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Effects of Clove Leaf Essential Oil (*Syzygium aromaticum*) in Inhibiting Biofilm Formation on *Candida albicans* Isolate

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ABSTRACT

Introduction: *Candida albicans* has a virulence factor, like biofilm formation. Biofilm is a three-dimensional structure that plays a role in antimicrobial resistance, thus requiring antibiofilm agents to address this problem. One of them is clove leaf (*S. aromaticum*). *S. aromaticum* has active phytochemicals (eugenol, β-caryophyllene, and others) that can inhibit biofilm formation in microorganisms, including fungi. This study aimed to prove the effect and to find a concentration of clove leaf essential oil that affects the biofilm formation of *C. albicans* isolate.

Methods: This study used a microtiter plate with a two-fold dilution technique. The tested concentrations were 6.25%, 3.125%, and 1.5625%. The positive control was 200 µL of *C. albicans* biofilm suspension, and the negative control was 200 µL of tryptic soy broth (TSB) media, and all were repeated four times. The biofilm was observed using crystal violet and evaluated using optical density (O.D.). The O.D. data was analyzed statistically using the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) version 26.

Results: The O.D. of the isolate was 2.039, while the negative control was 0.349, indicating that the isolate was a strong biofilm former. The concentrations of 6.25%, 3.125%, and 1.5625% showed inhibition percentages of 8.533%, 17.214%, and 8.484%, respectively. The O.D. was found to be normally distributed but not homogeneous. The Kruskal-Wallis's test was significant, and the Mann-Whitney test was not significant between test groups and positive control.

Conclusion: Clove leaf essential oil has inhibitory effects on *C. albicans* biofilm isolates. However, statistically, there was no significant difference between the test groups and the positive control.

Highlights:

1. *Candida albicans* is capable of forming biofilm, which can lead to resistance to antifungal treatments.
2. Clove leaf essential oil contains eugenol, which can inhibit biofilm formation, making it a potential anti-biofilm agent.

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Introduction

Infection remains a problem in tropical and subtropical countries, including Indonesia. This country has high air temperature and humidity, as well as biodiversity and diseases, making it vulnerable to infection by microorganisms, whether caused by bacteria, viruses, or fungi that are part of the normal flora or pathogens.[1,2] The human body contains normal flora, such as bacteria and fungi, but under certain conditions, they can become opportunistic infections due to immune system disturbances.[3–5] Fungal infections are most often caused by *Candida sp.* and manifest as candidiasis, whether it is local/superficial or systemic (candidemia) to invasive candidiasis affecting internal organs.[6,7] In Indonesia, around 2.89% of the population suffers from serious fungal infections each year,[2] and the prevalence of candidiasis in Indonesia ranges from 20% to 25%. It affects locally (skin, nails, mucous membranes) and even systemically.[1] *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* are the most common etiologies of these fungal infections.[1,8,9] Due to the virulence and pathogenicity factors of *C. albicans*, almost 50% of cases can lead to resistance to some treatments, such as fluconazole, amphotericin B, and echinocandins.[6]

The virulence factors of this species include its polymorphic ability, the presence of adhesion molecules and invasion to adhere to cell surfaces, biofilm formation, and phenotypic changes in releasing various hydrolytic enzymes.[10] *C. albicans* is a polymorphic organism, meaning it can change its form/structure to yeast, hyphae, and pseudohyphae depending on its environment. A biofilm is an organized collection of microbes surrounded by matrices produced by the microbes, acting as a shield from the host's immune system.[3] It is estimated that 80% of microbial infections in humans are due to biofilm formation.[3,11] The presence of biofilm can increase the morbidity and mortality of individuals infected with *C. albicans*, as well as resistance to antimicrobial agents.[3,9,10] Some antifungal agents are resistant to biofilms formed by *C. albicans*, necessitating the discovery of new active substances/drugs to address this issue.[3,9]

Approximately 80% of the world's population believes that traditional herbal medicine can be used in healthcare services.[12] This is because herbal ingredients have activities such as fungicidal, bactericidal, and virucidal, which can treat various infectious diseases caused by fungi, bacteria, and viruses. Typically, these herbal ingredients contain several active compounds that can be used for treatment, such as terpenes, alkaloids, steroids, tannins, saponins, glycosides, flavonoids, and others.[12] Additionally, there are several plants with potential and activity as antifungals, such as cloves (*S. aromaticum*),[13,14] lemongrass (*Cymbopogon citratus*),[15,16] lemon fruit (*Citrus limon*)[17] and others. One natural substance found in Indonesia is the clove plant, or *S. aromaticum*, which is still limited in active ingredient testing for its leaf extract's effect on biofilm formation in *C. albicans*. However, the plant has numerous benefits, including anti-inflammatory, antifungal, and antibacterial.[12] Based on gas chromatography-mass spectrometry analysis, the active potential antifungal compounds in *S. aromaticum* are eugenol, eugenol acetate, β-caryophyllene, and others.[12,18] Additionally, clove leaf essential oil and its eugenol content have a more significant antifungal effect than fluconazole, as evidenced by a lower inhibition zone with clove leaf essential oil compared to the antifungal.[18] The effectiveness of eugenol from clove plants as an antifungal can combat filamentous cells, yeast, and various human pathogenic fungi,[12] which play a role in the formation of *C. albicans* biofilm. This study was conducted in response to the formation of biofilm by *C. albicans*, which can lead to resistance to antifungal agents. This situation presents an opportunity for herbal substances to be studied for their effects on biofilm formation. The results of this study are expected to provide insights and scientific evidence regarding the active ingredients in clove leaf essential oil that are capable of inhibiting biofilm formation by *C. albicans*.[12,13,16]

Methods

This was an experimental study and a post-test-only control group design. Post-test-only control group design is a data collection method and is conducted only at the end of the study period after administering treatments.[19] This study was conducted in the Laboratory of Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, and Universitas Airlangga Hospital, Surabaya, from September 2022 to September 2023. The authors used one isolate of *C. albicans* biofilm, and the experiment was replicated four times. This study had received ethical clearance from the Ethics Committee for Health Research, Faculty of Medicine, Universitas Airlangga, Surabaya (No. 184/EC/KEPK/FKUA/2022) on 24-10-2022.

Production of Clove Leaf Essential Oil (*S. aromaticum*)

In this study, the steam distillation process was used to obtain clove leaf essential oil. The distillate, consisting of water and essential oil, was then separated using a funnel. The resulting essential oil was tightly sealed and stored in a place that was not exposed to sunlight, or it could be stored in a refrigerator. To create various concentrations of clove leaf essential oil, starting from 6.25%, 3.125%, and 1.5625%, it was dissolved in a dimethyl sulfoxide (DMSO) solvent. The highest concentration was first prepared using the two-fold dilution method by mixing the essential oil with the DMSO solvent at a 1:16 ratio. Subsequently, the next concentration levels were obtained from the initial concentration and homogenized using microtips, and so on, until the final concentration was discarded according to the initial volume taken.

Production of *C. albicans* Suspension Isolate for Biofilm and Antibiofilm Test

The *C. albicans* isolate was inoculated onto Sabouraud dextrose agar (SDA) medium using the streaking method on agar plates, followed by macroscopic observation. To create a *C. albicans* suspension, the fungus from the SDA medium was mixed into the tryptic soy broth (TSB) medium. Using a density check, the mixed suspension was

measured to be equivalent to 0.5 McFarland. Using the microtiter plate method, this suspension was then used to test the effect of clove leaf essential oil (*S. aromaticum*) in inhibiting biofilm formation in *C. albicans* isolate.

Biofilm Test of *C. albicans* Isolate

The biofilm detection test used the microtiter plate method, a quantitative method for determining biofilm production using an enzyme-linked immunosorbent assay (ELISA) microplate reader. The negative control was a well-containing media that was used as the blank value. The optical density (O.D.) blank value was used to identify biofilm formation. The suspension from the sample dilution buffer (SDB) and the negative control were then measured for O.D. using an ELISA reader. If the microbial O.D. value was higher than the blank O.D. value, the microbe produced biofilm. The test was performed four times, and the results are grouped based on the following criteria (Table 1).

Table 1. Classification of biofilm former[20]

Average of Optical Density	Biofilm Former
O.D. Microbes ≤ O.D. Control	Biofilm not produced
O.D. Control < O.D. Microbes ≤ 2× O.D. Control	Weak biofilm former
2× O.D. Control < O.D. Microbes ≤ 4× O.D. Control	Moderate biofilm former
4× O.D. Control < O.D. Microbes	Strong biofilm former

Source: Research data, processed

Antibiofilm Test of *C. albicans* Isolate

The clove leaf essential oil antibiofilm test on *C. albicans* biofilm was conducted using a microtiter plate. The microtiter plate comprised 96 wells divided into 12 columns and 8 rows. The authors assigned codes, such as wells (D1-D4, E1-E4, and F1-F4) as treatment groups with various concentrations of clove leaf essential oil (6.25%, 3.125%, and 1.5625%), wells (G1-G4) as the positive control (*C. albicans* biofilm isolate suspension), and wells (H1-H4) as the negative control (TSB only). A 100 µL of 6.25% essential oil concentration was added to well D1. Wells E1 and F1 were supplemented with sterile distilled water. Then, 100 µL of essential oil from well D1 was transferred to well E1 and homogenized. Subsequently, 100 µL of essential oil from well E1 was transferred to well F1. This process was repeated for D2-D4, E2-E4, and F2-F4. Then, each well (D1-D4, E1-E4, and F1-F4) had 100 µL of *C. albicans* biofilm isolate added to each well and homogenized. Meanwhile, wells G1-G4 were filled with 200 µL of *C. albicans* biofilm isolate suspension, and wells H1-H4 were filled with 200 µL of TSB media. Thus, the final volume of each well became 200 µL. The microplate was then incubated for 2 days at 37ºC. Then, flipping the microplate and washing it with phosphate-buffered saline (PBS) twice to prevent cell adhesion to the microplate. Then, 150 µL of methanol was added to fix the formed biofilm in the wells and incubated for 10-20 minutes. Subsequently, 150 µL of 0.5% crystal violet solution was added to each well and incubated for 10-15 minutes. The microplate was rewashed with distilled water three times.

The final step was reading the microplate using an ELISA reader at a wavelength of 595 nm. Then, to determine the percentage inhibition of clove leaf essential oil (*S. aromaticum*) on the growth of *C. albicans* biofilm using the formula as follows:

$$\text{Inhibition percentage} = \frac{O.D.Control\ (+) - O.D.Experimental}{O.D.Control\ (+)} \times 100\%$$

Data Analysis

The data collected from this study were analyzed using a normality test using the Shapiro-Wilk test, a homogeneity test using the Levene test, and a post-hoc test using the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) version 26 for Windows.[20–22] If the result of the normality and homogeneity test were normal and homogenous, then it would be analyzed by the one-way analysis of variance (ANOVA) test to see the difference between groups.[20] Nevertheless, if there were no normal or homogenous results between the normality or homogeneity test, then the Kruskal Wallis test and Mann-Whitney would continue as the posthoc test. All of these tests used IBM SPSS version 26 for Windows.[20–22]

Results

The isolate was sub-cultured using SDA media and incubated for 2 days. Subsequently, the isolate was transferred to TSB media, thoroughly mixed using a vortex to achieve a 0.5 McFarland standard, and evaluated using density-check. Next, 200 µL of the suspension was added to the wells of a microplate, and this process was replicated four times. The microplate was then incubated for 2 days, and the washing results are shown in Figure 1. Several wells absorbed crystal violet, indicating biofilm formation, while the negative control wells did, serving as a comparison with the positive control. However, to classify the biofilm formers, readings were taken using an ELISA reader to measure O.D. (Table 2).

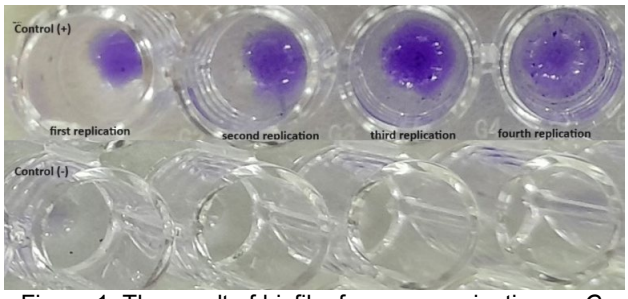

Figure 1. The result of biofilm former examination on *C. albicans*

Based on Figure 1, there were four wells for positive control and negative control that indicated the replication. The positive control wells absorbed a crystal violet, indicating biofilm formation, while the negative control did not absorb a crystal violet, indicating that this study was under control. Table 2 shows the results of O.D. readings using an ELISA reader.

Table 2. Result of biofilm examination on *C. albicans* isolates

Replication/Experimental	Isolate	Control (-)
1st replication	1.268	0.337
2st replication	1.810	0.352
3st replication	2.686	0.311
4st replication	2.390	0.396
Average	2.039	0.349
Biofilm former classification	Strong	

Source: Research data, processed

In this study, the test concentrations should start at 50%, 25%, and 12.5%. However, preliminary research showed that at these concentrations, the essential oil could form a layer on the microplate well walls, leading to false-positive results in the crystal violet staining test shown in Figure 2. Therefore, the tested concentrations in this study were 6.25%, 3.125%, and 1.5625%.

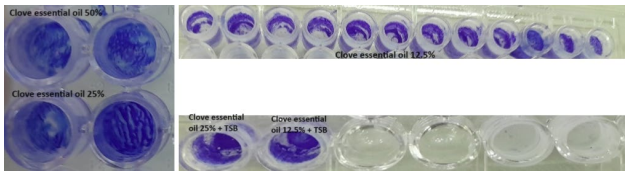

Figure 2. Preliminary research using concentration starting 50% - 12.5% with a two-fold dilution technique

Data about O.D. value was analyzed statistically using IBM SPSS version 26 for Windows, with the results showing that the normality test was normal (p-value > 0.05) and the homogeneity test was not homogenous (p-value < 0.05).[22] Then, the data was analyzed using the Kruskal Wallis test, which was significant (Asymp. Sig. 0.045), with a post-hoc test using Mann-Whitney in Table 4.

Table 3. O.D. value on antibiofilm examination of clove leaf essential oil in biofilm former on *C. albicans* isolates

Replication/ Experimental	E6.25 %	E3.125 %	E1.5625 %	Control (+)	Control (-)
1st replication	1.887	1.361	1.532	1.268	0.337
2st replication	2.031	1.933	1.608	1.810	0.352
3st replication	1.737	1.597	1.880	2.686	0.311
4st replication	1.805	1.861	2.442	2.390	0.396
Average	1.865	1.688	1.866	2.039	0.349
Inhibition percentage (%)	8.533	17.214	8.484		

Source: Research data, processed

Based on Table 4, significant differences were found between the negative control group and all concentrations and the positive control group. However, there were no significant differences between the positive control and all concentrations or among the concentrations.

Table 4. Mann-Whitney of O.D. value

Group		p-value of Mann-Whitney
Control (+)	Control (-)	0.021
	E1.5625%	0.773
	E3.125%	0.564
	E6.25%	
Control (-)	E1.5625%	0.021
	E3.125%	
	E6.25%	
E1.5625%	E3.125%	0.564
	E6.25%	0.386

Source: Research data, processed

Discussion

The clove leaf used in this study was sourced from Purwodadi. The essential oil of clove leaf (*S. aromaticum*) was characterized for its compound content using gas chromatography-mass spectroscopy at the Pharmacy Research Service Unit of Universitas Airlangga, Surabaya. The characterization results revealed the presence of isohexane, chavicol, α-Cubebene, 1,3,4-Eugenol, 4-Allyl-2-methoxy-phenol, 2-Methoxy-4-formylphenol, β-caryophyllene, (E)-isoeugenol, α-caryophyllene; 5-Oxatricyclo[8.2.0.0(4,6)-]dodecane, 4,12,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)], delta-Cadinene. Additionally, the eugenol content in the clove leaf essential oil was found to be 84%.[18]

The average O.D. of *C. albicans* isolate was 2.039, and the negative control was 0.349, resulting in a strong biofilm-former.[23] Biofilm is a three-dimensional structure produced by a microorganism that attaches its cells to the host surface and is surrounded by an extracellular matrix.[10] In *C. albicans*, there are several stages in biofilm formation, including the adhesion phase (attachment of yeast cells to the host substrate to form its basal layer), proliferation phase (adhered yeast cells forming hyphal filaments), maturation phase (hyphal production followed by extracellular polymeric substances (EPS) secretion related to antifungal resistance of the formed biofilm), and dispersion phase (farnesol production allowing yeast cells to spread and form new biofilms).[3,10]

This study used clove leaf essential oil with an 84% eugenol content. This compound is known to have antifungal and antibiofilm effects on several species, one of which is *C. albicans*.[14] This study dissolved the essential oil with DMSO solvent, obtaining an initial concentration of 50%. However, concentrations of 50%, 25%, and 12.5% resulted in false positive effects as they could form a layer that absorbs crystal violet. Therefore, the study started with a concentration of 6.25%. Clove extract with ethyl acetate solvent exhibited the highest antifungal activity against *C. albicans*, *C. glabrata*, and *C. tropicalis*.[13] The inhibition zone against yeast and fungi ranged from 8.22 to 18.56

mm.[13] However, the inhibition zone for *C. albicans* was approximately 8.22 ± 1.53 mm, indicating weak antimicrobial activity as the inhibition zone fell within the 5-10 mm range.[24] Phytochemicals such as phenols and tannins can inhibit substrates, destroy cell walls, prevent adhesion, and reduce exopolysaccharide production in biofilm formation.[25]

Additionally, antimicrobial peptides inhibit microbial biofilms by down-regulating quorum sensing, inhibiting surface attachment, and modulating the body's immune response.[25] In this study, eugenol, the essential oil component of clove leaf (*S. aromaticum*), is a phytochemical compound whose mechanism of action as an anti-biofilm agent includes inhibiting substrates, destroying cell walls, preventing adhesion, and reducing exopolysaccharide production in biofilm formation.[13,14,25,26]

The antibiofilm test using a microtiter plate was determined based on the turbidity of the wells and their O.D. values. An increase in turbidity after adding a dye such as crystal violet will result in a higher O.D., and higher O.D. values in the concentration groups will lead to lower inhibition percentage values.[27] The inhibition percentages obtained from concentrations of 6.25%, 3.125%, and 1.5625% were 8.533%, 17.214%, and 8.484%, respectively. In other studies, the antimicrobial activity of clove leaf essential oil against yeast *C. albicans* was at 0.5%, leading to a decrease in morphotype mass in the *C. albicans* control group at a concentration of 0.25% and a change in its protein profile at concentrations of 0.125%-0.25%.[14,26] Meanwhile, in the biofilm of *C. tropicalis*, clove leaf essential oil can inhibit 50% of its inhibition, thus potentially serving as an anti-biofilm agent.[26] In fact, this study also found that at concentrations of 12.5%, 25%, and 50% in the preliminary study, after washing, the biofilm appeared to absorb purple color from crystal violet, forming a layer resembling a biofilm. This was evidenced by higher O.D. values compared to the control group, with some even reaching a value of (+) above 3.5, making it unreadable by the ELISA reader.[28,29]

Several compounds can support biofilm formation, such as chitosan, a partially deacetylated form of chitin, which can mediate biofilm formation by initiating its formation phase.[28] Fungi such as *C. albicans* can release compounds like prostaglandins from their host cell membranes, potent metabolites that induce biofilm formation, morphological changes, and colonization within the host's body. Furthermore, their production is higher compared to that of their planktonic counterparts.[28] Meanwhile, research using the crystal violet parameter, based on the interaction of the dye with negative charges, shows false positives in biofilm mass after antibiotic administration, depolymerase treatment, and similar interventions. This occurs because antimicrobials increase negative charge residues, which form crystal violet complexes, potentially resulting in misleading interpretations of biofilm mass.[29]

Based on the statistical analysis conducted using IBM SPSS version 26, it was found that the O.D. data in this study followed a normal distribution (p-value > 0.05) but was not homogenous (p-value < 0.05).[22] The Mann-Whitney test followed the Kruskal-Wallis's test to determine the significant differences between groups. In this study, significant differences were observed between groups (p-value < 0.05), but there were no significant differences between the test group and the positive control group.[20,21]

Strength and Limitations

The strength of this study lay in its novelty, as it investigates the effects of clove leaf essential oil on biofilm formation in *C. albicans* isolates. Furthermore, it was observed that even at low concentrations, essential oils could inhibit *C. albicans* biofilm formation. However, this study has limitations, such as testing only a single isolate, resulting in a limited and less diverse dataset. Additionally, the tested concentrations were limited. Therefore, the minimum concentration required to inhibit 50% of the biofilm formation was unknown.

Conclusion

Based on the results, it was concluded that clove leaf essential oil (*S. aromaticum*) has inhibitory effects on the biofilm formation of *C. albicans*.

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Conflict of Interest

The authors declared there is no conflict of interest.

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Ethical Clearance

This study had received ethical clearance from the Ethics Committee for Health Research, Universitas Airlangga, Surabaya (No. 184/EC/KEPK/FKUA/2022) on 24-10-2022.

Authors' Contributions

Designed the study and drafted the manuscript: AR and PDE. Collected data and performed background literature review: AR, PDE, and YS. Performed statistical analysis: AR. Supervised results and discussion: PDE, YS, and EBK. All authors reviewed and approved the final version of the manuscript.

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