

ERADICATION EFFECT OF JAVANESE TURMERIC (*CURCUMA XANTHORRHIZA. ROXB.*) EXTRACT ON THE EARLY PHASE OF *CANDIDA ALBICANS* BIOFILM

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ABSTRACT

Objectives: To analyze the effect of Javanese turmeric extract on eradicating the initial phase of *C. albicans* biofilm formation.

Methods: A 100 µl of *C. albicans* suspension 10^{-4} was cultured on a 96 - well plate, incubated for 1.5 and 3 h at 37° C and then aspirated and washed by phosphate buffer saline before exposure to 100 µl of 5%, 10%, 15%, 20% 25%, 30%, and 35% Javanese turmeric extract for 24 h. The positive control was 100 µl of nystatin 100,000 IU.

Results: The eradication effect of 35% Javanese turmeric extract at the two stages of initial phase biofilm was comparable with those of nystatin. The viability of *C. albicans* exposed to the extract at an advanced stage of the early phase of biofilm formation tended to be higher than that at the early stage, but the difference was not significant.

Conclusion: The *in vitro* antifungal effect of Javanese turmeric extract against *C. albicans* could be effective in eradicating yeast in the early phase of biofilm formation.

Keywords: *Candida albicans* biofilm, Javanese turmeric extract, *Curcuma xanthorrhiza* Roxb, Viability.

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INTRODUCTION

Candida albicans is a commensal microorganism, which, in planctonic form, is commonly present in a healthy oral cavity without causing any inflammation or pathological condition in the surrounding tissues [1]. The planctonic form of *C. albicans* does not adhere to the adjacent tissue surface. However, any environmental changes that lead to increased growth and development of the yeast could alter its virulence factors. The alteration would result in the development of the yeast from a commensal microorganism to an opportunistic pathogen [2]. *C. albicans* is known to be responsible for oral candidiasis, which is suffered mainly by subjects with weakened immune systems, such as HIV/AIDS patients, the elderly, and denture wearers with bad oral hygiene [3]. About 74% of fungal infections in the oral cavity are caused by *C. albicans* [4].

The key virulent characteristic of *C. albicans* is its ability to develop oral biofilm so that it becomes more resistant to antifungal agents [5]. *C. albicans* develops into an oral biofilm in three phases: Early, intermediate, and maturation [6]. The early phase of biofilm formation is initiated by the adhesion of the yeast to the surface of oral tissues or dentures, where it forms colonies, which is followed by the proliferation of yeast cells. In the intermediate phase, the yeast cells undergo a morphological change from yeast cells to filamentous cells, which indicate the changes from a commensal microorganism to a pathogen. As filamentous cells, *C. albicans* might penetrate the epithelial tissue and increase its resistance to the function of the host immune system [7,8]. The last phase of biofilm formation is maturation, which occurs by extracellular matrix accumulation and increased resistance to antifungal agents [7]. Compared to its planctonic form, the biofilm form of the microorganism has increased stability and resistance to threatening environments, such as the limited availability of nutrition and the presence of antimicroorganism agents [9].

The adhesion and proliferation stages that occur in the early phase of *C. albicans* biofilm formation are important initiation stages that allow

the next phases of biofilm formation to occur. The adhesion of yeast cells on the surface of host tissues, which is the first stage of biofilm formation, occurs in 1–2 h. At 3 h, the yeast starts to proliferate and form colonies. At 6–11 h, *C. albicans* develops a complex structure with hyphal formation [7]. The common clinical procedure in the treatment of oral candidiasis is to apply a topical antifungal agent such as nystatin. The molecular binding between nystatin and the sterol component of the *C. albicans* cell membrane could lead to the instability of the membrane, which in turn would result in ion leakage and cell lysis [10]. Nystatin at a dose of 100,000 IU is commonly applied topically in treating oral candidiasis and eliminating any *Candida* species involved in denture stomatitis [11]. Indonesian communities have a long tradition of using herbs as medicine. However, the development of herbal medicine in using an appropriate scientific procedure began only in the past decade. One of the nine herbal plants that are intensively studied by the Indonesian Ministry of Health is Javanese turmeric (*Curcuma xanthorrhiza* Roxb.) [12]. The root of Javanese turmeric is known to have various medicinal effects, which have been reported to have anti-inflammation, antioxidant, antimicrobe, and antifungal effects [13]. Javanese turmeric root is comprised various components that have medicinal effects, including volatile oil, curcumin, starch, protein, cellulose fat, and minerals. Chemical analysis has shown that the main substances in Javanese turmeric are starch, fibers, volatile oil as phelandren, camphor, tumerol, sineol, borneol, xanthorrhizol, and curcuminoid as curcumine, and desmetoksicurcumin [14]. Among these components, the active substance is xanthorrhizol which is contained in the volatile oil. Xanthorrhizol had been known to have antimicrobial and antifungal effects [15,16] Xanthorrhizol is a colorless, non-aromatic, and non-volatile compound that has been reported to be capable of inhibiting the growth of planctonic *C. albicans* [17]. However, previous studies reported that the Javanese turmeric root even in the form of an extract or volatile oil had antifungal effects against planctonic *C. albicans* [18].

The aim of this study was to investigate the effects of Javanese turmeric root extract on eradicating the early phase of *C. albicans* biofilm.

MATERIALS AND METHODS

Determination of *C. albicans* suspension concentration

The sample used in this study was laboratory strain *C. albicans* ATCC 10231, which was obtained from the Oral Biology Laboratory in the Faculty of Dentistry at Universitas Indonesia. The yeast was cultured in Sabouraud dextrose agar and incubated at 37°C for 48 h. The cultured yeast was then diluted in 1 ml Sabouraud dextrose broth and homogenized for 20 s to obtain the master suspension of *C. albicans*. A serial dilution of the master suspension of *C. albicans* was conducted to obtain 10⁻², 10⁻⁴, 10⁻⁶, and 10⁻⁸ suspensions by adding PBS. Then, *C. albicans* was cultured on Sabouraud dextrose agar (SDA) which was made from each concentration of *C. albicans* suspension in Duplo (37°C for 72 h). Based on the observation of colony formation in the 72-h *C. albicans* cultures (the number of colonies that could still be counted accurately), it was determined that the suspension used in this study was a concentration of 10⁻⁴.

Handling of Javanese turmeric extract

Javanese turmeric root extract was processed by the Research Center of Tropical Herbal Plants (BALITTRO) in Bogor, Indonesia. The Javanese turmeric root was extracted by the maceration technique using 96% ethanol. In this study, 500 ml of extract was derived from 6 kg of Javanese turmeric root, which then was centrifuged at 1300 rpm for 20 min so that in the resulting extract the pellets were separated from the supernatant. The supernatant, which contained 41.78% xanthorrhizol, was then used in this study and diluted in 10% dimethyl sulfoxide (DMSO).

A previous test confirmed that 10% DMSO did not significantly influence the growth of planctonic *C. albicans*. To obtain the concentrations of Javanese turmeric root extract that would be analyzed in this study, the diluted supernatant of the extract was then further diluted by adding 10% DMSO.

Minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC)

The concentrations of Javanese turmeric extract that were used in this study were 0.25%, 0.5%, 1%, 5%, 10%, 15%, 20%, and 25%. The positive control was exposed to 100 µl of 10,000 IU of nystatin, and the negative control was exposed to SDB. Into each well of 96-well plate, 100 µl of the 10⁻⁴ *C. albicans* suspension was placed, to which was added 100 µl of various concentrations of the extract, nystatin, or SDB. As a standard of the extract color, a blank group was made by filling each well with 100 µl SDB and 100 µl of the same various concentrations of the extract or nystatin but without the *C. albicans* suspension.

The 96-well plate was then homogenized in an orbital shaker and incubated in anerobic condition for 48 h. The optical density (OD) was read by a microplate reader at a wavelength of 450 nm. The MIC was calculated by the following formula:

$$\%MIC = 1 - \left(\frac{OD_{\text{sample}} - OD_{\text{blank sample}}}{OD_{\text{negative control}} - OD_{\text{blank negative control}}} \times 100\% \right)$$

In this study, the result of more than 90% was considered as the MIC. To determine the MFC, the samples that had been exposed to the Javanese turmeric extract started in the MIC concentration were inoculated on an SDA medium and incubated at 37°C for 72 h. The experiment was conducted in Duplo. The lowest concentration of Javanese turmeric extract on which *Candida* culture showed no colony formation was considered as the MFC.

C. albicans biofilm formation and exposure of Javanese turmeric root extract

In this experiment, two concentrations of Javanese turmeric extract below the MIC up to two concentrations above the MFC were used: 1%, 5%, 10%, 15%, 20%, 25%, 30%, and 35%. The *C. albicans* biofilm was grown by dripping 100 µl of the 10⁻⁴ *C. albicans* suspension on to the surface of a 96-well plate, which was then incubated at 37°C in an aerobic environment for 1.5 and 3 h. Before exposure to the extract, the wells were aspirated and washed by PBS 3 times to free the wells from the planctonic *C. albicans*. Each experimental well was exposed to 100 µl of various concentrations of Javanese turmeric extract. The positive control wells were exposed to 100 µl of 10,000 IU nystatin, and the negative control wells were exposed to 100 µl SDB and incubated at 37°C for 24 h. The experiment was conducted in Duplo.

Viability test analysis of *C. albicans* biofilm

The viability of *C. albicans* in the early phase of the biofilm formation was assessed by the methylthiazol tetrazolium (MTT) assay. Into each well of *C. albicans* biofilm, 10 µl of 5 mg/ml MTT solution was added and then incubated for 2 h at 37°C in an aerobic condition. Then, 100 µl of acidified isopropanol was added into each well, and the 96-well plate was placed in an orbital shaker for 1 h. The OD was read by microplate reader at a wavelength of 490 nm.

Data Analysis

The data collected about the viability of *C. albicans* biofilm were analyzed using a two-way ANOVA followed by a *post hoc* Tukey test. The correlations between the variables were tested using Pearson's correlation coefficient.

RESULTS

The results of determining the MIC of the Javanese turmeric extract are shown in Table 1.

The minimum concentration that inhibited the growth of *C. albicans* equal to or more than 90% was determined as the MIC.

Therefore, as shown in Table 1, the MIC of the Javanese turmeric extract against planctonic *C. albicans* was 10%. The MFC was determined based on the minimum concentration that resulted in no colony formation of *C. albicans* cultured on the SDA medium.

As shown in Table 2, the MFC of the Javanese turmeric extract against planctonic *C. albicans* was 25%.

The results of the MTT assay regarding the OD of *C. albicans* biofilm both with and without various concentrations of Javanese turmeric extract exposure were then converted into the percentage of viability. The results showed trends of the decreased viability of *C. albicans*

Table 1: The MIC of Javanese turmeric extract against planctonic *C. albicans*

No.	Javanese turmeric extract concentration (%)	Inhibition (%)		X
		1	2	
1	0.25	74.09	94.41	84.25
2	0.5	90.38	68.93	79.66
3	1	89.57	82.35	85.96
4	5	90.74	61.88	76.31
5	10	95.90	92.22	94.06
6	15	81.88	73.11	77.49
7	20	100	68.77	84.39
8	25	100	100	100
9	Positive control	100	100	100
10	Negative control	0	0	0

MIC: Minimum inhibition concentration, *C. albicans*: *Candida albicans*

biofilm along with the increased concentration of the Javanese turmeric extract. It could be seen on Table 3 that at 1.5 h, which is considered as the adhesion stage in the early phase of the formation of *C. albicans* biofilm, the viability of the fungi decreased significantly compared to the negative control even in those exposed to 1% Javanese turmeric extract.

However, the 69,013% *C. albicans* viability in those exposed to 1% extract was also significantly different from those in the positive control at 13,5204%. This trend continued in *C. albicans* culture groups exposed to higher concentrations of Javanese extract up to 25%.

A nonsignificant difference of *C. albicans* viability was shown between those exposed to 35% extract (17,728%) and the positive control (13,5204%) with $p > 0.05$. The result of the Pearson correlation analysis showed -0.959 , indicating an inverse strong correlation between *C. albicans* viability and Javanese turmeric extract concentration. The higher the extract concentration was the lower the viability of the *C. albicans* biofilm.

Table 4 shows that at 3 h, which is considered the beginning of the proliferation stage of *C. albicans* yeast cells, a trend similar to the results shown in Table 1 was observed. All *C. albicans* biofilm exposed to 1–30% Javanese turmeric extract had significantly different viability compared to both the negative and positive controls.

Table 2: The MFC of Javanese turmeric extract against planktonic *C. albicans*

No.	Javanese turmeric extract concentration (%)	Colony formation unit		
		1	2	X
1	5	79	497	288
2	10	25	105	65
3	15	31	282	156.5
4	20	0	31	15.5
5	25	0	0	0
6	Positive control	0	0	0
7	Negative control	∞	∞	∞

MFC: Minimum fungicidal concentration, *C. albicans*: *Candida albicans*

Table 3: The viability of *C. albicans* at 1.5 h in biofilm exposed to various concentrations of Javanese turmeric extract

Extract concentration (%)	Mean of viability (% ± SD)	p value to positive control	p value to negative control
1	69.9131±2.01452	0.000	0.000
5	74.2896±2.13688	0.000	0.000
10	56.982±1.78775	0.000	0.000
15	53.7146±0.78927	0.000	0.000
20	52.8825±1.40054	0.000	0.000
25	37.056±0.60285	0.000	0.000
30	20.1203±2.42488	0.002	0.000
35	17.728±2.99728	0.237	0.000
Positive control	13.5204±2.59803	-	0.000
Negative control	100±0	0.000	-

C. albicans: *Candida albicans*, SD: Standard deviation

Table 4: Viability of *C. albicans* biofilm at 3 h of exposure to various concentrations of Javanese turmeric extract

Extract concentration (%)	Mean of viability (%±SD)	p value to positive control	p value to negative control
1	77.4401±0.49371	0.000	0.000
5	73.8000±1.62356	0.000	0.000
10	59.5841±2.01595	0.000	0.000
15	56.0247±1.18029	0.000	0.000
20	39.3691±1.72952	0.000	0.000
25	41.2655±0.48739	0.000	0.000
30	20.1693±2.87002	0.002	0.000
35	17.0322±0.72569	0.342	0.000
Kontrol positif	14.0497±1.27843	-	0.000
Kontrol negatif	100±0	0.000	-

C. albicans: *Candida albicans*, SD: Standard deviation

However, in the exposure to 35% extract, the viability of *C. albicans* biofilm did not differ from the viability of the positive control. Similar to the results of the analysis of *C. albicans* biofilm at 1.5 h, the results of Pearson's correlation analysis of the data *C. albicans* biofilm at 3 h was -0.981 , which showed an inverse strong correlation between *C. albicans* viability and the concentration of the Javanese turmeric extract. The higher the extract concentration was the lower the viability of the *C. albicans* biofilm.

DISCUSSION

In this study, SDA and SDB were chosen as the medium for growing *C. albicans* because they are the standard medium of fungi, and they contain peptone and dextrose, which support the growth of yeast. In addition, the medium has a low pH, which is suitable for growing fungi and preventing bacterial contamination [18].

The MIC and MFC of Javanese turmeric extract against planktonic *C. albicans* shown by this study were 10% and 25%, respectively. These results aligned with previous studies that examined the efficacy of Javanese turmeric extract against *C. albicans*. xanthorrhizol, which is the active component in the volatile oil found in Javanese turmeric extract, was reported to have an MIC of 2.5–15 mg/L and an MFC of 2.5–15 mg/L [17].

In this study, the results of the MTT assay showed that Javanese turmeric extract had a significant effect on reducing the viability of *C. albicans* in the early stages of biofilm formation. The results of the ANOVA analysis confirmed that even in a 1% concentration, the Javanese turmeric extract exposed to *C. albicans* biofilm at both 1.5 and 3 h reduced the viability of the yeast to a significantly lower percentage compared to the negative control. However, although at concentrations of 1–30%, exposure to the Javanese turmeric extract led to the decreased viability of *C. albicans* biofilm, nonetheless, it was still significantly different from the viability of the positive control, which was exposed to nystatin. A similar efficacy of Javanese turmeric extract to nystatin in reducing the viability of *C. albicans* biofilm could be seen in 35% concentration.

The results of this study also showed the same pattern in the adhesion and proliferation stages of the early phase of *C. albicans* biofilm, that

is, the higher the concentration of the extract, the lower the viability of *C. albicans* in the biofilm. The dose-dependent effect of Javanese turmeric extract in reducing the viability of *C. albicans* biofilm might be due to the higher concentration of xanthorrhizol in the higher concentration of the extract, which then led to a stronger, destructive effect on the cell membranes of the fungi. Because xanthorrhizol contains (-OH), it penetrated the biofilm and bound to the *C. albicans* cell membrane, which caused cell degradation [12]. In this study, the comparison of MIC and MFC with the eradication effect of the extract showed that against the planktonic *C. albicans*, the extract inhibited the growth of *C. albicans* at 10%, and it was able to stop the fungi growth at 25%. However, against *C. albicans* in the early phase of biofilm formation, at a concentration of 35%, the extract was capable of reducing 90% of the yeast's viability, but it was not able to eradicate it completely. A higher concentration of the extract was required to inhibit the growth of *-icans* in its early phase of biofilm formation than in the planktonic form. This result might have been due to the limited activity of the xanthorrhizol contained in the extract, which could only erode the superficial part of the biofilm. Therefore, the affected *C. albicans*, which then underwent cell lysis, colonized only the superficial part of the biofilm. The lysis yeast cells on the superficial part of the biofilm then degraded from the biofilm and reduced the percentage of the viability of the living cells in the biofilm [19]. The adhesion stage is important in initiating the formation of biofilm and inducing the growth of the yeast by proliferating [6]. The ability of the yeast to adhere to the host tissues and denture surface was supported by the activity of the adhesion protein in the *C. albicans* cell membrane [1,8].

The results of this study showed that the eradication effect of Javanese turmeric extract against *C. albicans* in the adhesion phase and the proliferation phase was both at a concentration of 35%. This result indicates that the resistance of the yeast from the adhesion stage (at 1–2 h) to the proliferation stage (at 3–6 h) in the early phase of biofilm formation remained the same. The proliferation of *C. albicans* cells did not affect their resistance to the surrounding environment. Another possibility is that at 3 h, the yeast had just begun the proliferation stage, which continued to 11 h [20]. Thus, at 3 h, there were no significant changes from the condition at 1, 5 h in the condition of the biofilm.

CONCLUSION

Javanese turmeric extract has an inhibiting effect against *C. albicans* both in planktonic form and in the early phase of biofilm formation. The antifungal effect of Javanese turmeric extract against planktonic *C. albicans* is stronger than its eradication effect against *C. albicans* biofilm. At a concentration of 35%, Javanese turmeric extract reduced 90% of the viability of *C. albicans* in the adhesion and proliferation stages of the early phase of biofilm formation. No differences were found in the eradication effects of Javanese turmeric extract between the adhesion and proliferation stages of the early phase the formation of *C. albicans* biofilm.

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