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Research Article

THE POTENCY OF JAVANESE TURMERIC (*CURCUMA XANTHORRHIZA* ROXB.) ETHANOL EXTRACT TO ERADICATE WILD STRAIN *CANDIDA ALBICANS* BIOFILM

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ABSTRACT

Objective: The pathogenic yeast *Candida albicans* forms biofilm to increase its resistance toward antifungal agents. Javanese turmeric is an Indonesian medicinal plant reported to have antifungal effects due to the active component, xanthorrhizol. The objective of this study was to measure the *in vitro* potential of Javanese turmeric ethanol extract to eradicate *C. albicans* biofilm.

Methods: *C. albicans* was exposed to Javanese turmeric ethanol extract for 1 h during biofilm formation phases. MTT assay was used to test the percentage of biofilm eradication.

Results: The minimum inhibitory concentration and minimum fungicidal concentration of Javanese turmeric ethanol extract against planktonic *C. albicans* were 15%. The minimum biofilm eradication concentration ($MBEC_{50}$) was 25% in the early phase and 15% in the intermediate and maturation phases.

Conclusions: Javanese turmeric ethanol extract is effective at eradicating clinical isolate of C. albicans biofilm.

Keywords: biofilm, *Candida albicans* clinical isolate, Javanese turmeric ethanol extract, MTT assay, Percentage of eradication.

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INTRODUCTION

Oral candidiasis is an opportunistic infection caused by *Candida albicans* fungal infection. *C. albicans* is part of the normal oral flora and normally lives commensally within its host, but the changes in the oral cavity environment can cause *C. albicans* to become pathogenic. Changes in *C. albicans* characteristics can be caused by predisposing factors in the host, such as use of broad-spectrum antibiotics or corticosteroids, systemic conditions such as HIV/AIDS and diabetes mellitus, malnutrition, removable dental appliances, and decreases in saliva flow [1].

Alternatively, some virulence factors of *C. albicans* can increase its pathogenic characteristics. One of these is the ability to adhere and form biofilms in host tissue. The biofilm formation process comprises three development phases, namely, the early phase (0–11 h), intermediate phase (12–30 h), and maturation phase (38–72 h). For planktonic *C. albicans*, the adhesion process to the substrate occurs for 0–2 h and then proceeds to proliferation and biofilm formation. During the intermediate phase, the biofilm structures consist of yeast, germ tubes, and young hypha with an extracellular matrix. During the maturation phase, the extracellular matrix expands; the incubation time increases; and increasing yeast, pseudohypha, and hypha are planted in the matrix [2].

Biofilm development in *C. albicans* is also associated with increased resistance to antifungal drugs. The biofilm resistance mechanism is unknown, but likely results from the extracellular matrix that inhibits antifungal drug penetration, changes in sterol membrane composition during growth that cause phenotypic changes, and increased expression of drug efflux pump protein [2,3].

Standard therapy for oral candidiasis is 100,000 IU nystatin oral suspension. The antifungal binds to ergosterol in *C. albicans* cell membranes, causing cell membrane leakage and cell death [4,5]. The minimum inhibitory concentration (MIC) of nystatin for treating *C. albicans* was 0.615 mg/mL [6].

Medicinal plants may have similar properties to synthetic drugs, but are less expensive, easier to obtain, and have fewer side effects. One medicinal plant known to have antifungal effects is Javanese turmeric (*Curcuma zanthorrhiza* Roxb.). The rhizomes of Javanese turmeric contain protein, starch, yellow curcuminoid substance, and essential oils. One of the active substances found only in Javanese turmeric essential oil is xanthorrhizol [7-9].

Previous *in vitro* research found that xanthorrhizol isolates can inhibit *C. albicans* growth and kill its clinical isolates at MIC 1–5 and 5–10 mg/L of minimum fungicidal concentration (MFC) [10]. The ability of xanthorrhizol to inhibit growth and kill *C. albicans* was suspected because it has a hydroxide group (−OH) similar to nystatin and may, thus, have similar antifungal action as the synthetic drug [11].

Antimicrobial drugs may be bacteriostatic or bactericidal. Bacteriostatic drugs produce inhibitory effects, whereas bactericidal drugs have eradication effects on microorganisms [12]. The antifungal effects of Javanese turmeric have been studied at the Faculty of Dentistry, University of Indonesia, since 2009. A previous study noted that the MIC and MFC of Javanese turmeric ethanol extract against *C. albicans* ATCC 10231 were 5–30 and >30 mg/L, respectively [11]. Another study found that Javanese turmeric ethanol extract could inhibit phospholipase enzyme activity in *C. albicans* at a concentration of 2.5 mg/L [13]. Recent studies showed that Javanese turmeric ethanol extract with a xanthorrhizol content of 41.78% had an inhibitory effect and decreased *C. albicans* ATCC 10231 biofilm viability during the adhesion, proliferation, filamentation, and maturation phases at 35% test concentration [14,15].

Despite these findings, the questions remain whether Javanese turmeric ethanol extract has inhibitory or lethal effects on clinical isolates of

planktonic *C. albicans in vitro*, and if the effects are greatest during the early, immediate, or maturation phase of biofilm production. This research aimed to answer these questions.

METHODS

In this *in vitro* laboratory experiment, clinical isolates of *C. albicans* were exposed to Javanese turmeric ethanol extract at various concentrations. The samples of *C. albicans* used in the experiments were obtained from tongue swabs from patients aged 56–65 with removable dental prostheses. Participants with a history of oral antifungal treatment were excluded. Samples of *C. albicans* were obtained from individuals who met the criteria exposed to 100,000 IU nystatin as a positive control and from individuals who met the criteria not exposed to Javanese turmeric ethanol extract or nystatin as a negative control. All participants provided informed consent.

Equipment used in this research included sterile cotton buds, CHROMagar, microscope slides and glass objects, autoclave, incubator, vortex mixer, centrifuge, 15 mL centrifugation tubes, water bath, refrigerator at 4°C, scales, Petri dishes, 1.5 mL Eppendorf tubes, test tubes, Erlenmeyer flasks, measuring cups, coil, aluminum foil, orbital shaker, cotton, jars, glass bottles, Bunsen burner, and microplate reader. *C. albicans* specimens were maintained using CHROMagar, fetal bovine serum (FBS), Sabouraud dextrose agar (SDA) as a solid-growing medium, Sabouraud dextrose broth (SDB) as a liquid-growing medium, phosphate-buffered saline (PBS), 70% alcohol, sterile distillate water, MTT, and acidified isopropanol. Javanese turmeric ethanol extract was obtained from BALITTRO (Balai Penelitian Tanaman Obat dan Aromatik), Bogor. Nystatin (100,000 IU) was used as the positive control.

Sampling began with the preparation of sterile cotton buds in test tubes labeled with an ID and date of sampling. Sampling was performed by swabbing the dorsum of the patient's tongue in one direction with slight pressure without injuring the tissue. The cotton bud was then inserted into a container containing 1 mL of PBS and taken to the laboratory for identification.

Divortex was added to the sample for 20 s. As much as 10 µL of the sample was then inoculated to CHROMagar medium and incubated at 37°C for 48 h. After 48 h, the colonies were formed. *C. albicans* ATCC 10231 stock from the Oral Biology Laboratory of FKG UI received the same treatment for confirmation. For identification, a small colony of *C. albicans* was taken from CHROMagar, mixed with 10 µL FBS, and covered with a glass cap. The preparation was incubated for 2 h at 37°C. After 2 h, the preparation was observed under a light microscope at 100X and then confirmed by 450X magnification to observe germ tube formation.

Positively identified *C. albicans* were then bred in a Petri dish containing SDA. Colony was taken a bit and then distributed onto the Petri dish and closed again. Petri dishes were incubated at 37°C incubators for 72 h. For the preparation of the *C. albicans* main solution, sterile Eppendorf tubes were prepared and filled with 1 mL SDB. The Petri dish containing *C. albicans* was removed from the incubator, retrieved using an oasis needle, heated until red, and injected into Eppendorf tubes. Retrieval was repeated three times. The Eppendorf tube was homogenized with a vortex mixer for 20 s. The concentration of *C. albicans* was determined by making dilutions of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}; culturing in SDA media; and calculating the number of colonies (CFU/10 µL).

Incubation under aerobic conditions and 37°C was the optimal condition for the growth of C. albicans and suitable with oral cavity as well as triggering the growth of hyphae [2]. The medium used was SDA and SDB which is a standard medium for growth of *C. albicans* because it contains glucose and acidic pH, so it can accelerate the growth of *C. albicans* [16].

Javanese turmeric ethanol extract was centrifuged at 1300 rpm for 20 min to form three layers. The top layer was considered to be 100% and was diluted to concentrations (% V/V) of 0.5%, 1%, 10%, 15%, 25%, 30%, 35%, and 40% using SDB. The dilutions were made using the formula $C_1 \times V_1 = C_2 \times V_2$, where C_1 and C_2 are the first and final ethanol extract concentrations, and V_1 and V_2 are the first and last ethanol extract volumes. The mixtures were homogenized using a vortex mixer for 20 s and stored in a refrigerator at 4°C until use.

For the determination of MIC and MFC, well plates were filled with up to 100 µL of a suspension of *C. albicans* at a concentration of 10^{-4}. Various concentrations of Javanese turmeric ethanol extract were added to the treatment groups, nystatin (100,000 IU) was added to the positive controls, and the negative controls contained *C. albicans* only. A blank was also prepared with SDB and Javanese turmeric ethanol extract. The well plate was then homogenized using an orbital shaker. Next, optical density (OD) was determined using a microplate reader at 450 nm wavelength. To determine the effect of Javanese turmeric ethanol extract on *C. albicans*, the inhibition percentage was calculated using the formula:

$$\%\text{inhibition} = \left(1 - \left[\frac{\text{OD sample} - \text{OD blank sample}}{\text{OD negative control} - \text{OD blank negative control}}\right]\right) \times 100\%$$

The MIC was determined as the lowest concentration of Javanese turmeric ethanol extract that achieved 90% or greater inhibition. The suspension was then inoculated to SDA media and incubated for 48 h. The MFC was determined as the lowest concentration of Javanese turmeric ethanol extract that did not show growth of *C. albicans* on the SDA.

To determine eradication of the biofilm of *C. albicans* by Javanese turmeric ethanol extract, biofilm was first prepared using 100 µL of *C. albicans* suspension incubated at different durations, that is, early phase (6 h), intermediate phase (24 h), and maturation phase (48 h). Biofilm that formed on the well base was rinsed with 100 µL PBS solution to remove planktonic *C. albicans*. Next, 100 µL of Javanese turmeric ethanol extract at various concentrations was added. In the positive control group, 100 µL of nystatin was added, whereas in the negative control group, the biofilm was not treated. The well plate was incubated for 60 min at 37°C and then rinsed once with 100 µL PBS solution. Next, 10 µL of 5 mg/mL MTT solution was added to each well, and the well plate was incubated for 3 h at 37°C. A further 100 µL of acidified isopropanol was added to each well. The well plate was placed on an 80 rpm shaker at room temperature for 1 h. The well plate was then read using a microplate reader at 570 nm. Biofilm eradication was expressed as a percent against the control using the formula:

$$\left(1 - \left[\frac{\text{OD sample} - \text{OD blank sample}}{\text{OD control} - \text{OD blank control}}\right]\right) \times 100\%$$

The minimal biofilm eradication concentration was determined as the concentration that reached 50% ($MBEC_{50}$) or 90% ($MBEC_{90}$). Statistical analysis was conducted to determine whether there was a difference in the percentage eradication between the positive control and treatment groups. The percentage eradication was analyzed using the analysis of variance (ANOVA) test with *post hoc* comparisons. Correlation tests were used to assess the relationship between Javanese turmeric ethanol extract concentration and biofilm percentage eradication. $p<0.05$ were considered statistically significant.

RESULTS

The presence of *C. albicans* on patients' tongue swabs was confirmed by CHROMagar and the germ tube formation test. The results showed germ tube formation in clinical isolates after incubation at 37°C for 2 h (Figs. 1 and 2). A concentration of 10^{-4} was determined to be sufficient for evaluating growth differences between the treatment and control groups.

MIC and MFC tests for Javanese turmeric ethanol extract against *C. albicans* were performed. Microplate reader data were converted to percentage inhibition, as presented in Table 1. A concentration

Table 1: Minimum inhibitory concentration of Javanese turmeric ethanol extract on clinical isolate of planktonic *Candida albicans*

Number	Concentration of Javanese turmeric extract (%)	Inhibition (%)		
		I	II	X
1	1	88.48	88.31	88.40
2	5	80.87	79.45	80.16
3	10	82.16	86.89	84.53
4	15*	92.73	96.99	94.86
5	20	55.15	60.85	58.00
6	25	61.39	68.91	65.15
7	30	95.50	89.64	92.57
8	35	95.50	93.53	94.52
9	40	96.88	96.37	96.63
10	45	96.28	94.95	95.61
11	Positive control	94.03	95.48	94.75
12	Negative control	0	0	0

Table 2: Minimum fungicidal concentration of Javanese turmeric ethanol extract on clinical isolates of planktonic *Candida albicans*

Number	Concentration of Javanese turmeric ethanol extract (%)	Number of colonies		
		I	II	X
1	15	0	0	0
2	20	101	5	53
3	25	3	9	6
4	30	0	0	0
5	35	0	0	0
6	40	0	0	0
7	45	0	0	0
8	Positive control	0	0	0
9	Negative control	∞	∞	∞

of 15% was determined to be the MIC. On the basis of the number of colonies present (Table 2), 15% was determined to be the MFC against planktonic *C. albicans*.

On the basis of the OD converted to eradication percentage, all concentrations of Javanese turmeric ethanol extract eradicated the middle phase biofilm of *C. albicans* (24 h) at an eradication percentage >50%. In the early phase (6 h) and maturation phase (48 h), an eradication percentage >50% was found for concentrations of Javanese turmeric ethanol extract of 25%, 30%, and 35% and 15%, 20%, 30%, 35%, and 40%, respectively. Thus, the $MBEC_{50}$ of Javanese turmeric ethanol extract on biofilm *C. albicans* was 25% at 6 h and 15% at 24 and 48 h. In this study, the $MBEC_{90}$ at 6, 24, and 48 h could not be established. There was no significant difference ($p>0.05$, ANOVA) between the eradication percentage in the positive control and treatment groups at 6 h (15%, 25%, 30%, and 35% concentration), 24 h (15%–40% concentration), or 48 h (40% concentration).

The correlation test showed a strong positive correlation ($p>0.5$) between increasing Javanese turmeric ethanol extract concentration and eradication percentage of biofilm at 6 and 24 h with p values of 0.626 and 0.566, respectively. At 48 h, there was a moderate positive correlation ($0<p<0.5$) with a p value of 0.381. This result indicates that the percentage of biofilm eradication at various phases increased along with increasing Javanese turmeric ethanol extract concentration but, as the biofilm incubation time increased, the eradication ability decreased.

DISCUSSION

Clinical isolates of *C. albicans* used in this study were derived from tongue swabs of elderly patients with removable denture, which

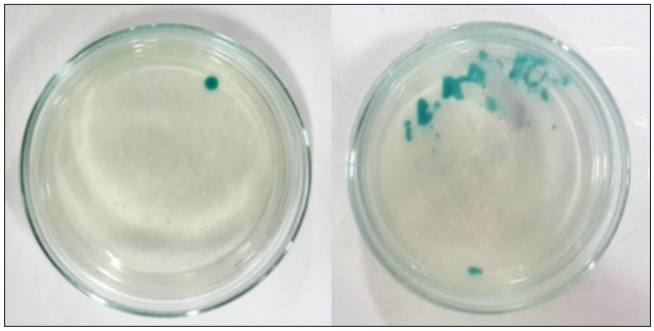

Fig. 1: Breeding results of *Candida albicans* (left) and ATCC 10231 control (right) on CHROMagar (48 h) showing green colonies

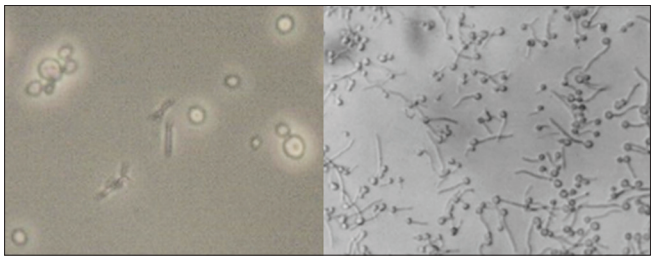

Fig. 2: Results of germ tube formation of *Candida albicans* clinical samples after 2 h. Exposure at ×40 magnification (left) and germ tube formation for reference (right)

are risk factors for oral candidiasis. Identification of *C. albicans* was performed by CHROMagar because this method is fast, accurate, easy, and inexpensive. Confirmation of *C. albicans* species was performed by germ tube formation tests, because *C. albicans* incubated in serum produce germ tubes visible by light microscopy [17,18].

The Javanese turmeric plant in this study was processed into extract to obtain the nutritious biological material as well as the general form of the dosage. Javanese turmeric ethanol extract was the result of *Curcuma zanthorrhiza* Roxb extraction by maceration method with stirring using 96% ethanol solvent. This method was chosen because it reduces the possibility of component degradation that occurs because of high temperatures and because it is faster [19]. Ethanol solvent was chosen because it has a high polarity for removing active substances and is nontoxic [20]. Using this method, 3 kg of Javanese turmeric plant produced liquid extract of 350.9 g and viscous extract of 139.4 g. The liquid extract consists of two layers of solid black and dark brown, whereas the viscous extract has a consistency of clay, is hard to stir, and a brownish-yellow color. Despite using the same method and solvent, the Javanese turmeric extract ethanol used in our study had an organoleptic difference from that used in research by Lewiyonah, which was reported to be a dark brown liquid extract with a distinctive aroma of Javanese turmeric that did not separate [14].

Xanthorrhizol levels in the top layer of centrifuged Javanese turmeric ethanol extract also differed between our research and that of Lewiyonah, at 9.38% in our study and 41.78% in Lewiyonah's [14]. This is assumed to be due to the organoleptic differences, where the active substances settled in the viscous extract and did not mix in the liquid extract in our study.

Different solvents were used in this study and Lewiyonah's, namely, SDB and DMSO [14]. DMSO is known to increase the effectiveness of xanthorrhizol in inhibiting the development of *C. albicans.* The solvent used should not affect the effectiveness of Javanese turmeric ethanol extract [20].

Javanese turmeric ethanol is thought to inhibit the growth of *C. albicans* through the active substance xanthorrhizol. Xanthorrhizol

is a phenol with a hydroxide group (−OH) similar to the drug nystatin. The hydroxide group present in nystatin can lead to destruction of the plasma membrane of *C. albicans* cells by binding to ergosterol and oxidizing the lipid layer of the cell plasma membrane. This causes leakage of potassium ions (K+) out of the cells and ultimately cell death [5]. Xanthorrhizol is assumed to have a similar effect on *C. albicans* cell membranes.

Our study showed that Javanese turmeric ethanol extract is effective in inhibiting growth and killing *C. albicans* at MIC and MFC 15%. Another study reported that Javanese turmeric ethanol extract was equally effective in inhibiting growth and killing *C. albicans* strain ATCC 10231 at MIC and MFC 20% [21]. Both of these studies reported a similar trend where the percentage of inhibition increases with increasing concentration of Javanese turmeric ethanol extract. However, at some concentrations, the results are inconsistent, suggesting that the percentage of inhibition is not dose dependent. This may occur because the Javanese turmeric ethanol extract also contains starch, fiber, and curcumin [22].

The MIC and MFC values obtained in this study for clinical isolates of *C. albicans* were lower than those for ATCC strains, indicating that clinical isolates are more susceptible to the antifungal activity of Javanese turmeric ethanol extract. We used clinical isolates because it was assumed that the characteristics of *C. albicans* could change in response to differences in the environmental conditions of individual oral cavities. The higher susceptibility to Javanese turmeric extract in the clinical isolates of *C. albicans* was probably due to the selected patients never receiving antifungal treatment in the oral cavity and the *C. albicans* sample not being pathogenic.

Compared to previous studies by Lewiyonah on the effects of antifungal Javanese turmeric ethanol extract on *C. albicans* ATCC 10231, the MIC was notably lower [14]. This is likely due to differences in xanthorrhizol levels in Javanese turmeric ethanol extract and the use of DMSO as a solvent.

In this study, *C. albicans* biofilm was exposed to Javanese turmeric ethanol extract for 1 h. In a study by Rukayadi (2006), exposure of *Streptococcus mutans* biofilm to xanthorrhizol for 1 h was able to remove as much as 76% of biofilm compared with the control group [10]. Although they were not completely eliminated, bacteria were absent in the adhesion phase and the early accumulation of biofilm development. The same mechanism is assumed to occur between Javanese turmeric ethanol extract and *C. albicans* biofilm as the biofilm structures are similar. In addition, Javanese turmeric ethanol extract at 15 µg/mL concentration is known to cause deformation of *C. albicans* cells after 1 h [23].

A lower concentration of Javanese turmeric ethanol extract was required to eradicate 50% of biofilm in the intermediate and maturation phases compared with the early phase. This is assumed to be due to the dense biofilm at this phase, the symbiotic relationship between the species components, and the increasing competition for resources (space, nutrition, and light). The high population density and limited ability for diffusion of the polymer matrix support the assumption that biofilms can be affected by even small chemical components [24].

In this study, the concentration of Javanese turmeric ethanol extract capable of degrading 90% of *C. albicans* ($MBEC_{90}$) biofilm could not be determined in all three phases. Although there were some eradication percentage values of 100% in the maturation phase (48 h), these values are not representative because nonconformity of the measuring instrument was assumed. Maximum OD values may have occurred because of remnants of the Javanese turmeric ethanol extract on the well walls that affected reduction of the MTT solution. This may also be due to the maturation phase (48 h) when biofilms were more concentrated and had higher OD values, as evidenced by the higher OD values in the negative control with increasing incubation time. Other contamination factors related to microorganisms and pipetting errors may have affected the OD values.

Based on the correlation test, there was a trend of increasing eradication percentage with increasing concentration of Javanese turmeric ethanol extract. However, when the correlation between the phases was compared, there was a decrease in correlation value along with an increase in incubation time, meaning that an increase in biofilm age is expected to decrease the eradication ability of Javanese turmeric ethanol extract.

CONCLUSIONS

Javanese turmeric ethanol extract inhibited the growth of planktonic *C. albicans* with MIC and MFC of 15% and has the potential to eradicate *C. albicans* after 1 h of exposure in all three phases of biofilm formation.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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