

Anti-glucan effects of propolis ethanol extract on *Lactobacillus acidophilus*

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ABSTRACT

Background: In deep dentinal caries cases, bacteria mostly found are *Lactobacillus acidophilus* classified as gram positive bacteria and as facultative aerobes producing glucosyltransferase (GTF) enzyme. GTF enzyme can alter sucrose into glucans. Glucan is sticky and insoluble in water. As a result, GTF enzyme can facilitate plaque formation and microorganism colonization on tooth surface. In addition, *Lactobacillus acidophilus* also can form acid leading to demineralization of organic and inorganic materials, resulting in dental caries. Multidrug-resistant phenomena, on the other hand, have led to the use of natural resources, one of which is propolis as an antimicrobial material and as a new anti-infective therapeutic strategy. Propolis is a resinous substances collected by worker bees (*Apis mellifera*) from barks and leaves of plants. Propolis has a complex chemical composition and biological properties, such as antibacterial, antiviral, antifungal, anti-inflammatory, and antitumor. **Purpose:** This research aimed to reveal anti-glucan effects of propolis ethanol extract generated from honey bee, *Apis mellifera* spp on *Lactobacillus acidophilus* bacteria. **Method:** Before anti-glucan test was conducted, glucan-formation test was performed on *Lactobacillus acidophilus* bacteria using SDSPage. Meanwhile, anti-glucan adhesion test on *Lactobacillus acidophilus* bacteria was carried by culturing the bacteria at 37°C temperature in a jar with 10% CO₂. Test tubes were placed at an angle of 30° for 18 hours to review the attachment of bacteria at the glass surfaces. After the incubation, the culture of bacteria was vibrated using a mixer vortex for a few minutes, and then cultured in solid MRS A media. Bacteria grown were measured by using colony counter. **Result:** The ethanol extract of propolis with a concentration of 1.56% was the lowest concentration inhibiting the attachment of glucan to *Lactobacillus acidophilus* bacteria. **Conclusion:** The ethanol extract of propolis with a concentration of 1.56% can be used as an anti-glucan material for *Lactobacillus acidophilus* bacteria.

Keywords: insoluble glucan; *Lactobacillus acidophilus*; propolis extract ethanol

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INTRODUCTION

Dental caries is a multifactorial infectious disease caused by an interaction between teeth, biofilms, carbohydrates, and time, resulting in severe tissue damage. The process of damage to the hard tissue of a tooth triggered by a chemical reaction of bacteria begins with an inorganic breakdown, and then continues in the organic part.¹ Bacteria, as a result, can be considered to play an important role in the process of dental caries since the absence of bacteria will not trigger dental caries. Various species of bacteria colonize in the oral

cavity, especially on dental plaque. Those bacteria are able to produce acid, causing the process of demineralization of dental hard tissues. Caries begins from the enamel surface, and then will progress into deep dentinal caries.

Microorganisms involved in the caries process are complex. Bacterial transition occurs in the development of carious lesions. In early caries lesions, bacteria are in the form of aerobic facultative one, while anaerobic bacteria in deeper caries lesions. In a study of 65 deep dentinal caries samples, the most common bacteria found were *Lactobacillus acidophilus* bacteria.²

Several researches have shown a positive correlation between the number of *Lactobacillus acidophilus* bacteria in dental plaque and the prevalence of dental caries. This is due to some characteristics of the *Lactobacillus acidophilus* bacteria that are *acidophilus*, able to synthesize insoluble polysaccharide of the extracellular glucan bond, α (1-3), produce lactic acid through homofermentation process, and form a colony attached tightly to the surface of the tooth. *Lactobacillus acidophilus* bacteria also produce glucosyltransferase (GTF) enzyme that can convert sucrose and produce glucans. Glucans are sticky and insoluble in water. Glucans can facilitate formation of plaque and colonization of microorganisms on the tooth surface.

Lactobacillus acidophilus bacteria can form acids that result in demineralization of organic and inorganic materials in tooth. If caries reaches to dentine and leaves a thin layer of dentine or pulp perforation, it is necessary to take care of the capsule pulp. Pulp capping is a treatment using a biocompatible material that serves to protect the pulp from mechanical, chemical, and bacterial irritants, so the inflammatory pulp tissue can be repaired and recovered, as well as healthy.³ Nevertheless, fault rate of pulp capping is still high due to opened pulp triggered by caries that is equal to 66.7%, whereas 7.8% is caused by mechanical ones.⁴⁻⁶ Medicine commonly used is Ca (OH)₂, but this material still has deficiency in dentin bridge formation as well as tunnel defect resulting in re-infection of bacteria leading to tooth necrosis.

Emergence of multidrug-resistant phenomena has led to increased attention to find new antimicrobial agents and new anti-infective therapeutic strategies. In recent years, many direct resources from nature have been explored, one of which is propolis. Propolis is a resin substance collected by worker bees (*Apis mellifera*) from barks and leaves of trees. Propolis has a complex chemical composition as well as biological properties, such as antibacterial, antiviral, antifungal, anti-inflammatory, and antitumor. However, these compositions depend on the surrounding plants. Propolis also has strong local antibiotics as well as antifungal properties.

In addition, propolis has antibacterial activities against Gram-positive and Gram-negative bacteria. Propolis also has been known to be effective against gram-positive and gram-negative bacteria. A previous research shows that the antibacterial compounds of propolis are also effective against oral bacteria, such as *Peptostreptococcus anaerobius*, *Lactobacillus acidophilus*, *Actinomyces naeslundii*, *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*.⁷ Another previous research conducted by Parolia et al also claims that the antibacterial compounds of propolis also can be effective for *Veillonella parvula*.⁸ Propolis extract derived from different extraction methods can provide minimal inhibitory concentration and minimal bactericidal concentration.⁹ In the other words, those previous researches have proven that propolis has antibacterial activities

against specific caries bacteria. Based on the properties of propolis, resin contained in propolis is expected to prevent colonization of *Lactobacillus acidophilus* on the surface of teeth. Therefore, this research aimed to reveal anti-glucan effects of propolis ethanol extract generated from honey bee, *Apis mellifera* spp on the attachment of *Lactobacillus acidophilus* bacteria.

MATERIALS AND METHOD

This research used propolis generated from *Apis mellifera* spp bees. Tools used in research were sterilized in autoclave at a temperature of 121°C for 30 minutes. Ethanol extract of propolis was obtained by using maceration method with 70% ethanol solvent.

Before revealing inhibitory effects of the ethanol extract of propolis on glucan formation in *Lactobacillus acidophilus*, glucan formation test was conducted using SDS page. *Lactobacillus acidophilus* was isolated from frozen stock, grown in MRS-B with 5% glucose at 37°C for 24 hours. The SDS-PAGE test then was performed in several stages.

Gel preparation was conducted, gel plates were made by arranging two glass plates with a spacing of 1 mm plate. Gel was made with two layers, namely gel as a place of collection of samples (stacking gel) and gel as a medium

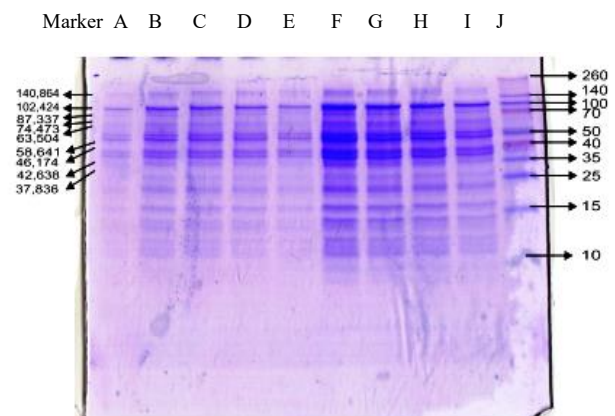


Figure 1. Results of the glucan measurement using SDS-PAGE.

Table 1. Results of the anti-glucan test of the ethanol propolis extract on the number of *Lactobacillus acidophilus* bacteria

Group	Mean \bar{x} (cfu)	Standard Deviation	p
Control	100	1.988	p= 0.000
1.56% EEP	5.57	4.082	

Note: EEP: ethanol extract of Propolis; p<0.005 is significantly different

for separation of protein (separating gel). The separating gel mixture was carefully inserted into the plates using the micropipette. Those plates were settled for 10-30 minutes until the gel was formed. The stacking gel was poured over the separating gel while fitted with a comb until gel and its well were formed. They were settled for for 30 minutes. After the gel was formed, the comb was removed. The plates were mounted on electrophoresis device. The buffer was poured on the electrophoresis vessel.

Afterwards, preparation for sample injection was performed by providing 10 μ L of protein isolate samples added with 10 μ L of Tris-Cl + 20 μ L of reducing sample buffer (RSB), inserted into microtube and then heated in a water bath at 100 °C for 3 minutes. After cooled, the samples were put into the gel wells, about 20 μ L for each well. The anode was connected to the lower reservoir, while the cathode was connected to the upper reservoir. The power supply was turned on with an electric current of 30 mA and 130 V. The running process then was stopped after the blue color of the marker reached a height of 0.5 cm from the bottom of the gel plate.

Staining was conducted by soaking the gel in staining solution for 30-60 minutes. Color removal then was performed by soaking the gel in the destaining solution while shaking with an automatic shake until the gel became clear. Results of the electrophoresis process was scanned. Determination of molecular weight then was carried out by comparing the results of sample electrophoresis with protein marker.

Minimum inhibitory concentration (MIC) of the ethanol extract of propolis was used to determine concentration level of propolis which could be used as an antigen (concentration 1.56%, 1.95% and 2.34%). Anti-glucan adhesion test was performed by culturing the bacteria in a jar at 37°C with 10% CO₂. Reaction tubes containing the bacterial media and propolis then were placed at 30° angle for 18 hours.¹¹ MIC could be used to detect bacterial growth. After incubation, the bacterial culture was vibrated using a vortex mixer for several minutes, and cultured on solid MRS A media. The bacteria grown then were measured by using colony counter. The concentration level of propolis that could inhibit bacterial attachment was defined as the lowest concentration where there was no visible attachment to the glass surface.¹⁰

RESULTS

Based on results of the preliminary research on the measurement of the resistance zone of *Lactobacillus acidophilus* growth by the ethanol extract of propolis, the ethanol extract of propolis is known to have inhibitory effects on the growth of *Lactobacillus acidophilus* bacteria. In addition, based on results of sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) test, the total amount of *Lactobacillus acidophilus* protein molecules was 140,864 kDa (Figure 1). This result is closed to the

result of a previous research conducted by Mattos-Graner et al¹², showing that the formation of insoluble glucan has a molecular weight in the range between 150-160 kDa. As a result, insoluble glucans formed in this research can be considered as places of bacterial attachment.

The number of *Lactobacillus acidophilus* bacteria was calculated based on the anti-glucan effects of the propolis ethanol extract on the number of *Lactobacillus acidophilus* bacteria (Table 1). Based on the research data, the ethanol extract of propolis with a concentration of 1.56% can inhibit the formation of glucan in *Lactobacillus acidophilus* bacteria.

DISCUSSION

Dental biofilms containing 99% of bacteria consist of various types of bacterial cell species attached to the surface, binding to form a series of matrixes. Biofilm formation is triggered by population density. Various mechanisms can occur through adhesion, generally considered as reversible and irreversible stages. The reversible stage is an early stage that begins with hydrophobic interactions, electrostatic interactions, or van der Waals bonds, influenced by hydrodynamic temperature or bonds.¹³ Such activity is sufficient to create bacterial bonds and usually followed by irreversible adherence that begins with a specific host cell or bacterial cell receptor. The attachment of proteins to receptors is generally and clinically associated with biofilm-forming bacteria and also associated with early attachment. In addition, this attachment also is considered as bacterial co-aggregation in the same or different sequences. Cell surface proteins, such as pili, fimbriae, or flagella, are generally adhesion proteins and may bind to specific receptors or form hydrophobic bonds with surfaces.¹⁴ The attachment of bacteria to a surface, then forming biofilms, will be affected by the physical properties of the surface. Rough surface causes more bacterial colonization.¹⁵

Mechanism of *Lactobacillus acidophilus* in attaching to tooth enamel or tooth-plaque surfaces consists of two attachments, namely sucrose-independent attachment and sucrose-dependent attachment. The sucrose-dependent attachment on the glass surface is mediated by GtfI and GtfSI. *Lactobacillus acidophilus* actually produces three Gtf enzymes, namely GtfI encoded as glucosyltransferase B (gtfB), GtfSI encoded as glucosyltransferase C (gtfC), and GtfS encoded as glucosyltransferase D (gtfD).

The attachment of *Lactobacillus acidophilus* onto tooth surfaces with glucan intermediates, in which insoluble glucan production (insoluble in water) serves as an important virulence factor. It indicates that *Lactobacillus acidophilus* has a function in the accumulation and formation of plaque. *Lactobacillus acidophilus* has acidogenic and aciduric properties triggering an ability to synthesize glucan as a major factor in the formation of cariogenic biofilms. Glucan is synthesized from sucrose by *Lactobacillus acidophilus* on tooth surfaces. *Streptococcus mutants* and *Lactobacillus*

acidophilus produce three types of GTF, namely GTF-B synthesizing most of insoluble glucans, GTF-C synthesizing soluble and insoluble glucans, and GTF-D synthesizing soluble glucans. The inhibition of glucosyltransferase enzyme activity then causes *Lactobacillus acidophilus* cannot convert glucose, especially sucrose to glucan, so it cannot be attached to tooth surface.¹¹

Based on the results of this research, the ethanol extract of propolis with a concentration of 1.56% could inhibit glucan adhesion on glass surface. Propolis is a natural ingredient that has antimicrobial properties, namely apigenin and tt-farnesol. Apigenin has a role in inhibiting the activities of GTF B and GTF C, but apigenin has no antibacterial properties. By inhibiting the activities of GTF B and GTF C, apigenin can affect the activity of fructosyltransferase. Besides, apigenin can effectively inhibit insoluble glucan synthesis. In other words, apigenin is a unique therapeutic substance that affects the activity and expression of the GTF enzyme without showing antibacterial activity. Apigenin is a potent non-competitive inhibitor against the activities of GTF B and GTF C.¹¹ On the other hand, tt-farnesol exhibits barriers to bacterial growth and metabolism by destroying bacterial cell membranes, thereby affecting the process of glucan synthesis. If apigenin affects the permeability of the cell membrane, how tt-farnesol in the propolis extract inhibits the synthesis of glucan may be triggered by its effects on the cell membranes more than its effects on enzyme activity since tt-farnesol is a poor GTF inhibitor. Nevertheless, the chemical structure and lipophilic properties of tt-farnesol that support membrane localization can trigger changes in the permeability and instability of cell membranes. Consequently, cell membrane will damage, not only reducing bacterial metabolism, but also affecting the synthesis of glucan by *Lactobacillus acidophilus*.

Apigenin and tt-farnesol can be considered as non-toxic materials, both in vitro and in vivo. To suppress the amount of glucan production, some experts then recommend the use of natural ingredients rather than broad-spectrum antimicrobial agents since they will affect the normal flora of the oral cavity. This is also supported by the results of various previous researches determining the effectiveness of anti-plaque or anti-caries.¹¹ It can be concluded that ethanol extract of propolis with a concentration of 1.56% can be used as an anti-glucan material for *Lactobacillus acidophilus* bacteria.

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