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Effectiveness of Ethanol Extract Clove Leaves (*Syzygium aromaticum*) In Inhibiting Biofilm of Candida Albicans ATCC 14053

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Abstract

Candida albicans is the most common type of *Candida*. It is a normal microbiota in the healthy human body that can become pathogenic when its balance is disturbed, causing an infection referred to as candidiasis. Antifungal resistance to biofilms is estimated to be 10,000 times that in planktonic form. Clove leaves (*Syzygium aromatium)* can treat infectious diseases, including candidiasis. This study aimed to analyze whether the ethanol extract of clove leaves (*S. aromaticum*) destroys the biofilm of *C. albicans* ATCC 14053. The research design–was pure experimental with a *post test control group only design* approach. The extraction method used was maceration. Test antibiofilm activity of ethanol extract of clove leaves using *microtiter plate biofilm assay* method. Measurement of results with a *microplate reader* was carried out using a wavelength of 595nm in accordance by the wavelength of crystal violet used as a colouring agent. The results showed that ethanol extract of clove leaves (*S. aromaticum*) has significant effectiveness against the maturation of *C. albicans* biofilm ($p<0.05$) with the highest percentage of activity at 20% concentration. The $MBEC_{50}$ was determined by probit analysis, so the concentration of clove leaf ethanol extract (*S. aromaticum)* that can eradicate 50% of *C. albicans* biofilm is located at a concentration of 0.45%. The clinical benefits that can be developed from the results of this study are the potential use of clove leaf ethanol extract as an alternative therapy for *C. albicans* infection in the form of soap or ointment.

Keywords: Biofilm; *Candida albicans*; Candidiasis; Clove leaf.

Abbreviations: MBEC Minimum Biofilm Eradication Concentration; ATCC American Type Culture Collection

INTRODUCTION

Candida is a genus of fungi often found in the environment, especially in tropical climates (Mbatu *et al.,* 2018). Candida *albicans* is the most commonly found type of *Candida* and is a normal microbiota in the healthy human body, for example, on the skin, mouth, intestines, genital tract, and under the nails (Khafidhoh *et al.,* 2015). *C. albicans* can become pathogenic when its balance is disturbed, which will accelerate its proliferation and cause infection. Fungal infections caused by *Candida are* referred to as candidiasis (Masfufatun *et al.,* 2014).

In Indonesia, candidiasis is around 20-25% (Puspitasari *et al.,* 2019*).* The occurrence of candidiasis recurrence is related to the pathogenicity of *C. albicans*, one of which is supported by the ability to form biofilms. The stages of biofilm formation include attachment, microcolony formation, biofilm formation, maturation, and cell dispersion (Purbowati, 2016). Biofilms are composed of various types of cells (yeast-shaped, having round buds, oval pseudohyphal cells, and long hyphal cells), attached to biotic and abiotic surfaces and embedded in a layer called the exopolysaccharide matrix (Gulati *et al.,* 2016). The presence of this matrix causes resistance to antifungals and hinders phagocytosis by host cells (Tsui *et al.,* 2016).

Currently used antifungals include azoles, polyenes, echinocadin, allylamine, and fluoropyrimidine (Kabir & Ahmad, 2013). Although azoles have long provided effective treatment, recent epidemiological studies have shown azole resistance in some cases of candidiasis (Whaley *et al.,* 2017). Antifungal resistance to biofilms is estimated to be 10,000 times that in planktonic form (Rabin *et al.,* 2015). Antifungal resistance occurs due to complex biofilm-forming components, making it more difficult for antifungals to work (Sharma *et al.,* 2016). In addition to causing resistance, prolonged use of synthetic drugs can result in respiratory depression, uritaccharia, hepatotoxicity, decreased platelet levels, and gastrointestinal disorders (Lestari *et al.,* 2019).

An effort is needed to inhibit growth in both planktonic and biofilm forms. One of them is by using

natural materials. According to research that has been done, there is an antibiofilm effect of clove (*S. aromaticum*) essential oil nanoemulsion against *Staphylococcus aureus* with an MIC of 0.62 mg/mL, while against *Cryptococcus neoformans* with an MIC of 3.12 mg/mL, and *against* with an MIC of 50 mg/mL (Shehabeldine *et al.,* 2023).

Clove is a spice plant native to Indonesia. Almost all parts of cloves can be utilized, starting from the leaves, flowers, and twigs. Clove leaves (*Syzygium aromatium)* can be used to treat infectious diseases, including candidiasis. In the phytochemistry of clove leaf extract, both crude and purified extracts contain flavonoids, tannins, saponins, terpenoids, and alkaloids (Novema *et al.,* 2022).

MATERIALS AND METHODS

Research design

The research design used was pure experimental with a *post test control group only design* approach. Test the antibiofilm activity of clove leaf ethanol extract using the *microtiter plate biofilm assay* method.

Extraction of clove leaves

The method used to perform the extraction is maceration. Prepare 995 grams of simplistic powder and then put it in a macerator. Adding 96% ethanol solvent into the macerator. After that, let stand until all the simplistic powder is submerged for 3x24 hours while stirring periodically. Then the mixture is filtered and the pulp is soaked again using new 96% ethanol. The further filtering process will be carried out twice using 96% ethanol. Liquid extracts are put together and concentrated using a vacuum rotary evaporator at 78ºC and concentrated again using a water bath at 40ºC to produce thick extracts (Wahyulianingsih *et al.,* 2016).

Concentrations of extract

Each treatment requires 4 repetitions in 8 test groups, so the concentrations used in each treatment are 20%, 10%, 5%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%. The finished thick extract of clove leaves (*S. aromaticum*) was weighed by starting the largest concentration of 20%. Then put into a vial bottle. The thick clove leaf extract solution was diluted using *dimethyl sulfoxide* (DMSO) solvent according to the desired concentration using the following dilution formula:

$$M1\ x\ V1\ =\ M2\ x\ V2$$

$M1$: Concentration of solution before dilution
$V1$: Volume of solution before dilution
$M2$: Concentration of solution after dilution
$V2$: Volume of solution after dilution (Iqhasari R, 2017).

Regeneration and preparation of *C. albicans* inoculum

C. albicans isolates were inoculated as much as 1 ose by streak plate method or scraping the colony. Furthermore, the new SDA media is incubated using an incubator for 2-3 days so that a single colony of *C. albicans* will form. Inoculation of *C. albicans* on SDB media for 18-20 hours at room temperature using a shaker at 120 rpm produces inoculum (Naitullah *et al.,* 2014).

Harvesting of inoculum and preparation of *C. albicans* suspension

Single colonies of *C. albicans* grown on SDA for 2-3 days were put into Erlenmeyer flasks containing 10 ml of SDB. SDB media was used as a dilution solution because it supports biofilm growth. Next, the flask was shaken for 24 hours at 120 rpm in an Erlenmeyer flask. Performed centrifugation by moving the *C. albicans* inoculum into a centrifuge tube for 15 minutes until a pallet was produced. The pallet formed was separated from the filtrate using PBS and resuspended to 1 x 10^6 CFU/ml with RPMI media. The Optical Density (OD) of the *C. albicans* suspension will be calculated using a microplate reader. If the OD > 0.5, continue diluting until OD = 0.5 is obtained. *C. albicans* OD 0.5 suspension is ready for an antibiofilm test (Fauzan *et al.,* 2023).

Mature biofilm formation

100 µl of *C.albicans* suspension was put into the wells starting from rows A-D, columns 1-6, 8, 10, and 12. Then it was incubated for 2 hours at 37º C for the *C.albicans* attachment stage. Next, 100 µl RPMI media was added to all wells and incubated for 48 hours at 37º C for the growth stage of *C. albicans*. Then, added extracts in concentrations of 20%, 10%, 5%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%, RPMI (for positive, negative, and blank media controls), and fluconazole (for positive control). For blank samples, 50 µl of *C. albicans* suspension was also placed into the wells in rows G and H. Incubated at 37°C for 24 hours for the *C.albicans* maturation stage (Zhu *et al., 2023)*.

Mature biofilm eradication test

After incubation at 37°C for 24 hours for the *C.albicans* maturation stage. Each well was washed twice using PBS. The unattached planktonic cells of *C. albicans were* gently removed by inverting and pouring on a tissue to obtain *C. albicans* attached to the *microplate*. The biofilm was fixed using methanol for 15 minutes and then dried at room temperature. Next, 0.1% crystal violet solution was added to each well for staining for 30 minutes at room temperature. Next, wash 3 times using PBS. Adding 96% ethanol to each well then incubated for 1 hour at room temperature and measured using a *microplate reader* (Zhu *et al., 2023).*

Minimum biofilm eradication concentration ($MBEC_{50}$)

Minimum Biofilm Eradication Concentration ($MBEC_{50}$) is the lowest clove leaf ethanol extract concentration that can eradicate 50% of *C. albicans* biofilm. The $MBEC_{50}$ value was determined using OD maturation data converted to % eradication and analyzed SPSS probit (Gupta *et al.*, 2016).

Data analysis

The data analysis method used SPSS (Statistical Product of Service Solution) statistical analysis for Windows. The normality test used if the data is less than 50 is the Shapiro-wilk test. Furthermore, a homogeneity test was carried out using the Levene test if it was proven that the data was typically distributed or close to normal. Data that are normally distributed and homogeneous (P>0.05) will be analyzed using the One-Way Analysis of Variance (Anova) test, which is a parametric test to compare differences in the means of data from more than two groups. The significance level was set at P<0.05. After the One-Way ANOVA test was conducted, the Post Hoc test was followed. The Post Hoc test is an advanced test that analyzes which test group has a significant difference. Furthermore, the $MBEC_{50}$, it was determined using SPSS probit analysis.

RESULT AND DISCUSSION

RESULT

Characteristics of Clove Leaf

This study aimed to test the antibiofilm effect of ethanol extract of clove leaves (*S. aromaticum*) at the maturation stage. Clove leaves were obtained from Jatirejo District, Mojokerto. The clove leaves were picked, washed, and aerated for one week. After drying, the clove leaves were pulverized using a blender until they became powder (simplistic). Clove leaf simply obtained as much as 995 grams. The clove leaf ethanol extract was prepared by maceration method, namely by soaking the fine simplistic using 96% ethanol solvent and filtering using filter paper for 3x24 hours while stirring periodically. The liquid extract was combined and concentrated using a *vacuum rotary evaporator* at a temperature of 78° C and concentrated again using a *waterbath* with a temperature of 40° C to produce 156 grams of clove leaf ethanol extract with a greenish brown color.

Biofilm Maturation Detection Test

To see the biofilm maturation, *C. albicans* was incubated at 37°C for 2 hours for the attachment stage. Furthermore, another incubation was carried out for 48 hours at 37° C for the growth stage of *C. albicans.* Then, the ethanol extract of clove leaves (*S. aromaticum*) was added. The wells that have been treated are incubated for 24 hours at 37°C. The results of the *C. albicans* biofilm maturation test can be seen in Figure 1

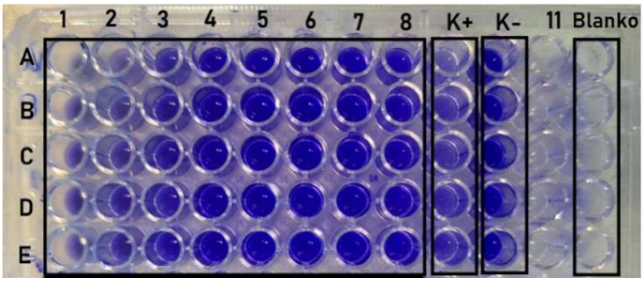

Figure 1. Mature Biofilm Eradication Test Results with Crystal Violet Staining.
Column 1_{A-E} = concentration 20%
Column 2_{A-E} = concentration 10%
Column 3_{A-E} = concentration 5%
Column 4_{A-E} = concentration 2.5%
Column 5_{A-E} = concentration 1.25%
Column 6_{A-E} = concentration 0.63%
Column 7_{A-E} = concentration 0.31%
Column 8_{A-E} = concentration 0.16%
Column 9_{A-E} = positive control
Column 10_{A-E} = negative control
Column 12_{A-E} = media blank

Based on Figure 1., the results show that clove leaf extract at a concentration of 20% after giving crystal violet has a faded purple color and the smaller the concentration, the more intense the purple color produced, meaning the higher the density of biofilm biomass.

The most intense purple color was found in the negative control group. Furthermore, the absorbance/Optical Density (OD) value was measured which can be seen in Table 1.

Table 1. OD Mature Biofilm Eradication Test Results with Clove Leaf Extract.

Replication	Clove Leaf Extract Concentration								Control	
	20%	10%	5%	2.5%	1.25%	0.63%	0.31%	0.16%	K (+)	K (-)
1	0.29	0.60	0.66	0.76	0.84	0.92	1.56	3.36	1.92	3.27
2	0.13	0.46	0.59	0.85	0.68	0.96	1.24	3.22	2.06	3.16
3	0.20	0.61	0.54	0.68	0.73	0.62	1.28	3.15	2.08	3.06
4	0.19	0.50	0.41	0.63	0.60	0.64	0.84	3.07	1.80	3.32
Average	0.20	0.54	0.55	0.73	0.71	0.79	1.23	3.20	1.97	3.20
Std	0.06	0.07	0.09	0.08	0.09	0.16	0.26	0.10	0.11	0.10

In Table 1, the *C. albicans* mature biofilm test results were replicated four times, with the highest average OD value obtained at a concentration of 0.16%, namely 3.20, and the weakest at a concentration of 20%, namely 0.20. At a concentration of 0.16%, the highest OD value is obtained, meaning that the density of the biofilm biomass is the highest so that the ability of clove leaf extract to eradicate *C. albicans* biofilm is lower and at a concentration of 20% has the lowest OD, meaning that the density of the biofilm biomass is the lowest so that the ability of clove leaf extract to eradicate *C. albicans* biofilm is higher. Meanwhile, for the control group, the highest OD value was found in the negative control (K-) because the negative control group was not given any treatment for *C. albicans* cells that matured on the microplate. Table 1 shows a decrease in the average maturation of *C. albicans* biofilm and an increase in concentration.

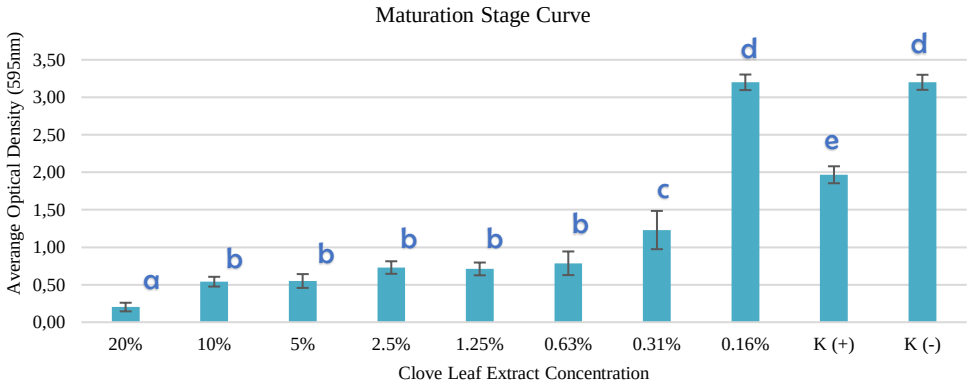

Figure 2. Post Hoc Analysis Results of Mature Biofilm Eradication with Clove Leaf Extract.
Superscript abc, if it contains the same letters means there is no significant difference.
If it contains different letters, it means there is a significant difference between the treatment groups based on the Post hoc test (P value <0.05 shows a significant difference)

Based on the Post hoc test results in Figure 2, significantly different groups were at a concentrations of 20%, 0.31% and positive control while groups that did not have significant differences were at concentrations of 10%, 5%, 2.5%, 1.25%, 0.63%, 0.16% and negative control.

$MBEC_{50}$ Value Determination Results

The Optical Density of the Table was used to determine the percent of *C. albicans* biofilm eradication testing using the formula below:

$$\% \text{ Biofilm maturation rate} = \frac{(\text{OD negative control} - \text{OD sample})}{(\text{OD negative control})} \text{x } 100\%$$

Table 2. Eradikasi Biofilm.

	Clove Leaf Extract Concentration							
% Eradikasi	20%	10%	5%	2.5%	1.25%	0.63%	0.31%	0.16%
	93.67	83.10	82.82	77.21	77.78	75.43	61.58	0.01

From table 2, the highest eradication results are in the clove leaf ethanol extract group with a concentration of 20% having the highest eradication ability, meaning that the growth of *C. albicans* biofilm is getting lower and vice versa at a concentration of 0.16% has the lowest eradication ability, meaning that the growth of *C. albicans* biofilm is getting higher.

Determination of $MBEC_{50}$ using probit analysis obtained the results of the concentration of clove leaf extract that can eradicate 50% of *C. albicans* biofilm is located at a concentration of 0.45%, meaning that clove leaf ethanol extract can eradicate 50% of *C. albicans* biofilm at a concentration of 0.45%.

DISCUSSION

Clove leaf extract yield

The clove leaves used in this study are about 6-8 months old, green , and fresh. Clove leaves that have been picked must be washed first to remove dirt, then aerated for one

week to reduce the water content so that it can inhibit microbial growth. After drying, the clove leaves are mashed using a blender until they become powder (simplisia) (Warnis, *et al.,* 2020). The particle size of the sample is related to the surface area that will contact the extraction solvent. The finer the simple, the easier the compounds in the leaves move into the solvent (Aji, 2018).

The extraction method used in this research is maceration using 96% ethanol solvent. The 96% ethanol solvent was chosen because it is a universal solvent that can dissolve organic compounds in samples of both polar and non-polar compounds (Munte *et al.,* 2015). The use of 96% ethanol as a solvent because ethanol is more accessible to enter the cell membrane so that it can extract secondary metabolite compounds such as flavonoids, tannins, saponins, terpenoids, and phenols (Dewi *et al.,* 2021). From the maceration results of 995 grams of clove leaf simplisia, 156 grams of thick extract were obtained with a percentage yield of 16%. This research is in line with that conducted by Suhendar and Sogandi (2019) who used the maceration method extract clove leaves. However, the solvent in the study used 70% methanol. The results of this method from 1 kg of clove leaf simplistic obtained a thick extract of 100.08 grams with a percent yield of 10%. Where good yield results have a value of> 10% (Novema *et al.,* 2022). 96% ethanol solvent has a lower polarity than 70% methanol. Methanol 70% also contains 30% water, which can reduce the extraction efficiency of some lipophilic compounds. Water can cause hydration or precipitation of specific components that should be extracted. Thus, compounds in clove leaves with lipophilic properties (like fat) will dissolve more easily in 96% ethanol. Organoleptic results show that the methanol extract of clove leaves has a thick form, reddish brown color, bitter taste, and smells typical of cloves. Meanwhile, the ethanol extract of clove leaves also has a thick texture, greenish brown color, and smells typical of cloves.

Dimethyl sulfoxide (DMSO) is used to dissolve organic compounds that are less soluble when using distilled water. DMSO is a solvent that can dissolve almost all compounds both polar and non-polar. DMSO has low toxicity, has anti-inflammatory, and analgesic effects. In research conducted by Rahmi & Putri (2020), it was found that the use of DMSO as a solvent had no effect on the test results carried out using the disc paper method against *C. albicans*. In this study, DMSO solvent was used because clove leaf ethanol extract does not have an aqueous phase.

Effect of Clove leaf extract on mature biofilm

Based on the results of the mature biofilm eradication test in Table 2. the OD value of the test group of Clove Leaf Extract is lower than the OD of the positive control except at a concentration of 0.16%. This indicates that at concentrations of 0.31% to 20%, clove leaf ethanol extract has a better mature biofilm effect than fluconazole antifungal. The lowest OD value was found at 20% concentration. The OD value from concentrations of 0.16% to 20% at the stage of mature biofilm of *C. albicans is* decreasing, which shows that with the increasing concentration of extracts used, the ability to eradicate biofilms is increasing. Based on the results of anova analysis, indicate that there is an effect of giving clove leaf ethanol extract (*S. aromaticum*) on the maturation stage of *C. albicans* biofilm significantly ($p<0.05$). In Figure 2, Groups that have significant differences are at concentrations of 20%, 0.31%, and positive control, while groups that do not have substantial differences are at concentrations of 10%, 5%, 2.5%, 1.25%, 0.63%, 0.16%, and negative control.

Clove leaf can eradicate mature biofilm because it contain active compounds such as saponins, alkaloids, tannins, flavonoids, and terpenoids. Tannins will inhibit the icaA and icaD genes. These genes will synthesize PIA, which plays a role in EPS formation and cell aggregation. The action of saponins is to increase cell wall permeability by reducing surface tension, so that cells leak and intracellular components come out. The role of saponins as antibiofilm monospecies of *C. albicans is* at the stage of biofilm adherence and maturation (Hamzah *et al.,* 2021).

From Table 2. the most effective test group was at 20% concentration because the percentage was the highest at 93.67%. Thus, the antibiofilm activity of clove leaf ethanol extract to eradicate *C. albicans* biofilm is high because the percentage of antibiofilm activity is ≥50% (Famuyide *et al.,* 2019).

At the maturation stage, research that supports this research is research conducted by Mirzaei et al., (2022), where melittin, vancomycin, and rifampicin against *Staphylococcus epidermidis* biofilms produced $MBEC_{50}$ for melittin, rifampin, and vancomycin, are 0.002%, 0.0512%, and 0.1024%. $_{50}$ According to Abidah's research, (2020), black mulberry leaf extract (*Morus nigra L.*) against *Escherichia coli* biofilm has an $MBEC_{50}$ of 0.016%. Meanwhile, in this study the $MBEC_{50}$ is 0.45%.

As the mature biofilm, the synergy and communication between the fungi in the biofilm becomes more complex. The EPS matrix layer that is formed is more numerous and thicker. The EPS matrix can increase the defense of fungi compared to still in planktonic form. The eradication ability of an extract is related to the ability of the compounds contained in the extract to penetrate the EPS matrix layer that envelops the bacteria and destroys the EPS matrix in the biofilm. Microcolonies that have formed will develop and undergo maturation. maturation is influenced by electrolyte concentration, carbon source, pH, surface type, temperature, and osmolarity. Maturation is characterized by an increase in biofilm density and complexity (Kumar *et al.,* 2017).

CONCLUSIONS

Based on the results of the research that has been done, it can be concluded that the ethanol extract of clove leaves (*S. aromaticum*) has an antibiofilm effect on the maturation stage of *C. albicans* significantly. At the highest concentration, 20% was able to eradicate 93.67%. the Minimum Biofilm Eradication Concentration ($MBEC_{50}$) in eradicating of 50% *C. albicans* biofilm is 0.45%. Thus, clove leaf ethanol extract has the potential to be an alternative therapy for *C. albicans* infection.

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***Competing Interests*:** The authors declare that there are no competing interests.

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