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Original article

An effective concentration of propolis extract to inhibit the activity of *Streptococcus mutans* glucosyltransferase enzyme

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ABSTRACT

Background: *According to Riset Kesehatan Dasar (Riskesdas) (2013) and the World Health Organisation (WHO), caries is still a global problem and highly prevalent in Indonesia. Caries is mainly caused by Streptococcus mutans with virulence factors known as glucosyltransferase (GTF). The GTF enzyme contribute to the pathogenesis of caries by converting sucrose to fructose and glucan, which are then used in the formation of biofilms and dental plaques. Natural propolis compounds containing flavonoids, terpenoids, saponins and tannins, can inhibit GTF enzyme activity.* ***Purpose:*** *This study aimed to determine an effective concentration of propolis extract for inhibiting the S. mutans GTF enzyme activity.* ***Methods:*** *This study used propolis extract at 14 µg/ mL, 16 µg/m and 1 µg/ mL to determine the inhibitory effect on S. mutans GTF enzyme activity. The GTF enzyme were obtained from the supernatant from S. mutans culture centrifugation. The GTF enzyme activity was measured using high-performance liquid chromatography (HPLC) to calculate the fructose level.* ***Results:*** *The mean fructose concentration at 14 µg/mL, 16 µg/mL, and 18µg/mL were 3.31%, 1.56%, and 0.29%, respectively.* ***Conclusion:*** *The most effective concentration of propolis extract for inhibiting the effect of S. mutans GTF enzyme activity is 14 µg/mL.*

Keywords: *Glucosyltransferase enzyme; medicine; propolis extract; Streptococcus mutans*

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INTRODUCTION

Dental caries is local damage to the teeth's hard tissue from acid products produced by bacteria during carbohydrate fermentation. Caries is generally chronic and progress slowly as a result of an imbalance between tooth minerals and biofilm.[1] Data from WHO show that 60–90% of children have dental caries, and almost 100% of adults have decay in their teeth.[2] According to Riskesdas (2013) , the national prevalence of dental and oral problems in Indonesia was 25.9%, with the national DMF-T index reaching 4.6, which WHO categorises as 'high'.[3]

Streptococcus mutans (S. mutans) is the oral cavity's normal flora and can be the main cause of caries formation and development due to the ability of biofilm formation by producing extracellular glucosyltransferase (GTF) enzyme, which are a virulence factor in the dental caries pathogenesis.[4] The GTF enzyme convert sucrose into glucose and fructose and catalyses the formation of glucan from sucrose. Glucan increases the adhesion and build-up of *S. mutans* bacteria on the enamel surface and is a crucial factor in forming dental plaque.[5] Inhibiting the activity of the GTF enzyme is one way to prevent caries using natural ingredients as an alternative. The development of natural ingredients is expected to have a more effective antibacterial ability and minimal side effects.[6] GTF enzyme activity can be measured by the level of free fructose produced during the catalytic reaction. In contrast, glucose cannot be measured using high-performance liquid chromatography (HPLC) because it is bound back by the GTF enzyme to form glucans. Several methods can be used to measure the activity of the *S. mutans* GTF enzyme, such as using HPLC or a fluorescence system to measure fructose levels.[7,8]

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Propolis is a natural resinous material collected by honeybees (*Apis mellifera*) from various plants and mixed with their saliva and different other enzyme to build nests.[9] There has been much research conducted on propolis. The outcomes show that propolis has good antibacterial and anti-inflammatory properties. It increases the body's resistance to self-healing and phagocytic activity, stimulates immune cells, and inhibits growth prostaglandin synthesis.[10] Propolis has antimicrobial properties and can withstand more than 100 types of bacteria, viruses and fungi, including those that cause influenza, diphtheria, syphilis and tuberculosis.[11] Indonesia is considered to have a diverse range of local bee species, including the honey bee or *Apis mellifera* that produce most of Indonesia's propolis.[12] The properties of propolis compounds depend on the polyphenol and content of polyphenols, both of which are influenced by seasonal factors, local vegetation, place of origin, bee species and the condition of propolis in either fresh or preserved form.[13]

Propolis is made up of resin and balsam (50–70%), wax and essential oils (30–50%), pollen (5–10%), and other components such as minerals, amino acids, vitamins A, B complex, E and biochemically active ingredients including bioflavonoids (vitamin P), phenols, and aromatic compounds.[13] The active ingredients in propolis extract have an antibacterial ability that hinders the enzymatic activity of GTFs, such as flavonoids, tannins, terpenoids, and saponins. According to research conducted by Achmad et al.,[14] flavonoids can inhibit GTF activity from *S. mutans* bacteria. Based on this research, further analysis is required to determine the effective concentration of propolis extract that can inhibit the activity of *S. mutans* GTF enzyme.

MATERIALS AND METHODS

Before conducting the experiment, ethical clearance was obtained from the Health Research Ethical Clearance Commission Universitas Airlangga Faculty of Dental Medicine (186/HRECC.FODM/IX/2017). The *S. mutans* bacteria used in this study were stock obtained from Universitas Airlangga Faculty of Dental Medicine Research Centre. This *S. mutans* stock was inoculated into 7 ml of Brain Heart Infusion Broth (BHIB) media, followed by a 24-hour incubation process at 37°C.[8] The *S. mutans* culture in BHIB was then centrifuged at 1500 rpm for 10 minutes at 4°C to produce the supernatant from which the GTF enzyme were extracted.[11] Meanwhile, propolis extract obtained from *Apis mellifera* plantation beehives in Lawang, Malang Regency, was extracted by a maceration method using 70% ethanol. It was then diluted with aquadest.[12]

In this research, 16 samples were split into three treatment groups and one control group. Each group received four test tubes containing 0.875 ml of 0.25 M sucrose in pH 7, 0.2 M phosphate buffer, and 0.1 ml of GTF enzyme solution. In the control group, 0.025 ml of Aqua Dest was added, while in the treatment group, 0.025 ml propolis extract was added in 14 µg/ml, 16 µg/ml, and 18 µg/ml. An incubation process was carried out in all control and treatment groups at 37°C for 2 hours.

Enzyme activity testing was carried out at the Faculty of Pharmacy's Testing Service Unit, Universitas Airlangga. After incubation and a filtration process using 0.45 µm filter paper, the fructose level was determined using HPLC by injecting 10 µl of treatment or control solution. Before calculating the fructose level, the retention time of the fructose standard solution was measured. The retention time can be used as a guide for reading the chromatogram results. With knowledge of the solution's area, a specific formula can be used to calculate the fructose level of the sample solution. Furthermore, the fructose level is obtained by applying the following formula to read the area of fructose in the standard solution:[8]

$$\text{Concentration (\%)} = \frac{\left\{\left(\frac{AC}{AS}\right) x \left(\frac{VIS}{VIC}\right) x FP\right\} x 100\%}{KS}$$

Notes:
AC = sample area
AS = standard area
VIC = volume of sample injection
VIS = volume of standard injection
KS = standard concentration
FP = diluted factor

The study measured means and standard deviations. Data analysis was based on One-Way ANOVA testing, demonstrating a significance level of 0.05, followed by the Post-Hoc Tukey HSD test.

RESULTS

The outcome of the HPLC instrument's calculations of the fructose levels revealed that the treatment group had lower fructose levels than the control group, as shown in Table 1. The decrease in fructose levels occurred after the administration of propolis extract with concentrations of 14 µg/ml, 16 µg/ml, and 18 µg/ml. This indicates that the increase in the concentration of the propolis extract is inversely proportional to the decrease in fructose level material. The lowest fructose level compared to other treatment groups was found at 18 µg/ml concentration.

Table 1. The mean and standard deviation of the tested groups' fructose level

Tested groups	N	Mean (%)	SD
Control group (Aqua Dest)	4	6.09	0.67014
14 µg/ml propolis extract	4	3.31	0.30561
16 µg/ml propolis extract	4	1.56	0.21313
18 µg/ml propolis extract	4	0.29	0.06238

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Table 2. Post-Hoc Tukey HSD analysis test results

Tested groups	Control group	14 µg/ml group	16 µg/ml group	18 µg/ml group
Control group		0.000*	0.000*	0.000*
14 µg/ml group			0.000*	0.000*
16 µg/ml group				0.003*
18 µg/ml group				

Note: * indicates that the tested groups have meaningful differences

When performing the One-Way ANOVA analysis, there was a significant difference between treatment groups with a value of $p = 0.00$ ($p < 0.05$). In the Post-Hoc Tukey HSD analysis, all treatment groups had a p-value < 0.05, suggesting significant differences between treatment groups, as shown in Table 2. This indicated that propolis extracts at doses of 14 µg/ml, 16 µg/ml, and 18 µg/ml were found to suppress the activity of the *S. mutans* GTF enzyme.

DISCUSSION

The control group had the highest fructose levels, with an average of 6.09%. For the control group, the study used Aqua Dest, which tends to be neutral, so that the enzymatic reaction of the GTF was not inhibited. The increased activity of the GTF enzyme is related to the increased fructose levels. Meanwhile, in the treatment groups, with 14 µg/ml, 16 µg/ml, and 18 µg/ml propolis extract, a decline in fructose level was detected. This was inversely associated with increased propolis extract concentration. This shows that GTF enzyme activity had been inhibited.

The most significant level of inhibition was at a concentration of 18 µg/ml because, at this concentration, the lowest fructose level was obtained (0.29%). The most effective concentration of propolis extract in this experiment was 14 µg/ml, which was the lowest concentration level that could inhibit the *S. mutans* GTF enzyme activity. Propolis extract has an inhibitory effect on *S. mutans* GTF enzyme activity because it contains active ingredients that inhibit enzymatic reactions.

Temperature, pH, the amount of enzyme and substrate present, and the presence of inhibitors and activators all seem to be factors that can alter enzyme activity level. Enzymatic inhibitors are molecules that interact with enzyme and decrease enzyme activity. The decrease in enzyme activity occurs because of a metabolic imbalance that reduces enzyme reactions.[15]

This research was conducted using propolis extract as an inhibitor for GTF enzyme activity in *S. mutans*. At a concentration of 18 µg/ml, the lowest fructose level was obtained because the higher levels of active ingredients inhibited the GTF enzyme activity. The phytochemical tests showed that saponins were the active element with the highest concentration (2.48%).

Saponins have detergent-like properties and can increase cell membrane permeability without causing bacterial lysis. The increase of cell membrane permeability causes interference with the substances that pass through the cell membrane. Saponins interact with sterols and form single ion channels that destabilise cell membranes. This results in the release of cellular proteins and enzyme, thereby inhibiting enzyme activity.[16]

Furthermore, according to Veloz et al.,[17] flavonoids like pinocembrin and apigenin alter the arrangement of *S. mutans* biofilm structures. Previous research has shown that polyphenols have an additive effect in small dosages, inhibiting biofilm production. The biofilm thickness was likewise reduced by pinocembrin and apigenin, which was linked to enzymatic GTF suppression. Flavonoids have A and B rings that play a role in the hydroxylation of hydroxyl groups so that the basic arrangement of DNA and tRNA is disrupted which results in the inhibition of nucleic acid and cell protein synthesis.[18]

The inhibition of propolis extract against the *S. mutans* GTF enzyme was also due to the terpenoid content as the active ingredient. However, the concentration of terpenoids was not as high as that of saponins and flavonoids (2.05%). These compounds were found to be effective inhibitors of GTF enzyme activity. Terpenoids and saponins have the same target of action on cell membranes. Terpenoids react with porins to form strong polymeric bonds, thus causing damage. Porins act as transmembrane proteins, so that damage to this structure causes membrane permeability disorders. If protons enter the cell easily, it can trigger a decrease in pH and acid sensitisation. This condition can hinder the activity of GTF enzyme.[19]

In addition to saponins, flavonoids, and terpenoids, tannins are active components in propolis extract that inhibit GTF enzyme activity in *S. mutans*. The inhibition mechanism of these compounds is the result of taking over the substrate needed by bacteria, inhibiting energy metabolism, triggering redox reactions, and causing protein precipitation which can suppress the number of enzyme.[6,20] Tannin contains a hydroxyl group in its structure that can trigger a redox reaction, which is an oxidation-reduction reaction in which electrons are exchanged between two chemical structures, and thus inhibits GTF enzyme activity.[21] However, based on phytochemical tests, the concentration of tannins in propolis extract was only 0.24%. Therefore tannin-inhibited GTF enzyme

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activity is less significant when compared to other active ingredients.

Based on the research conducted, it was found that a decrease in fructose levels indicated inhibition of *S. mutans* GTF enzyme activity caused by the active ingredients in propolis extract as an inhibitor of enzymatic activity. Therefore, it can be concluded that propolis extract at a concentration of 14 µg/mL effectively inhibits GTF enzyme activity in *S. mutans*. However, further research is needed into the role of each fraction of active ingredient in inhibiting the activity of the GTF enzyme. Additionally, clinical research on propolis extract in the form of a topical agent or mouthwash for inhibiting the formation of dental plaque should be considered.

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