ORIGINAL ARTICLE

Decreased Activity of Phospholipase and Proteinase Enzymes of *Candida albicans* Biofilm Inhibited by Javanese Turmeric (*Curcuma xanthorrhiza Roxb.*) Ethanol Extract

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ABSTRACT

Introduction: The efficacy of Javanese turmeric (*Curcuma xanthorrhiza Roxb.*) extract in inhibiting the growth of *C. albicans* biofilm decreases as the biofilm gets mature. phospholipase and proteinase are *C. albicans* hydrolytic enzymes which facilitate the adhesion and invasion of the fungi into the host cell. Objective: Analyzing the activity of phospholipase and proteinase enzymes on three phases of *C. albicans* biofilm whose growth has been inhibitted by Javanese turmeric ethanol extract. **Methods:** After 1,5 hour incubation, the *C. albicans* biofilm in the treatment group were exposed to Javanese turmeric ethanol extract at its Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$) which was determined by MTT assay, the negative controls were exposed to nothing, while the positive controls were exposed to Nystatin. *C. albicans* biofilm were further incubated for 6, 24, and 48 hours to achieve initial, intermediate, and maturation phases. Activity of the hydrolytic enzymes were analyzed by measuring the diameter of precipitation/ proteolytic zone that were seen surrounding the colony zone of *C. albicans* on Egg yolk Agar (for observing Phospholipase activity) and Bovine Serum Albumin agar (for observing Proteinase activity)**. Results:** In early, intermediate, and mature phases *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol extract, both the diameters of precipitation / proteolytic zone of phospholipase and proteinase were smaller compared to negative controls. **Conclusion:** Inhibition effect of Javanese turmeric ethanol extract toward the growth of three phases of *C. albicans* biofilm could decrease the activity of phospholipase and proteinase enzymes of *C. albicans.*
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Keywords: *C. albicans, Curcuma xanthorrhiza Roxb.*, Phospholipase, Proteinase, Biofilm

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INTRODUCTION

Javanese turmeric (C*urcuma xanthrorrhiza Roxb.*) is one among many original Indonesian plants that mostly used as the basic component of traditional medicine. (1) The root of Javanese turmeric plant contains curcumin, essential oil, starch, protein, lipid (fixed oil), cellulose, and minerals. Among those, the active components with medicinal effect are curcumin and essential oil. (2, 3, 4) The active component of the Javanese turmeric root which is contained in its essential oil is xanthorrhizol. Xanthorrhizol which has many medicinal effects is the characteristic of Javanese turmeric because it is detected only in this plant. (5, 6) Various *in vitro* studies had reported that xanthorrhizol isolated from Javanese turmeric root showed anti-bacterial and anti-fungal effects against various oral microbes including *C. albicans.* (5) More recently, it had also been reported that the ethanol extract of Javanese turmeric root has potential in inhibiting *C. albicans* growth *in vitro.* (7)

C. albicans biofilm develops through three phases. At the initial phase the *C. albicans* cells start to adhere on the surface of host tissue (1-2 hours), continue to develop and form microcolony (3-4 hours), and eventually aggregate one to another forming a dense one-layer biofilm (11 hours). At intermediate phase (12-30 hours) the *C. albicans* biofilm is already developed to become bilayer consisting of yeast, germ tubes, and young hypha covered by the extracellular matrix. At the maturation phase (38-72 hours) the thickness of the extra

cellular materials increases so that yeast, pseudo hypha, and hypha are completely embedded in the matrix. (8) Phospholipase and proteinase are two hydrolytic enzymes secreted by *C. albicans* which have a role in destroying the epithelial cell membrane of the host. Phospholipase can hydrolyze the ester bond on the glycerophospholipids so that the cell membrane become unstable and eventually leads to cell lysis. (9) The activity of phospholipase could be detected since the formation of *C. albicans* germ tube. However, the activity of this enzyme is higher at the hyphal form rather than at the mycelium stage. (10) It had also been reported that the highest activity of phospholipase is detected when the yeast has the strongest adherence with buccal epithelial cells. (11)

In general, the role of *C. albicans* proteinase is to support the adherence of the yeast on the surface of the host cells. There are four hypothetical mechanisms of *C. albicans* proteinase virulence. First, the enzyme facilitates adhesion by destructing the surface of host cell membrane which leads to destruction of the host infected tissue. Secondly, the enzyme causes defects in the immune response of the host. Thirdly the enzyme increases the nitrogen resources of the yeast via degradation of peptide, and fourthly this enzyme can destroy the endothelial cells. (12)

Both biofilm development and the activity of proteolytic enzymes are very important virulence factors of *C. albicans*. Previous studies had reported that the activity of *C. albicans* phospholipase and proteinase is influenced by the maturity of the biofilm. The Javanese turmeric ethanol extract had been reported to inhibit the development of *C. albicans* biofilm since the initial, intermediate, and maturation phases. Therefore, it will be evaluated how the activity of these two proteolytic enzymes on various phases of *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol extract.

MATERIALS AND METHODS

Preparation of Javanese turmeric ethanol extract

The Javanese turmeric ethanol extract (which was cultivated and prepared by BALITTRO/Research Center for Medicinal Tropical Plants, Bogor, West Java, Indonesia) was centrifuged at 3.700 rpm for 20 minutes so that it was dispersed into four layers. From those 4 layers, the top layer which has the clearest color and highest concentration of xanthorrhizol was used in this study. The extract was diluted by 10% Dimethyl Sulfate Dioxide (DMSO) to become various concentrations as follows; 15%; 20%; 25%; 30%; 35%; 40%; dan 45%. After this, the extracts were homogenized by vortex for 20 seconds and kept in 4^0C.

Preparation of C. albicans suspension

Inoculation of *C. albicans* ATCC 10231 (Laboratory strain cultured at Laboratory of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Indonesia) was conducted by culturing 10 µL *C. albicans* suspension (in SDB) on Saburou Dextrose Agar (SDA) which was then incubated for 48 hours at 37°C. After 48 hours, a colony of *C. albicans* was suspended in 1mL SDB, homogenized by vortex for 20 seconds, and serially diluted into various concentrations by adding SDB into *C. albicans* suspension. In this study the *C. albicans* suspension was diluted into 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}.

Determination of concentration of *C. albicans* suspension to be used in this study

From each dilution of *C. albicans* suspension, 1ml was cultured on SDA, and incubated at 37°C. This step was conducted triply. After 72 hours, the colonies grew on each SDA plates were counted. The concentration chosen for this study was 10-4 because in this concentration the number of grown colonies are in ranges of (1-5) x 10^6CFU/ml. (13)

Determination of Minimum Biofilm Inhibition Concentration (MBIC)

Into 96-well-plate, 100 µL of 10^{-4} *C. albicans* suspension (experimental group), nystatin (positive control), and SDB (negative control) were added and incubated for 90 minutes at 37˚C. After incubation, the inoculation were washed once by 100 µL PBS to shed the detached *C. albicans* cells. After being washed, 100 µL SDB were added into each well and into wells contained blank solution. Blank solution for experimental group contained 100 µL Javanese turmeric extract, for positive control contained 100 µL nystatin, and for negative control contained 100 µL SDB. These inoculations were further incubated at 37˚C to reach the targeted biofilm phase (6 hour for initial phase, 24 hours for intermediate phase and 48 hours for maturation phase). After incubation periods were completed, the well contained *C. albicans* biofilm were washed twice using 100 µL PBS. The determination of MBIC was based on viability analysis using MTT assay. Into each well 50 µL of MTT solution (5 mg/mL) was added, incubated at 37˚C for 3 hours. Afterward, 100 µL acidified isopropanol was added into each well, and put on an orbital shaker at 80 rpm for 1 hour. The optical density was read using ELISA reader (Metertech) with 600 nm wavelength. The MBIC was determined using the following formula.

$$\% \text{ inhibisi} = \left(1 - \left(\frac{\text{OD Sample - OD Sample Blank}}{\text{OD Control - OD Control Blank}} \right) \right) \times 100\%$$

Preparation of Egg Yolk Agar (EYA) as the test medium for phospholipase activity

The medium used to analyze the activity of *C. albicans* phospholipase was Egg Yolk Agar (EYA). The EYA contained 13 grams of SDA, 11.7-gram NaCl, 0.11-gram $CaCl_2$ and 20 ml of 50% sterile egg yolk emulsion diluted in 184ml aquades. This mixture of SDA, NaCl, and $CaCl_2$ were incubated to reach 50°C before the 20ml of egg yolk emulsion was added, poured onto petri-

dishes, and waited until set. The procedure of pouring the mixture from Erlenmeyer glass onto petri-dishes was conducted close to the Bunsen fire.

Preparation of Bovine Serum Albumin Agar (BSAA) as the test medium for proteinase activity

The composition of BSAA was 0,2% yeast extract [Merck Milipore]; 0,4% BSA [Thermo Scientific]; 4% Agar [Titan Media].28 The yeast extract and agar were mixed with aquades and being autoclaved for 15 minutes at 121°C. After the agar mixture reached room temperature, BSA was added and poured into patry-dishes.

Analysis of Hydrolytic enzymes activity on *C. albicans* biofilm inhibited by Javanese turmeric extract

C. albicans biofilm were grown in 96-well-plate. Sterile paper discs (Oxoid) were placed on the bottom of each well. Into each well, 100 µl ($2x10^6$ CFU/ml) *C. albicans* suspension were added and incubated for 1.5 hour at 37˚C to form the initial phase of *C. albicans* biofilm. After which, the SDB in each well was aspirated to remove the planktonic *C. albicans*, followed by addition of 100 µl SDB. Afterward, 100 µl Javanese turmeric ethanol extract at MBIC or 100 µl Nystatin were added onto each well of experimental and positive control groups. The wells contained *C. albicans* biofilm were further inoculated at 37˚C for a various duration of time to reach the initial, intermediate, or maturation phase. After incubation period, the remains of SDB were aspirated, the sterile paper disc on the bottom of the well were taken out and placed on the EYA or BSAA. These steps were conducted triply. The inoculation on the EYA or BSAA were then further incubated for four days at 37°C.

Measurement of phospholipase activity

The activity of phospholipase is positive when a zone of precipitation could be seen surrounding the paper disc which contained *C. albicans* biofilm. The fungi were inoculated on the Egg Yolk Agar (EYA) and the diameters of precipitation zone, *C. albicans* biofilm, and the summation of the two diameters were measured. The value of enzyme activity was formulated by the following equation.

$$\text{Phospholipase activity (Pz)} = \frac{\text{Diameter of colony}}{\text{Diameter of colony + presipitation zone}}$$

When the result of the equation (Pz) between diameters is equal 1, it means there is no enzyme activity. The lower the result of equation, means the stronger the activity of phospholipase on the colony.

The activity of *C. albicans* phospholipase formulated by this equation could be categorized as follows;(14) Pz 1 = no activity of phospholipase; Pz 0,90-0,99 = phospholipase activity is very low; Pz 0,80-0,89 = phospholipase activity is low; Pz 0,70-0,79 = phospholipase activity is moderate; Pz < 0,70 = phospholipase activity is high

Measurement of proteinase activity

The activity of *C. albicans* proteinase could be analyzed by measuring the diameter of proteolytic zone surrounding the *C. albicans* colony which was cultured on Bovine Serum Albumin Agar (BSAA) medium. The measurement of the diameter of the proteinase proteolytic zone was based on the farthest distance between the outer border of *C. albicans* colony and the outer border of the proteolytic zone. The measurement result was categorized as follows;(15) Negative (-) if no proteolytic zone could be detected; Positive (+) means light activity if the distance between the two borders is 0,1 – 1,1 mm; Two positive (++) means strong activity if the distance between the two borders is 1,2 – 3,3 mm; Three positive (+++) means very strong activity if the distance between the two borders is >3,3 mm

RESULT

Determination of *C. albicans* suspension concentration

The results of colony counting from various concentrations of *C. albicans* suspension showed that the dilution that results in (1-5) x 10^6 CFU/mL colony number is 10^{-4}. At this concentration, there was adequate growth of *C. albicans* colony, but the number could still be counted accurately.

Determination of MBIC of Javanese turmeric ethanol extract

The MBIC of the Javanese turmeric ethanol extract was analyzed for the three different phases of *C. albicans* biofilm. The MBIC was determined by measuring the viability of *C. albicans* cells in the biofilm exposed to the extract using MTT assay. The results of MTT assay which had been converted by a formula to get the inhibition percentage of the extract could be seen in Figure 1.

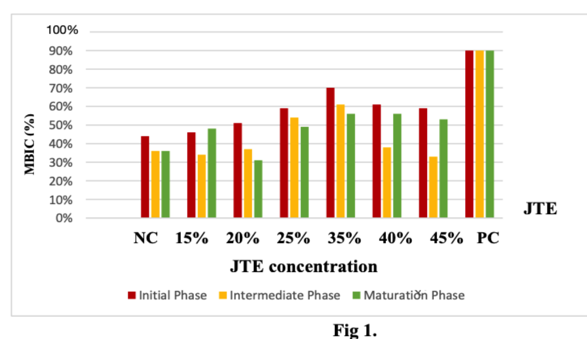

Fig 1. The MBIC (inhibition percentage) of Javanese turmeric ethanol extract against initial, intermediate, and maturation phases biofilm of *C. albicans* ATCC 10231

It could also be seen in Figure 1, Javanese turmeric ethanol extract from 25%, 30%, 35%, 40%, and 45% could inhibit the initial phase of *C. albicans* biofilm > 50%. Therefore, it could be determined that the $MBIC_{50}$ of Javanese turmeric ethanol extract against initial phase of *C. albicans* biofilm is 25%. Whereas the $MBIC_{50}$ of the extract toward intermediate and mature phase *C.*

albicans biofilm are 30% and 35% respectively. In this study the $MBIC_{90}$ could not be determined.

Phospholipase activity of *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol extract

It could be seen on Figure 2A that the trend of phospholipase activity of *C. albicans* which had been exposed to Javanese turmeric ethanol extract were similar in all the three phases of biofilm. The activity of phospholipase of *C. albicans* was low (0,84 at initial phase, 0,8 at intermediate phase and 0,83 at maturation phase). While the negative control showed high phospholipase activity (initial phase 0,55; intermediate phase 0,57; maturation phase 0,6). Conversely the positive control showed no phospholipase activity.

On Figure 2B it could be seen that the diameter of precipitation zone which was formed on the negative control (a) was the largest, followed by the experimental (b) and not visible on the positive control (c).

Figures 2C and 2D show the activity of phospholipase of *C. albicans* biofilm at intermediate phase and maturation phase respectively. Like what can be seen at the initial phase, at these intermediate and maturation phases of the *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol Extract (b) or Nystatin (c), also showed lower phospholipase activity compared to the negative controls (a).

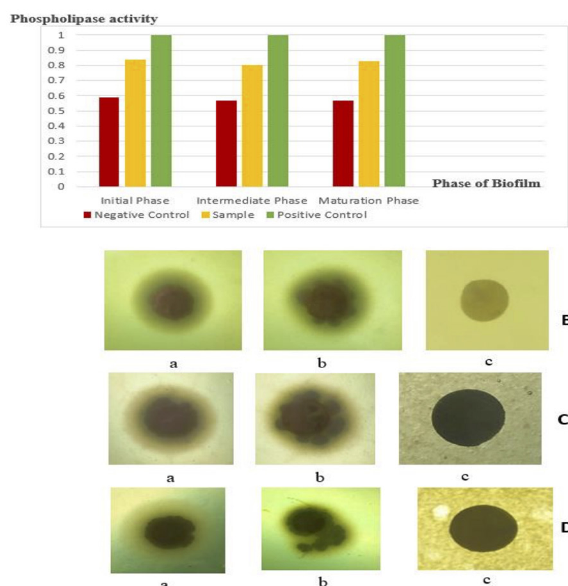

Fig 2. Phospholipase activity of C. albicans biofilm at various phases and groups
A.Graph of phospholipase activity at the initial, intermediate, and maturation phases of C. albicans biofilm in the experimental and control groups
B.Photograph of phospholipase activity of C. albicans biofilm at initial phase.
(a) Negative control, (b) Experimental group, (c) Positive control
C.Photograph of phospholipase activity of C. albicans biofilm at intermediate phase.
(a) Negative control (b) Experimental group (c) Positive control
D.Photograph of phospholipase activity of C. albicans biofilm at maturation phase
(a) Negative control (b) Experimental group (c) Positive control

Proteinase activity of *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol extract

Figure 3A shows that with no treatment/negative control (left group of bars), the more mature the *C. albicans* biofilm (24 hours and 48 hours), the stronger the activity of proteinase. The inhibition effect of Javanese turmeric ethanol extract could stop the activity of proteinase at the initial phase and decrease the proteinase activity at the intermediate (24 hours) and maturation phases (48 hours). At the initial phase, the diameter of proteolytic zone at the negative control was 0,75mm and not visible at the experimental group. At the intermediate and maturation phases, the diameter of the proteolytic zone at the negative control were 4,5mm dan 5 mm respectively. While at the experimental group were 2mm and 3,5mm. No activity of proteinase could be seen at the positive control.

The activity of proteinase of *C. albican*s biofilm which had been inhibited by Javanese turmeric ethanol extract in this study was analyzed by the formation of proteolytic zone on the BSAA. The proteolytic zone appears as a clear zone surrounding the colony of the yeast.

Figure 3B, 3C, 3D respectively shows the activity of proteinase of *C. albicans* biofilm at initial, intermediate and maturation phases. At all the three phases the negative control (a) showed the largest proteolytic zones compared to biofilm which had been exposed to

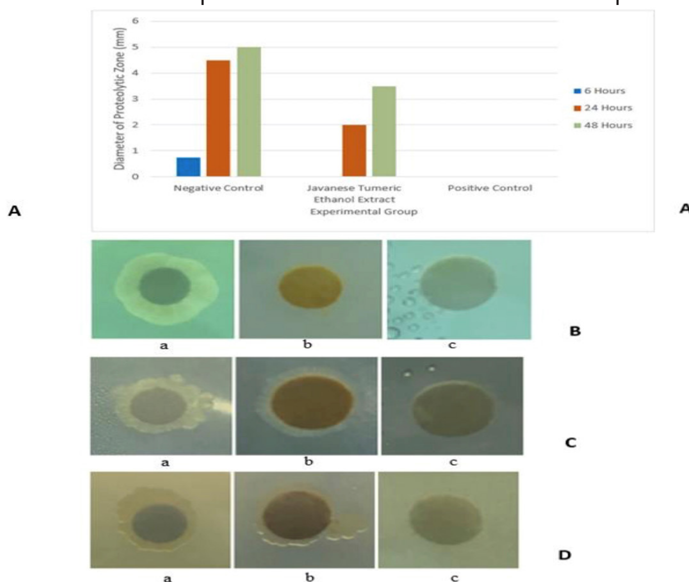

Fig 3. Proteinase activity of C. albicans biofilm at various phases and groups
A.Graph of proteinase activity at the initial, intermediate, and maturation phases of C. albicans biofilm in the experimental and control groups
B.Photograph of proteinase activity of C. albicans biofilm at initial phase (6 hours).
(a)Negative control (b) Experimental group (c) Positive control
C.Photograph of proteinase activity of C. albicans biofilm at intermediate phase (24 hours).
(a)Negative control (b) Experimental group (c) Positive control
D.Photograph of proteinase activity of C. albicans biofilm at maturation phase (48 hours).
(a) Negative control (b) Experimental group (c) Positive control

Javanese turmeric extract (b). Conversely, there was no activity of proteinase at the initial phase (3B), both on the experimental (b) and on the positive control groups (c). At the intermediate (3C) and maturation phases (3D), the *C. albicans* biofilm which had been inhibited by the extract (b) showed proteinase activity but lower than what could be seen at the negative control (a). The positive control (c) did not show proteinase activity at all three phases.

DISCUSSION

In this study the Javanese turmeric ethanol extract showed inhibition effect toward *C. albicans* biofilm in all initial, intermediate, and maturation phases. This result confirms previous report of the inhibition effect of the extract at all *C. albicans* biofilm phases. (16, 17) Based on the observation on the negative control of this study which is the *C. albicans* biofilm without any treatment, it could be seen that the activity of phospholipase and proteinase are increasing following the more mature development of the biofilm. This phenomenon might be due to the increased density of *C. albicans* hypha in the more mature biofilm. A previous in-vitro study reported that *C. albicans* phospholipase is produced higher by *C. albicans* in form of hypha compared to those in form of yeast cells. (18) The activity of *C. albicans* phospholipase has been reported to be more active when the biofilm is at the stage of hyphal form and are predominantly produced at the end of the hyphal formation. (19)

The positive control in this study, which is *C. albicans* biofilm inhibited by nystatin, showed no activity of either phospholipase or proteinase. It is known that Nystatin could destroy the permeability of cell membrane of the yeast by interaction with the cell membrane ergosterol of *C. albicans*. (9, 20) The broken *C. albicans* cell membrane was assumed could decrease the activity of phospholipase because this enzyme is secreted on the membrane cell to become active at the extracellular environment. (21)

The decreased activity of proteolytic enzymes of *C. albicans* biofilm which had been inhibited by Javanese turmeric ethanol extract as shown in this study might be due to the activity of xanthorrhizol which like Nystatin could destruct the *C. albicans* membrane cell. (5) It had been reported that *C. albicans* phospholipase activity is expressed on the fungi cell membrane either in form of yeast or hypha. The activity is centralized on the peripheral of yeast cell membrane or localized at the tip of hypha. Therefore, it might be assumed that any cell membrane destruction could affect the activity of the enzyme. (19)

Based on the observation on the negative control in this study, it could be seen that the activity of proteinase at the initial phase *C. albicans* biofilm is categorized as low, while at the intermediate and maturation phase the activity of this enzyme is categorized as high. In a previous study, it was reported that planktonic *C. albican*s have lower proteinase activity compared to biofilm *C. albicans*. However, that study focused on the proteinase activity of mature *C. albicans* biofilm and no report yet about such condition on the initial and intermediate phase of *C. albicans* biofilm. (22)

In this study, the observation of proteinase activity on *C. albicans* biofilm was conducted every day until there was no more formation of proteolytic zone. In a previous study it was reported that the formation of the proteolytic zone stopped at day six while in this study there was no more formation of proteolytic zone after day three. The formation and development of the proteolytic zone of the proteinase activity might be influenced by the absence of yeast carbon base in the medium used to inoculate the yeast in this study. Yeast carbon base has role in facilitating the nitrogen assimilation. Therefore, the absence of this substance might result in insufficiency of nutrients after several days of inoculation and decreased secretion of proteinase. (23)

Results of this study showed that exposure of Javanese turmeric ethanol extract at concentration that could inhibit ≥50% of *C. albicans* biofilm could also lead to decreased activity of *C. albicans* proteinase at all three biofilm phases. This result might be not only because xanthorrhizol is the main active component of the extract, but also could be due to curcumin which had been reported to have potency in decreasing the activity of proteinase without decreasing the secreted enzyme. (24)

CONCLUSION

The inhibition effect of Javanese turmeric ethanol extract on *C. albicans* biofilm could result in decreased activity of phospholipase and proteinase of *C. albicans* biofilm. The activity of both phospholipase and proteinase could be weakened at all the initial, intermediate, and maturation phases of *C. albicans* biofilm. The influence of Javanese turmeric ethanol extract in weakening the activity of phospholipase and proteinase is stronger at the earlier than at the later phase of *C. albicans* biofilm. At the initial phase of *C. albicans* biofilm, the inhibition effect of Javanese turmeric ethanol extract could even stop the activity of proteinase.

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