The effect of CPP-ACP-propolis chewing gum on calcium and phosphate ion release on caries-active subjects’ saliva and the formation of Streptococcus mutans biofilm

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Abstract. The aim of this study was to analyze the effect of CPP-APP and propolis wax if they are combined in a chewing gum formulation, observed from the calcium and phosphate ion level released by CPP-ACP and the emphasis of Streptococcus mutans mass in the biofilm by propolis wax on caries-active subjects’ saliva. Chewing gum simulation was done in vitro on 25 caries-active subjects’ saliva using five concentrations of chewing gum (0% propolis + 0% CPP-ACP, 0% propolis + CPP-ACP, 2% propolis + CPP-ACP, 4% propolis + CPP-ACP, and 6% propolis + CPP-ACP) and was then tested using an atomic absorption spectrophotometer to analyze calcium ion levels, an ultraviolet-visible spectrophotometer to analyze phosphate ion levels, and a biofilm assay using crystal violet to analyze the decline in biofilm mass. After the chewing simulation, calcium ion levels on saliva+gum eluent increased significantly compared to the saliva control, with the highest calcium level released by CPP-ACP + 2% propolis chewing gum. There was an insignificant phosphate level change between the saliva control and saliva+gum eluent. There was also a significant decline of S. mutans biofilm mass in the saliva+gum eluent, mostly by the CPP-ACP chewing gum and CPP-ACP + 6% propolis. The CPP-ACP-propolis chewing gum simulation generated the largest increase in calcium and phosphate ion level and the largest decline in S. mutans biofilm mass.

1. Introduction
Dental caries are the most common oral disease in Indonesia [1]. According to a 2014 health survey by the Ministry of Health, the prevalence of dental caries in Indonesia reached 90.05%, up from 53.2% in 2013 [2,3], an increase of 43.4%. Dental caries are lesions found in calcified dental tissues caused by bacterial activities that result in the dissolution of minerals and localized tissue damage. Dental caries are a multifactorial disease with four etiological factors: host (tooth structure, saliva), microorganisms (cariogenic bacteria), substrate (low-molecular-weight carbohydrates), and time [4-6]. Saliva is a body defense against the occurrence of dental caries, as it produces the calcium ions, phosphorus, and fluoride used for remineralizations. The increase in salivary flow rate will maximize the role of saliva as an agent of the body’s defenses. Salivary flow rate can be stimulated by food mastication, as well as by chewing gum [5-7]. Biofilm is a soft layer consisting of 70% bacterial colonies and 30% matrix. The most commonly found cariogenic bacteria in the mouth is Streptococcus mutans [5,8].

One alternative biological resource in Indonesia that can be used as an antibacterial agent is propolis wax. Propolis wax is a residue of the refining process of honeybees [9]. Aside from fluoride,
there is one alternative agent that can be used to promote remineralization, casein phosphopeptide-amorphous calcium nanocomplexes phosphate (CPP-ACP) [10]. CPP-ACP releases calcium ions and phosphate, which are the main components of enamel hydroxyapatite that can help remineralization when teeth are in acidic conditions [11].

The combination of propolis wax, which has antibacterial properties, and CPP-ACP, which is a remineralization agent, as an active component in chewing gum medium is a new innovation in the prevention of caries. Benefits of propolis wax and CPP-ACP in the prevention of caries have been investigated, but the results when both are combined in a single medium have not been known. Currently, it is not yet known whether there is an influence of the concentration number of propolis in chewing gum on the release of ions from the CPP-ACP, or vice-versa. The purpose of this study was to determine the levels of calcium and phosphate ions as well as S. mutans suppression in biofilm that contained the saliva of caries subjects after being given CPP-ACP-propolis chewing gum.

2. Materials and Method

This research is an experimental study using sugar-free gum samples containing five concentrations of propolis wax with only 5% of CPP-ACP without any variation, using 0% negative control of propolis and 0% CPP-ACP. There were 25 subjects. After taking caries-active subjects’ saliva samples, tests of calcium and phosphate ion release were executed. This was the saliva control. Before simulation of masticating chewing gum started, five CPP-ACP-propolis gums were taken from five concentrations and mashed with saliva samples for 5 min. The samples were then collected inside a graduated test tube and cooled in a –20°C refrigerator. The calcium and phosphate release test and biofilm test were done using these samples of saliva. The results of the samples are the saliva+gum eluent. When the samples were ready, the test to measure calcium ions was executed using an atomic absorption spectrophotometer (AAS), while an ultraviolet-visible spectrophotometer (UV-Vis) was used to measure phosphate ions. The process of calculating the number of colonies and execution of the biofilm test was started by taking S. mutans that had been incubated for 48 h and using an ELISA reader to get the value of biofilm mass through optical dentistry (OD) at a wavelength of 490 nm. The biofilm test was done for the saliva control and saliva+gum eluent.

3. Results and Discussion

3.1 Results

Analysis of the release of calcium and phosphate ions was carried out in the control saliva and saliva+eluente gum. The results of the average value of calcium ions released can be seen in Figure 1.

![Figure 1](image-url)

**Figure 1.** The changes in calcium ions in the control saliva and saliva+gum eluent

Based on Figure 1, there are changes in calcium ion levels between control saliva and saliva+eluente CPP-ACP-propolis gum in all concentrations. The calcium ions were released in greatest numbers in CPP-ACP + 2% propolis gum and least numbers by the negative control. After analysis using a normality test, paired t-test, one-way ANOVA, and post-hoc LSD, it was concluded that there are significant differences in calcium ions level in the concentration of the negative control with CPP-ACP, negative control with CPP-ACP + 2% propolis, and CPP-ACP + 2% propolis with CPP-ACP +
6% propolis (p ≤ 0.05). The average levels of phosphate ions released from the chewing gum in all concentrations can be seen in Figure 2.

![Figure 2](image)

**Figure 2.** The changes in phosphate ions in the control saliva and saliva+gum eluent

Based on Figure 2, there are changes in the levels of phosphate ions between control saliva and saliva+eluent CPP-ACP-propolis gum in all concentrations, where the greatest release was in CPP-ACP + 2% propolis and the least from CPP-ACP + 4% propolis. After analysis using normality test, paired t-test, one-way ANOVA, and post-hoc LSD, it was concluded that there are no significant differences between the mean of phosphate in all concentrations.

A biofilm test was performed in control saliva and saliva+eluent gum. Controls used in this study were 2%, 4%, and 6% pure propolis. The average value of *S. mutans* biofilm mass in control saliva and saliva+eluent chewing gum, as well as *S. mutans* biofilm mass in the propolis control, can be seen in Figure 3.

![Figure 3](image)

**Figure 3.** The change in biofilm mass of *S. mutans* (OD 490 nm) in the saliva control, saliva + gum eluent, and propolis control

Based on Figure 3, there is a change in the mass of *S. mutans* biofilm between control saliva and saliva+eluent CPP-ACP-propolis gum in all concentrations. The lowest reduction is in eluent gum (CPP-ACP + 6% propolis) and CPP-ACP gum. All types of biofilm saliva+eluent gum showed a lower number of biofilm mass than the propolis control biofilm. After analysis using a normality test, paired t-test, one-way ANOVA, and post-hoc LSD, it was concluded that there is a statistically significantly different biofilm mass between saliva+eluent CPP-ACP gum and all another concentrations. No other types of chewing gum have a significant difference in biofilm mass of *S. mutans*.

3.2 Discussion

The results showed that there are significant changes between levels of calcium ions contained in control saliva and saliva+eluent gum. The highest increase was found in saliva+eluent gum CPP-ACP
+ 2% propolis, followed by saliva+elucent gum CPP-ACP (0% propolis + CPP-ACP). Although in this study the highest levels of calcium ion release were found in saliva+elucent gum CPP-ACP + 2% propolis, the level is not significantly different from calcium ions in CPP-ACP gum. This shows that the addition of 2% propolis did not impede the release of calcium ions from the gum. The propolis wax was not a pure propolis, where the main composition of propolis is wax with an oil-based substance [8], while CPP-ACP is a water-based substance [1]. From its differences of nature, those substances are not able to be united, and when it is released in the mouth, it will undergo its own functions without interfering with each other, but, somehow, the two substances are combined together in an emulsion of gum. By the mechanical movement of the simulated chewing gum, propolis wax and CPP-ACP detached and split in the mouth. There is a possibility that propolis wax envelops the ions that split from the CPP-ACP in chewing gum. This can cause calcium ions that may not be detected by AAS, thus causing lower levels of calcium ions.

The level of phosphate ions released from the gum showed that the levels of phosphate ions in saliva+elucent gum are not significantly different from the saliva control. However, there are elevated levels of phosphate ions in all concentrations of chewing gum, with the highest increase in CPP-ACP (0% propolis + CPP-ACP) gum. The data showed that the lowest release was from CPP-ACP + 4% Propolis gum. The insignificant increase in phosphate ion release can be caused by the differences in length of time while taking the saliva samples. Aside from that, the mechanical motion of chewing gum simulation allows ion phosphates inside CPP-ACP to split into phosphorus and oxygen ions. In this study, however, control saliva and saliva+elucent gum could only be detected by using a UV-Vis instrument with a wavelength to detect the phosphate ions at 700 nm. This means that the phosphorus ions, a fraction of the phosphate ions released by CPP-ACP in chewing gum, were not detected in the mouth so that the levels of phosphate ions did not change significantly. The combination of CPP-ACP with propolis wax in gum indicates that the release of calcium and phosphate ions occurs similarly to gum that contains only CPP-ACP. This shows that the anticariogenic mechanism of CPP-ACP remains, where CPP is able to stabilize and localize ACP at the tooth surface and suppress demineralization and improve remineralization. However, according to research by Hidaka et al. (2008), it is known that flavonoids can inhibit the precipitation of calcium phosphate to hard tissues of the tooth and inhibit the formation of hydroxyapatite. This inhibition occurs because flavonoids can disallow the growth of hydroxyapatite crystals directly or slow their induction process [12].

The biofilm test result showed that there is a significant decrease in biofilm mass (OD 490 nm) throughout the biofilm when compared to control saliva. Significant biofilm mass reduction in saliva+elucent gum occurred because of the content of polyphenol compounds, especially flavonoids [13]. Although the concentration of the entire chewing gum showed a decrease in biofilm mass, the decline is not in line with the increasing content of propolis or is not dose-dependent.

Based on the results of this study, formulations of CPP-ACP-propolis gum worked well with the release of calcium and phosphate ions, as well as increasing its antibacterial effects. The results of this study are in line with the previous studies that only tested the effects of each active component separately, as the maximum effect of each active component can be accomplished if it is not combined with other components. This is particularly observable on the possibility of its antagonistic properties between CPP-ACP and propolis wax, in that flavonoids in propolis wax can inhibit the precipitation of calcium phosphate to the enamel and the formation of hydroxyapatite during remineralization.

4. Conclusion

Based on the results of research on the release of calcium and phosphate ions and S. mutans mass suppression by the CPP-ACP-propolis chewing gum conducted in caries subjects, it can be concluded that there is a possibility that the release of calcium and phosphate ions by CPP-ACP is influenced by the content of propolis wax in the CPP-ACP-propolis gum. The decrease of S. mutans biofilm mass that occurs is not in line with the increasing content of propolis or is not dose-dependent. The combination of CPP-ACP and propolis wax in the chewing gum medium is likely not better than the use of each active component separately.
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