Javanese Turmeric (*Curcuma Xanthorrhixza* Roxb.) Ethanol Extract Has Inhibitory Effect on The Development of Intermediate Phase of *Candida Albicans* Biofilm

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Abstract

Javanese turmeric (*Curcuma canthorrhiza* Roxb.) is an Indonesian native medicinal plant that contains xanthorrhizol, which is known to have antifungal effect. After adhering to a surface in the early phase, the *C. albicans* biofilm develops into intermediate phase during which it grows filaments and transforms from yeast cells into hypha, so that it becomes more virulent. We aimed to analyze the potential effect of Javanese turmeric (*Cucurma xanthorrhiza* Roxb.) extract in inhibiting the intermediate phase of *C. albicans* biofilm.

The Javanese turmeric ethanol extract was exposed to *C. albicans* ATCC 10231 biofilm aged 1.5 h and incubated in 37 °C for 24 h to achieve intermediate phase. MTT *assay* was used to assess the viability of *C. albicans* biofilm.

The minimum biofilm inhibitory concentration ($MBIC_{50}$) of Javanese turmeric ethanol extract against *C. albicans* ATCC 10231 in the intermediate phase was 35%.

Javanese turmeric ethanol extract showed potential to inhibit *C. albicans* biofilm formation in the intermediate phase.

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Introduction

Oral candidiasis is most commonly caused by *Candida albicans* (*C. albicans*).[1] This yeast is found in the oral cavity of approximately 80% of healthy subjects.[2] In most people, this yeast lives as commensal microorganism. However, this microorganism is an opportunistic pathogen that may cause disease in immunocompromised subjects such as patients with diabetes or human immunodeficiency virus infection, elderly people, and chronic denture wearers with poor oral hygiene.[3]

C. albicans is a polymorph microorganism that exists as an ovoid yeast, as an elongated oval cell with a constriction at their septum (pseudohypha), or as a true hypha with parallel membrane.[4] Survival of *C. albicans* both as yeast and as hypha depends on environmental factors such as temperature, pH, level of carbon dioxide, and the presence of serum that stimulates the growth of the hypha. The switch between yeast and hypha forms has been shown to be important for virulence of the yeast. The hyphal form of *C. albicans* is known to be more pathogenic than the yeast form.[3] The dimorphism (switch from yeast to hypha) enhances the ability to attach to the surface of host tissue or dental appliance worn by the host.[5]

Biofilm formation by *C. albicans* is one of the mechanisms that supports the yeast to become more resistant to antifungal agents.[6] The process of development of *C. albicans* biofilm comprises of three phases, i.e., initial, intermediate, and maturation phases. The initial phase refers to the first 4–11 hours (h) during which the formation of micro-colony is visible and *C. albicans* begin to aggregate. The intermediate phase occurs during 12–36 h during which the biofilm structure exhibits two layers comprising yeast, germ tubes, and young hypha with extracellular matrix. In the maturation phase (37-72 h), the biofilm structure exhibits multilayers with yeast, pseudohyphae, and hyphae embedded in the matrix.[7,8]

Javanese turmeric (*Curcuma xanthorrhiza* Roxb.) is an original Indonesian plant, which together

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with eight other plants has been scientifically developed as a potential herb by the Indonesian Ministry of Health since 2003.[9]

Curcumin and *Xanthorrhizol* are among the major components of Javanese turmeric volatile oil which is known to have antimicrobial properties. *Xanthorrhizol* content in the Javanese turmeric volatile oil is around 64.8%.[10] *Xanthorrhizol* was shown to have antibacterial, antifungal, antiseptic, and antibiotic effects.[11] Inhibitory effect of *Xanthorrhizol* against planctonic *C. albicans* was demonstrated at concentrations of 1–15 μg/mL.[12] Another report described the efficacy of *Xanthorrhizol* in reducing *C. albicans* biofilm formation at the adhesion, intermediate, and maturation phases. [7] In our previous studies, we demonstrated that the Javanese turmeric ethanol extract inhibits the growth of dual *Streptococcus* species biofilm and eradicates the initial phase of *C. albicans* biofilm.[13,14] Inhibition of the development of *C. albicans* biofilm from reaching the more mature phase is known to weaken the virulence of the yeast and increase the efficacy of antifungal agents. Therefore, it is important to further study the potency of Javanese turmeric extract against intermediate and maturation phases of *C. albicans* biofilm.

The aim of this study was to investigate the inhibitory effect of Javanese turmeric ethanol extract against the intermediate phase *C. albicans* biofilm.

Materials and methods

C. albicans sample

In this study, we used the laboratory strain of *C. albicans* American Type Culture Cell (ATCC) 10231, which belongs to the Oral Biology Laboratory, Faculty of Dentistry, University of Indonesia. The planctonic *C. albicans* was cultured in Sabouraud Dextrose Broth (SDB) in tubes and incubated at 37 °C for 48 h. The *C. albicans* biofilm was cultured in SDB in a 96-well plate and incubated for 24 h (to mimic an intermediate phase biofilm) at 37 °C.

C. albicans suspension

C. albicans suspension was made from the 48 h *C. albicans* cultures that were further inoculated in Eppendorf tubes containing 1 mL SDB and homogenized by placing it in a vortex mixer for 20 seconds (s). Serial dilution of *C. albicans* suspension (10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}) was performed by initially mixing 990 μL SDB with 10 μL *C. albicans* master suspension to prepare 10^{-2} suspension; subsequently, the suspension was further sequentially mixed with 10 μL of *C. albicans* suspension to prepare the thinner suspensions. From each concentration of *C. albicans* suspension, 10 μL was cultured in SDA and incubated at 37 °C. This step was performed in duplo. After 72 h, the *C. albicans* colonies in SDA medium were counted. The concentration of *C. albicans* suspension used in this study was 10^{-4}.

Javanese turmeric ethanol extract

The Javanese turmeric ethanol extract used in this study was derived from Javanese turmeric root that was extracted by maceration technique using 96% ethanol as solvent and was processed at BALITTRO (Research Centre for Aromatic Herbal Plant) in Bogor, Indonesia. The extract was then centrifuged at 3700 rpm for 20 min to separate it into three layers. The top layer that contained 19.5% *Xanthorrhizol* which is the highest concentration of *Xanthorrhizol* content compared to the other two layers was used in this study. To obtain various concentrations of the extract, the SDB was used as the solvent. In this study, the concentrations of the extract used to determine the respective minimum inhibitory concentration (MIC) against planctonic *C. albicans* were 5%, 10%, 15%, 25%, 30%, 35%, 40%, and 45%.

MIC

The MIC of the Javanese turmeric ethanol extract was determined for both the planctonic and biofilm *C. albicans*. The MIC against planctonic *C. albicans* is the lowest concentration of the extract that inhibits ≥90% growth of planctonic *C. albicans*. The inhibitory percentage was calculated (following Queve, Plano, Pantuso, and Bennet study, 2008) on the basis of the optical density (OD) of the yeast cultures exposed to various concentrations of the Javanese turmeric ethanol extract measured by microplate reader at 450 nm wave length. The formula is as follows:

$$\%\ inhibisi = \left(1 - \left(\frac{OD\ sampel - OD\ sampel\ blank}{OD\ kontrol - OD\ kontrol\ blank}\right)\right) x\ 100\%$$

The $MBIC_{50}$ and $MBIC_{90}$ (lowest concentrations of the extract that inhibit 50% and 90% growth, respectively) against *C. albicans* biofilm were determined.

The *C. albicans* biofilm was prepared by dripping 100 µL *C. albicans* suspension into each well of the 96-well plate, followed by incubation for 1.5 h at 37 °C to allow *C. albicans* to adhere on the well surface and initiate biofilm formation. After 1.5 h, the biofilm model on the well surface was washed by 100 µL PBS to render the biofilm model free from planctonic *C. albicans*.

Exposure of Javanese turmeric ethanol extract on the *C. albicans* biofilm model

After washing, 100 µL of various concentrations of Javanese turmeric ethanol extract was added to each well containing *C. albicans* biofilm, which was categorized as the experimental group. In the positive control group, the same amount of 100.000 IU nystatin was added, whereas nothing was added in the negative control group. The plate was incubated for 24 h (intermediate phase) at 37 C.

MTT *assay*

After incubation for 24 h, each well was washed once with 100 µL PBS solution. Ten microliters of MTT solution (concentration: 5 mg/mL) was added into each well and incubated for 3 h at 37 °C. Then 100 µL acidified isopropanol was added into each well. The well plate was then placed in an orbital shaker for 1 h at room temperature. The OD value was read on a micro-plate reader with 570 nm wavelength. The inhibitory percentage was calculated using the formula mentioned above.

$$\%\ inhibisi\ biofilm = \left(1 - \left(\frac{OD\ sampel - OD\ sampel\ blank}{OD\ kontrol\ negatif - OD\ kontrol\ blank}\right)\right) x\ 100\%$$

Data analysis

ANOVA with *post hoc* test was used to assess the differences between the MBIC of various concentrations of the extract against various groups of *C. albicans* biofilm. Corelation test was used to analyze the relationship between increased concentration of the extract and the inhibitory effect against *C. albicans* biofilm.

Results

The MIC of Javanese turmeric ethanol extract against planctonic *C. albicans* in this study was 20% (Table 1). At this concentration, the extract inhibited >90% growth of planctonic *C. albicans*.

Using the MIC value as the second lowest concentration to be tested, the minimum fungicidal concentration (MFC) of the extract against planctonic *C. albicans* was examined. The MFC of Javanese turmeric ethanol extract against planktonic laboratoric strain *C. albicans* (ATCC 10231) was 35% (Table 2).

Concentration of Javanese turmeric ethanol extract (%)	Inhibition Percentage (%) of the extract against planctonic *C. albicans*
0.25	55.30
0.5	63.65
1	60.92
5	74.41
10	68.72
15	74.57
20	**97.50**
25	82.68
30	77.77
35	89.31
40	94.85
45	83.23
Positive control	100
Negative control	0

Table 1. Minimum inhibitory concentrations of Javanese turmeric ethanol extract against planctonic *C. albicans* ATCC 10231.

Concentration of Javanese turmeric ethanol extract (%)	Inhibition Percentage (%) of the extract against planctonic *C. albicans*
15	-
20	94
25	15
30	2
35	**0**
40	0
45	0
Positive control	0
Negative control	∞

Table 2. The MFC of Javanese turmeric ethanol extract against planctonic *C. albicans.* CFU=Colony-Forming Unit.

Minimum Biofilm Inhibition Concentration (MBIC) of Javanese turmeric ethanol extract against intermediate phase *C. albicans* biofilm

As shown in Figure 1, Javanese turmeric ethanol extract at concentrations of 35%, 40%, and 45% inhibited >50% growth of intermediate phase *C. albicans* biofilm. Therefore, the $MBIC_{50}$ of the extract against intermediate *C. albicans* biofilm was 35%.

Statistical analysis revealed significant differences between the experimental group and the negative control group with respect to the MBIC of the extract against intermediate phase *C. albicans* biofilm at all concentrations and between experimental groups and positive control at concentrations of 20%, 25%, 30%, 35%, and 40% ($p < 0.05$). The correlation test showed a strong positive correlation between the increment of Javanese turmeric ethanol extract concentration and the inhibition percentage of the extract against *C. albicans* biofilm (r = 0.880).

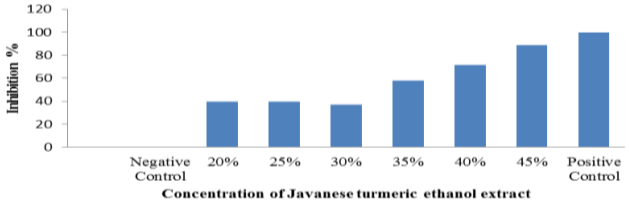

Figure 1. MBIC of Javanese turmeric ethanol extract against intermediate phase biofilm of *C. albicans*.

Discussion

This study demonstrates the antifungal efficacy of Xanthorrhizol and Javanese turmeric extract against both planctonic *C. albicans* and biofilm, which is consistent with the results of previous studies.[7,12,14] In this study, the MIC of the extract was 20% against planctonic *C. albicans*, whereas the $MBIC_{50}$ was 35% against intermediate phase biofilm of *C. albicans*. These results reflect increase in resistance of *C. albicans* in parallel with its development from yeast form into fungi and biofilm. At 35% concentration, the Javanese turmeric ethanol extract completely aborted the growth of planctonic *C. albicans* but only inhibited >50% growth of *C. albicans* intermediate phase biofilm. This is consistent with the results of a previous study in which the MIC was found to be lower than the MBIC. [14]

Planctonic *C. albicans* lives freely in the environment. Once it transforms into biofilm, the yeast is already adherent to the host surface and begins to develop thicker and more complex structure that renders it more impregnable to antifungal agents. [15] By the intermediate phase, which occurs at 12–37 h, the *C. albicans* biofilm is already strongly adhered onto the host surface, aggregated to one another, and forms double layer of biofilm comprising yeast, germ tube, pseudohyphae, and young hyphae, all covered in extracellular matrix.[7,8,15]

The effective concentration of the Javanese turmeric extract against planctonic *C. albicans* varies between studies. In this study, the MIC of the extract was 20%, whereas in previous studies, the reported MIC against planctonic *C. albicans* was 10% and 15%. [15,16] One among the several possible reasons for this discrepancy is the different concentrations of *Xanthorrhizol* contained in the Javanese turmeric ethanol extract used in those studies. The Javanese turmeric ethanol extract used in this study contained 19.5% *Xanthorrhizol*, whereas the extract used in the other two studies contained 41.78% and 9.38% *Xanthorrhizol*. [15,16] This may be attributable to the different physical characteristics of the extract and different condition of the plants from which it is extracted. The environment of the growing field, the soil composition, the strain of the plant, and age of the plant at the time of harvesting are liable to influence the concentration of the active component. [17]

Similar differences were observed with respect to the efficacy of the extract against *C. albicans* biofilm. In this study, 35% extract inhibited >50% growth of *C. albicans* intermediate biofilm. However, 35% concentration of the extract was shown to eradicate >90% growth of the early phase biofilm in a previous study. [15] In another study, 15% extract was shown to eradicate >50% growth of the intermediate phase biofilm of *C. albicans*. [16] These results indicate the need for further studies to standardize the preparation of the Javanese turmeric extract and at the same time provide further insights into the mechanism of interaction between the extract and the biofilm of *C. albicans*. Inhibition effect of antifungal agent refers to the ability of the agent to inhibit the development of the biofilm because the agent is being exposed to the biofilm since the beginning

of biofilm formation. However, eradication effect refers to the ability of the agent in disrupting the already formed biofilm. Considering the development phases of the biofilm in which the increased resistance of the biofilm is in line with the maturity of the biofilm, the effective concentration of antifungal agent to inhibit is supposed to be lower than its eradication concentration.

As shown in Figure 1, compared with nystatin as the positive control, the inhibition percentage of the Javanese turmeric ethanol extract against intermediate phase biofilm of *C. albicans* is lower than that of nystatin at all concentrations tested. However, statistical analysis showed no significant difference ($p > 0.05$) in inhibition percentage between nystatin and the extract at 35%, 40%, and 45% concentrations. Therefore, it could be considered that starting at 35%, the Javanese turmeric ethanol extract has a similar effect as that of nystatin in inhibiting the growth of intermediate phase biofilm of *C. albicans*.

The potency of Javanese turmeric extract as antifungal agent against *C. albicans* might be due to its *Xanthorrhizol* content. *Xanthorrhizol* is a phenolic compound that has (−OH), which is also present in nystatin. [18] Phenolic compound has strong penetration effect, and it binds with the cell membrane of *C. albicans* through the formation of hydrogen bond between hydroxyl group in the compound and proteins in the cell membrane, which leads to unstable permeability of the membrane and eventually cell lysis. [19]

Conclusions

The Javanese turmeric ethanol extract has potency to be developed as an antifungal agent against *C. albicans*. The extract is more effective in inhibiting the growth of planctonic *C. albicans* than in inhibiting the development of *C. albicans* biofilm. However, the Javanese turmeric ethanol extract is still capable of inhibiting the development of *C. albicans* biofilm even at its intermediate phase.

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Declaration of Interest

The authors report no conflict of interest.

References

1. Garcia-Cuesta C, Sarrion-Perez MG, Bagan JV. Current treatment of oral candidiasis: A literature review. J Clin Exp Dent 2014;6:576-82.
2. Coronado-Castellote L, Jiménez-Soriano Y. Clinical and microbiological diagnosis of oral candidiasis. J Clin Exp Dent 2013;5:279-86.
3. Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence 2013;4:119-28.
4. Berman J, Sudbery PE. Candida albicans: A molecular revolution built on lessons from budding yeast. Nat Rev Genet J 2002;3:918-32.
5. Abaci O. Investigation of extracellular phospholipase and proteinase activities of candida species isolated from individuals denture wearers and genotypic distribution of Candida albicans strains. Curr Microbiol J 2011;62:1308-14.
6. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167-93.
7. Rukayadi Y, Hwang JK. In vitro activity of xanthorrhizol isolated from the rhizome of Javanese turmeric (Curcuma xanthorrhiza Roxb.) against Candida albicans biofilms. J Phyther Res 2013;27:1061-6.
8. Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence 2013;4:119-28.
9. BPOM. Gerakan Nasional Minum Temulawak. Info POM. 2005;6:1-3.
10. Salleh NAM, Ismail S, Ab Halim MR. Effects of Curcuma xanthorrhiza extracts and their constituents on phase ii drug-metabolizing enzymes activity. J Pharmacognosy Res 2016;8:309-15.
11. Nur SW. Comparison of extraction system and validation technique to determine xanthorrhizol from temulawak (Curcuma xanthorrhiza) using high performance liquid chromatography (HPLC). Bogor: Faculty of Mathematics and Agricultural Institute of Bogor. 2014.
12. Rukayadi Y, Hwang JK. In vitro anticandidal activity of xanthorrhizol isolated from Curcuma xanthorrhiza Roxb. J Antimicrob Chemother 2006;57:1231-4.
13. Ajrina Busri, Ria Puspitawati, Sri Utami. Antibacterial effect of Java turmeric ethanol extract against dual-species Streptococcus mutans and Streptococcus sanguinis biofilm (in Vitro). Asian J Pharm Clin Res 2017;10:57-60.
14. Ria Puspitawati, Rista Lewiyonah, Ranny Rahaningrum Herdiantoputri, Ferry P Gultom, Dewi F Suniarti. Eradication effect of Javanese turmeric (*Curcuma xnthorrhiza* Roxb.) extract on the early phase of *Candida albicans* biofilm. Int J Appl Pharm 2017;9(Special issue 2):117-20.
15. Ryan KE. Candida albicans adhesion and biofilm formation on phosphated and non-phosphate containing poly (methylmethacrylate) polymers. Marquette Univ 2010;16-9.
16. Lestari AD. Potential use of java turmeric ethanol extract in eradicating *C. albicans* clinical isolate biofilm. (Thesis). Jakarta: Universitas Indonesia; 2016.
17. Halim MRA, Tan MSMZ, Ismail S, Mahmud R. Standardization and phytochemical studies of Curcuma xanthorrhiza Roxb. Int J Pharm Sci 2012;4:606-10.
18. Rukayadi Y, Han S, Yong D, Hwang JK. In vitro activity of xanthorrhizol against Candida glabrata, C. guilliermondii, and C. parapsilosis biofilms. J Med Mycol 2011;49:1-9.
19. Lyu X, Zhao C, Hua H, Yan Z. Efficacy of nystatin for the treatment of oral Candidiasis: A systematic review and meta-analysis. Drug Des Devel Ther 2016;1:1161-71.