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ANTIMICROBIAL EFFECTIVENESS OF CALCIUM HYDROXIDE, PROPOLIS, AND THEIR COMBINATION ON THE GROWTH OF *Streptococcus mutans*

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ABSTRACT
Background: *Dental caries is a global oral health issue associated with the dominance of Streptococcus mutans in dental plaque. Calcium hydroxide is a commonly used dental material; however, there are increasing concerns regarding its adhesion and dissolution issues. Propolis, a natural compound with antimicrobial properties, promotes fibroblast proliferation, releases antioxidants, and reduces oxidative stress when combined with calcium hydroxide. This combination shows potential for enhancing antibacterial efficacy and pulp preservation in restorative dentistry.* ***Purpose:*** *This study aims to evaluate the antimicrobial effect of a combination of calcium hydroxide and propolis in inhibiting the growth of Streptococcus mutans.* ***Methods:*** *The experimental study assessed the antimicrobial activity of Streptococcus mutans ATCC 25175 using the agar disk-diffusion method. Five treatments with five repetition groups were tested: 2% chlorhexidine (positive control), propolis, calcium hydroxide, a calcium hydroxide-propolis mixture, and saline (negative control). The inhibition zone was measured after 24 hours of incubation at 37°C. Data were analyzed using one-way ANOVA and Tukey's post hoc test in IBM SPSS 25.* ***Results:*** *The positive control (chlorhexidine) showed the largest inhibitory zone (1.488±0.11), followed by propolis (0.862±0.06), calcium hydroxide combined with propolis (0.746±0.05), and calcium hydroxide suspension which suggested the smallest inhibitory zone (0.684±0.09).* ***Conclusion:*** *The combination of calcium hydroxide and propolis demonstrated superior antibacterial activity against Streptococcus mutans only when compared to calcium hydroxide suspension alone.*

Keywords: *Calcium hydroxide, Caries, Propolis, Streptococcus mutans*

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INTRODUCTION

Dental caries is a prevalent chronic oral disease and a significant global health concern. The disease affects over 2 billion adults and 520 million children worldwide, significantly impacting public health.[1] This condition is inseparable from the presence and dominance of pathogenic bacteria in dental plaque, where caries lesions have a high percentage of the presence of certain bacterial species such as *Streptococcus mutans*, *Streptococcus sobrinus*, and Lactobacilli.[2] Previous studies on caries lesions found that the presence of *Streptococcus mutans* and *Streptococcus sobrinus* was higher in tooth enamel that had caries when compared to healthy enamel, while Lactobacilli bacteria were found in advanced lesions.[3] Traditionally, deep caries management involves the complete (nonselective) and destructive removal of infected dentin. However, minimally invasive treatment strategies are now recommended to selectively (partially) eliminate caries and reduce the risk of pulp exposure.[4]

Thorough removal of infected dentin plays an important role in the successful treatment of dental caries. However, under certain conditions, some affected dentin is intentionally left behind. One of them is in the condition of total removal of dentin in deep cavities, which can result in the pulp opening, so some dentin is left to protect the teeth and pulp. Therefore, to preserve the pulp from secondary infections caused by microleakage or bacterial residues, the use of capping pulp material with antibacterial properties under permanent restoration is an ideal choice and an important step to avoid pulp damage.[5] The use of natural substances as an alternative to pulp capping materials has great potential and is beneficial for health. Previous research shows that the combination of propolis and probiotics are two natural substances that are promising as antimicrobials. [6]

Calcium hydroxide is the gold standard for direct pulp capping treatment.[7] Although calcium hydroxide-based cementing materials have been used for many years, they have some drawbacks, including poor adhesion to dentin, dissolution over time, and some tunnel defects in the formed dentin.[8] Therefore, a natural additive is needed that has effective antibacterial properties but is not toxic to tissues. In restorative dentistry, the combination of propolis and calcium hydroxide is increasingly being studied and is expected to provide better results than calcium hydroxide alone. Based on previous research, it is known that water-soluble propolis extract shows effective antibacterial ability by not showing toxic effects on HDFa cells so that it has the potential to increase the proliferation of fibroblast cells.[9] In combination with calcium hydroxide and propolis, it is known to produce caffeic acid phenethyl ester (CAPE) as an antioxidant substance that can bind free radicals, especially hydroxyl ions (OH−), which can prevent lipid peroxidation and cell death.[10] Based on the previous exposure, this study was carried out to determine the effect and effectiveness of antimicrobial from the combination of calcium hydroxide and propolis when compared to pure calcium hydroxide on the growth of *Streptococcus mutans* bacteria.

METHODS

This research is based on a laboratory experiment conducted *in vitro* at the Molecular Medicine and Therapy (MMT) Laboratory, Institute of Research and Innovation, Universitas Muhammadiyah Yogyakarta. This study has received ethical approval under No. 052/EC-EXEM-KEPK FKIK UMY/II/2023.

Bacterial Culture and Strains

The bacteria used in this study were Streptococcus mutans ATCC 25175, cultured aerobically in Brain Heart Infusion (BHI) broth at 37°C for 24–48 hours. The formed bacterial colonies were adjusted for turbidity to the 1 McFarland standard (3 x 10^8 CFU/mL) to obtain an equivalent number of colonies in each treatment.

Research Design

In this study, five different sample groups were used with five repetitions for each treatment group. Group A contained 2% chlorhexidine dissolved in distilled water and used as a positive control. Group B is in the form of pure Propolis as much as 1 ml. Group C contains 0.1 grams of calcium hydroxide supplemented with 400 µL of glycerin and dissolved in 600 µL distilled water. Group D is a sample containing a mixture of 0.1 grams of calcium hydroxide, 50 µL of propolis, plus 400 µL of glycerin and dissolved in 600 µL of distilled water. As for group E in the form of Saline NaCl solution as a negative control.

Agar disk-diffusion assay

In vitro antimicrobial evaluation was conducted using the agar diffusion method, with slight modifications to treatment volume and incubation time.[11] 100 µL of *Streptococcus mutans* ATCC 25175 bacterial culture was inoculated onto the surface of Brain Heart Infusion (BHI) agar by the plating spreader method. Furthermore, the disc disc (about 6 mm in diameter), was dripped with each test compound, namely group A (CHX/positive control), B (propolis), C (calcium hydroxide), D (calcium hydroxide and propolis) and E (saline/negative control solution). The disc was left submerged in the treatment solution for approximately 1 hour. Then, the disc that has contained the treatment group is placed on the agar surface and incubated for 24 hours at a temperature of 37° C under aerobic conditions. The inhibitory (radial) zone of each group was then measured using a sliding caliper with a precision level of 0.01 mm.

Statistics Analysis

All data was processed using IBM SPSS 25. The normality of the obtained data was analyzed using the Shapiro-Wilk test, Levene test followed by ANOVA and Tukey's post hoc test.

RESULTS

The results showed antibacterial activity, as evidenced by the formation of inhibitory zones of varying sizes in each sample group. (figure 1).

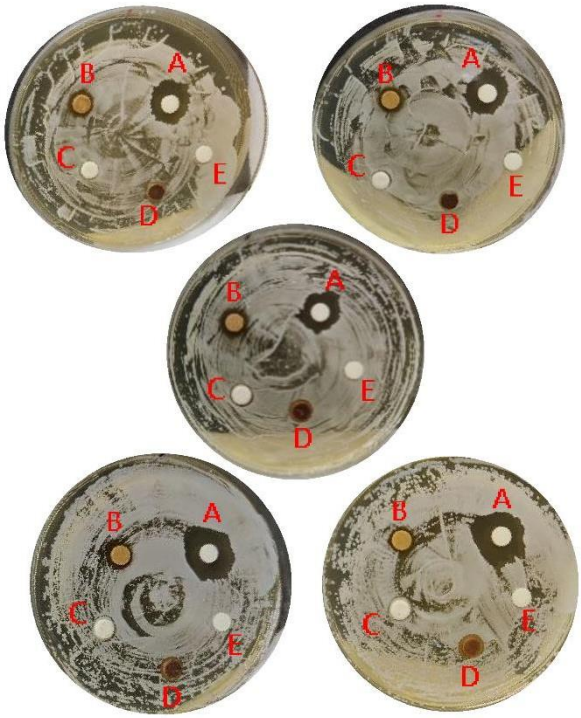

Figure 1. The inhibition zone is formed. A: Chlorhexidine (positive control), B: Propolis, C: Calcium hydroxide, D: Calcium hydroxide and Propolis, E: Saline (negative control).

The ANOVA test results ($p < 0.0001$) indicate a significant effect of the treatment in inhibiting the growth of *Streptococcus mutans*. A one-way ANOVA was followed by Tukey's post hoc test to determine significant differences in antibacterial effectiveness between treatment groups, as presented in Figure 2.

Figure 2 shows the average results of the measurement of the inhibition zone formed from the treatment group. The results indicate that the largest inhibition zone was observed in the positive control (chlorhexidine), with an average of 1.488 ± 0.11 cm. Propolis has an inhibition zone with an average of 0.862 ± 0.06 cm. Calcium hydroxide with an average inhibition zone of 0.684 ± 0.09 cm. Calcium hydroxide combined with propolis has an average value of 0.746 ± 0.05 cm. Negative control (saline) showed no any inhibition zone.

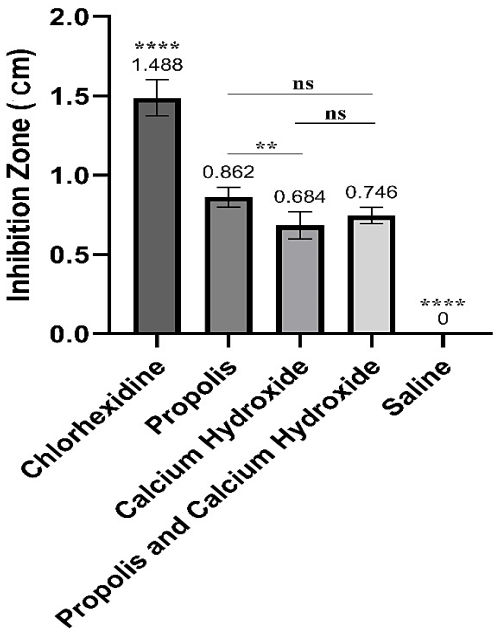

Figure 2. Mean inhibition zone for different treatments; ns indicate no significant differences; asterisks indicate statistically significant differences: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Based on Figure 2, there was no significant difference between the calcium hydroxide group and the calcium hydroxide combined with the propolis group. Similarly, no significant difference was observed between the propolis group and the calcium hydroxide combined with the propolis group. In contrast, a significant difference was found between the propolis and calcium hydroxide groups.

DISCUSSION

This study was carried out to evaluate the antimicrobial effect of the combination of calcium hydroxide and propolis in inhibiting the growth of *Streptococcus mutans* bacteria. The bacteria used are pure strains of *Streptococcus mutans* bacteria (ATCC 25175) in the Molecular Medicine and Therapy (MMT) Laboratory, Faculty of Dentistry, University of Muhammadiyah Yogyakarta. S. *mutans* is a bacterium that lives in plaque on the surface of teeth and is the main etiological agent of human dental caries. The materials used as influence variables are calcium hydroxide suspension (calcium hydroxide powder with glycerin and water), propolis products, combination of calcium hydroxide suspension and propolis as well as Chlorhexidine as a positive control and saline as a negative control.

In this study, calcium hydroxide showed a smaller inhibition zone compared to propolis products. The antimicrobial properties of calcium hydroxide come from the dissociation of calcium and hydroxyl ions. Hydroxyl ions create an alkaline pH that is unfavorable to bacteria. The antimicrobial properties of hydroxyl ions in microorganisms affect the growth, structure, metabolism, and division of bacterial cells.[12]

Chemically, calcium hydroxide is known as a strong base when mixed with liquids (pH around 12.5–12.8). Calcium hydroxide provides antimicrobial activity by dissociating into calcium and hydroxyl ions that increase pH. The alkaline environment detoxifies bacterial lipopolysaccharides (LPS) by removing esterified fatty acids and chemical arrangements that cause the integrity of the bacterial cytoplasmic membrane to be destroyed.[13]

Chlorhexidine, used as a positive control in this study, exhibited the largest inhibition zone. Chlorhexidine is a synthetic cationic biguanide used in endodontic treatment.[14] Chlorhexidine is used as an irrigant and as a component in intracanal medicaments in endodontics..[15] It has a direct antimicrobial effect, a broad spectrum of action, substance, and relatively lower toxicity, but organic residues present in the dental canal cannot be dissolved and when this material is mixed with sodium hypochlorite, it can stain the dentin which may interfere with the sealing of the root filling [14].

Based on the results of this study, the inhibition zone produced by the calcium hydroxide-propolis combination did not significantly differ from that of propolis alone. Calcium hydroxide has a low solubility in water (about 1.2 gr/L) at a temperature of 25 which decreases with increasing temperature. The main role comes from the ionic dissociation of Ca^{2+} and OH^- ions. The effect on vital tissues is to induce the deposition of hard tissues and become antibacterial.[16] Propolis contains acidic components, such as phenolic acids such as vanillic acid, cubic acid, ferulic acid, cinnamic acid, gallic acid, chlorogenic acid, rosmarinic acid, and ellagic acid as well as flavonoids such as hesperidin, epicatechin, rutin, apigenin, quercetin, naringin, and kaempferol.[17] This research is supported by previous research that shows that the flavonoid content in propolis extract is able to show a inhibitory capacity against the proteolytic activity of subgingiva and root canal bacteria, and also shows beneficial properties for health through the antioxidant capacity contained in it.[18,19] The pH measurement results for the combination of calcium hydroxide with aquades ranged from 12, for the combination of calcium hydroxide with propolis ranged from 8.[20] This shift in pH is likely to cause the inhibition zone produced by the combination of calcium hydroxide and propolis not much different from calcium hydroxide suspension. In conclusion, the combination of calcium hydroxide and propolis demonstrated superior antibacterial capacity against Streptococcus mutans only when compared to calcium hydroxide suspension alone.

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