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**Research Article** 

# Potential of 10% propolis-based toothpaste on the inhibition of biofilm-forming bacteria growth *in vitro*

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# Abstract

Aim of the study: To investigate the potential of 10% propolis-based toothpaste on inhibiting biofilm-forming bacteria growth in vitro.

**Material and method**: Organoleptic properties are evaluated, considering color, odor, and taste. Antibacterial tests use a disc diffusion method against *Streptococcus mutans*, *Staphylococcus aureus* and *Porphyromonas gingivalis* bacteria, while cytotoxicity is assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay on fibroblast cells. Statistical analysis involves mean  $\pm$  standard deviation. The data were then tested using a one-way analysis of variance and Kruskal-Wallis, followed by post-hoc test (p < 0.05).

**Results**: The organoleptic evaluation of 10% propolis toothpaste reveals a visually clear appearance, consistent orange flavor, and aroma lasting 30 days. Based on the antibacterial results, a 10% level of propolis toothpaste sample inhibited the growth of *Streptococcus mutans*, *Staphylococcus aureus* and *Porphyromonas gingivalis* bacteria. The post-hoc test showed that toothpaste demonstrated significant inhibition on *S. mutans* and *S. aureus* compared to the negative control (p < 0.05). The toothpaste showed a larger inhibitory zone towards *P. gingivalis* compared to the adverse control; however, no significant differences were observed (p > 0.05). Cytotoxicity assessment on fibroblast cells shows a high percentage (85.31%) of viable cells. The findings highlight the 10% of propolis toothpaste's potential and non-toxic as oral care product.

Conclusions: 10% propolis toothpaste inhibits S. mutans, S. aureus, P. gingivalis growth, and not toxic on fibroblast.

### Keywords

propolis, toothpaste, organoleptic, antibacterial, cytotoxicity

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# Introduction

Dental caries remains the most prevalent chronic oral disease and a worldwide oral health concern (Wen et al. 2022). Caries of deciduous teeth affect 46.9% and 39.3% of children aged 1 to 4 and 5 to 9 years, respectively, while 2.3 billion people worldwide have permanent tooth caries, making it the most common health disease. It is also regarded as an irreversible microbial disease that affects the hard tissue of teeth and is defined by the destruction of the inorganic and organic components of the tooth, which may result in cavitation and tooth loss (de Sousa et al. 2022). Early stages of caries formation are characterized by the presence of visible signs of demineralization, such as white spots, in the mineralized tooth tissues. However, it is important to note that the genesis of this disease mostly occurs within the dental plaque that resides on the surface of the teeth (de Sousa et al. 2023).

A regulated diet with less carbs and good dental hygiene can disaggregate the cariogenic biofilm on the tooth surface and control the disease (Takahashi et al. 2019). Throughout ancient times, people have taken care of their teeth, demonstrating the significance of maintaining oral health. Because of advances in technology and medicine, oral health is continuously evolving. In recent years, natural treatments for oral cavity diseases have gained popularity due to their reduced side effects (Otręba et al. 2022). Humans began using propolis more than 2,000 years ago for a variety of reasons, the most prominent of which was to treat wounds against infection. The modest anti-infective effects of the substance contribute to the facilitation of the healing process. Throughout the course of time, several iterations of this product have been introduced to the market, encompassing toothpaste, mouth rinses, and lozenges (Rezende et al. 2006). Propolis extract exhibits antimicrobial efficacy against Streptococcus mutans, a Gram-positive cocci commonly found in the human oral cavity, contributing significantly to plaque formation. Its in vitro antimicrobial activity extends to bacteria associated with periodontal diseases and potential superinfectious microorganisms, including Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, Prevotella melaninogenica, Aggregatibacter actinomycetemcomitans, Capnocytophaga gingivalis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans (Gebara et al. 2002; Saputra et al. 2019). In case studies and pilot clinical studies, propolis was also found to be effective in the treatment of gingivitis and oral mucosa lesions (Silveira et al. 1988).

Due to their demonstrated therapeutic efficacy, the use of natural products for pharmacological purposes has become widespread over the past few decades. Propolis, a substance produced by honeybees that has been widely used in folk medicine since antiquity, appears to be a promising agent for addition to topical formulations due to its multidirectional properties (Sforcin and Bankova 2011) in addition to antioxidant and anti-inflammatory activity (Czuba and Krol 1996). Epidemiological studies have identified propolis's antibacterial, antifungal, antiviral, and antitumor properties (Kujumgiev et al. 1999). Ethanolic extract of propolis solutions have been used commercially as an effective antimicrobial and anti-inflammatory agent in toothpaste, mouthwash, lozenges, etc. (Moreno et al. 1999).

Nevertheless, it is still undervalued in academic medicine and dentistry. In general, propolis consists of fifty percent resin vegetable balsam, thirty percent wax, ten percent essential and aromatic oils, five percent pollen, and five percent various other substances, including organic debris, depending on the place and time of collection (Bankova et al. 1995). Climate, season, location, and year have a significant impact on the composition of propolis, and its chemical formula is unstable (Seidel et al. 2008). Flavonoids, the primary component of propolis, inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, as well as the activity of enzyme systems such as cyclooxygenase and lipoxygenase (Viuda-Martos et al. 2008). The present study aimed to investigate the potential of propolis-based toothpaste on the inhibition of biofilm-forming bacteria growth in vitro.

# Materials and methods

Antibacterial and cytotoxicity analysis are presented in this study. The protocol of this study was approved by the Research Ethics Committee of the Faculty of Dentistry-Prof. Soedomo Dental Hospital UGM (reference number 211/KE/FKG-UGM/EC/2022).

#### Preparation of 10% propolis toothpaste

Previous studies have shown that 10% propolis is effective in inhibiting the growth of several types of bacteria species with no colony growth on petri dishes (Nam et al. 2016; Faizah et al. 2017). Propolis was obtained from Inbinusantara Corporation (Indonesia). To prepare 10% propolis toothpaste, start by heating distilled water. Then,



Figure 1. Toothpaste appearance.

dissolve sorbitol, hyaluronic acid, propolis, and glycerin into the mixture and stir the liquid homogeneously for 15 minutes using a hotplate stirrer. Next, incorporate orange flavoring and sodium benzoate into the mixture, ensuring thorough mixing for a duration of 30 minutes until a uniform paste is achieved.

#### Organoleptic properties

The organoleptic properties such as color, odor and flavor were evaluated. Color of the prepared toothpaste was evaluated for its color, the colur was checked visually. Odor was found by smelling the product. Taste was checked manually by tasting the product (Rahman et al. 2023).

#### Antibacterial test

A disc diffusion method was performed to measure the potential of the propolis 10% toothpaste on inhibition of S. mutans ATCC 25175, S. aureus ATCC 25923, P. gingivalis ATCC 33277 growth. 0,2% chlorhexidine solution was used as positive control and distilled water was used as negative control. Bacterial microorganisms: S. mutans, S. aureus, P. gingivalis were used throughout the study. Bacterial strain from stock cultures was cultivated in Brain Heart Infusion broth (Oxoid, USA) at 37 °C for 24 hours, corresponding to 108 CFU/mL using the 0.5 Mc-Farland standard. All bacteria were spread on the surface of Mueller Hinton Agar petri dish and incubated at 37 °C for 24 h. Especially for P. gingivalis spread on Blood Agar and incubated on anaerobic condition. After 24 h, the paper blank disc (6 mm diameter) is dipped into the toothpaste solution agent and planted at the petri dish which contains the bacterial culture. The plates were then incubated at 37 °C 48 h. The inhibitory zone was evaluated by determining the diameter (mm) of inhibition zones around each disc.

#### Cytotoxicity test

The MTS assay was performed to measure the cytotoxic effect of 10% propolis toothpaste on fibroblast cell line from human dermal fibroblasts-adult (HDFa) cell (Gibco C-013-5C, USA). The principle of the MTS Assay based on the previous research (Felicia et al. 2018; Goenka and Lee 2023). This method is a colorimetric measurement based on the formation of a purple insoluble formazan salt from the reduction reaction of tetrazolium which is soluble in water to produce a yellow solution. The MTS reagent only reacts with living cells and then it is broken down through a reduction reaction by the tetrazolium succinate reductase system to form formazan. Fibroblast cells cultured on a 96 well microplate was prepared to be treated using a 10% propolis toothpaste. Untreated cells were used as the blanco group. The 96 well microplate was then incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 24 hours. After completion of incubation, the MTS test was carried out by adding 100 µl of MTS solution (Promega CellTiter 96, USA) into

each well and incubation back into the 5%  $CO_2$  incubator at 37 °C for 4 hours. The MTS reaction was stopped by adding 100 µl of stopper (solubilizer) to each well, then shaking it for 1 hour. Absorbance was measured at 490 nm on ELISA reader. The average percentage of viable cells is calculated from the optical density (absorbance) value of each sample at each concentration against the blanco value with the following formula:

#### **Statistical analysis**

The results were expressed as the mean  $\pm$  standard deviation (SD) for triplicate. One-way ANOVA was used to compare values, followed by post-hoc least significant difference (LSD) test. Kruskal-Walis was performed for non-parametric data, followed by Mann-Whitney posthoc test. All data analysis was performed using the IBM SPSS statistics.

#### Results

#### Organoleptic properties

Visually, toothpaste presented a consistent color and clear appearance with orange flavor that was unaltered for up to 30 days of storage in room temperature (Table 1).

Table 1. Organoleptic test results.

Parameters	Observation
Color	Clearly
Odor	Orange flavor
Taste	Orange flavor

#### Antibacterial results

Research on the potential inhibition test of toothpaste containing 10% propolis on the growth of *S. mutans* has been carried out using the disc diffusion method. The mean and standard deviation of the inhibition of *S. mutans* can be seen in Figs 2, 3. The One-Way ANOVA results show a statistically significant difference in the mean data between groups (p<0.05). The results of the LSD post-hoc showed the significance of the difference between 10% propolis toothpaste and the negative control.

The results of the Kruskal-Wallis test show a significance (p < 0.05), its means there is a statistically significant difference in the mean data between groups (Figs 2, 4). A post-hoc test was then carried out to find out the details of the significance of the difference in inhibition of *S. aureus* between groups. The results of the Mann Whitney Post Hoc test showed a significant difference between 10% propolis toothpaste and the negative control but did not provide a statistically significant difference to the positive control. This indicates that 10% propolis toothpaste was effective as an antibacterial agent against *S. aureus*.

The results of the Kruskal-Wallis show a significance p < 0.05, which means that there is a statistically significant



Figure 2. Diameter zones inhibition of S. mutans, S. aureus, P. gingivalis.









difference in the mean data between groups (Figs 2, 5). A post-hoc test was then carried out to find out the details of the significance of the differences in inhibition against *P. gingivalis* bacteria between groups. The results of the Mann Whitney Post Hoc test indicated that there was no

Figure 4. Inhibition of S.aureus.

significant difference (p > 0.05) between the 10% propolis toothpaste and the negative control group. Nevertheless, there was a statistically significant difference between the positive control group and the 10% propolis toothpaste (p < 0.05).



Figure 5. Inhibition of P. gingivalis.

#### Cytotoxicity results

The cytotoxicity of 10% propolis toothpaste was assessed against fibroblast cells. The percentage cell viability was 85.31%. These results revealed that 10% propolis has a high percentage of viable cells (Fig. 6).



Figure 6. The absorbance of MST assay.

# Discussion

The organoleptic test findings emphasize the sensory aspects of the propolis-infused toothpaste, showcasing its visually clear appearance, distinct orange aroma, and consistent orange flavor. The evaluation determined that the toothpaste's color was described as "clear," suggesting a visually attractive product with transparency, likely linked to propolis processing and refinement. This clarity holds significance in consumer perception, conveying purity and quality in the formulation (Mendes et al. 2016). The assessment also detected an "orange flavor" in the toothpaste, indicating the inclusion of an aromatic component reminiscent of orange, enhancing the olfactory experience. The orange flavor may result from propolis derived from plants with orange-like aromatic profiles or intentional addition of natural orange flavoring agents (Cedeño-Pinos et al. 2021).

The integration of an orange flavor in both odor and taste contributes to an overall enhanced sensory experience, potentially boosting user satisfaction. The consistency between observed orange flavor in the odor and the identification of "orange flavor" in taste suggests a harmonious sensory encounter, aligning oral perception with olfactory expectations (El-Sakhawy et al. 2023). These sensory attributes may impact consumer preferences, influencing the marketability of the propolis-based toothpaste. Understanding and optimizing the organoleptic qualities in oral care product development are crucial, ensuring consumers not only receive desired oral health benefits but also enjoy a positive and appealing user experience (Ibrahim and Alqurashi 2022).

Depending on the location and timing of collection, propolis is made up of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% miscellaneous materials, including organic debris. It's an antimicrobial in nature. The flavonoids, phenolics, and other aromatic chemicals are responsible for the therapeutic qualities. Antibacterial, antifungal, antiviral, antioxidant, and anti-inflammatory qualities are possessed by flavonoids. According to research, the most potent flavonoid agents against bacteria are pinocembrin, pinostrobin, and galangin. Propolis also has antibacterial properties due to the presence of ferulic and caffeic acids. Dental caries is indirectly decreased by propolis extract, which inhibits plaque growth on the tooth's surface. Propolis's fatty acids have a cariostatic effect by reducing microbes' ability to tolerate low pH and delaying the generation of acid. Propolis's bactericidal, bacteriostatic, and anti-adherent properties work against microbes linked to dental cavities. Studies on propolis's antibacterial properties, however, yield inconsistent findings. Its chemical components may differ, which could be the cause of this. Additionally, it has been found that the antibacterial activity of samples gathered from various geographic origins with varying climates and vegetation vary. In addition, the inhibition zone value calculation is dependent on technical specifications that differ throughout labs. Propolis exhibits antimicrobial properties against both Gram-positive and Gram-negative bacteria, as well as Candida. Propolis has certain chemical constituents that destroy the structural and functional integrity of bacteria' cell walls. Because of its mucoprotective properties, it can be effectively employed in the oral cavity (Dualibe et al. 2007; Hegde et al. 2013; Djais et al. 2019). Propolis and its derivatives have the ability to directly impact bacteria through several methods or by influencing the host's immune system. Propolis is believed to impact the permeability of the cell membrane, resulting in a decrease in membrane-related functions such as adenosine triphosphate generation. This, in turn, limits bacterial movement and other activities. The increased effectiveness of propolis against Gram-positive bacteria compared to Gram-negative bacteria may be attributed to the hydrolytic enzymes produced in the outer membrane protein structure of Gram-negative bacteria. These enzymes have the ability to impair the function of the active components present in propolis (Sforcin and Bankova 2011)

An inhibition zone formed, indicating that the positive control, 0.2% chlorhexidine solution, could stop the growth of *S. mutans*, *S. aureus*, *P. gingivalis* bacteria. In comparison to the 10% propolis toothpaste sample solution and the distilled water negative control solution, the inhibition zone diameter of the 0.2% chlorhexidine solution was the largest. This was brought about by the chemical antibacterial agent, specifically a broad spectrum antibacterial, present in the 0.2% chlorhexidine solution. High concentrations of 0.2% chlorhexidine solution, the gold standard for antibacterial agents, have bactericidal properties (Fiorillo et al. 2019; Brookes et al. 2020).

There are contradictory results in previous studies regarding the level of cytotoxicity of propolis against human cells, especially HGF (human gingival fibroblast) cells. Several studies state that low concentrations of propolis show a low level of toxicity to normal cells. The toxicity level of propolis extract from the west pomeranian region in Poland on normal cells, namely HGF, shows that at low concentrations (10 µg/mL and 100 µg/mL) the results obtained are non-toxic, while toxic results are obtained at high concentrations (500 µg/mL or 1000 µg/mL). Very different results were obtained for HGF with propolis residue that has been incubated for a prolonged time which actually showed a proliferated condition of HGF. The same results were presented by Puspasari et al. (2018) who stated that topical application of propolis can increase fibroblast growth factor-2 and induce fibroblast cell proliferation in experimental rat's oral mucosa that has experienced traumatic ulceration. These conditions can also be found in fibroblast cells in the periodontal ligament. Additionally, a study regarding the toxicity of mouth rinse with various concentrations of propolis extract on HGF states that with concentrations of 5%, 2.5% and 1.25%, mouth rinse is classified as non-toxic, while at a concentration of 10% it is classified as mild-toxic. However, all concentrations showed a significantly lower level of toxicity compared to the control group using 0.2% chlorhexidine (Ozan et al. 2007; Gjertsen et al. 2011; Wieczynska et al. 2017; Puspasari et al. 2018).

However, many other literatures state that the level of propolis toxicity to cells is high, especially in cells that proliferate rapidly. A study states that propolis extract from the Stingless Bee *Trigona Sirindhornae* has a cytotoxic effect on head and neck squamous cell carcinoma. Additionally, anti-proliferative activities on human colon carcinoma cell lines were demonstrated by the ethanolic extracts of propolis from Trigona laeviceps. The cytotoxic effect of ethanol extract of propolis was also observed against K562 erythroleukemic cells. However, other studies say that propolis has

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a selective cytotoxic effect on cells, such as the results of research using Sonoran propolis extract which indicates that the toxicity of propolis on normal cells is lower than on cancer cells (Ishihara et al. 2009; Valencia et al. 2012; Bonamigo et al. 2017; Utispan et al. 2017). Variations in study results regarding the cytotoxic effects of propolis on cells are due to variations in concentration, length of incubation time, and also variations in the methods used. The complexity of the substances contained in propolis varies greatly in different types of propolis so that differences in the use of propolis toxicity identification methods can affect its biological properties (Tyszka-Czochara et al. 2014; Wieczynska et al. 2017).

Our study's limitation is that we did not explore antibacterial activity using assays for minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC). Furthermore, it is imperative that we take into account the techniques employed for plant extraction, as historically plant extracts were commonly prepared using aqueous methods such as poultices, decoctions, and infusions.

# Conclusion

10% propolis toothpaste was shown to inhibit *S. mutans*, *S. aureus*, *P. gingivalis* growth and be non-toxic on fibroblast cells. The application of toothpaste containing 10% propolis formulations exhibits promising potential in the prevention of caries and gingivitis. Consequently, it is imperative to do additional *in vitro* research and clinical trials to thoroughly evaluate its efficacy.

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