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by Muhammad Luthfi

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Inhibitory effect of jengkol leaf (*Pithecellobium jiringa*) extract to inhibit *Candida albicans* biofilm

Muhammad Luthfi, Ira Arundina, and Nizamiar Hanmi

Department of Oral Biology
Faculty of Dental Medicine, Universitas Airlangga
Surabaya-Indonesia

ABSTRACT

Background: *Candida albicans* (*C. albicans*) are dimorphic fungi in oral cavity, considered not only as normal flora, but also as pathogens. *C. albicans* have an ability to grow biofilm, which has a thick layer of outer skin structure, called as extracellular matrix. Jengkol leaves (*Pithecellobium jiringa*) contain alkaloids, flavonoids, terpenoids, and lectins, which have an ability as antifungal agent. **Purpose:** This study aimed to analyze the optimum dose of jengkol leaf extract as antibiofilm against *C. albicans* biofilms. **Method:** *C. albicans* were cultured on yeast peptone dextrosa (YPD) media in 96-well microtiter plate flat bottom plates. There were one control group (without treatment) and three treatment groups. The first treatment group was given jengkol leaf extract at a dose of 100 mg/ml. The second treatment group was given jengkol leaf extract at a dose of 200 mg/ml. And, the third treatment group was given jengkol leaf extract at a dose of 400 mg/ml. Semi quantitative method was applied to determine *C. albicans* biofilms using Crystal Violet staining technique. The absorbance of the cells then was calculated using a spectrophotometer with a wavelength of 570 nm. **Result:** The mean value of optical density in the control group was 1.23. The mean value of optical density in the treatment group with a dose of 100 mg/ml was 0.2. Meanwhile, the mean value of optical density in the treatment group with a dose of 200 mg/ml was 0.2, and 0.21 in the treatment group with a dose of 400 mg/ml. The results also showed that there were significant differences between the control group and all of the treatment groups ($p < 0.05$), but there was no significant difference between the treatment groups ($p > 0.05$). **Conclusion:** The optimum dose of jengkol leaf extract used as antibiofilm against *C. albicans* biofilms is 100 mg/ml with an inhibitory percentage of 83.7%.

Keywords: jengkol leaf extract; antibiofilm; *Candida albicans*

Correspondence: Muhammad Luthfi, Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Jl Mayjend. Prof. Dr. Moestopo no. 47 Surabaya 60132, Indonesia. E-mail: m.luthfi7@yahoo.com

INTRODUCTION

Candida albicans (*C. albicans*) are dimorphic fungi in oral cavity, considered not only as normal flora, but also as pathogens. If the growth of *C. albicans* in the oral cavity is excessive, it can trigger certain diseases.¹ Those diseases are usually such fungal infections that are known as oral candidiasis.² According to a research conducted by Pamman, the prevalence of fungal infections caused by *C. albicans* is high, ie 55 cases out of 100 patients. Similarly, based on data from the Directorate General of Disease Control and Environmental Health of the Ministry of Health of the Republic of Indonesia, there were 21,591 cases in 2010,

21,031 cases in 2011, and 21,511 cases in 2012 related to Human immunodeficiency Virus (HIV) in Indonesia, 10,689 of which are also classified as oral candidiasis caused by *C. albicans*.^{3,4}

Candida is a type of fungus that has a high prevalence in the formation of biofilms, particularly *C. albicans*. *C. albicans* biofilms have the outermost layer structure, called as extracellular matrix. Extracellular matrix is formed by polysaccharide that serves to maintain stability of *C. albicans* while growing and developing.⁵ Oral candidiasis, can be classified based on clinical symptoms. Classic type commonly occurs is pseudomembranous candidiasis. Pseudomembranous candidiasis patients

have certain clinical symptoms, such as disturbing pain in the area of oral infections. Bleeding then can occur when pseudomembranous open. This disease even occurs potentially in patients who have been infected with HIV.²

Actually, there have been many choices for antifungal drugs on markets today, one of which widely used is azole class with certain side effects when consumed in long term. One of the side effects is in the form of hepatotoxicity. A research using experimental animals given fluconazole at a dose of 12.26 mg (the therapeutic dose in humans) per animal per day for 20 weeks, liver tumors even can emerge at week 10 after the administration.⁶

Jengkol (*Pithecellobium jiringa*) is a typical plant of Southeast Asia. Jengkol leaves contain flavonoids, tannins, alkaloids, steroids, glycosides, lectins, and steroids/terpenoid.^{7,8} Alkaloids are able to impair mitochondrial function and consistency of the cell wall. Lectin has an ability to attach to mannose and hyphae. Lectins and flavonoids, furthermore, have an ability to disrupt cell interaction process.^{9,10} Terpenoid has an active role in inhibiting cell cycle process of *C. albicans*.¹¹ Similarly, a research conducted by Bakar¹² shows that the methanol extract of jengkol leaves (*Pithecellobium jiringa*) has antifungal power against *C. albicans* cells with minimum inhibitory concentration (MIC) of 100 mg/ml.

Unfortunately, there have not many researches on the effects of antibiofilm derived from jengkol leaf extract, especially against *C. albicans* biofilms. Jengkol leaves have some natural chemical compounds considered to be able to work synergistically as antibiofilm. Thus, the results of this research can be considered as a scientific study for the development of traditional medicine expected to be applied as a candidate of antifungal drug selection.

MATERIALS AND METHOD

This research was an in vitro laboratory experimental research. *C. albicans* were cultured at the Laboratory of Microbiology in Faculty of Dentistry, Universitas Airlangga. Meanwhile, jengkol leaves were extracted at the Laboratory of Pharmacognosy in Faculty of Pharmacy, Universitas Airlangga. And, antibiofilm test on *C. albicans* were conducted at the Laboratory of Microbiology in Faculty of Medicine, University of Brawijaya from June to July 2015.

Jengkol leaf extract at various concentrations were carried out in several stages. Jengkol leaves were taken from a cultivating garden in Ngantang, Malang. Jengkol leaves then were identified in the Technical Implementation Unit of Plant Conservation Center in Purwodadi (Unit Pelaksana Teknis Balai Konservasi Tumbuhan Kebun Raya Purwodadi Lembaga Ilmu Pengetahuan Indonesia) as a part of Indonesian Institute of Sciences in Pasuruan. Sampling of jengkol leaves was conducted with certain criteria, such as fresh, green, and planted at least for 5 years. Jengkol leaves were separated from the stems and

stalks. Those leaves then were dried up through wind, and crushed with a blender. 50 grams of jengkol leaf powder was put in an Erlenmeyer flask sized 250 ml, added with 150 ml of methanol, and then macerated for 24 hours using a shaker. The results of this maceration were filtered with filter paper to obtain a filtrate.

Jengkol leaf powder residue was added to the methanol and then re-macerated for 24 hours. Steps 4 and 5 were repeated until there was no color (clear) in thin layer chromatography (TLC) check. The solution derived from the filtration process was evaporated with rotary vacuum evaporator. The extract resulted was in the form of condensed extract weighed 5 grams. It was diluted at doses of 100 mg/ml, 200 mg/ml, and 400 mg/ml.

C. albicans used this research were cultured on yeast peptone dextrosa (YPD) media in a 96-well microtiter plate flat bottom plate. *Ca. albicans* biofilms then were made in several stages. 100 L suspension of *C. albicans* as the test concentration and sucrose 2% were added using a multichannel pipette to induce biofilm formation into 96-well microtiter flat bottom plate. The entire microtiter plates were covered, wrapped with cling wrap, and then incubated for 24 hours at a temperature of 37⁰ C. Multichannel pipette was used to rinse the plates repeatedly 3 times with sterile PBS (200 mL per well). Alternatively, automated microtiter plate washer can be used. During flushing and after the last rinsing, the plates were placed upside down and then closed with a paper towel to remove any residual PBS. In these circumstances, biofilms formed could be seen by inverted microscope. Biofilms then were ready to proceed to the antibiofilm testing process.

Afterwards, the samples were divided into four groups, consisted of one control group (no treatment) and three treatment groups. Those three treatment groups were given jengkol leaf extract at doses of 100 mg/ml, 200 mg/ml, and 400 mg/ml. Replication was performed in each treatment five times.

Jengkol leaf extract with doses of 100 mg/ml, 200 mg/ml, and 400 mg/ml were applied to *C. albicans* biofilms that had been cultured in a 96-well microtiter flat bottom plate. By using multichannel pipette, jengkol leaf extract at a dose of 100 mg/mL was added to column 1, a dose of 200 mg/mL to column 2, and a dose of 400 mg/mL to column 3. Column 4 as a control was not given the extract. The plates then were covered and incubated for 48 hours at a temperature of 37⁰ C. After that, the plates were rinsed three times with sterile PBS (200 mL per well). Staining then was performed using crystal violet, and incubated for 45 minutes. The whole wells were rinsed four times with sterile distilled water, and immediately given 200 mL of 95% methanol. 100 µL of the methanol was taken using pipette to be replaced to a new well. Afterwards, absorbance of each well was measured using a spectrophotometer with a wavelength 570 nm.¹³

Data of the mean value of optical density then were derived from spectrophotometric readings. Optical density (OD) is a unit used to look turbidity of *C. albicans* biofilms

on microtiter plates. Turbidity illustrates the number of *C. albicans* biofilms attached to microtiter plates.¹⁴ The mean optical density value of each treatment group then was converted into an inhibitory percentage using the following formula:

$$\text{Inhibitory percentage} = \frac{(\text{OD control at 570 nm} - \text{OD treatment at 570 nm})}{\text{OD control at 570 nm}} \times 100$$

RESULTS

In this research, there were four groups, one control group (no treatment) and three treatment groups. Replication of each group was performed 6 times. Table 1 shows the values of optical density in each group, the control group and the treatment groups.

In Table 1, column with control information indicates the optical density value of the control group. Column with a caption 1 shows the optical density value of the jengkol leaf extract at a dose of 100 mg/ml. Meanwhile, column with a caption 2 shows the optical density value of the jengkol leaf extract at a dose of 200 mg/ml. Column with a caption 3 shows the optical density value of the jengkol leaf extract at a dose of 400 mg/ml.

Table 2 shows the mean optical density values of the research groups. In the control group, the mean OD value was 1.23 with a standard deviation of 0.53. In the group with jengkol leaf extract at a dose of 100 mg/ml, the mean OD value was 0.2 with a standard deviation of 0.01. Meanwhile, in the group with jengkol leaf extract at a dose of 200 mg/ml OD, the mean OD value was 0.2 with a standard deviation

of 0.02. In the group with jengkol leaf extract at a dose of 400 mg/ml, the mean OD value was 0.21 with a standard deviation of 0.03.

Normality test was performed using one-sample Kolmogorov-Smirnov test. P value obtained for each sample was more than 0.05. It means that the data had normal distribution. Furthermore, after the homogeneity test (Levene test) was conducted, p value obtained was 0.00 (p<0.05). It indicates that the data were homogeneous. Afterwards, to know the differences between the groups, Kruskal test was carried out. The significance value obtained was 0.003 (p<0.05). It means that there was a difference between the groups. Mann Whitney test then was performed to find significant differences between two groups. The results of Kruskal Wallis test also showed that there was significant differences between groups since the significance value obtained was less than 0.05 (0.003) as shown in Table 3.

Based on the results of Mann Whitney test, there was a significant difference between the control group and the treatment group with a dose of 100 mg/ml (0.004). There was also a significant difference between the control group and the treatment group with a dose of 200 mg/ml (0.004). Similarly, there was a significant difference between the control group and the treatment group with a dose of 400 mg/ml (0.004).

However, there was no significant difference between the treatment group with a dose of 100 mg/ml and the treatment group with a dose of 200 mg/ml (0.371). Similarly, there was no significant difference between the treatment group with a dose of 100 mg/ml and the treatment group with a dose of 400 mg/ml (0.421). There was also no significant difference between the treatment group with

Table 1. OD values of *Candida albicans* biofilms after the administration of jengkol leaf extract (*Pithecellobium jiringa*) at a dose of 100 mg/ml, 200 mg/ml, and 400 mg/ml in the treatment groups as well as in the control group (without the administration)

Description	Doses of Jengkol Leaf Extract (mg/ml)	Replication					
		I	II	III	IV	V	VI
Control	Control	1.972	1.012	0.887	1.731	0.556	1.249
1	100	0.196	0.184	0.181	0.190	0.214	0.220
2	200	0.195	0.177	0.183	0.172	0.201	0.235
3	400	0.248	0.206	0.175	0.239	0.175	0.230

Table 2. The mean and standard deviation of OD values of *Candida albicans* biofilms after the administration of jengkol leaf extract (*Pithecellobium jiringa*) at a dose of 100 mg/ml, 200 mg/ml, and 400 mg/ml in the treatment groups as well as in the control group (without the administration)

Data	n	\bar{X}	SD
Control	8	1.23	0.53
Jengkol leaf extract (<i>Pithecellobium jiringa</i>) (100 mg/ml)	8	0.2	0.01
Jengkol leaf extract (<i>Pithecellobium jiringa</i>) (200 mg/ml)	8	0.2	0.02
Jengkol leaf extract (<i>Pithecellobium jiringa</i>) (400 mg/ml)	8	0.21	0.03

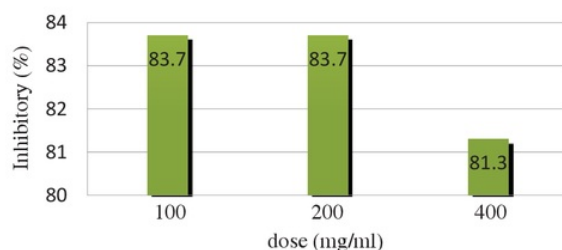


Figure 1. Diagram of the inhibitory percentages in each treatment group against *Candida albicans* biofilms.

Table 3. Differences between groups based on the results of Mann Whitney test

	Control	100mg/ml	200mg/ml	400mg/ml
Control		0.004*	0.004*	0.004*
100 mg/ml			0.371	0.421
200 mg/ml				0.257
400 mg/ml				

a dose of 200mg/ml and the treatment group with a dose of 400 mg/ml.

In addition, the diagram below (Figure 1) shows the inhibitory percentages of the whole groups against *C. albicans* biofilms. In the groups with doses of 100 mg/ml and 200 mg/ml, the inhibitory percentage for each was 83.7%. Meanwhile, in the group with a dose of 400 mg/ml, the inhibitory percentage was 81.3%. It means that there was a decrease of 2.4% in the inhibitory percentage of the treatment groups, from a dose of 400 mg/ml to a dose of 200 mg/ml.

DISCUSSION

In this study, jengkol leaf extract used was at doses of 100 mg/ml, 200 mg/ml, and 400 mg/ml. The determination of these doses was based on a preliminary study stating that jengkol leaf extract containing antifungal materials that has a minimum inhibition concentration (MIC) of 100 mg/ml against *C. albicans*.¹² In Table 2, the mean value of the optical density (OD) in the control group (untreated group) was 1.23, higher than the groups treated with doses of 100 mg/ml and 200 mg/ml generating 0.2 for each, and a dose of 400 mg/ml deriving 0.21. It means that the number of *C. albicans* biofilms in the treatment groups was fewer than in the control group (untreated group). It also indicates that there was no decline in the mean value of OD as the dose increased. There was an increase in the mean value of OD in the treatment group with a dose of 400 mg/ml compared to the treatment group with a dose of 200 mg/ml.

The increase in the mean value of OD at a dose of 400 mg/ml is likely to be caused by the resistance of *C. albicans* biofilms against jengkol leaves (*Pithecellobium jiringa*). The resistance of *C. albicans* biofilms to antifungal drugs is due to complex and multifactorial causes. Efflux pump is one of factors causing the resistance of *C. albicans* biofilms.¹⁵ Efflux pump is a form of fungal cell's defense against agent/ antifungal drugs. Efflux pump occurs as a result of huge pressure from jengkol leaf extract at a dose of 400 mg/ml, which affects osmolarity of the fungal cells. Efflux pump mechanism occurs by pumping out or secreting the antifungal agents through the fungal cell membrane using adenosine triphosphatase of binding cassette transporter (ATP-binding cassette transporter). As a result, the antifungal drugs that are already in the cells pumped out without making any contact with the drug targets.¹⁶

The inhibitory percentage of jengkol leaf extract against *C. albicans* biofilms (Figure 1) at a dose of 100 mg/ml was 83.7%. Meanwhile, at a dose of 200 mg/ml, the inhibitory percentage was 83.7%, and at a dose of 400 mg/ml the inhibitory percentage was 81.3%. It indicates that jengkol leaf extract had inhibitory effects on *C. albicans* biofilm formation. Similarly, an initial research claims that jengkol leaf extract is antifungal against *C. albicans* cells.¹² Based on phytochemical examination in the laboratory of the Faculty of Pharmacy, Universitas Airlangga, jengkol leaf extract positively contains alkaloids, flavonoids, terpenoids, and polyphenols. Jengkol also contains lectin compounds.^{17,18}

Alkaloids are antifungal derived from organic plants. In a research conducted by Dhangaye, alkaloid undermines the integrity of the cell walls of *C. albicans* as well as interferes calcineurin pathway system. Such damage then causes mitochondrial dysfunction, and *C. albicans* ultimately will die.¹⁹ Lectin, on the other hand, has an ability to bind to hyphae and mannose. Hyphae in *C. albicans* is a structure that serves as a major tool in the absorption of nutrients. Therefore, impairing absorption of nutrients can cause disruption of spore germination of *C. albicans*. The binding of lectin - mannose that is elements of the extracellular matrix of the biofilm, furthermore,

will trigger antibiofilm activities.⁹ Meanwhile, lectin and flavonoids have an ability to disrupt the interaction of cells (cell signaling pathway). The interaction of cells is a process of interaction between *C. albicans* and host cells that occurs in the attachment stage. The interaction stage, consequently, can be considered as an important stage in the colonization and penetration of *C. albicans*.^{9,10} Terpenoid has an ability to damage cell cycle system of *C. albicans* by binding proteins on the cell membrane of *C. albicans*, resulting in disruption of cell cycle leading to the death of *C. albicans*.¹¹

In other words, jengkol leaves (*Pithecellobium jiringa*) contain alkaloids, flavonoids, terpenoids, and lectin that work synergistically as antibiofilm of *C. albicans*, ultimately degrading *C. albicans* biofilms. The degradation of *C. albicans* biofilms is described by a decrease in the mean value of OD at doses of 100 mg/ml, 200 mg/ml, and 400 mg/ml (Tabel 2). It may be concluded that the optimum dose of jengkol leaf extract (*Pithecellobium jiringa*) in inhibiting *C. albicans* biofilms is 100 mg/ml with an inhibitory percentage of 83.7%.

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