Tooth Coating Gel with Casein Phosphopeptide–Amorphous Calcium Phosphate and Propolis to Prevent Dental Caries

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Abstract

Dental caries is an irreversible condition where tooth cavities occur because of *Streptococcus mutans*. It can be prevented using casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) to re-calcify and inhibit the demineralization of dentin. Propolis is used to prevent *S. mutans* growth. We aimed to observe the efficiency and stability of CPP–ACP with ethanol extract propolis (EEP) in tooth coating gel to prevent caries.

The CPP–ACP complex preparation was adapted from the US patent for calcium phosphopeptide complexes. Three EEP concentrations (2%, 4%, and 6%) and a control (0%) were used.

An organoleptic study was used to qualitatively measure the appearance and texture of the tooth coating gel. Then, bacterial growth was evaluated to analyze antibacterial activity. The morphology of all tooth samples was evaluated by scanning electron microscopy.

The tooth coating gel containing CPP–ACP and 2% EEP had excellent stability, and the treated tooth had a good prism pattern and smooth uniform pores. The tooth coating gel containing CPP–ACP and 6% EEP inhibited 73.78% growth of *S. mutans* compared with the control.

We suggest the combination of CPP–ACP and propolis in the tooth gel can support remineralization and be used to prevent dental caries.

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Introduction

Dental caries, otherwise known as tooth decay, is one of the most prevalent chronic diseases worldwide that people are susceptible to throughout their lifetime. Dental caries is the result of a complex interaction over time between acid-producing bacteria, fermentable carbohydrates, and many host factors, including teeth and saliva.[1] According to Riskesdas, the prevalence of active caries in the population of Pusdatin, Indonesia, has increased from 43.4% in 2007 to 53.2% in 2013.[2] This indicates that dental caries is a common disease in this community.

Dental caries begins by demineralization of tooth minerals. Bacteria ferment the carbohydrates in the mouth, producing organic acids and decreasing the pH in the mouth. When the pH in the mouth reaches a critical 5.5, the organic acid penetrates the pellicle and diffuses into the enamel surface of the tooth, causing the dissolution of minerals.[3]

The natural response to demineralization is remineralization, which involves the transfer of minerals from the saliva to the demineralized lesions. Under physiological conditions, saliva contains supersaturated concentrations of calcium and phosphate that are continuously deposited onto the surface of the enamel[4] to remineralize the demineralized areas. Tooth surface remineralization begins when the oral pH becomes acidic. The resulting tooth structure consists of fluoridated hydroxyapatite and fluorapatite, which is more resistant to an acidic environment than the original structure.[3]

Casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) prevents dental caries in 2 ways: re-calcification and

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remineralization of dentin. Re-calcification is initiated by supplying calcium and phosphorus ions to a demineralized lesion under an enamel surface. Low pH conditions fully supply the enamel with calcium while also reducing the demineralization of dentin. Dentin demineralization occurs when the solubility of calcium phosphate in the enamel-dentin surface layer is significantly decreased, and the tooth starts to dissolve. As a result, when supplying CPP–ACP, the calcium and phosphorus ion concentrations remain supersaturated in the saliva.[5]

According to Soekanto *et al.*, propolis fluoride has been shown to reduce bacterial adhesion on the surface of the oral cavity due to antibacterial activity.[6] In addition, CPP–ACP chewing gum has exhibited the ability to prevent caries by inhibiting *Streptococcus mutans* and remineralizing the tooth, thereby increasing the amount of calcium and phosphate ions.[7,8] Propolis in the form of ethanol extract of propolis (EEP) inhibits the activity of *S. mutans* bacteria, which is a common cause of dental caries.[7,8]

Gels are used in water-based drugs in a semi-solid state, with colloidal particle interactions. In this study, we chose to use gel in a tooth coating to prevent dental caries because it has a fast release mechanism and high stability. A gel can form a film and prevent dental caries over an extended period.

Materials and methods

Casein Isolation

The isolation of casein from pasteurized skim milk (Diamond, Indonesia) was adapted from Sahlan's method.[9] Briefly, a liter of milk was used as the source of casein and warmed to 35 ºC. The pH was adjusted to 4.6 by adding acetic acid. Rennet Fromase® (DSM Food Specialities, Netherland) was used to coagulate the milk. Briefly, rennet tablets were crushed to a powder, and 50 mg was diluted in 10 ml of distilled water, stirred at 500 rpm until thoroughly homogenized, and added to the milk. The mixture was then stirred at 200 rpm for 45 min and incubated at room temperature for another 45 min. Then 1 l of distilled-water is added with temperature of 60ºC to inactivate the chymosin enzyme. Incubation is done to the precipitated milk at room temperature until its completely precipitated, then separate the sediment (casein) from the whey solution with decantation. The casein was washed with distilled water 3 times and filtered with Whatman micro-filter paper (Sigma-Aldrich, Singapore). Water content was removed using a freeze dryer FDR-101 (Labconco Corporation, Missouri USA). Drying process proceeds for 6 hours to obtain dried casein. Then it reduced in size by using a grinder to a powder form.

Preparing the CPP–ACP Complex

The preparation method of the CPP–ACP complex was adapted from the US patent 2005/003798 for calcium phosphopeptide complexes.[10] For the CPP–ACP formulation, 1.6 M $CaCl_2$, 1 M Na_2HPO_4, 1 M NaOH, Paya papain enzyme (Enzyme Development Enterprise, Indonesia), and distilled water were required. Casein (10% w/v; Murray Goulburn, Victoria, Australia) or caseinate solution was digested with trypsin (0.2% w/w). In this study, trypsin from pork was substituted by papain to qualify as a halal product. Six milligrams of Paya papain enzyme is comparable to 1 mg of pure papain. Casein was digested with papain for 2 h at 50 ºC at a of pH 8.0 ± 0.1, adjusted using 1 M NaOH. After digestion, the solution was adjusted to pH 4.6 using CH_3COOH to meet the qualification of a food grade product. The precipitate was removed using a thermo shaker, then the supernatant was obtained and adjusted to pH 9.0 by adding NaOH. Then 1.6 M $CaCl_2$ and 1 M Na_2HPO_4 were added slowly (≤1% vol. per min) at constant agitation to reach pH 9.0. The pH was held constant at 9.0 ± 0.1 using NaOH. $CaCl_2$ and sodium phosphate were added to the final concentrations of 100 and 60 mM, respectively. The retentate was freeze-dried using a freeze dryer FDR-101 (Labconco Corporation, Missouri USA) to produce a white powder that was 50% CPP and 40% ACP, with residual water.

Tooth Coating Gel Formulations

For the tooth coating gel formulation, glycerin, gelatin, EEP, propylene glycol, sodium carboxymethylcellulose, and distilled water were purchased from Bratachem Company, Indonesia. Table 1 shows the formulation of CPP–ACP combined with the EEP tooth coating gel. EEP was added at 3 variation concentrations of 2%, 4%, and 6% and a control at 0% was used.

Organoleptic and pH Analysis

An organoleptic study was used to qualitatively measure the appearance and texture

of the tooth coating gel. The pH stability was evaluated to make sure the pH of the tooth gel was above the critical pH of 5.5. A non-acidic pH is indispensable because the tooth gel was applied directly to the enamel. Colloid stability was evaluated by centrifugation in 2,500 rpm for 5 hours.

Antibacterial Activity

Bacterial growth was evaluated to analyze antibacterial activity. For total plate count methods, plate count agar, brain heart infusion (BHI) agar, alcohol were purchased from Sigma Aldrich, Singapore. First, *S. mutans* (ATCC, Manassas, VA, USA) was cultured on BHI agar. Colonies were diluted in 10^{-1}, 10^{-2}, and 10^{-3} dilutions and counted in sterile Ringer's solution using a Bio-rad microplate reader model 2550 on λ = 515 nm (Bio-Rad, California, USA).

Scanning Electron Microscopy

The efficacy of the CPP–ACP and EEP tooth coating gel used to remineralize the enamel was evaluated using scanning electron microscopy (SEM). This method was adapted from Clark.[3] Briefly, 7 samples of maxillary premolar teeth that showed no evidence of white spots or lesions, enamel cracks, or caries on visual inspection were selected for evaluation. All samples were cut using a diamond disc burr (3 mm × 5 mm × 5 mm thickness). Each sample was rubbed with the tooth coating gel for 3 min using a gloved finger and rinsed with deionized water. A wash with deionized water followed every application of the tooth gel. The tooth gel was applied 26 times to simulate equivalent tooth gel usage. Each tooth was then immersed in 50% phosphoric acid as a demineralizing solution to simulate tooth demineralization. The morphology of all teeth samples was evaluated by SEM.

Results

Organoleptic and pH Analysis

The organoleptic study evaluated the color, viscosity, and homogeneity of the samples (Figure 1). The qualitative organoleptic test result show that entire tooth gel variations have good texture and color as the expectation of this research. The results of the organoleptic study are summarized in Table 2. Furthermore, the solubility of tooth gel with a combination of CPP-ACP and EEP do not produce sediment so the stability is accepted. Table 3 shows the pH of each sample and the placebo gel. It shown that gel CPP-ACP have obtained in a condition of around pH 7.

The colloidal stability of the tooth gel was evaluated by centrifugation in 2,500 rpm until 5 hours. The best colloidal stability was seen with CPP–ACP gel + 2% EEP. Precipitation did not occur, and the gel was viscous. Calcium was precipitated with CPP–ACP + 4% EEP and CPP–ACP + 6% EEP tooth gel at rates of 0.06% and 0.2%, respectively.

Antibacterial Activity

Bacterial activity decreased with the increase in EEP in the formulation. Figure 2 shows the inhibition trend of each sample. The result of in vitro test of tooth gel showed that the concentration of CPP-ACP and EEP 6% prove their capabilities to inhibit S. mutans, which reached 73.7% more effective relative to negative control.

SEM Analysis

The results of SEM imaging are shown in Figure 3. In Figure 3F, with CPP–ACP and 2% EEP, demineralization did not occur on the tooth surface, as can be seen from the patterns formed. The surface looked smooth and had a uniform pattern. No basins were seen, if it indicates an occurrence of demineralization.

Materials	Function	% w/w
Glycerol/glycerine	Humectant, gel base	28.0
AquaDes	Solvent	44.7
Gelatin	Thickener	1.0
Propolis extract (EEP 20%)	Antibacterial agent	13.0
Propylene glycol	Humectant, coating film polymer	3.0
Na-CMC	Thickener and stabilizer	1.0
CPP–ACP	Remineralization agent	9.3

Table 1. Formulation of casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) and ethanol extract of propolis (EEP) tooth coating gel

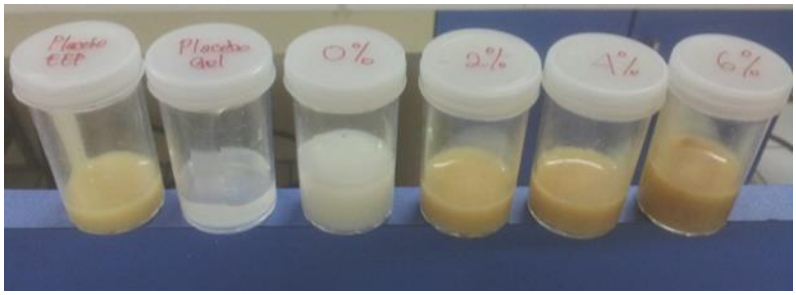

Figure 1. The appearance of each sample and placebos

No.	Sample	Texture	Color
1	Placebo gel	viscous and homogenous	Transparent
2	Gel and 2% EEP	viscous and homogenous	Light brown
3	Gel and CPP–ACP	viscous and homogenous	Compact white
4	Gel, CPP–ACP, and 2% EEP	viscous and homogenous	Pale brown
5	Gel, CPP–ACP, and 4% EEP	viscous and homogenous	Brown
6	Gel, CPP–ACP, and 6% EEP	viscous and homogenous	Dark brown

Table 2. Organoleptic analysis of samples.

No.	Sample	pH
1	Placebo gel	7.20 ± 0.10
2	Gel and 2% EEP	6.93 ± 0.02
3	Gel and CPP–ACP	6.82 ± 0.03
4	Gel, CPP–ACP, and 2% EEP	6.91 ± 0.05
5	Gel, CPP–ACP, and 4% EEP	6.99 ± 0.06
6	Gel, CPP–ACP, and 6% EEP	7.01 ± 0.10

Table 3. pH of each formula.

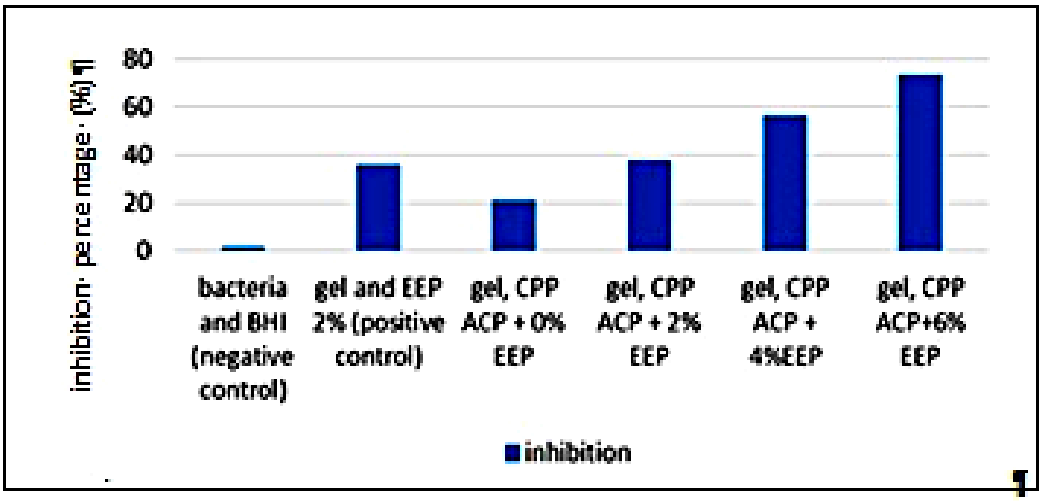

Figure 2. Inhibition trend of each sample

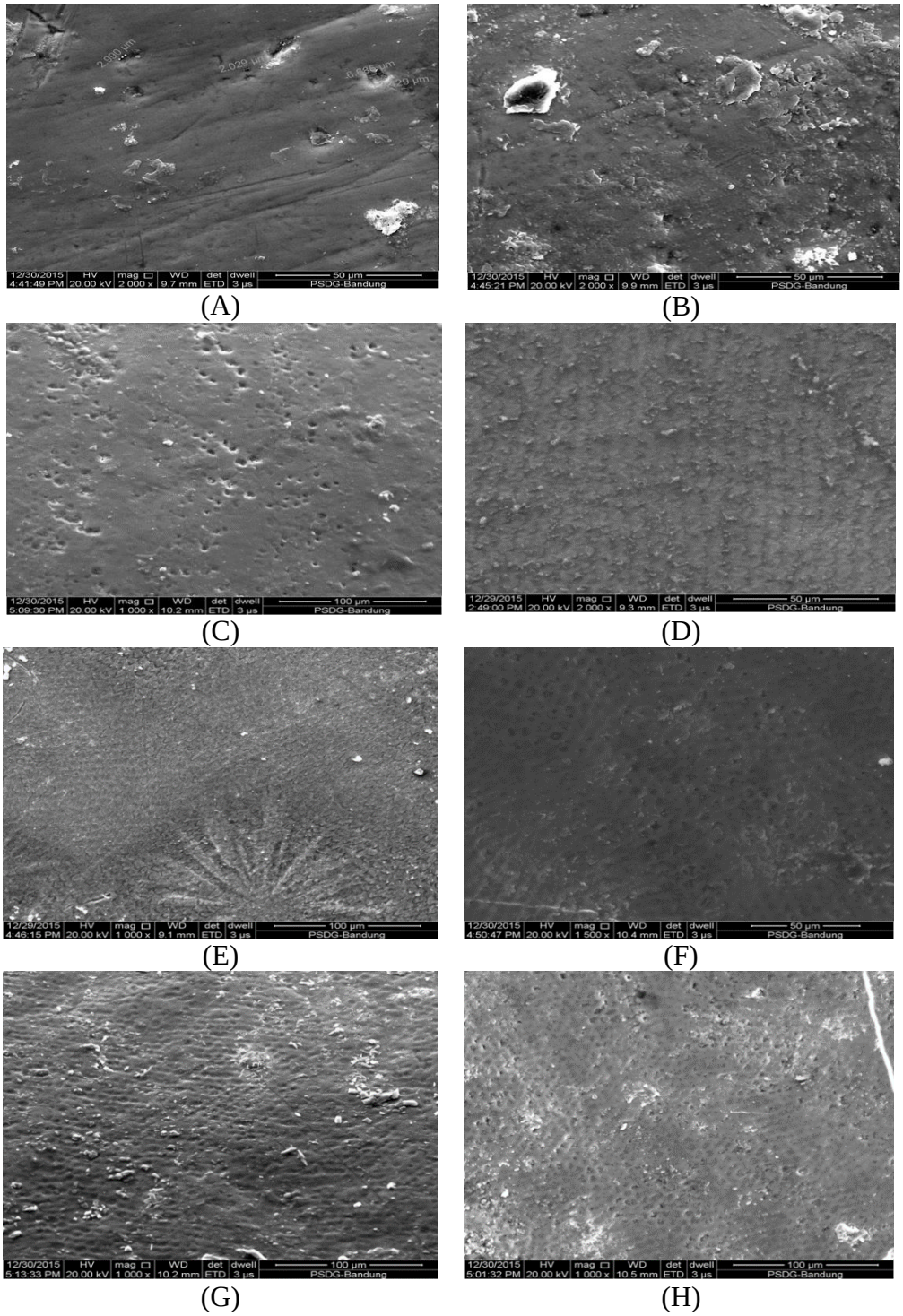

Figure 3. Scanning electron micrographs of the enamel morphology of tooth samples: (A) positive control; (B) negative control; (C) tooth gel placebo; (D) tooth gel + EEP; (E) tooth gel CPP–ACP; (F) tooth gel CPP–ACP and 2% EEP; (G) tooth gel CPP–ACP and 4% EEP; (H) tooth gel CPP–ACP and 6% EEP.

Discussion

Dental caries can be prevented by inhibiting the bacteria that causes it and increasing the pH in the mouth to above 5.5. For 3 decades, dentists have been using calcium and phosphate ions for remineralization. However, this has not been effective because of their insoluble properties, the effort required for a clinical application, and the concentration of

calcium and phosphate ions found in saliva is low. An insufficient concentration gradient cannot remineralize the enamel's surface. These limitations are the reasons why the CPP–ACP complex technology was created. The CPP–ACP complex could stabilize a high concentration of calcium and phosphate ions on the tooth surface, creating supersaturation conditions. In acidic conditions, the CPP–ACP complex acts as a buffer and dissolves into diffusible calcium and phosphate ions. These ions are deposited on the enamel subsurface, remineralizing the lesions.

We evaluated the pH stability by measuring the pH of each sample. All samples had a pH above the critical pH of 5.5, so biofilms and plaques on the enamel were not affected by the tooth gel CPP–ACP and EEP. Minerals in the saliva are saturated when the pH is above 5.5, so they are precipitated onto the enamel, resulting in remineralization.[11,12]

Tooth gel CPP–ACP and EEP need to have appropriate organoleptic features, including accepted color. The organoleptic analysis was performed by evaluating the color, viscosity, and homogeneity of the samples. The color of tooth gel CPP–ACP and EEP ranged from pale brown to dark brown. The higher the propolis concentration, the darker the tooth gel. All concentrations of the samples were viscous and homogeneous. This result showed that applying the tooth gel containing CPP–ACP and EEP is possible in a clinical setting.[13]

Complementary medicine and alternative treatment methods are now being considered due to antibiotic resistance. Until now, only a few studies have investigated the antibacterial properties of EEP, notably several bacterial studies on periodontitis and caries.[14,15] Propolis samples were found to be active mainly against Gram-positive bacteria and some fungi.[16] The pharmacologically active constituents against oral bacteria in Brazilian propolis are flavonoids (flavones, flavanols, and flavanones), phenolics, and aromatics, including p-coumaric acid, ferulic acid, cinnamic acid, and its derivatives (drupanin, baccharin, and artepillin C), chrysin, tectochrysin, pinocembrin, pinobanksin, isosakuranetin, kaempferol, kaempferide, and quercetin.[17,18] Polyphenol compounds in propolis has antibacterial activity and inhibits the enzymatic activity of glucosyltransferase and amylase.[19-21]

The best inhibition activity was seen with the CPP–ACP + 2% EEP tooth coating gel. This formula can inhibit 73.78% of *S. mutans* growth compared with the negative control. Tooth gels with CPP–ACP and EEP deposit calcium phosphate in dentin, thereby closing holes in the dentinal tubules. By maintaining a condition saturated with hydroxyapatite, calcium and phosphate ions from the CPP–ACP deposition will reduce demineralization. This finding is in accordance with Amalina *et al.*, who used CCP–ACP with propolis to remineralize dentin surfaces by releasing calcium, phosphate, and fluoride ions.[22] The enamel surface was flat when compared with the negative control (Figure 3). Propolis acts as an antibacterial that inhibits the performance of biofilm-forming bacteria that cause dental caries. EEP stimulates the formation of reparative dentin that reduces permeability. Propolis has adhesive properties that extend the contact time with the teeth and increase resistance to the acid solubility of enamel.

The results showed that 2% of propolis in the formula is the best composition for antibacterial activity and the inhibition of demineralization. Higher propolis concentrations might be able to interact with tooth minerals and inhibit the remineralization of teeth by CPP–ACP. Further studies are needed to confirm this hypothesis. The tooth gel formulation with CPP–ACP and 6% EEP could decrease bacterial growth by 73.7% bacteria relative to the negative control. Although antibacterial activity of the tooth gel containing CPP–ACP and 2% EEP is lower than that with 6% EEP, the former showed an excellent row pattern prism with a smooth and flat surface as identified by SEM, which has been proven to prevent dental caries.

Conclusions

The formulation of the CPP–ACP and propolis tooth gel inhibited *S. mutans* and supported remineralization. In addition, the tooth gel can be used as an alternative to prevent dental caries.

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Declaration of Interest

Declared none.

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