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Research Article

BIOFILM FORMATION OF *CANDIDA ALBICANS* EXPOSED TO ETHANOL EXTRACT OF PROPOLIS

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ABSTRACT

Objective: Propolis extract showed an excellent *in vitro* performance against yeast and was additionally found to be fungistatic and fungicidal. Propolis extract is also used for treatment and prevention of fungal infections. However, its effectiveness against *Candida albicans* biofilm formation requires investigation. The study evaluated the ability of propolis to inhibit *C. albicans* while the fungus is growing as a biofilm *in vitro.*

Methods: Two reference strains, *C. albicans* ATCC 25923 and a clinical strain (laboratory stock), were used in this study. For the biofilm experiment, the fungi were cultured in Tryptic Soy Broth medium with 1% sucrose and incubated at 37°C for 24 h, and different concentrations of ethanol extract of propolis were used as the inhibitor agents. Biofilm assays were performed in 96-well microtiter plates, quantification of the total biofilm biomass was performed using a crystal violet staining method, and the Student's t-test was chosen for statistical analyses.

Results: Our data showed that 3 h incubation with propolis did not affect the biomass in the experimental group compared to the control. When the incubation time was extended to 18 h, the biomass increased significantly compared to the control.

Conclusion: This study showed that several concentrations of propolis did not inhibit biofilm. However, in each incubation time, we observed no hyphal morphology in the biofilm mass. Propolis might attenuate the opportunistic virulence of fungus growing as a biofilm *in vitro*. Further studies are necessary to confirm this phenomenon.

Keywords: Ethanolic extract of propolis, *Candida albicans*, Crystal violet assay.

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INTRODUCTION

The yeast *Candida albicans* is an oral commensal microorganism that occurs in the oral cavity of 50%–70% of healthy individuals. This microbial can grow under anaerobic, aerobic, or aerobic conditions with 5% CO_2 [1]. It is a polymorphic yeast and an opportunistic fungal pathogen that causes oral infections [1,2]. *C. albicans* has several gene families that encode proteins involved in pathogenesis and biofilm formation. Among these Aglutinin-like sequence (ALS) family that encodes cell-wall glycoproteins, some of which are involved in adherence to host surface [2]. *C. albicans* is distinguished from many other fungal species by its ability to form both yeast cells and hyphae under many different environmental conditions. Hyphae are an important structural component of *C. albicans* biofilms. The hyphae in biofilms contribute to the overall architectural stability of the biofilm, acting as a support scaffold for yeast cells and other hyphae. Thus, the ability to form hyphae and the ability of these hyphae to adhere to one another and to yeast cells are critical for normal biofilm development and maintenance [3].

Propolis is a honeybee product that is used to cover hive walls and fill gaps [4]. It consists of resins, balsams, bee wax essential oils, and other organic compounds collected from plants, buds, and exudates by honey bees. Its chemical composition includes 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances including minerals and organic compounds such as phenolic acids or their esters, flavonoids, terpenes, aromatic aldehyde and alcohol, fatty acids, stilbenes, and B-steroids [5]. The chemical composition of propolis is highly variable and depends on the local flora at the site of collection as well as on the season of collection [4,5]. Propolis also exhibits antimicrobial, anticancer, antifungal, antiviral, and anti-inflammatory properties that have gained attention in both the dental and medical fields. Propolis extract shows excellent performance against yeast during *in vitro* testing. In low concentrations, propolis extract is both fungistatic and fungicidal. Antifungals are used to treat and prevent fungal infections [6].

In spite of this, the efficacy of ethanol extract of propolis for inhibition of *C. albicans* biofilm formation remains unclear. Therefore, the aim of the present study was to investigate the effectiveness of propolis on the inhibition of *C. albicans* biofilm.

METHODS

Strains, growth conditions, and propolis extract

Two reference strains were used in this study, *C. albicans* ATCC 25923 and an oral isolate obtained from the laboratory stock at the Oral Biology Laboratory, Faculty of Dentistry, University of Indonesia. In these experiments, all strains kept in a glycerol stock at −80°C were subcultured in Tryptic Soy Broth (TSB) medium with 1% sucrose and incubated at 37°C for 24 h.

Several concentrations of the ethanol extract of propolis (10%, 5%, and 2.5%) were used in this study and were kindly provided by Dr. Sahlan from the Chemical Engineering Laboratory of the Engineering Faculty, University of Indonesia.

Biofilm formation in 96-well microtiter plates

Biofilm assays were carried out on 96-well microplates (TPP, Switzerland). Before the biofilm assay, the bottom of the microplate wells was coated with 100 µL of saliva sterilized by filtration containing 200 µg/mL of protein that was measured with the Bradford method.

The microplate was then agitated for 5 min in a shaker (60 rpm) and incubated for 60 min at 37°C. The non-adherent salivary proteins were then removed. Subsequently, 200 µL of *C. albicans* (10^7 cells; optical density [OD]: 0.1) was added and the solution was incubated for 90 min at 37°C. The non-adherent cells were then removed by pipetting. Furthermore, 200 µL of different concentrations of ethanol extract of propolis (10%, 5%, and 2.5%) were added to the preformed biofilm in each well and incubated for 3 and 18 h at 37°C. After each incubation, the supernatants were removed and the formed biofilms were gently washed with 200 µL of PBS (pH 7.2).

Quantification of the total biofilm biomass

The total biofilm biomass was measured using the crystal violet (CV) staining method [7]. Briefly, after the incubation period wherein the biofilm was exposed to several propolis concentrations, the supernatants were removed and washed with 200 µL of PBS. At this point, the biofilms on the microplates were allowed to dry at room temperature, 200 µL of CV (0.5% v/v) was added to each well, and the microplate was then incubated for 15 min. The unabsorbed CV solution was removed and the absorbed CV was extracted using 96% ethanol. Finally, the absorbance was read using a microtiter plate reader (M965+Microplate Reader, Mertech Inc.) at 600 nm [7].

The inhibition and cell mass percentage were calculated using the following respective formulas [8]:

$$\%\text{Inhibition}=\frac{\text{Control absorbance-Treatment absorbance}}{\text{Control absorbance}}\times 100\%$$

$$\%\text{Cell Mass}=\frac{\text{Treatment absorbance}}{\text{Control absorbance}}\times 100$$

Statistical analysis

All obtained data were expressed as mean ± standard deviation of triplicate experiments. A two-tailed Student's t-test was used to determine the differences in biofilm formation between the control and treatment groups. $p<0.05$ was considered to be statistically significant.

RESULTS

The results of the biofilm assay showed that, when ethanol extract of propolis was added to the biofilm performed on the well and incubated for 3 h, the biofilm mass in both *C. albicans* strains tested decreased (Fig. 1). The absorbance value of group 0.784 of *C. albicans* ATCC 25923 was 3.6 times increase from 0.216, while 0.706 of *C. albicans* wild type was 3.4 times increase from 0.209. However, compared to the control, there was no significant reduction in the biofilm mass in either experimental group ($p>0.05$). When the incubation time was extended to 18 h, the biomass in each treatment group increased significantly ($p>0.05$). The ODs of the 18-h incubation period were 3.777 and 3.649 for the *C. albicans* ATCC 25923 and *C. albicans* wild type, respectively.

These phenomena were observed for all concentrations of propolis tested. On treatment with 2.5% ethanol extract of propolis, we observed an OD of 0.717 and 0.587 for *C. albicans* ATCC 25923 and *C. albicans* wild type, respectively, after the 3-h incubation. After the 18-h incubation, we observed an OD of 3.329 for *C. albicans* ATCC 25923 and 3.427 for *C. albicans* wild type (Fig. 1).

This study found that there was no inhibitory effect of all the selected concentrations of propolis on the *C. albicans* biofilm as the microbes were still viable (Table 1) during all the experimental time periods. The results of the exposure to 10% ethanol extract of propolis showed a growth of 72.44% and 94.46% for *C. albicans* ATCC 25923 and 69.12% and 94.90% for the *C. albicans* wild type following incubation for 3 and 18 h, respectively. The results of the incubation with 5% ethanol extract of propolis showed a 67% and 93.80% growth for the *C. albicans* ATCC 25923 and 64.84% and 94.95% for the *C. albicans* wild type after incubation for 3 and 18 h, respectively. Concurrently, the 2.5% ethanol extract of propolis incubation showed a growth of 63.87% and 93.90% for *C. albicans* ATCC 25923 and 61.15% and 94.22% for wild-type after incubation for 3 and 18 h, respectively (Table 2).

The percentage of *C. albicans* biofilm cells after exposure to 10% ethanol extract of propolis was 362.96% and 1.807% for *C. albicans* ATCC 25923 and 323.85% and 1.961% for the *C. albicans* wild type after incubation for 3 and 18 h, respectively. The percentage of cells following incubation with 5% ethanol extract of propolis was 303.03% and 1.614% for *C. albicans* ATCC 25923 and 284.47% and 1.984% for *C. albicans* wild type and that for the 2.5% ethanol extract of propolis was 276.83% and 1.639% for *C. albicans* ATCC 25923 and 257.45% and 1.730% for *C. albicans* wild type after incubation for 3 and 18 h, respectively (Table 1).

DISCUSSION

In this study, biofilm inhibition following propolis exposure was observed after incubation for 3 and 18 h. The 3-h incubation period was chosen to represent the adhesion stage during biofilm formation. This is the first phase of supragingival biofilm formation on the coating of salivary components, known as acquired pellicle, on teeth surfaces [9]. The initial step of biofilm formation is adhesion [10]. This process relies on several cell wall proteins (CWPs) called adhesins. These proteins

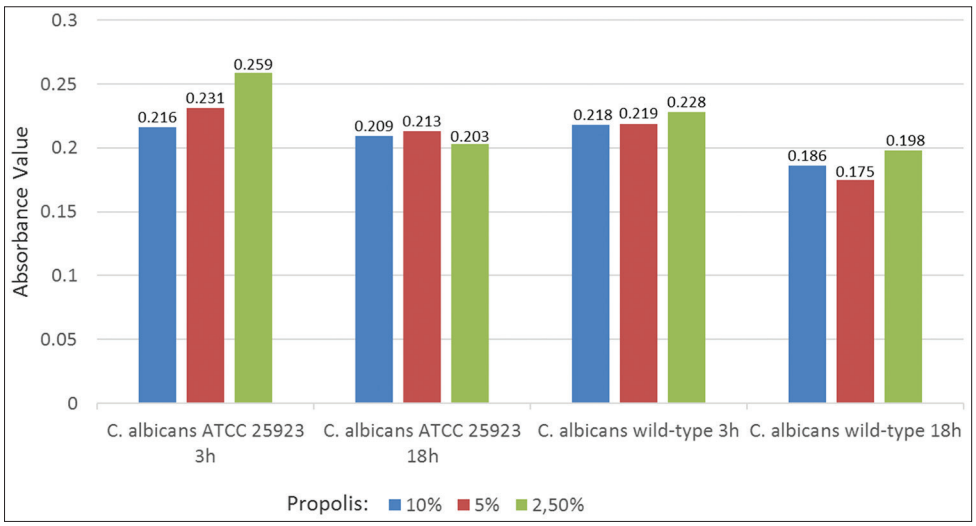

Fig. 1: Absorbance values at 3 and 18 h for several propolis concentrations of the *Candida albicans* ATCC 25923 and *C. albicans* wild type

Table 1: Percentage of biomass cells associated with *C. albicans* biofilms

Propolis (%)	*C. albicans* ATCC 25923 (%)		*C. albicans* wild type (%)	
	3 h	18 h	3 h	18 h
10	362.96	1.807	323.85	1.961
5	303.03	1.614	284.47	1.984
2.5	276.83	1.639	257.45	1.730

C. albicans: Candida albicans

Table 2: Percentage of the growth of *C. albicans* biofilms

Propolis (%)	*C. albicans* ATCC 25923 (%)		*C. albicans* wild type (%)	
	3 h	18 h	3 h	18 h
10	72.44	94.46	69.12	94.90
5	67	93.80	64.84	94.95
2.5	63.87	93.90	61.15	94.22

C. albicans: Candida albicans

promote attachment to other epithelial and microbial cells or abiotic surfaces by binding to specific amino acid or sugar residues [10,11]. In general, adhesins are glycosylphosphatidylinositol (GPI)-dependent CWPs comprising a GPI anchor, serine/threonine domain, and carbohydrate or peptide domain [12]. In *C. albicans,* several adhesins belong to the ALS family [10,12], which is part of the GPI-CWP family. Another important family of adhesins in *C. albicans* is the hyphal wall protein (Hwp) family, including Hwp1 [10].

In addition to the 3-h incubation period, we observed the biofilm maturation stage after 18 h. Here, 18 h represents the intermediate (premature phase) of the biofilm formation. After adhesion, the biofilm development continues through morphologic modifications and increases in cell number and production of expandable polystyrene (EPS), which influence the biofilm architecture [10]. *C. albicans* alone produces matrix materials such as β-glucans, chitins, and β-N-acetylglucosamine during biofilm formation in this time period. This appears to confer protection from antifungal agents. β-glucan is a component of the cell wall and is actively secreted by *C. albicans* during biofilm formation on silicone or polystyrene surfaces [13]. Thus, *C. albicans* biofilms have a variety of possible architectures, adhesion properties, cellular morphologies, and EPS compositions. For *C. albicans* biofilm formation *in vitro*, the early phase takes approximately 11 h, culminating in the formation of distinct microcolonies. The intermediate phase of the biofilm formation may last for 12–30 h and is characterized by the production of EPS and formation of a bilayer consisting of yeast, germ tubes, and/or young hyphal. The maturation process usually takes approximately 38–72 h [10].

We selected *C. albicans* for this study because its biofilm formation plays an essential role in the pathogenicity of *C. albicans* [12]. *C. albicans* infections rely on the organism ability to switch morphology between yeast cells and hyphae forms to adhere to surfaces, form biofilms, and penetrate tissues. Hyphae are believed to be invasive and the pathogenic forms of *Candida* species, whereas yeast is the commensal nonpathogenic form [14]. Biofilm is a cell mass that is surrounded by an extracellular matrix consisting of yeast cells and filaments. It has also a relationship with the surface and exhibits different phenotypic features than planktonic cells [10].

Biofilm model research findings vary depending on the laboratory materials and techniques used. Biofilm models can be difficult to compare due to differences in the biofilm formation times, growth mediums, and microbial species used in different environments [15]. Moreover, the medium used to evaluate biofilm formation *in vitro* is responsible to affect biofilm morphology [10].

In our study, TSB with 1% sucrose was used as nutritional support for *C. albicans*. This resulted in microbial growth after incubation for 3 and 18 h. A sudden increase or decrease in the relative abundance of one or more species in a microbial community often indicates a homeostatic breakdown of the community. A common feature is a significant change in the nutrient status, such as the introduction of an excess of substrate such as a sugar or a chemical compound, which can disturb the ecosystem [16].

C. albicans has been detected more frequently in the oral cavities of children with ECC than in children without caries [1,17,18]. *C. albicans* and *Streptococcus mutans* are found together and interact within the conducive conditions of ECC [19]. Metabolic interactions are found between *C. albicans* and *S. mutans*. The lactic acid produced by *S. mutans* can be metabolized by *C. albicans*. *C. albicans* also increases the external pH by producing ammonia, leading to less cariogenic conditions [1]. However, the presence of *C. albicans* enhances the formation of oral biofilms [1,17].

C. albicans has the ability to interact with many bacterial species on different levels. The latter produce glucan that binds to the cell walls of *C. albicans*. Thus, yeast provides adhesion sites for bacteria, increasing dual-species biofilm formation. *C. albicans* was also found to exhibit a complex interaction with the cariogenic organism *S. mutans* [1]. In the oral cavity, *C. albicans* can interact and develop biofilms with *S. mutans,* greatly enhancing colonization by this organism on the teeth surfaces. A number of *C. albicans* and *S. mutans* were clearly higher than those observed in subjects with *S. mutans* only. This suggests that *C. albicans* might escalate the severity of ECC [17]. *C. albicans* also interacts with bacteria through chemical signaling in a process called quorum sensing (QS). *S. mutans* produces a small peptide called competence-stimulating peptide, which inhibits *C. albicans* hyphal formation during the early stages of biofilm formation [1].

To respond the changes in the biofilm environmental conditions after exposure to ethanol extract of propolis, it is necessary to examine the biomolecular level, including the QS molecule. Farnesol is a QS molecule that acts as an anti-biofilm agent [10,18]. It is produced by *C. albicans* and blocks the morphological transition from yeast to hyphae [10,18]. Hyphal development is important for the formation of substantial biofilm biomass [18]. In general, farnesol is mostly secreted in the later stages of biofilm formation by *C. albicans* [10]. Farnesol is an autoinducer molecule that influences the expression of genes involved in antifungal resistance, cell-wall maintenance, phagocytic responses, surface hydrophobicity, iron metabolism, and heat shock [10].

In this study, the growth of *C. albicans* was not inhibited by ethanol extract of propolis. This may be due to the diverse chemical composition of propolis. The bees of the subfamily Meliponinae, popularly known as stingless bees, are widespread over tropical and subtropical areas of the world [17]. Meliponinae are valuable pollinators of many crops, and most species produce honey together with wax and propolis. In spite of this, its chemical composition and biological activity are largely unknown. The composition of Meliponinae propolis samples is complex due to the heterogeneity of their chemical pattern. For instance, Meliponinae honey bee propolis had weak or no activity against the Gram-negative test strain *Escherichia coli*, and its action against *C. albicans* was also weak [19]. The chemical composition of propolis is highly variable and depends on the local flora at the site of collection and on the season of collection [4,5]. Despite potential intrinsic differences, which may depend on the propolis origin, it has been proven that most propolis variants have a wide-ranging therapeutic biological effects such as antimicrobial, antifungal, and antiviral activity [4].

CONCLUSION

This study showed that ethanol extract of propolis did not exert an inhibitory effect with respect to *C. albicans* growth. This held true for all concentrations of propolis tested. Further studies should be carried out to observe gene factors that contribute to the formation

of *C. albicans* biofilms, as influenced by propolis concentration. One example is the gene encoding farnesol, which is associated with QS, and the *ALS* gene, which is in turn associated with glucans formation. Furthermore, additional research is needed to validate the clinical results from examinations of dual oral microorganism biofilms, such those of *C. albicans* and *S. mutans*, which are closely associated with dental caries, exposed to ethanol extract of propolis.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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