains, as indicated by the smaller OD value, than in the positive control (a less virulent *S. mutans* laboratory strain). This OD value decreased due to the antibacterial effect of the propolis flavonoids, which inhibited the formation of biofilms by the *S. mutans* clinical strains. Therefore, propolis flavonoid can be deemed effective against the growth of *S. mutans* clinical strains.

The results shown in Table 2 indicate no significant difference between propolis flavonoid concentrations of 0.05 and 0.1% in terms of *in vitro* biofilm formation by *S. mutans* isolated from children's dental plaque. Therefore, both concentrations of propolis flavonoids were equally effective at inhibiting biofilm formation. This finding agrees with those of the previous studies reporting that propolis flavonoids at various concentrations (0.5, 0.25, 0.1, 0.075, and 0.05%) had the same antibacterial effect in terms of inhibiting the *S. mutans* ATPase. These five levels of propolis flavonoid concentrations also gave the same antibacterial effect as observed with a 10% ethanol control [19].

Previous studies also indicated that a propolis flavonoids concentration of 0.1% was the most effective concentration for inhibiting the growth of *S. mutans* bacteria after 24 h incubation, whereas a 0.05% concentration gave a smaller effect [8]. In the present study, with the same incubation period of 24 h, the 0.05% concentration was just as effective as the 0.1% concentration at inhibiting *S. mutans* biofilm formation. The differences in the findings of these two studies might reflect differences in the bacterial culture medium used. Selective TYS20B and TYS broth media were used in the present study, whereas using glucose nutrient agar, medium was used in the previous study [8].

The results of this study support the conclusion that the inhibition of *S. mutans* biofilm formation by propolis flavonoids could involve inhibition of the GTF enzyme, as previously postulated [10-12]. This inhibition of GTF would reduce glucan production, thereby disrupting the coadhesion process and biofilm maturation of *S. mutans*. A propolis flavonoids concentration of 0.05% is recommended for antibacterial purposes because this was the smallest effective concentration and would therefore be expected to have the fewest pharmacological side effects.

Toxicity tests on the flavonoids derived from propolis are still necessary, even though flavonoids are generally considered to be low-toxicity compounds and seldom trigger allergic reactions. This

Table 1: Mean OD treatment and control group

Treatment and control group	Mean OD
0.05% propolis flavonoids	0.047
0.1% propolis flavonoids	0.049
Positive control	0.061
Negative control	0

OD: Optical density

Table 2: *S. Mutans* biofilm formation

Propolis flavonoids (%)	n	Mean OD value±SD	p
0.05	16	0.047±0.025	0.810
0.1	16	0.049±0.023	

Unpaired t-test; meaning value based on p<0.01.
S. mutans: Streptococcus mutans, OD: Optical density

research represents a next step in the future use of propolis flavonoids as antibacterials, in the form of mouthwashes, toothpastes, or other preparations, for the prevention of caries in humans, and especially in children.

CONCLUSIONS

These research findings support the conclusion that propolis flavonoids suppress *in vitro* biofilm formation by *S. mutans* isolated from children's dental plaque. Notably, a 0.05% concentration was equally as effective as a 0.1% concentration at suppressing biofilm formation by the clinical *S. mutans* strains investigated. Further research is needed to determine the toxicity and sensitivity responses of the oral mucosa to propolis flavonoids to support the use of these flavonoids as antibacterial agents in humans.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

This study was designed, conducted, and coordinated by Mochamad Fahlevi Rizal, Eva Fauziah, Margaretha Suharsini as the principal investigator, provided conceptual and technical guidance for all aspects of the project. Agnes Linggriani performed, analyzed the data and write the manuscript.

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