

EFFICACY OF PROPOLIS FLUORIDE AND NANO SILVER FLUORIDE FOR INHIBITION OF *STREPTOCOCCUS MUTANS* AND *ENTEROCOCCUS FAECALIS* BIOFILM FORMATION

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ABSTRACT

Objective: The objective of this study is to investigate the potency of propolis fluoride (PPF) and nano silver fluoride (NSF) as fluoride-based varnishes for inhibiting *Streptococcus mutans* and *Enterococcus faecalis* biofilm formation. In this study, both varnishes were compared to silver diamine fluoride (SDF) varnish, the gold standard for anticariogenic agents.

Methods: The suspensions of *S. mutans* and *E. faecalis* were cultured and then plated into 96 - well plates and combined with SDF (38%), NSF (3.16, 3.66, and 4.16%), or PPF (3, 6, and 10%). *E. faecalis* was incubated in an anaerobic environment for 24 h, and the same protocol was used for *S. mutans*. The amount of biofilm inhibition was evaluated by optical density measurements at 570 nm using a microplate reader. Data were analyzed using one-way ANOVA.

Results: The minimum inhibitory concentration (MIC) of PPF for *S. mutans* was 3%, and minimum bactericidal concentration (MBC) was 10%. The MIC of PPF for *E. faecalis* was at 6%, but no MBC was established. The MIC of NSF for *S. mutans* was 3.16%, and the MBC was 4.16%. The MIC of NSF for *E. faecalis* was 3.16%, while the MBC was 4.16%. Biofilm formation was inhibited dose-dependently by both NSF and PPF.

Conclusion: NSF and PPF fluoride-based varnishes show clear antibacterial effects that are comparable to those obtained with SDF fluoride-based varnish.

Keywords: Propolis fluoride, Nano silver fluoride, *Streptococcus mutans*, *Enterococcus faecalis*, Biofilm formation.

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INTRODUCTION

Dental caries, a multifactorial disease, is defined as a chronic and dynamic disease of the teeth that is characterized by the mineral loss. Some contributing factors are carbohydrate or acid consumption, saliva as natural protective factor, fluoride, and dental plaque. Plaque accumulation and retention can lead to increased retention of early colonizer bacteria, such as *Streptococcus mutans*. This species plays a major role in caries formation [1,2], as it produces more acid when compared to other streptococci. It is a Gram-positive and facultative anaerobic species that normally exists in low quantities under oral conditions.

However, when *S. mutans* develops as a single species biofilm, it can produce a protein chain that helps secondary colonizers to form a multispecies biofilm [3]. One of these secondary colonizers is *Enterococcus faecalis*, a bacterial species found in secondary root canal infection but not in the normal oral condition. This species is persistent and difficult to eliminate in root canal treatment. It is also a Gram-positive facultative anaerobe that forms single-species biofilms, but it can also coaggregate with other bacteria to form multispecies biofilms. It has a known resistance to antibacterial agents because of proteins in its cell wall and cell membrane, and it also has resistance genes against antibacterial agents. In addition, *E. faecalis* tends to survive longer under various conditions when compared to other Gram-positive bacteria [4].

For these reasons, persons with high quantities of oral *S. mutans* also have a high risk of dental caries. Some innovations have been advanced to cure and prevent dental caries by invasive or non-invasive mechanisms. One innovation has been the introduction of a dental

varnish, which can prevent and even stop active caries. A study initiated in 1970 examined the efficacy of silver diamine fluoride (SDF) [3-5], a varnish made of ammonium and silver fluoride. The ammonia ions bind to silver ions to form a complex ion called silver diamine. The silver diamine then binds to fluoride and forms SDF. The silver ion acts as an antibacterial agent, while the fluoride balances the bonding and helps with remineralization. However, much clinical research has indicated that SDF has some undesirable side effects, such as discoloration of the organic tissue of teeth, a metallic taste, and irritation following contact with soft tissues [3-5]. The discoloration by SDF tends to happen in primary teeth and arises because SDF is photosensitive [6]. Positive silver ions react with oxygen to form Ag₂O, which then discolors organic tissue, especially the collagen tissue in dentine.

Another anticariogenic agent, nano silver fluoride (NSF), has been formulated to overcome the limitations of SDF, especially the black stain formation on caries lesions and irritation of soft tissues [7]. Previous paper stated that application of NSF once a year had the same effectiveness as SDF in preventing dental caries but caused no tooth discoloration [8]. Targino *et al.* stated that NSF could inhibit *S. mutans* growth, as the nanoparticles could penetrate the cell wall and destroy the bacterial cell membrane [9]. No research has been published regarding possible side effects of NSF, but some studies have examined nanosilver, which is the main component of NSF. A study by Braidich-Stolle *et al.* demonstrated a change in mitochondrial function in rat liver [10]. Another study indicated that nanosilver could have serious toxic effects on men's reproductive systems [11]. However, no previous research has demonstrated an effect of NSF on oral *S. mutans* biofilm formation.

Another varnish component is propolis, which was used in ancient Egypt and is still in use as a remedy for pain and infection [12]. The propolis used in the present study was obtained from *Tetragonula* sp., a stingless bee that is farmed at Universitas Indonesia. Potency of propolis fluoride (PPF) was prepared by the Faculty of Engineering, Universitas Indonesia, in the Bioprocessing Laboratory. The PPF varnish was newly developed by combining fluoride and propolis. Propolis has a flavonoid component that can inhibit the attachment of pioneer bacteria, as well as kill bacteria, while the fluoride acts as a remineralization and antibacterial agent. The fluoride ion in PPF acts as a remineralization agent because of its ability to increase tooth endurance to acid by the formation of fluoroapatite [13]. Consequently, the use of PPF would be expected to overcome the undesired side effects of SDF and NSF.

The aim of the present study was, therefore, to investigate the potency of PPF and NSF in inhibiting *S. mutans* and *E. faecalis* biofilm formation, both as single species and dual species (combination) biofilms. SDF (38%) served as the gold standard for comparisons of biofilm inhibition in this research.

MATERIALS AND METHODS

Materials

SDF was prepared by combining 6.816 g of Silver nitrate (AgNO_3) in a vial with 5 ml of distilled water, then homogenized by vortexing. After addition of 1.47 g of ammonium fluoride (NH_4F), the vial contents were again homogenized. Ammonia was added until a final mixture volume of 13.2 and pH 7 was reached. The vial was covered with black plastic and stored at 8°C in the refrigerator until use.

NSF was made at three different concentrations (3.16, 3.66, and 4.16%) by adding AgNO_3 . Gelatin (5 ml) was added as a stabilizer to each concentration to avoid sedimentation by mixing it at 70°C. Glucose (13.3 g in 40 ml distilled water) was then mixed into this solution, and nanoparticles were formed. The nanoparticle suspension was combined with 4.4 g of NH_4F for 1 min and then maintained at 8°C.

PPF was made from 100 g propolis after extraction in 500 ml ethanol (96%). PPF solutions were prepared at three different concentrations 3, 6, and 10%. A 5 g sample of NH_4F was added to 50 ml distilled water and mixed until homogeneous. A surfactant solution, made from 96% ethanol and tween 80, and propolis at the three different concentrations were then added, and the resulting solutions were stored at 8°C. The SDF, NSF, and PPF solutions were made by the Bioprocessing Laboratory, Faculty of Engineering, Universitas Indonesia.

Methods

Bacterial culture

This research was an *in vitro* experimental laboratory study. The bacterial samples were *S. mutans* (strain ATCC 31397) and *E. faecalis* (strain ATCC 29212) from the Oral Biology Laboratory, Faculty of Dentistry Universitas Indonesia. Bacterial suspensions were cultured and incubated in brain heart infusion (BHI) broth (BD Difco; San Jose, USA) in an aerobic atmosphere at 37°C for 24 h. The bacterial cultures were transferred onto mitis salivarius agar plates and incubated at 37°C for 24 h. Before use, both bacterial strains were subcultured and prepared on BHI.

Antibacterial study

A bacterial inoculum (100 μl) of approximately 10^{-7} CFU/ml was transferred into each well of a round-bottomed 96-well plate (Corning; Oneonta, NY, USA). Control and test groups were incubated at 37°C for 24 h in anaerobic conditions for single and combination biofilms of *S. mutans* and *E. faecalis*. The inhibition of biofilm formation was tested using the methylthiazole tetrazolium assay and crystal violet using a microplate reader at 570 nm. The optical density value was converted to biofilm viability percentage using the following formula:

$$\% \text{Cell viability} = \frac{\text{OD of treated groups} - \text{OD blank}}{\text{OD of treated group negative controls} - \text{OD blank}} \times 100\%$$

Data for the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) were then analyzed using a microplate reader.

Statistical analyses

Data were analyzed statistically with the Shapiro-Wilk to test the normality of the biofilm inhibition scores. After the normality test, one-way ANOVA and a *post hoc* test were used to determine the mean differences between the control and test groups. A correlation test was used between the concentration and the percentage of inhibition. The effect of concentration on the percentage of inhibition was analyzed using linear regression. Values of $p < 0.05$ were treated as statistically significant.

RESULTS AND DISCUSSION

Propolis, such as honey and royal jelly, is a product obtained from beehives. Different resins from tree buds, exudates, or saps are collected by the bees and mixed together to make propolis. During the gathering process, the bees using their saliva to blend the components together and metabolize them [12]. Takaisi-Kikuni and Schilcher in 1994 were the first to describe the antibacterial properties of propolis [13]. They found that propolis could suppress bacterial development by blocking the process of cell mitosis and protein synthesis, and it also had a direct bactericidal activity by destroying the bacterial cytoplasm, cell membrane, and cell walls. Flavonoids, which are the biologically active substances present in propolis, are proven to be strongly antibacterial [14]. The present study confirmed that the propolis extracts from the *Tetragonula* sp. stingless bee that is farmed at Universitas Indonesia had a strong antibacterial effect on both *S. mutans* and *E. faecalis*. Ethanolic extract was found to have the highest amount of flavonoid [15].

Many studies have shown that SDF varnish, which is considered the gold standard, unfortunately, causes a black staining of the teeth [16]. Our control group treated with 38% SDF showed a strong inhibition of *S. mutans* and *E. faecalis*, but the effectiveness of PPF (Table 1) was not significantly different. The MIC for PPF was 3% for *S. mutans*, and the MBC was 10%. *E. faecalis* was inhibited in the same dose-dependent manner as *S. mutans*, and the MIC was 3%, but no MBC was established. This finding agreed with a previous study that found *E. faecalis* to be more resistant to antibacterial agents when compared to *S. mutans*. This is because the cell wall of *E. faecalis* consists of three components: 40% peptidoglycan, teichoic acid, and polysaccharide. The polysaccharide and peptidoglycan act as defense agents that regulate the entry of foreign substances into the cell. Hence, a higher concentration of PPF was needed to kill *E. faecalis* [17].

Table 1: Percentage inhibition of growth of *S. mutans* and *E. faecalis* by PPF compared with SDF

Concentration (%)	Inhibition (%) \pm SD	
	<i>S. mutans</i>	<i>E. faecalis</i>
SDF 38	104.22 \pm 2.54	110.57 \pm 6.7
PPF 3	94.16 \pm 2.46*	88.22 \pm 0.77*
PPF 6	94.93 \pm 3.54	91.00 \pm 0.94
PPF 10	97.08 \pm 7.04**	95.30 \pm 0.25

*MIC, **MBC. *S. mutans*: *Streptococcus mutans*, *E. faecalis*: *Enterococcus faecalis*, PPF: Potency of propolis fluoride, SDF: Silver diamine fluoride, SD: Standard deviation, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

In this study, NSF was prepared by a modification of the procedure of Santos *et al.*, with silver nanoparticles, fluoride, and chitosan [8] but gelatin replaced the chitosan as a stabilizer for making the silver nanoparticles. AgNO₃ was used as the source of silver ion in this study and NH₄F as the source of fluoride ions for NSF. Green synthesis methods were used, so three rules required evaluation: The choice of solvent, the reducing factor, and the non-toxic stabilizer that was safe for the environment. Distilled water was chosen as the solvent, gelatin as the stabilizer, and glucose as the reducing agent [18].

Gelatin is a natural protein with a triple helix chain; it is nontoxic, biocompatible, and can be ionized. Gelatin molecules, therefore, have the potential for the synthesis of silver nanoparticles. Gelatin also contains both positive and negative charges and hydrophobic domains that give it stability. Gelatin can stabilize a surface by the spatial arrangement of the atoms inside a molecule, so its main function is as a stabilizer. Glucose was used as a reducing agent to reduce silver cations to silver atoms. A sign that the solution was forming nanoparticles was the gradual change in color from a light brown to a darker brown [18,19].

The silver nanoparticles in NSF exert their antibacterial action by a mechanism that involves penetration of the cell wall and disruption of the cell membrane, as well as damage to DNA by binding to nucleic acids. The fluoride in NSF serves as an anticariogenic agent. Fluoride was added to reduce biofilm formation and adhesion, as well as to reduce acid production and prevent demineralization. Fluoride can balance demineralization and remineralization to strengthen the NSF antimicrobial action [20].

NSF is known to be a good antibacterial agent [21], and it gave better results than SDF. The present study confirmed the antibacterial effect of NSF on *S. mutans* but also showed its effectiveness on *E. faecalis*, which is known to be more resistant than *S. mutans*. The MIC in this study using NSF was 3.16% for both *S. mutans* and *E. faecalis*, and an MBC was reached at 4.16%. This research also confirmed that NSF was more effective than SDF (38%), due to both the concentration and the components of NSF. The components were AgNO₃, NH₄F, gelatin as a stabilizer agent, and glucose as a reducing agent. The production process also affected the efficacy. A previous study also showed that NSF was a good antibacterial and left no black staining on the teeth, in agreement with the results of the present study, where NSF was better at inhibiting both bacterial species (Table 2) [21].

Biofilm formation by *S. mutans* was also inhibited in a dose-dependent manner by PPF (Table 3). A 3% concentration of PPF could give a >50% inhibition of *S. mutans* biofilm formation, so 3% was the MBIC₅₀. A 10% concentration inhibited *S. mutans* biofilm formation by more than 90%, so 10% was the MBIC₉₀. Similar inhibition was seen for *E. faecalis*. PPF was not as potent in inhibiting *E. faecalis* biofilm. Linear regression using the equation $Y = 57.617 + 1.325 X$ showed that a 1% increase in PPF could increase the percentage inhibition by 1.325%.

The potency of PPF in inhibiting *E. faecalis* biofilm formation showed a MBIC₉₀ of 10%. Linear regression using the equation $Y = 54.900 + 1.434 X$ showed that a 1% increase in PPF increased the percentage inhibition by 1.434%. *E. faecalis* is a facultative anaerobe and a Gram-positive bacteria. Therefore, the cell wall and cell membrane of *E. faecalis* have specific proteins that allow *E. faecalis* to penetrate the dentine tubule and form a single species biofilm in any environmental condition and destroy antibacterial defences [22].

E. faecalis maintains a homeostatic pH across the intracellular membranes through a proton pumping mechanism, so PPF does not work as effectively as its mechanism involves changes in the intracellular pH. PPF could inhibit *E. faecalis* biofilm formation, but it was not as effective as SDF.

In this research, NSF was a better inhibitor of *S. mutans* biofilm formation than was the gold standard, SDF (38%). This was because

Table 2: Percentage inhibition of growth of *S. mutans* and *E. faecalis* by NSF compared with SDF

Concentration of NSF and control	Inhibition (%) ± SD	
	<i>S. mutans</i>	<i>E. faecalis</i>
SDF 38%	90.84±0.19	90.68±1.05
NSF 3.16%	91.73±1.16*	91.06±1.03*
NSF 3.66%	92.85±1.25	93.05±0.46
NSF 4.16%	102.37±1.12**	102.43±2.8**

*MIC, **MBC, *S. mutans*: *Streptococcus mutans*, *E. faecalis*: *Enterococcus faecalis*, NSF: Nano silver fluoride, SDF: Silver diamine fluoride, SD: Standard deviation, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

Table 3: PPF Compared with SDF in Inhibiting Biofilm Formation by *S. mutans* and *E. faecalis*

Concentration	Inhibition (%) ± SD	
	<i>S. mutans</i>	<i>E. faecalis</i>
SDF 38%	102.06±0.68	101.89±1.48
PPF 3%	78.58±2.38*	79.19±3.44*
PPF 6%	89.62±0.96*	85.67±0.85*
PPF 10%	98.87±1.26*	90.92±2.41*

One way ANOVA test result *p<0.05, *S. mutans*: *Streptococcus mutans*, *E. faecalis*: *Enterococcus faecalis*, PPF: Propolis fluoride, SDF: Silver diamine fluoride, SD; Standard deviation.

Table 4: NSF Compared with SDF in Inhibiting Biofilm Formation by *S. mutans* and *E. faecalis*

Concentration	Inhibition (%) ± SD	
	<i>S. mutans</i>	<i>E. faecalis</i>
SDF 38%	84.10±0.27	69.13±0.05
NSF 3.16%	87.27±0.16*	75.91±0.07*
NSF 3.66%	87.43±0.18*	81.53±0.09*
NSF 4.16%	94.34±0.15*	93.04±0.12*

One way ANOVA test result *p<0.05, *S. mutans*: *Streptococcus mutans*, *E. faecalis*: *Enterococcus faecalis*, NSF: Nano silver fluoride, SDF: Silver diamine fluoride, SD; Standard deviation.

the nano-sized particles of NSF are much smaller than SDF, so the of the bacteria with silver was much greater. Therefore, even small concentrations of silver could give great antibacterial effects with NSF, so the toxic effects of NSF could be reduced (Table 4). The silver nanoparticles in NSF could inhibit biofilm formation and maturation through inhibition of exopolysaccharide synthesis, as these nanoparticles would readily penetrate the polysaccharide matrix inside the cell. Silver nanoparticles at high concentrations could inhibit bacterial growth by more than 98% [23].

The present research showed that NSF was better than SDF at inhibiting *E. faecalis* biofilm formation. As mentioned before, Gram-positive bacteria were more susceptible than Gram-negative bacteria, except for *E. faecalis*, which had the same MIC and MBC values as Gram-negative bacteria. *E. faecalis* in biofilms had an increasing capability to adapt to worsening conditions and showed increasing resistance to antimicrobial agents [24]. Effect of PPF and NSF on Dual Species Biofilm Formation by *S. mutans* and *E. faecalis* were observed (Figs. 1 and 2). The inhibition score for NSF was higher for single species biofilm formation than for a combined *S. mutans* and *E. faecalis* biofilm. This agreed with the previous research of Deng *et al.*, who reported that a combination of *S. mutans* and *E. faecalis* could increase biofilm formation. Hence, NSF was less effective at inhibiting the combined biofilm growth. Deng *et al.* also reported that the amount of *S. mutans* on hydroxyapatite could be five-fold higher in the presence of *E. faecalis* clinical strains, but the underlying mechanism was not clearly explained [24]. One possible

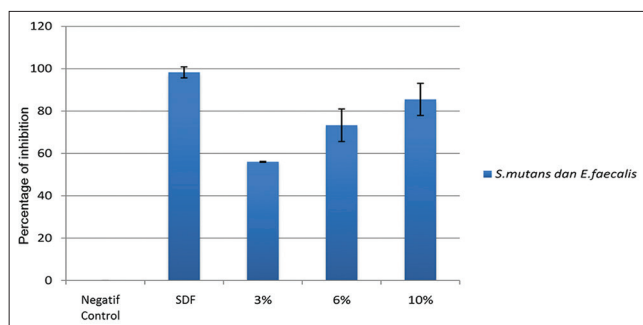


Fig. 1: Potency of PPF: Propolis fluoride to inhibit Streptococcus mutans and Enterococcus faecalis biofilm formation compared to SDF: Silver diamine fluoride

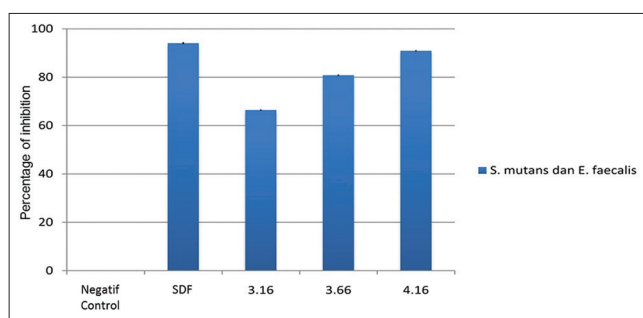


Fig. 2: Potency of NSF: Nano silver fluoride to inhibit Streptococcus mutans and Enterococcus faecalis biofilm formation compared to SDF: Silver diamine fluoride

explanation might be coaggregation, i.e. the ability of one species of bacteria to adhere to another bacterial species. The previous research also indicated that *E. faecalis* biofilm formation was strongly affected by other species within the root canal.

CONCLUSION

NSF and PPF fluoride-based varnishes both showed an expected antibacterial effect. The effects of NSF and PPF on biofilm formation were comparable to that of the gold standard SDF fluoride-based varnish. The antibacterial characteristic of the NSF and PPF varnish formulations tested here confirm that both fluoride-based varnishes are promising as useful agents for caries prevention and could be of clinical relevance.

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