


Inhibition and Eradication Effect of *Javanese turmeric (Curcuma xanthorrhiza Roxb.)* Extract Against Mature Phase Biofilm of *Candida albicans*

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Abstract

Objective: To analyze the potency of Javanese turmeric extract in inhibiting and eradicating the mature phase of *C. albicans* biofilm. **Material and Methods:** *C. albicans* ATCC 10231 was cultured in Sabouraud Dextrose Broth on a 96-well plate and was incubated at 37°C. To analyze its inhibitory effect, *C. albicans* cultures were incubated for 1.5 hours before being exposed to various concentrations of Javanese turmeric extract, followed by a further 48-hour incubation to reach the maturation phase. To analyze the eradication effect, the 48-hour *C. albicans* cultures were exposed to the extract and incubated further for 24 hours. Nystatin (100,000 IU) was used as a positive control. The percentage of viable *C. albicans* cells on the 48-hour biofilm was determined by a methylthiazol tetrazolium assay. This value was converted into the percentage of the extract's minimum inhibitory and eradication concentrations. **Results:** Against the mature phase *C. albicans* biofilm, the minimum biofilm inhibitory concentration of the Javanese turmeric extract was 40%, while the minimum biofilm eradication concentration was 45%. There were significant differences between the inhibition percentage of the positive control and that of the solutions exposed to Javanese turmeric with all the tested concentrations ($p < 0.05$). There was a strong positive correlation between the increase in extract concentration and the eradication percentage of the mature *C. albicans* biofilm ($r = 0.981$). **Conclusion:** Javanese turmeric extract is potential for inhibiting and eradicating mature phase *C. albicans* biofilm. The extract is more effective in inhibiting than in eradicating the biofilm.

Keywords: Complementary Therapies; Phytotherapy; Dental Plaque; *Candida albicans*.

Introduction

As an oral cavity commensal fungus, *C. albicans* can live in planktonic form in approximately 30-50% of population [1]. The development of *C. albicans* from a commensal fungus to a pathogen is initiated by a change in the homeostasis of the oral cavity's ecosystem, and by the yeast's virulence factors, including morphological dimorphism of the yeast into hypha and the formation of biofilm [2]. The development of *C. albicans* biofilms consists of 3 phases: adhesion phase, intermediate phase, and maturation phase. The adhesion phase is the first stage and lasts for 11 hours. During this phase, planktonic *C. albicans* begins to adhere on a surface. The next phase, which lasts for 25 hours, is the intermediate phase, during which *C. albicans* changes form from yeast to hypha. The last phase is the maturation phase, which lasts for 34 hours. In this phase, the biofilm becomes more complex and the extracellular matrix thickens, thereby increase the resistance of *C. albicans* biofilm so that the penetration by antifungal agents becomes more difficult [3].

Previous studies have reported the potency of Javanese turmeric extract as an antifungal agent against *C. albicans* in its planktonic form and in the initial biofilm phase. It was previously reported that *xanthorrhizol*, which is isolated from Javanese turmeric root, had an antifungal effect in inhibiting *C. albicans* biofilm with a minimum inhibitory concentration (MIC) of 1-5 mg/L and a minimum fungicidal concentration (MFC) of 5-10 mg/L [4,5]. Javanese turmeric ethanol extract had been reported to eradicate the early phase of *C. albicans* biofilm [6]. An agent's antifungal effect in penetrating and disrupting the development and morphology of the biofilm is known as its eradication effect [7].

It has been reported that Javanese turmeric ethanol extract has an antifungal effect against planktonic *C. albicans* and could eradicate *C. albicans* biofilm in the initial phase [4,6]. The aim of this study was to investigate the potency of Javanese turmeric ethanol extract in inhibiting and eradicating the mature phase of *C. albicans* biofilm.

Material and Methods

Candida albicans

For this study, we employed the laboratory strain of *C. albicans* (ATCC 10231). The negative control was the *C. albicans* biofilm unexposed to any antifungal agent, and the positive control was the *C. albicans* biofilm exposed to 100,000 IU of nystatin. The concentration of the *C. albicans* suspension employed in this study was determined by counting the yeast's colony-forming units (CFUs) after incubating the yeast in Sabouraud Dextrose Agar (SDA) for 24 hours at 37°C.

In this study, planktonic *C. albicans* refers to the *C. albicans* cultured in Sabouraud Dextrose Broth (SDB) in a 96-well plate, exposed to various concentrations of Javanese turmeric or nystatin (positive control) and incubated for 48 hours at 37°C. The *C. albicans* biofilm refers to the biofilm already cultured in SDB in a 96 well-plate and incubated for 48 hours at 37°C.

Exposure of *C. albicans* to Javanese turmeric Extract

To test the inhibitory effect of the Javanese turmeric extract, the *C. albicans* biofilm was exposed to the extract after the culture had been incubated for 1.5 hours and further incubated for 48 hours to reach the maturation phase. To test the extract's eradication effect, the 48-hour biofilm was exposed to the extract and incubated the biofilm for an additional 24 hours.

Javanese turmeric Extract

The Javanese turmeric ethanol extract was extracted by employing the maceration technique using 96% ethanol. We derived 4 kg of extract powder from 20 kg of Javanese turmeric root, which was further extracted to collect 672.5 mg of oil with a yield of 11.20%. To separate the oil into 3 layers, the extract oil was then centrifuged at 3700 rpm for 20 minutes. We used phytochemistry analysis to determine that the top supernatant layer contained 19.59% xanthorrhizol. We diluted the Javanese turmeric ethanol extract to various concentrations by adding SDB. For this study, the extract concentrations tested to achieve MICs were 0.25%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40% and 45%. The extract concentrations tested to achieve minimum biofilm inhibitory concentrations (MBICs) were 15%, 20%, 25%, 30%, 35%, 40%, and 45%.

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of the Extract Against planktonic *C. albicans*

The extract's MIC capable of inhibiting 90% or more of the growth of planktonic *C. albicans* was determined by calculating the optical density of the *C. albicans* culture exposed to Javanese turmeric extract [8]. A microplate reader with a wavelength of 595 nm was used to measure the optical density. The MFC was the minimum extract concentration capable of inhibiting the growth of planktonic *C. albicans* such that none of cultures presented even a single colony.

Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration

The extract's minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were defined as the minimum extract concentration capable of inhibiting 50% or more or 90% or more, respectively, of the viable *C. albicans* cells in the 48-hour (mature) biofilm and were determined using a methylthiazol tetrazolium (MTT) assay. An equation was used to determine the inhibition and eradication percentages [8]. The application of this equation required the optical density of the experimental groups, positive controls, negative controls, blank group, negative blank and positive blank. The experimental groups were the *C. albicans* suspension with various concentrations of Javanese turmeric extract. The negative controls consisted of *C. albicans* suspensions in isolation. The positive controls consisted of *C. albicans* suspensions plus 100 µL nystatin. The blank group consisted of 100 µL SDB plus 100 µL of various concentrations of Javanese turmeric extract. The negative blank group consisted of 100 µL SDB in isolation, and the positive blank groups consisted of 100 µL SDB plus 100 µL nystatin.

MTT Assay

The concentration of the MTT solution employed in this study was 5 mg/mL. The MTT solution was kept in 15 mL tubes and was wrapped with aluminum foil until used. The *C. albicans* biofilm in the 96-well plate was washed with a 100 µL phosphate-buffered saline solution, then added to a 50 µL MTT solution and wrapped in aluminum foil before incubating for 3 hours at 37°C. Then, the solutions were removed from the incubator, and 100 µL acidified isopropanol was added to each well. Next, the well plate was shaken by an orbital shaker at 80 rpm for 1 hour. Finally, the solutions' optical density was measured by a microplate reader at a wavelength of 570 nm.

Data Analysis

Data were analyzed using IBM SPSS Statistics for Windows Software, version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) and post-hoc tests were used. We used a Pearson correlation test to analyze the correlation between the increase in extract concentration and the increase in the inhibition and eradication percentage. For ANOVA and post-hoc tests the level of significance were $p < 0.05$, while for the Correlation test the level of significance was $p < 0.01$.

Results

Table 1 lists the results of the colony counts. The concentration of the *C. albicans* suspension that had an adequate number of colonies but could still be counted visually was 10^{-4} . Therefore, for this study, we employed the 10^{-4} concentration of *C. albicans* suspension.

Table 1. Quantity of *C. albicans* colony at various concentrations.

Dilution Concentration of <i>C. albicans</i>	Number of <i>C. albicans</i> Colonies (CFUs/10 µL)		
	I	II	Mean
10^{-2}	260	257	258.5
10^{-4}	2	30	16
10^{-6}	0	25	12.5
10^{-8}	0	3	1.5

The MIC of the Javanese turmeric extract against planktonic *C. albicans* was determined by measuring the cultures' optical density using a microplate reader with a wavelength of 595 nm, which was then converted into the inhibition percentage value. As Table 2 shows, the minimum concentration of the extract capable of inhibiting 90% or more of planktonic *C. albicans* was 20%.

Table 2. Minimum inhibitory concentration of the Javanese turmeric ethanol extract against planktonic *C. albicans*

No.	Concentration of Javanese turmeric ethanol extract (%)	Inhibition (%)
1	0.25	55.30
2	0.5	63.65
3	1	60.92
4	5	74.41

5	10	68.72
6	15	74.57
7	20	97.50
8	25	82.68
9	30	77.77
10	35	89.31
11	40	94.85
12	45	83.23
13	Positive Control	100.0
14	Negative Control	0

Table 3 shows that the MFC of Javanese turmeric ethanol extract against planktonic *C. albicans* is 35%. At this concentration, exposure to the extract resulted in zero growth of the *C. albicans* colony.

Table 3. Minimum fungicidal concentration of Javanese turmeric ethanol extract against planktonic *C. albicans*.

No.	Concentration of Javanese turmeric ethanol extract (%)	Number of <i>C. albicans</i> colonies, CFUs/10 µL
1	15	-
2	20	94
3	25	15
4	30	2
5	35	0
6	40	0
7	45	0
8	Positive Control	0
9	Negative Control	∞

As Figure 1 shows, exposure to 40% and 45% Javanese turmeric extract resulted in a >50% growth inhibition of the mature *C. albicans* biofilm. Therefore, the MBIC₅₀ of Javanese turmeric ethanol extract in this study was 40%. However, the extract's MBIC₉₀ could not be determined in this study.

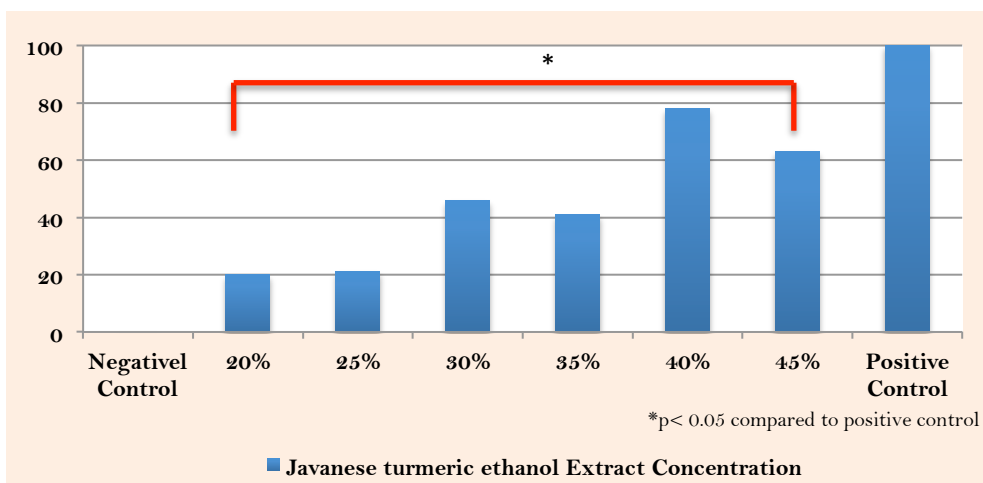


Figure 1. Minimum biofilm inhibition concentration of Javanese turmeric ethanol extract.

The post-hoc statistical analysis confirmed that there were significant differences between the inhibition percentage of the positive control and that of the solutions exposed to Javanese turmeric with all the tested concentrations ($p < 0.05$). The relationship between the Javanese turmeric extract concentration and the inhibition percentage effect showed a strong positive correlation ($r = 0.905$), demonstrating that the higher the extract concentration, the higher its inhibition percentage.

As Figure 2 shows, the 45% Javanese turmeric ethanol extract was able to eradicate more than 50% of the mature *C. albicans* biofilm. Therefore, this study showed that the MBEC₅₀ of the Javanese turmeric ethanol extract against mature *C. albicans* biofilm was 45%. However, the extract's MBEC₉₀ could not be determined.

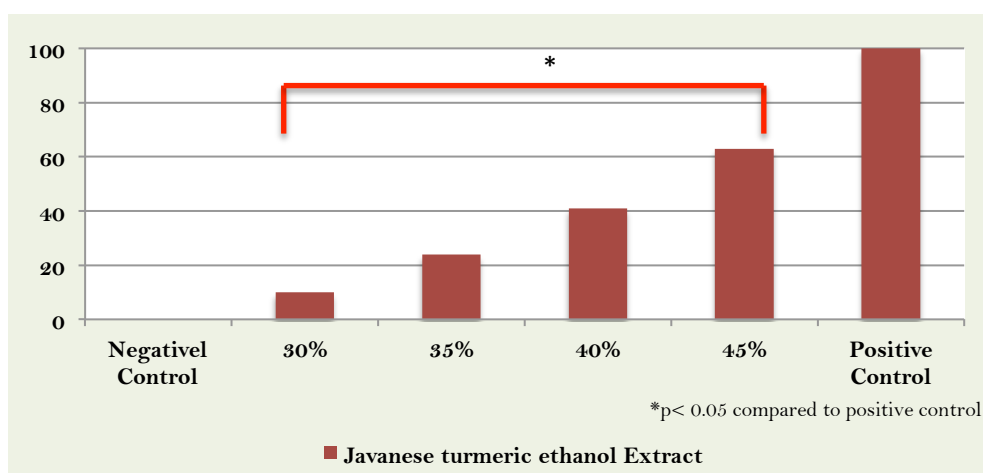


Figure 2. Minimum biofilm eradication concentration of Javanese turmeric ethanol extract.

The post-hoc statistical analysis showed significant differences between the eradication percentage of the positive control and that of the experimental groups ($p < 0.05$). There was a strong positive correlation between the increase in extract concentration and the eradication percentage of the mature *C. albicans* biofilm ($r = 0.981$), demonstrating that the extract's eradication percentage increases as the extract's concentration increases.

Discussion

The MIC and MFC of Javanese turmeric ethanol extract against planktonic *C. albicans* in this study were 20% and 35%, respectively. This study had higher MIC and MFC values than a previous study that reported extract MIC and MFC of 10% and 20%, respectively [6]. This difference could be due to the different xanthorrhizol content in the Javanese turmeric extracts used in the studies. However, the two studies showed similar results in that the MFC was higher than the MIC, and the extract concentration required to inhibit planktonic *C. albicans* is lower than the concentration required to inhibit the *C. albicans* biofilm.

Similar results were also reported by several previous studies on the antifungal agent [9-11]. In this study, the inhibitory effect of Javanese turmeric extract was examined by exposing the *C. albicans* culture to the extract after the culture had been incubated for 1.5 hours. By doing so, the

yeast culture was given the chance to adhere to the well surface and start the biofilm formation, given that planktonic *C. albicans* requires 1 to 2 hours to adhere to a surface [3,12].

The results of this study show that the extract concentration needed to inhibit (MBIC) and to eradicate (MBEC) more than 50% of the growth of the mature *C. albicans* biofilm were 40% and 45% respectively. However, the MBIC₉₀ and MBEC₉₀ could not be determined in this study. These results confirm that eradication of *C. albicans* biofilm is more difficult than its inhibition and, therefore, the extract concentration required to eradicate the biofilm was higher than the concentration needed to inhibit it. The results also showed that the extract could only inhibit or eradicate approximately 60% of the growth of the mature *C. albicans* biofilm, even at a 45% concentration. Conversely, the positive control (nystatin) was capable of inhibiting and eradicating 100% of the growth of the mature *C. albicans* biofilm. These results indicate that although it is not as strong as nystatin, Javanese turmeric ethanol extract has sufficient potency to inhibit or eradicate the *C. albicans* biofilm even during the maturation phase.

From a previous study, we know that at a 35% concentration, the Javanese turmeric extract can eradicate 90% of early-phase *C. albicans* biofilms, both during the adhesion phase (1.5 hours) and during the proliferation phase (3 hours) [6]. From these two studies, we can see that the more mature the *C. albicans* biofilm, the higher the extract concentration required to eradicate the biofilm and the weaker the extract's eradication effect. At a 35% concentration, Javanese turmeric extract can eradicate 90% of early-phase *C. albicans* biofilm, while at a 45% concentration, the extract can only eradicate approximately 60% of the mature *C. albicans* biofilm.

The activity of xanthorrhizol in inhibiting *C. albicans* biofilm is influenced by the extract concentration and the phase of the *C. albicans* biofilm. It has been reported that xanthorrhizol can eradicate 100% of early-phase *C. albicans* biofilm at 8 µg/mL but can only eradicate 67.48% of the mature biofilm at 32 µg/mL [4]. These results indicate that the efficacy of Javanese turmeric extract is in line with the efficacy of xanthorrhizol, which is its active antifungal component. During the maturation phase, *C. albicans* biofilm is more complex and its extracellular matrix is denser, which makes penetration more difficult [3,13]. Both nystatin and xanthorrhizol contain OH chains capable of disrupting the *C. albicans* cell membrane [14]. This antifungal mechanism could explain the antifungal effect of Javanese turmeric extract.

Conclusion

The antifungal effect of Javanese turmeric extract against mature *C. albicans* biofilm is not as strong as its effect on early-phase *C. albicans* biofilm. However, Javanese turmeric extract is potent in inhibiting and eradicating mature *C. albicans* biofilm. The inhibitory effect of Javanese turmeric ethanol extract is stronger than its eradication effect against mature *C. albicans* biofilm.

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Conflict of Interest: The authors declare no conflicts of interest.

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