



Effect of Propolis on the Adhesion Index, Morphology and Viability of *Candida albicans* Cells on Biofilm Formation

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Abstract: About 70-75% of reproductive women have experienced vulvovaginal candidiasis at least once and 40-45% will have recurrences. *Candida albicans* is the most etiology of vulvovaginal candidiasis and is able to form biofilm that can lead to antifungal resistances and recurrences. One of the natural products that have an antibiofilm effect is propolis. This study expected that propolis from Lawang can be the one of antibiofilm agent candidates to antifungi resistant cases. This study aimed to know the antibiofilm effect of propolis ethanol extract to cell adhesion index, morphology, and viability of *Candida albicans* cell on biofilm formation. The adhesion index was counted on 100 epithelial cells under light microscopy (1000x). Morphology was seen using light microscopy (400x). Cell viability was examined by CFU assay. At 12.5% concentration of propolis ethanol extract, adhesion index decreased ($p=0,000$) and hyphal growth was inhibited. Colony growth decreased at 2.5% concentration and was not seen at 10% concentration of propolis ethanol extract ($p=0,000$). This results indicated that propolis ethanol extract can decrease adhesion index, failed the *Candida albicans* morphology transition from yeast to hyphal, and decreased *Candida albicans* cell viability on biofilm formation. Propolis ethanol extract is likely to be one of alternatives to recurrent vulvovaginal candidiasis treatment, especially caused by *Candida albicans* biofilm formation.

Keyword: Propolis; adhesion index; morphology; cell viability; *Candida albicans*

INTRODUCTION

Vulvovaginal candidiasis is a disease that attacks the vulva and/or intravaginal (Gonçalves et al., 2016). Vulvovaginal candidiasis affects 70-75% of women's reproductive. About 40-45% of them experience vulvovaginal candidiasis for the second time. Recurrent infections (four episodes or more) can occur in about 5-8% of women (Sobel, 2016). *Candida albicans* (*C. albicans*) is the most common cause of vulvovaginal candidiasis with a percentage of 70-89% (Gonçalves et al., 2016). One of the virulence forms of *C. albicans* is biofilm formation. Biofilm is a community of microorganism cells that attach to a surface and encase in an extracellular matrix produced by the microorganisms themselves (Rodríguez-Cerdeira et al., 2018).

Biofilm formation occurs in 4 stages, namely cell adhesion, initiation, maturation, and dispersion (Ranjith et al., 2018). Adhesion of *C. albicans* cells to host cells is a

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prerequisite for biofilm formation (Dwivedi et al., 2019). Adhesion is a complex process resulting from the simultaneous interaction between fungal cell wall components and biomolecules on the surface of the host cell membrane (Nikou et al., 2019). McCall et al. (2019) showed that the adhesion of wild type *C. albicans* cells increased during growth along with the increase of Hyphal formation. Hyphal morphogenesis plays an important role in adhesion to biofilm development. The morphological transition from yeast to Hyphal or filaments are markers of invasion and the essence of pathogenesis, thereby increasing the ability of *C. albicans* to form biofilms (Cavalheiro and Teixeira, 2018). In the formation of *C. albicans* biofilm, Hyphal plays a role in providing structural stabilization, moreover, it keeps the yeast and cellular matrix in the maturation phase (Mutiawati, 2016). The formation of mature biofilm can increase cell viability and resistance to anti-fungal agents and antibiofilm (Lohse et al., 2018).

One of the alternative therapies from the natural product is propolis. Propolis is a natural product produced by honey bees (Capoci et al., 2015). Study conducted by de Castro et al. (2013) showed that Brazilian propolis inhibit *C. albicans* biofilm formation in vulvovaginal candidiasis; induces cell apoptosis (de Castro et al., 2013); and degrades the Extracellular Polymeric Substance (EPS). One of the compounds in propolis that has been known to act as an antibiofilm is a flavonoid (Przybylek and Karpinski, 2019). But, the composition of propolis can vary depending on the type of plant where the bees live (Silva-Carvalho et al., 2015), time of collection, and geographical origin (Anjum et al., 2018). Propolis used in this study was from Lawang, Malang, Indonesia. Hadi et al. (2019) showed that ethanolic extract of Propolis *Trigona* sp from Lawang Indonesia inhibited the production of *Staphylococcus aureus* biofilm but not known the antibiofilm effect of Propolis from Lawang, Indonesia to fungi, especially *C. albicans*. The purpose of this study was to know the effect of propolis ethanol extracts on the cell adhesion index, morphology, and viability of *C. albicans* cells on biofilm formation.

MATERIALS AND METHOD

Propolis Ethanol Extract

Propolis used in this experiment was honey bee production by *Trigona* sp. species which comes from Rimba Raya Wasp Farm, Lawang, Malang, Indonesia. Propolis was extracted by maceration method with 70% ethanol solvent. The concentrations of Propolis ethanol extract (PEE) include 2.5%; 5%; 7.5%; 10%; and 12.5%. Approval for this study was obtained from Ethics Commission of Medical Faculty, Brawijaya University No.88/EC/KEPK-S2/04/2020.

***C. albicans* cultures**

C. albicans isolates were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. *C. albicans* cells were cultured in SDA (Oxoid) medium at 37°C for 24 h. The results of the incubation were harvested for biofilm formation.

***C. albicans* Biofilm Formation**

C. albicans suspension was adjusted to 10⁷ CFU/mL. Then, the cells were added to 96-well flat-bottom plates (Costar Corning) that contain SDB medium (MERCK)+Glucose 2% (MERCK) and some concentrations of PEE. Then, the plate was incubated at 37°C for 48 h (Gulati et al., 2018). This assay was performed in four replications.

Adhesion Index

Adhesion index measurement was performed on vaginal epithelial cells of *Rattus norvegicus* Wistar strain. Epithelial cell isolation was performed according to

the Weisser method (Nagayama et al., 1995). Adhesion test used the method according to El-Din et al. (2012) with a modification of 0.5 mL of epithelial cells (10^5) and 0.5 mL of *C. albicans* cells (10^7) homogenized, added PEE, and incubated for 60 minutes on shaking water bath (Medlab Solution) at a temperature of 37°C. Then 10 µL of the suspension was dropped on object-glass, fixed, and Gram stained. This assay was performed in four replications.

$$\text{Adhesion Index} = \frac{\text{Total of } C. \textit{albicans} \text{ on epithelial cells} - 100}{100}$$

Morphology of *C. albicans* (Hyphal Growth)

C. albicans cell suspension (1×10^6 CFU/mL) was prepared in RPMI-1640 (Gibco) containing 10% Fetal Bovine Serum (FBS) (Sigma) and some concentrations of PEE. The sample was incubated at 37°C for 6 h. This assay was performed in four replications. The cell morphology was monitored by photographed (Olympus CH2, Olympus, Tokyo, Japan) at 400x magnification.

C. albicans Cell Viability

C. albicans cell viability on biofilm formation was performed by counting the colony growth. Briefly, the same procedure was performed as biofilm formation. After biofilm was formed, the suspension was diluted 1000x. Then, 10 µL suspension was streaked in the SDA medium (Oxoid) at 37°C for 24 h. This assay was performed in four replications. Colony growth was counted using a colony counter (Funke Gerber) (Capoci et al., 2015).

Statistical Analysis

Data of the results was performed in Mean \pm SD. Results were analyzed using one-way ANOVA with the Bonferroni test and Tukey Post-Hoc (SPSS version 23). Values of $p \leq 0.05$ were considered significant statistically.

RESULTS AND DISCUSSION

Propolis Ethanol Extract and *C. albicans* adhesion index to *Rattus norvegicus* vaginal epithelial cells

Adhesion assay was performed to determine the effect of propolis ethanol extract on the adhesion index of *C. albicans* to vaginal epithelial cells of *Rattus norvegicus*. The result showed that adhesion index decreased with the increase of propolis ethanol extract (PEE) concentration (Fig 1). The lowest average of the adhesion index was at the concentration of 12.5% propolis ethanol extract. At a concentration of 12.5% propolis ethanol extract, *C. albicans* attached to one vaginal epithelial cell of *Rattus norvegicus* is 3.37 ± 0.252 (3 cells of *C. albicans*). The highest average of the adhesion index was at the concentration of 2.5% propolis ethanol extract. At a concentration of 12.5% propolis ethanol extract, *C. albicans* attached to one vaginal epithelial cell of *Rattus norvegicus* is 10.12 ± 0.398 (10 cells of *C. albicans*). The analysis of the one-way ANOVA test showed a significant difference in the control and the treatment group (p -value = 0.000). The post-Hoc-Tukey test showed that the adhesion index on all treatment groups significantly decreased compared with the control. It is demonstrated that the increase of propolis ethanol extract concentration is associated with the decrease of *C. albicans* adhesion index to *Rattus norvegicus* vaginal epithelial cells. The results of the research have demonstrated the concentration of 12.5 % propolis ethanol extract is an effective dose to reduce the adhesion index of *C. albicans*.

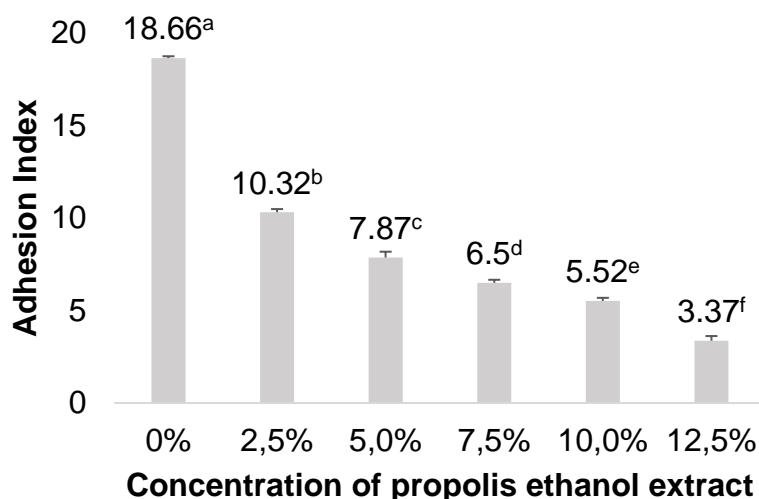


Figure 1. The Average of *C. albicans* Adhesion Index to *Rattus norvegicus* Vaginal Epithelial Cells after Treatment with PEE. The Histogram Showed that PEE Reduces the *C. albicans* Adhesion Index to *Rattus norvegicus* Vaginal Epithelial Cells.

Previous research reported the same result about PEE effect to adhesion of *C. albicans*. The study conducted by Nani et al. (2019) reported that 100 µg/ml of Brazilian Organic Propolis can reduced the adhesion of *C. albicans* to human keratynocytes. The percentage of *C. albicans* adhering to human keratinocyte cells after being treated with Brazilian Organic Propolis is approximately 30%. In our study, the adhered cells percentage is 18.06% at a concentration of 12.5%. This indicate that our propolis is probably has more flavonoid content. Adhesion inhibition can prevent fungi to interact with host cells and inhibit biofilm formation (Zhang et al., 2016). One of the main bioactive components contained in propolis is a flavonoid (Silva-Carvalho et al., 2015). Flavonoid can interfere with the formation of *C. albicans* cell wall by inhibiting the synthesis of β-glucan and chitin, which is the main component of the cell wall (Aboody et al., 2020). Antifungal agents will invade cells, such as through active transport, and interfere with the RNA synthesis. Therefore, it can cause RNA synthesis error, inhibit DNA transcription, and inhibit the protein synthesis process (Lagrough et al., 2017). Morphological changes also affect adhesion ability, such as *C. albicans* hyphal has stronger adhesion ability than the yeast form (Richardson et al., 2018). Okinczyc et al. (2020) reported that Nepal propolis can inhibit hyhal formation and decrease the virulence of *C. albicans* due to changes in cell hydrophobicity. Changes in cell surface hydrophobicity will affect the ability of *C. albicans* to adhere to epithelial cells (Richardson et al., 2018).

Propolis Ethanol Extract inhibit *C. albicans* Morphology

We evaluated propolis ethanol extract (PEE) to failure transition from yeast-like to hyphal growth in *C. albicans* culture (Fig 2). *C. albicans* cultures that were not treated with PEE showed that yeast cells were able to form and produce elongated and regular hyphal cells. At 7.5% concentration of PEE, yeast cells had inhibition of pseudohyphae formation. Treatment of PEE with a concentration of 12.5% showed yeast cells decrease. This indicates that yeast cells can not produce asexually and are unable to form blastospore (budding).

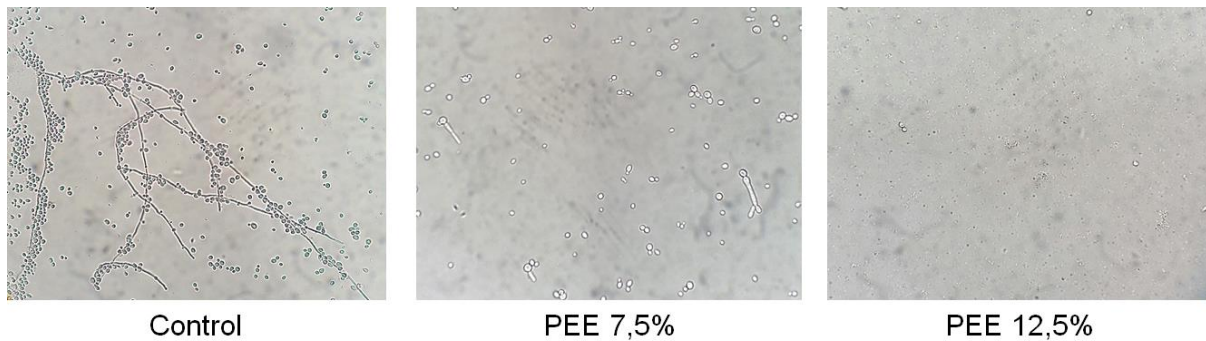


Figure 2. Effect of Propolis Ethanol Extract Inhibits on *C. albicans* Morphology. *C. albicans* Cells were Grown in RPMI-1640 Medium Containing 10% Fetal Bovine Serum (FBS) at Some Concentrations of Propolis at 37°C for 6 h. Samples were Withdrawn after Incubation and Photographed at 400x Magnification.

The ability of *C. albicans* to transition from yeast-like to hyphal growth will increase its virulence compared to the form of planktonic/yeast cells because hyphal has a larger size than spores which will complicate the phagocytosis process by macrophages (Li et al., 2019). The hyphal formation which has intensive ability to tissue invasion and infiltration plays an important role in the pathogenesis (Valerio et al, 2016). The experiment conducted by Valerio et al. (2016) has a similiar result with our study. They revealed that Green Brazilian propolis extract can inhibit the formation of hyphal at 450 µg/ml propolis extract (PE). At this concentration, PE was able to block 90% of transition from yeast-hyphal growth in *C. albicans*. Therefore, our study showed that hyphal growth did not seen at 7.5% concentration of PEE from Lawang, Malang, Indonesia. According to Chua et al. (2014), a flavonoid contained in propolis interacts with cellular sulfhydryl compounds on the cell wall, which causes detachment of the yeast cell wall and reduction of germ tube formation and hyphal length. As a result, it inhibits the yeast-mycelium transition which ultimately prevents cell division.

Propolis Ethanol Extract Can Decrease *C. albicans* Cell Viability on Biofilm Formation

C. albicans cell viability test was performed using a colony-forming unit (CFU) assay. The result showed that colony growth decreased with the increased of propolis ethanol extract (PEE) concentration (Fig 3). Colony growth started to decline at 2.5% of PEE (1069101 CFU/mL). Then, colony growth at 5% of PEE was 459402 CFU/mL and 13750 CFU/mL at 7.5% of PEE. Colony growth did not seen at 10% and 12.5% concentration of PEE (0 CFU/mL) (Fig 4). Therefore, 10% concentration of PEE had been able to inhibit *C. albicans* cell growth. It is indicated that PEE can decrease *C. albicans* cell viability on biofilm formation. Moreover, the result of the One-Way ANOVA statistic test showed a significant difference between colony growth on each concentration ($p=0,000$).

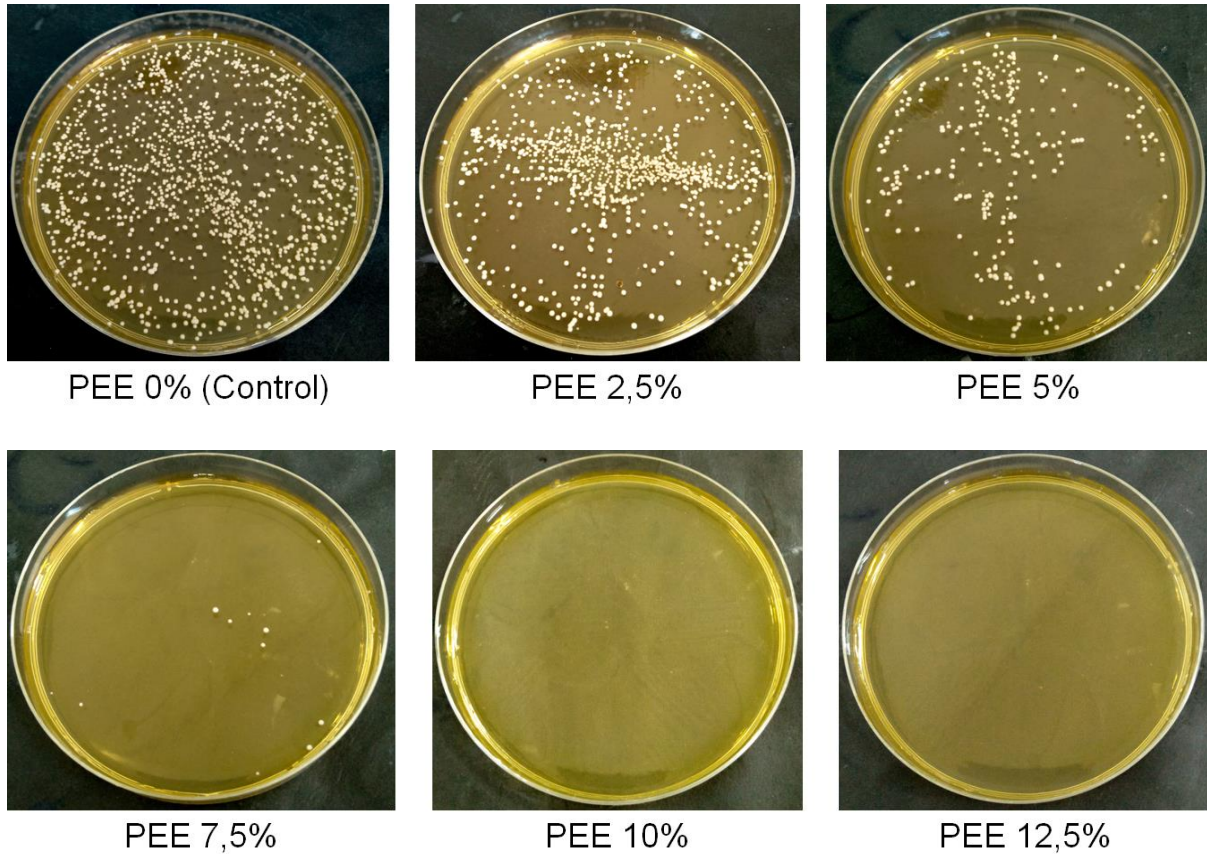


Figure 3. *C. albicans* Colony Growth in SDA Medium on Biofilm Formation with some Concentrations of Propolis Ethanol Extract Treatment (0%; 2.5%; 5%; 7.5%; 10%; 12.5%). Colony was Counted Using Colony Counter. This Showed that Colony Growth Decreased with the Increased of Propolis Ethanol Extract Concentration.

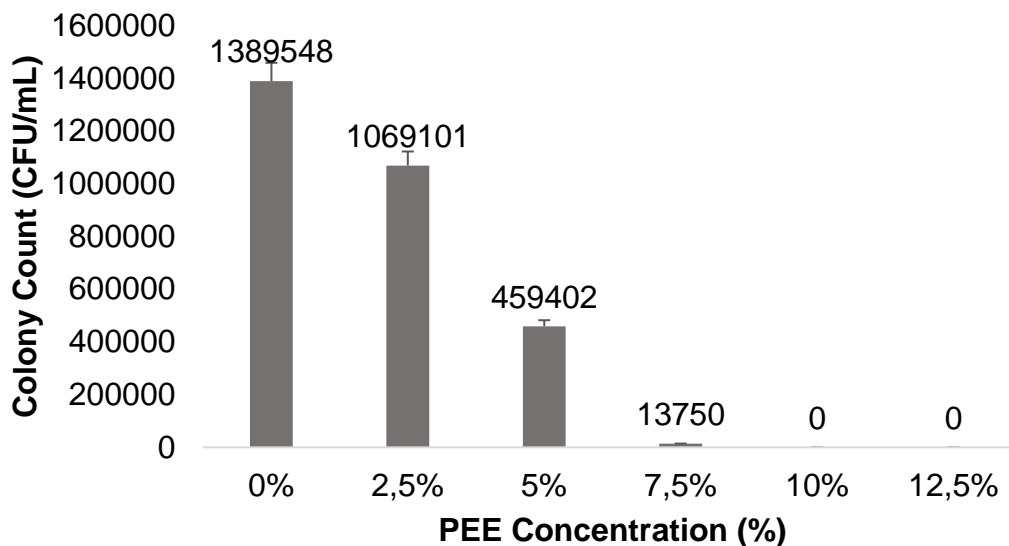


Figure 4. Colony Count Histogram of *C. albicans* Viable Cell on Biofilm Formation with some Concentrations of Propolis Ethanol Extract Treatment (0%; 2.5%; 5%; 7.5%; 10%; 12.5%). The Histogram Showed that Propolis Ethanol Extract can Decrease the *C. albicans* Cell Viability on Biofilm Formation.

Other research revealed the similar result about PEE effect to cell viability of *C. albicans* biofilm. The experiment conducted by Capoci et al. (2015) showed that there is a colony decrease with the increase of Brazilian propolis extractive solution (PES) concentration. They find out that cell viability at the highest concentration of PES in their study (546.87 µg/mL) was 42.24% (Capoci et al., 2015). Therefore, our study has revealed that 10% concentration of PEE can decreased the cell viability until 0 CFU/mL (0%). This can happen because propolis has a various chemical composition according to the geographical origin. Bees gather the resins and plant substances from plants around them, which vary from region to region (Cheng et al., 2013). Propolis used in this study was come from Lawang, Malang, Indonesia, that has a good plant sources, water availability, geographical and climatic condition.

There are several mechanisms of propolis in damaging cells. Propolis is known to damage the cell wall and cytoplasm (Tyagi et al., 2013). Even though, cell wall integrity has an important role in cell division. Then, the flavonoid in propolis can inhibit the oxidative phosphorylation process, consequently, the ATP formation is inhibited. It can cause the lack of energy to grow properly. Therefore, cell viability will decrease (Majiene et al., 2010).

The results obtained in this study show that PEE has antibiofilm effect against *C. albicans* biofilm formation in vitro. This indicates that PEE is likely to be one of alternatives to recurrent vulvovaginal candidiasis treatment, especially caused by *C. albicans* biofilm formation. But, this study has limitations. The limitation is this research has not been done in vivo. So that this research has not been able to determine the toxic dose of propolis. Moreover, this study did not measure the flavonoid content of propolis from Lawang, Indonesia.

CONCLUSION

The purpose of this study was to know the effect of propolis ethanol extracts on the cell adhesion index, morphology, and viability of *C. albicans* cells on biofilm formation. The adhesion index significantly decreased at the concentration of 12.5% PEE. Hyphal growth was optimally inhibited at the concentration of 12.5% PEE. The viability of *C. albicans* cells on biofilm formation decreased at 2.5% concentration and was not seen at 10% concentration of PEE. Therefore, PEE is likely to be one of alternatives to recurrent vulvovaginal candidiasis treatment, especially caused by *C. albicans* biofilm formation. Future study needs to examine the effect of Indonesian PEE from Lawang, Malang to *C. albicans* biofilm in vivo and its toxic dose. Besides that we recommend to measure the flavonoid content of propolis from Lawang, Indonesia.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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