




Effect of Propolis on *Streptococcus mutans* Biofilm Formation


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Abstract

Objective: To evaluate the susceptibility of *S. mutans* during growth as a biofilm in the presence of different concentrations of propolis. **Material and Methods:** Three different concentrations of ethanolic extract of propolis (10%, 5%, and 2.5%) were used to evaluate its potential to attenuate the biofilm formation of *S. mutans* (ATCC 25175) on microplates. A crystal violet staining method was performed to measure the optical density (OD) of the biofilm biomass after 3 h and 18 h time periods. All the experiments were performed in triplicate, and the obtained data were expressed as mean \pm standard deviation. A two-tailed Student's t-test was used to determine the different abilities of biofilm formation between the treated and control groups of the bacteria film in the presence of propolis. A p-value of <0.05 was taken as a significant value. **Results:** The OD levels (determined using an ELISA reader) obtained after growing *S. mutans* as a biofilm in the presence of propolis were similar ($p>0.05$) to those of the control (*S. mutans* grown in tryptic soy broth + 1% sucrose). **Conclusion:** All the tested concentrations of propolis added to the growth medium did not inhibit the biofilm formation of *S. mutans*. Since biofilms consist of bacterial cells and extracellular matrices, we hypothesize that the extracellular matrix may have interfered with the antimicrobial properties of the tested propolis.

Keywords: Plant Extracts; Propolis; Dental Plaque; Streptococcus mutans.

Introduction

Streptococcus mutans belongs to the phylum Firmicutes, a Gram-positive, facultative anaerobic oral bacterium that can ferment a large spectrum of dietary sugars [1,2]. The excreted organic acids cause a large localized drop in pH that can cause lesions of the dental enamel and thus initiate the development of caries [1]. *S. mutans* is a principal etiological agent that plays a significant role in the transition of nonpathogenic commensal oral microbiota to highly acidic and cariogenic biofilms, resulting in the development of dental caries [3-5]. When attaching to the dental surface, *S. mutans* is assembled as biofilm communities and forms matrix-embedded biofilms [2,3]. This caries-associated phenomenon is induced by dietary sugars that are transformed into extracellular polysaccharides by the enzyme glucosyltransferase (Gtf), an important target for anticaries strategies [3,5].

Propolis is a natural sticky substance collected by honeybees (*Apis mellifera* L.) from the buds of various plant species, depending on the climate zone. The chemical composition of propolis depends on its geographical origin, local flora, species of bee, and season. Generally, propolis is composed of 50% plant resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances, which include minerals and organic compounds such as phenolic acid, or their esters, flavonoids, terpenes, aromatic aldehyde and alcohol, fatty acids, stilbenes, and B-steroids [6-9]. The pharmacologically active constituents in propolis include various aromatic compounds, mainly flavonoids and phenolics [9,10].

Propolis is a nontoxic resinous natural substance exhibiting antimicrobial, anticancer, antifungal, antiviral, and anti-inflammatory properties, and has gained attention in both dentistry and medicine [7-9]. The chemical constituents of propolis found in temperate climates include chrysin, galagin, pinocembrin, and pinobaskin, and its major component is caffeic acid phenethyl ester [8]. Propolis extract limits plaque formation on the tooth surface, which indirectly reduces dental caries. The fatty acids in propolis provide a cariostatic effect by decreasing the tolerance of microorganisms to low pH and slowing acid production [8,9]. Ethanolic extract of propolis (EEP) was shown to be more effective against Gram-positive than Gram-negative bacteria during the planktonic state [8]. EEP is an effective antimicrobial and anti-inflammatory agent and is used commercially as a component of toothpaste, mouthwash, and lozenges [10]. However, the effects of propolis on the biofilm mass of *S. mutans* remain unclear.

A dental biofilm is a highly organized accumulation of microbial communities attached to the surface of an environment. Biofilms protect living bacteria within their structures and thereby provide an advantage over free-floating bacteria [11]. The slimy extracellular matrix produced by biofilm bacteria encloses the microbial community and protects them from their surrounding environment, including the attack by chemotherapeutic agents. The growth and development of biofilms are characterized by four stages: initial adherence, lag phase, rapid growth, and steady-state. Biofilm formation begins with adherence of the bacteria to a tooth surface, followed by a lag phase, in which changes in gene expression occur [11].

The bacteriostatic, bactericidal, and anti-adherent activities of propolis on caries-associated microorganisms [12,13], suggest its influence on the pathogenesis of caries. However, whether propolis can inhibit the biofilm mass of *S. mutans* remains unclear. Therefore, the present study aimed to evaluate the susceptibility of *S. mutans* during growth as a biofilm in the presence of different concentrations of propolis.

Material and Methods

S. mutans Strains and Growth Conditions

Two bacteria strains were used in this study. A laboratory stock of clinical isolate of *S. mutans* (Oral Biology Laboratory Faculty of Dentistry Universitas Indonesia) and a reference strain, *S. mutans* ATCC 25175, used as a control strain. All the bacteria strains were subcultured in tryptic soy broth (TSB, Oxoid Limited, Hampshire, UK) supplemented with 1% sucrose and incubated anaerobically at 37°C for 24 h.

Concentrations of 10%, 5%, and 2.5% of EEP were used in this study. The biomaterial was kindly provided by Dr. Sahlan from the Chemical Engineering Laboratory, Faculty of Engineering, Universitas Indonesia.

Biofilm Formation Using Crystal Violet Assay

Biofilm assays were carried out in 96-well microplates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) [14]. To grow the tested bacteria as a biofilm, we used filter-sterilized saliva and measured the protein concentration (200 µg/mL) using a spectrometer. Approximately 100 µL of the salivary proteins were added into each well, shaken for 5 min on a shaker (Certomat U, B Braun, Biotech International Ltd., New Delhi, India) at 60 rpm, and incubated for 60 min at 37°C; then, the unattached proteins were removed. Subsequently, 200 µL [around 10⁷ CFU/mL; measured at an optical density (OD) of 0.1] of each bacterial strain was inoculated into wells, then incubated for 90 min at 37°C in an anaerobic jar containing 80% N₂, 10% CO₂, and 10% H₂.

Then, an Eppendorf pipette was used to gently remove the nonadherent cells. About 200 µL of different concentrations of propolis (10%, 5%, and 2.5%) were added into each well and incubated for 3 h and 18 h at 37°C in the same anaerobic atmosphere described previously. After each experimental time period, the nonadherent bacteria were removed and the biofilm formed on the bottom microplate was gently washed with 200 µL of phosphate-buffered saline (PBS). Moreover, the biofilms were allowed to dry at room temperature, and 200 µL of crystal violet (CV; 0.5% v/v) was added to each well and was incubated for 15 min. The CV solution was removed, and microtiter plates were observed using an inverted microscope (PrimoVert, Carl Zeiss Inc., Oberkochen, Germany). Finally, 96% ethanol was added into the wells to extract the absorbed CV from bacterial cells, and the absorbance of the eluted solution was measured using a microtiter plate reader (M965+Microplate Reader, Mer Tech Inc., Houston, TX, USA) at 600 nm. A CV staining method was used to measure total biofilm biomass [15]. After 3 h and 18 h of biofilm formation, all the

medium was aspirated and the nonadherent cells were removed by washing the wells with 200 µL of PBS.

The percentage inhibition and percentage of cell mass were calculated using the following formula [16].

$$\% \text{ Inhibition} = \frac{\text{control absorbance} - \text{treatment absorbance}}{\text{control absorbance}} \times 100\%$$

$$\% \text{ Cell Mass} = \frac{\text{Treatment absorbance}}{\text{Control absorbance}} \times 100\%$$

Statistical Analysis

All the experiments were performed in triplicate, and the obtained data were expressed as mean ± standard deviation. A two-tailed Student's *t*-test was used to determine the different abilities of biofilm formation between the treated and control groups of the bacteria film in the presence of propolis. A *p*-value of <0.05 was taken as a significant value.

Results

The addition of EEP into the bacterial medium resulted in a decreased biofilm mass in both of the *S. mutans* strains tested. As Tables 1 and 2 show, after 3 h incubation period, there was no significant difference between the biomass in either experimental group (*p*>0.05). When the incubation time was extended to 18 h, the biomass increased significantly (*p*>0.05). This phenomenon was observed in biofilm masses formed by both bacterial strains tested, and with all concentrations of propolis used.

Table 1. Percentage of growth *S. mutans* biofilms.

Propolis	<i>S. mutans</i> (ATCC 25175)		<i>S. mutans</i> wild-type	
	3 hours	18 hours	3 hours	18 hours
10%	75.39	93.63	67.56	93.93
5%	67.89	92.89	62.99	93.11
2.5%	68.63	92.78	54.97	93.56

Table 2. Percentage biomass cell *S. mutans* biofilms.

Propolis	<i>S. mutans</i> (ATCC 25175)		<i>S. mutans</i> wild-type	
	3 hours	18 hours	3 hours	18 hours
10%	10%	406.40	1 571	308.33
5%	5%	311.51	1 406	270.25
2.5%	2.5%	318.86	1 386	222.11

The absorbance value of *S. mutans* ATCC 25175 was increased four-fold, from 0.203 to 0.825, while that of wild type *S. mutans* was increased three-fold, from 0.192 to 0.592, after 3h incubation by using 10% EEP concentration. Meanwhile, after 18 h, the OD was 3.393 for *S. mutans* ATCC 25175

and 3.554 for wild type *S. mutans*. Using 5% EEP concentrations, the ODs were 0.595 and 0.527 for *S. mutans* ATCC 25175 and *S. mutans* wild type, respectively, after incubation for 3 h. After an 18 h incubation, the ODs were 3.517 for *S. mutans* ATCC 25175 and 3.530 for wild type *S. mutans*. The results using 2.5% EEP showed OD values of 0.676 and 0.442 for *S. mutans* ATCC 25175 and wild type *S. mutans*, respectively, after incubation for 3 h. After 18 h incubation, the OD values were 3.384 for *S. mutans* ATCC 25175 and 3.342 for wild type *S. mutans* (Figure 1).

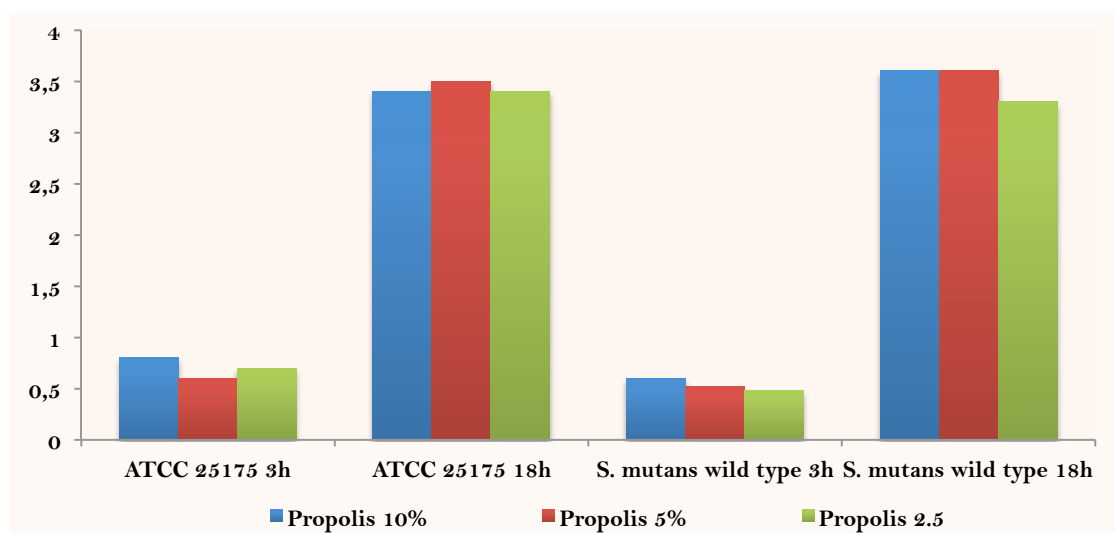


Figure 1. Absorbance value of 3 h and 18 h several propolis concentration on *S. mutans* (ATCC 25175) and *S. mutans* wild-type.

Our study found that there was no inhibitory effect of propolis on *S. mutans* biofilms using any of the selected concentrations since the bacteria were still viable (Table 2) after all the experimental periods. Exposure to 10% EEP resulted in 75.39% and 93.63% growth of *S. mutans* ATCC 25175 incubated for 3 h and 18 h, respectively, while the growth of wild type *S. mutans* was 67.56% and 93.93% after 3 h and 18 h, respectively. Exposure to 5% EEP showed 67.89% and 92.89% growth of *S. mutans* ATCC 25175, and 62.99% and 93.11% growth of wild type *S. mutans* after 3 h and 18 h, respectively. A concentration of 2.5% EEP resulted in 68.63% and 92.78% growth of *S. mutans* ATCC 25175, and 54.97% and 93.56% growth of wild type *S. mutans* after 3 h and 18 h, respectively (Table 1).

The percentage biomass cell of *S. mutans* biofilm using 10% EEP was 406.40% and 1517% for *S. mutans* (ATCC 25175), and 308.33% and 1575% for wild type *S. mutans*, after incubation for 3 h and 18 h, respectively. Exposure to 5% EEP resulted in 311.56% and 1406% growth for *S. mutans* (ATCC 25175), and 270.25% and 1450% for wild type *S. mutans* after incubation for 3 h and 18 h, respectively. Moreover, 2.5% EEP resulted in 318.86% and 1386% growth for *S. mutans* (ATCC 25175), and 222.11% and 1554% for wild type *S. mutans* after incubation for 3 h and 18 h, respectively (Table 2).

Discussion

In the present study, EEP was selected because the ethanolic extract was previously shown to be more effective than the water extract [7]. Furthermore, EEP was demonstrated to be more effective against Gram-positive than Gram-negative bacteria during the planktonic state [8]. Several studies showed a strong antimicrobial activity of propolis against several oral bacteria, including *S. mutans* [17]. Since biofilm formation is a phenotype of bacterial virulence, we evaluated whether propolis could inhibit *S. mutans* grown as a biofilm.

Closed-system models were used in our experiment because of their simplicity, high productivity, repeatability, controllability of the experimental conditions, lower risk of contamination, and cost-effectiveness, while salivary proteins were used as a silted pellicle [18].

CV assays were also used in our experiment as CV is a basic dye that binds nonspecifically to negatively charged surface molecules such as polysaccharides and environmental DNA in the extracellular matrix. However, CV stains both the matrix and the bacterial cells; however, it cannot differentiate between living and dead cells [19]. Therefore, it provides the general condition of the biofilm. Thus, the data of CV absorbance of *S. mutans* biofilms at 3 h and 18 h were increased. These conditions indicate the presence of biomass binding both dead and living cells of *S. mutans* ATCC 25175/wild type *S. mutans*. Since sucrose also serves as a substrate for the synthesis of extracellular and intracellular polysaccharides, it is possible that CV also stained this polysaccharide.

In the present study, both early (3 h incubation) and late (18 h incubation) settlers showed an increase in OD, and there was a statistically significant increase between the late and early settlers. It may be the role of sucrose in the TSB medium to grow the bacteria in our experiment. The initial stage of plaque formation can be sufficiently achieved by saliva, and oral streptococcus can grow continuously in human whole saliva at the expense of various organic components present in the saliva (glycoproteins) that support their growth [20]. Furthermore, sucrose can be utilized by oral streptococcus to produce extracellular polysaccharides in dental biofilms. Glucan plays a role in dental plaque formation and also facilitates bacterial attachment to the tooth surface.

Additionally, fructan also contributes to the virulence of the biofilm by acting as a binding site for the adhesion of *S. mutans*. Sucrose consumption can lead to a decrease in pH in both nonstarved and starved biofilms of *S. mutans* [21]. To ingest sucrose, *S. mutans* produces three types of Gtf that convert sucrose into glucan: (i) Gtf that synthesizes water-soluble glucan, (ii) Gtf that synthesizes water-insoluble glucan, and (iii) Gtf that synthesizes both types of glucan [2,5,20]. Consequently, sucrose plays a role in the development of late but not early settler oral biofilms [20].

Therefore, it is necessary to examine the components that play a role in the quorum sensing process that is closely related to dental biofilm formation. Many bacteria have been shown to regulate diverse physiological processes and group activities via quorum sensing. In particular, many bacteria are capable of using this mechanism to regulate biofilm formation and other social activities [12]. Quorum sensing systems in bacteria are generally divided into at least three classes: (i) LuxI/LuxR-type quorum sensing in Gram-negative bacteria that use acyl-homoserine lactones as a signal molecules, (ii) oligopeptide two-component-type quorum sensing in Gram-positive bacteria

that use small peptides as signal molecules, and (iii) LuxS-encoded autoinducer-2 (AI-2) quorum sensing in both Gram-negative and Gram-positive bacteria. AI-2 is produced by one species and influences the gene expression in another, and this signal can promote interspecies communication and enable bacteria to modify behaviors such as virulence, luminescence, and biofilm formation across different species [12,13]. In *S. mutans*, biofilm formation is regulated by quorum sensing involving the ComDe Two Component Signal Transduction System that regulates the expression of virulence factors in a cell density-dependent manner [4].

However, studies on the antimicrobial activity of propolis show conflicting results. This could be due to differences in its chemical components. It has also been reported that samples collected from different geographic origins with different climates and vegetation show different antibacterial activities [6,7,8,9,22]. Furthermore, the determination of the inhibition zone value depends on technical details that vary between laboratories. Moreover, the effect of EEP on biofilm needs to be evaluated in a vivo model [22].

Some authors showed similar results, showing that EEP extracted using pure ethanol showed no inhibition on any of five bacteria strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*), in spite of using different bacterial species. Similar results by other studies also showed that EEP extracted using pure, 70%, 50%, and 30% ethanol had no inhibitory effect on *P. aeruginosa*. Furthermore, Muli and Maingi showed that EEP extracted using pure ethanol had no inhibitory effect on *E. coli* and *S. typhi* [23].

Our study showed that EEP could not reduce the growth of *S. mutans*. This may be because EEP provides an environment conducive to the growth of *S. mutans* biofilm. In addition, it is also possible that the protein content of EEP supports *S. mutans* biofilm growth. Our results suggest that different extraction procedures lead to the extraction of different compounds, ultimately contributing to differences observed in the antibacterial activity of propolis [23].

Conclusion

Ethanol extract of propolis showed no inhibitory effects on the growth of *S. mutans* at any of the concentrations of propolis used in the present study. Hence, further studies are required to investigate the composition of the antibacterial components in the test material, and the effect of propolis concentration on the formation of *S. mutans* biofilms by evaluating bacterial gene expression, such as *LuxS* and *Gtf-B*, which are associated with quorum sensing and breakdown of sucrose by Gtf, respectively. Furthermore, additional studies are required to validate this in vitro study, such as the use of other oral microorganisms that are closely associated with dental caries, such as *Candida albicans* and *Veillonella* spp. exposed to ethanolic extract of propolis.

Authors' Contributions: AAD designed the study, performed data analysis and interpretation, and wrote the manuscript. NP, J and ARP performed the data collection, data analysis, and interpretation. RRD and BMB performed data analysis and reviewed the manuscript. All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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