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Research Article

EFFECT OF JAVANESE TURMERIC ETHANOL EXTRACT ON THE ERADICATION OF *CANDIDA ALBICANS* BIOFILMS IN EARLY, INTERMEDIATE, AND MATURATION PHASES

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ABSTRACT

Objectives: The aim of this research was to observe the effect of Javanese turmeric ethanol extract (JTEE) on the eradication of *Candida albicans* in various phases of biofilm development.

Methods: *C. albicans* biofilms were exposed to JTEE at a concentration 1–45% for 1 h. Cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the wavelength was read at 570 nm.

Results: The results showed that the 50% minimal biofilm eradication concentration was 30% in the early phase, 20% in the intermediate phase, and 25% in the maturation phase of the biofilm. The eradication percentage increased along with increasing JTEE concentration, but decreased with the age of the biofilm.

Conclusion: We concluded that JTEE has the potential to eradicate *C. albicans* biofilms in various phases of development.

Keywords: Biofilm, *Candida albicans*, Javanese turmeric ethanol extract.

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INTRODUCTION

Candida albicans is a commensal fungus in the oral cavity and is present as about 50% of the oral microflora [1]. However, in certain circumstances, such as during disturbances in the balance of oral microflora or decreased immune defenses, *C. albicans* may turn into an opportunistic pathogen [1]. The oral mucosa provides a good habitat for microorganisms including *Candida* to inhabit because of the availability of favorable environmental conditions for the colonization process [2]. *Candida* infections of the oral mucosa lead to the formation of white plaque lesions commonly known as candidal leukoplakia, but can also form reddish areas, such as in stomatitis, associated with the use of dentures [1].

Oral candidiasis is the most prevalent opportunistic infection of the oral mucosa, and *C. albicans* possesses various virulence factors that contribute to its ability to infect oral tissues [2]. These factors include phenotypic switching, adhesion, morphological dimorphism, and hydrolytic enzyme secretion [2,3].

Biofilms are microbial communities embedded in a polymer matrix that develop after attachment to a surface [4]. They protect microorganisms from host immune cells and antimicrobial agents [5]. *C. albicans* biofilms inhibit the penetration of antibiotics and prevent them from reaching their target cells [5]. The *C. albicans* biofilm formation occurs in several developmental stages: early (0–11 h), intermediate (12–30 h), and maturation (38–72 h) phases [6]. Biofilm extracellular matrix increases overtime to become multilayer consists of yeast cells, pseudohyphae, and hyphae. The hyphal cells play an important role because they can produce hydrolytic enzymes, such as secreted aspartyl proteinase and phospholipases that contribute to host-cell damage. It is also known that antifungal resistance increases with biofilm development, and this is associated with increasing metabolic activity during biofilm development, as revealed by a study using *C. albicans* biofilms with a high resistance to antifungal agents, such as fluconazole and nystatin [6].

Oral candidiasis can be treated locally, for example, using topical antibiotics to remove the white plaques or using nystatin or amphotericin lozenges to manipulate the oral microflora and return the balance [1]. Nystatin is the drug used as the gold standard for oral candidiasis, and fluconazole is an alternative option.

Javanese turmeric (*Curcuma xanthorrhiza* Roxb.) is one of Indonesia's leading medicinal plants and is currently being tested by the Food and Drug Supervisory Agency [7]. One of its active agents is xanthorrhizol [8]. A previous study showed that xanthorrhizol has many medical properties, including being an antimicrobial, anti-inflammatory, antioxidant, antihyperglycemic, antihypertensive, and anticoagulant [9]. Another study also showed that xanthorrhizol isolated from *C. zanthorrhiza* Roxb. has an antifungal effect with minimum inhibitory concentration (MIC) of 1–15 mg/l and minimum fungicidal concentration (MFC) of 200 mg/ml toward *C. albicans* [10].

Research on the medical effects of Javanese turmeric extract as a potential agent against *C. albicans* was initiated several years ago at the Faculty of Dentistry, University of Indonesia. In a research conducted by Pramudita, Javanese turmeric ethanol extract (JTEE) was shown to inhibit the activity of phospholipase enzymes produced by *C. albicans* at a concentration of 2.5 mg/l using shake handling [11]. Lewiyonah and Herdiantoputri reported the MIC and MFC of JTEE toward planktonic *C. albicans* and showed that, at a concentration of 35%, JTEE reduced the ability of *C. albicans* to enter the adhesion, proliferation, filamentation, and maturation phases within 24 h [12,13]. This study aimed to observe the effect of JTEE on the eradication of *C. albicans* in the early, intermediate, and maturation phases of biofilm development.

METHODS

C. albicans (ATCC 10231) was obtained from the Oral Biology Laboratory of the Faculty of Dentistry, University of Indonesia, and was grown in Petri dishes on Sabouraud dextrose agar (SDA) for 48 h at 37°C. Dishes

were stored at 4°C until use. *C. albicans* suspensions were prepared from stock dishes by inoculating 1 ml of Sabouraud dextrose broth (SDB) with three loops of an inoculation needle and homogenization by vortexing for 20 s.

JTEE (Balittro, Indonesia) was processed by centrifugation at 1300 rpm for 20 min at 4°C to form three layers. The upper layer, which contains the highest xanthorrhizol concentration, was used and diluted to 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 45%.(v/v) in SDB. After vortexing for 20 s, the JTEE samples were stored at 4°C until further use.

For biofilm preparation, three well plates were prepared, one for each biofilm phase. Each well was seeded with 100 µl of 10^4 cells. The plates were then incubated at 37°C for 6 h to produce the initial phase, 24 h for the intermediate phase, or 48 h for the maturation phase. Once biofilms were established, the medium was aspirated to remove planktonic C. *albicans*, and the plates were rinsed once with 100 µl of phosphate-buffered saline (PBS).

Various JTEE dilutions (100 µl) were added to the biofilms in the wells, and 100 µl of nystatin (10000 units in SDB) was used as a positive control. SDB was used as the negative control. The plates were then incubated for 1 h at 37°C and then rinsed once with 100 µl of PBS.

Following this, 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well, and the plates were incubated at 37°C for 2 h. Following incubation, 100 µl acidified isopropanol was added, and the plates were shaken on an orbital shaker at 80 rpm for 1 h. Optical absorbance was determined using a microplate reader a 570 nm, and the biofilm eradication was expressed as a percentage of the control using the formula:

$$\left(1-\left[\frac{\text{ODsample}-\text{ODblank sample}}{\text{ODcontrol}-\text{ODblank control}}\right]\right)\times 100\%$$

Minimal biofilm eradication concentration (MBEC) was determined as the concentration that produced eradication values of 50% ($MBEC_{50}$) or 90% ($MBEC_{90}$).

ANOVA with a *post hoc* test was used to analyze the data obtained. Data analysis was performed using SPSS Software to compare the difference between eradication percentage of biofilm treated with JTEE and the positive control (nystatin). A correlation test was used to determine whether there was a correlation between increasing JTEE concentration and biofilm eradication percentage.

RESULTS

Before JTEE was tested on *C. albicans* biofilms, the MIC and MFC toward planktonic *C. albicans* were determined by measuring optical densities using a microplate reader to determine the percentage inhibition at each JTEE concentration. MIC is the smallest concentration that can inhibit the growth of *C. albicans* by 90% or more, and this was found to be 20% JTEE (Table 1).

After determining MIC, 10 µl *C. albicans* that had been exposed to JTEE was inoculated into SDB agar medium and grown, and the number of colonies was counted to determine MFC, which was also found to be 20% (Table 2).

JTEE was able to eradicate more than 50% *C. albicans* in biofilms at all three stages of biofilm development at all concentrations, except at 20% on the 6-h biofilms. However, statistical analysis showed that not all of the concentrations were significantly different from the positive control. The results of ANOVA statistical analysis showed that there was no significant difference in the eradication percentage in the 6-h biofilms between the positive control and the 30% and 35% JTEE concentrations ($p>0.05$). The 24-h biofilms exposed to JTEE at 20%, 25%, 35%, 40%, and 45% gave similar results to 48-h biofilms exposed to JTTE at 25%, 35%, and 40% concentrations. Therefore, the $MBEC_{50}$ of JTTE was 30%, 20%, and 25% against the 6-h, 24-h, and 48-h *C. albicans* biofilms, respectively. The $MBEC_{90}$ could not be determined because it was considered biased.

Table 1: Inhibition percentage of planktonic *Candida albicans* growth by Javanese turmeric ethanol extract at various concentrations

Number	JTEE concentration (%)	Inhibition (%)		
		I	II	X
1	1	18.68	16.99	17.835
2	5	3.86	4	3.93
3	10	15.22	13.52	14.37
4	15	80.88	73.3	77.09
5	20*	95.51	98.91	97.21
6	25	31.85	30.89	31.37
7	30	98.61	84.08	91.345
8	35	90	90.81	90.405
9	40	95.62	92.63	94.125
10	45	98.61	88.36	93.485
11	Positive control	97.44	97.01	97.225
12	Negative control	0	0	0

*JTEE: Javanese turmeric ethanol extract

Table 2: Minimum fungicidal concentration of Javanese turmeric ethanol extract toward planktonic *Candida albicans*

Number	JTEE concentration (%)	Colonies (n)		
		I	II	X
1	20	0	0	0
2	25	4	61	32.5
3	30	0	0	0
4	35	0	0	0
5	40	0	0	0
6	45	0	0	0
7	Positive control	0	0	0
8	Negative control	∞	∞	∞

JTEE: Javanese turmeric ethanol extract

Linear regression fitting of the 6-h biofilm produced a correlation coefficient of $r=0.703$, indicating a strong relationship between concentration and eradication values. Correlation coefficients were smaller for the 24-h and 48-h biofilms with values of 0.296 and 0.524, respectively. The correlations were all positive although the increases in concentrations were not always followed by ideal eradicating values.

DISCUSSION

The high polarity of ethanol as well as its hydrophobic ethyl group makes it ideal for extracting the active substances of Javanese turmeric rhizomes, including polar, nonpolar, and phenolic components. The method used in manufacturing the ethanol extract was the stirring–maceration technique, which can speed up extraction times by 6–24 h and thus reduce decomposition due to temperature effects [14].

Although the ethanol extract used in this study was obtained from the same place and produced by the same method and with the same solvent, there were physical differences between it and that used in a previous study in 2015 [12,13]. In this previous study, JTEE was a homogenous, yellowish-brown liquid with a unique smell [12,13]. In contrast, JTEE in this study had two different appearances mixed into one. In one container, the ethanol extract obtained had clay consistency and yellowish-brown color. In the previous study, the ethanol extract was centrifuged at 3000 rpm for 20 min and yielded four layers with xanthorrhizol concentration of 41.8%, whereas, in this study, the extract was centrifuged at 3000 rpm but did not yield similar layers. Thus, the centrifugation of the ethanol extract was modified to 1300 rpm for

20 min, which yielded three layers with a xanthorrhizol concentration of 9.38%.

This difference of the Xanthorrhizol content in the Javanese turmeric extract used in the two studies was likely due to a difference in the amount of Javanese turmeric in kilograms demanded during extract preparation. It has been reported that the active substances in every Javanese turmeric extract differ because of differences in the fertilizer type used in cultivation, which can affect the content of essential oils, curcumin, and xanthorrhizol [15]. The higher the essential oil content of the simplicial, the better is its quality because all medicinally active substances are contained in the essential oils. In addition, the quality and active ingredients of Javanese turmeric plants are also influenced by other factors such as the growing environment, superior properties of the plants, availability of nutrients, protection provided against pest organisms, and postharvesting treatment [16].

To produce various concentrations of JTEE used in this study, SDB was used as a solvent, unlike a previous study by Lewiyonah that used dimethyl sulfoxide (DMSO) [13]. Even though 10% DMSO is known to be nontoxic, the extract could not be dissolved well in DMSO in this study and precipitated rapidly. The test was conducted on three phases of biofilm formation, i.e., the initial (6 h), medium (24 h), and maturation (24 h) phases, and the biofilms were exposed to JTEE for 1 h. SDA and SDB media were used because they are standard media for growing *C. albicans* because of the presence of glucose and acidic pH [17]. *C. albicans* were grown at 37°C for 48 h as this temperature reflected the temperature of the oral cavity and was able to stimulate hyphae formation [18].

The MIC and MFC of JTEE toward planktonic *C. albicans* in this study were at 20%. This differed from Lewiyonah, who reported an MIC and MFC of 10% and 25%, respectively [13]. These differences may have been due to the differences in the concentrations of the ethanol extracts and their xanthorrhizol contents.

The microtiter broth dilution method was chosen to determine the MIC and MFC of JTEE toward planktonic *C. albicans*. To obtain inhibition percentage values, proliferation of *C. albicans* starting with 10^4 cells was monitored by measuring optical density at 450 nm. This method was chosen because it is convenient and accurate when compared to the agar dilution method, which produces inconsistent results.

The concentrations of JTEE used in this study were 1–45%. Although the values for MIC and MFC determined in this study differed to those reported by Lewiyonah [13], similar trends in inhibition with increasing JTEE concentration were observed.

However, due to inconsistencies of eradication effect of the extract against *C. albicans* biofilm at some concentration, it is not possible to conclude that the inhibition effect of the extract was dose dependent. In a previous work, JTEE was produced from 6 kg of Javanese turmeric rhizomes that were dissolved in pure ethanol, whereas this study only used 3 kg of rhizome dissolved in 90% ethanol. The ethanol extract used by Lewiyonah in the previous study had a xanthorrhizol concentration of 41.8%. In contrast, in this study, the ethanol extract concentration was 9.38%. This may have occurred because, eventhough the Javanese turmeric extract used were processed at the same laboratory, different shapes and properties accepted due to the different amount of demanded rhizomes in kilograms, thereby affecting the final outcome of the study. The quality and active ingredients of Javanese turmeric plants are also influenced by other factors described above [16].

Xanthorrhizol is known to inhibit the growth of microorganisms. Hwang (2006) showed that xanthorrhizol isolated from Javanese turmeric is antifungal against planktonic *Streptococcus mutans*. The results of this study showed that the JTEE containing only 9.38% xanthorrhizol could still inhibit growth of *C. albicans* biofilm. The hydroxyl groups (-OH) found in nystatin are known to induce damage to the plasma membrane of *C. albicans* cells by binding to ergosterol and oxidizing the membrane lipids, leading to permeabilization, cell leakage, potassium ion release, and eventual cell death [19]. The same mechanism may be responsible for xanthorrhizol's antifungal activity.

In this study, JTEE eradicated *C. albicans* cells in all three phases of biofilm formation when compared to the controls. The $MBEC_{50}$ values of JTEE on 6-h, 24-h, and 48-h biofilms were 30%, 20%, and 25%, respectively. These results were assumed to occur via similar mechanisms to those that affected planktonic *C. albicans.* Thus, JTEE efficacy was high in the initial phase, decreased in the intermediate phase, and increased again in maturation phase. In contrast, previous studies have linked increased antifungal resistance with stages of biofilm development, suggesting that resistance progression develops over time along with biofilm maturation.

It is known that more mature biofilms produce more complex extracellular matrices, which allows them to become more resistant to antifungal agents. This was assumed to occur because, in the early phase of biofilm formation, *C. albicans* cells have a higher metabolic activity as they are actively growing; therefore, it took a higher concentration of JTEE to suppress the growth. This then decreased in the intermediate phase and increased again in the maturation phase. This may occur because, as reported by Henriques *et al.*, cell activity of a biofilm is not dependent on the number of cells present [20]. In a biofilm, the cells are surrounded by an extracellular matrix that limits the access of nutrients and oxygen, resulting in alterations to cellular metabolic activity. As biofilm mass increases over time while activity decreases, biofilms in the maturation phase may contain large numbers of cells, but these cells have low metabolic activity.

The eradication percentage of *C. albicans* biofilms by JTEE at 6-h, 24-h, and 48-h was lower than the positive control, but these differences were not statistically significant. Thus, JTEE has the potential to eradicating biofilms but not as well as nystatin. Previous study showed that the exposure of *S. mutans* biofilms to xanthorrhizol for 1 h was sufficient to eradicate as much as 76% of the biofilms [21].

Although not completely eliminated, bacteria were removed in the adhesion phase and early accumulation of biofilm development [21]. Killed cells will degrade and detach from the biofilm, resulting in a decrease in its cell content [21]. Henriques *et al.* suggested that *C. albicans* biofilm organization generally resembles the biofilm structures of oral bacterial species; therefore, a similar mechanism may occur in xanthorrhizol-mediated eradication of *C. albicans* biofilms [20].

CONCLUSION

The results of this study indicate that the MIC and MFC of JTEE toward planktonic *C. albicans* after 1 h exposure are 20%. JTEE had the potential to eradicate *C. albicans* biofilms at any of the three phases of biofilm formation after 1 h of exposure.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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