

ANTIFUNGAL EFFICACY OF SECANG HEARTWOOD (*CAESALPINIA SAPPAN L.*) SOLUTIONS ON BIOFILMS OF *CANDIDA ALBICANS* ATCC 10231

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ABSTRACT

Objective: *Candida albicans* is a predominant fungal species found in persistent root canal infection, and its virulence depends on the ability to form biofilms. To be able to eliminate this infection, a safe irrigation solution during root canal preparation is needed. This study is conducted to investigate the antifungal properties of secang heartwood extract solutions on *C. albicans* biofilm.

Methods: *C. albicans* biofilm was obtained from incubation of *C. albicans* ATCC 10231 at 96-well plates in 37°C for 24 h. These biofilms were exposed to various treatments: Secang heartwood in three different concentrations (25%, 33%, and 50%), sodium hypochlorite (NaOCl) 2.5%, and control group. The results of the treatment are to see the optical density (OD) value. The higher the OD values, the lower antifungal activity of the solution.

Results: The mean OD result from six samples obtained the mean value of various OD values showed in ELISA reader. Antifungal efficacy of 25% secang heartwood was lower than concentration of 33% and 50%. All concentrations of secang heartwood showed higher OD values than 2.5% NaOCl but lower than control group with $p=0.01$.

Conclusion: Secang heartwood possesses antifungal effects against *C. albicans* biofilm but is lower than 2.5% NaOCl. Concentration of 25% has the strongest effect against *C. albicans* among other concentrations. This was a preliminary study of the antifungal efficacy of secang heartwood extract solutions against *C. albicans* biofilm. The result indicates that this solution has the potential to be used as an herbal alternative in root canal irrigations.

Keywords: *Candida albicans*, Biofilm, Secang heartwood, Optical density.

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INTRODUCTION

A major consideration in the treatment of endodontic failure is the elimination of dominant bacteria such as *Enterococcus faecalis* and fungi such as *Candida albicans* [1]. The ability of *C. albicans* to form biofilms provides a protective barrier in the form of an extracellular matrix and makes *C. albicans* thousands of times more resistant to phagocytosis, antibodies, and antimicrobial agents [2,3]. The long-term success of root canal treatment is affected by the cleaning, shaping, and disinfection of the root canals. Effective irrigation is essential because the anatomy of the complex root canal system reduces the effectiveness of instrumentation in root canal treatment [4].

The commonly used root canal irrigation solutions include sodium hypochlorite (NaOCl), citric acid, and ethylenediaminetetraacetic acid (EDTA) [5]. In 2010, Fidalgo *et al.* studied the effectiveness of NaOCl, 17% EDTA, and citric acid on *E. faecalis*, *C. albicans*, and *Staphylococcus aureus*. Irrigation using 17% EDTA resulted in better antifungal activity than 0.5% NaOCl but showed the same effectiveness as 1% NaOCl [6]. NaOCl at concentrations of 2.5% and 5.25% was more effective than 17% EDTA, and 5.25% NaOCl had lethal effects on all microorganisms studied. Increasing the concentration of NaOCl not only increased its effectiveness but also resulted in increased toxicity. 2.5% NaOCl can be recommended, as it has the same effectiveness as 5.25% NaOCl but is less toxic [6]. There has been a report of NaOCl extrusion into periapical tissues including acute pain, swelling, hemorrhage, numbness, and secondary infection [7].

Caesalpinia sappan L., commonly referred to as Secang, is a medicinal plant widely used in Ayurveda and Chinese herbal medicine for treating tuberculosis, diarrhea, dysentery, skin infection, and anemia [8]. Brazilin, the major natural compound found in secang heartwood, has various biological activities, including antimicrobial activity [9-11]. In

2014, Kusmiati and Priadi studied the antimicrobial activity of secang heartwood, using ethanol as a solvent, at concentrations of 50%, 33%, and 25% against planktonic *C. albicans*, and showed a maximum zone of inhibition of 14.65 mm in diameter with a concentration of 50% [12]. Hence, the purpose of this study is to assess the efficacy of secang heartwood extract solutions to *C. albicans* biofilm.

The present study aimed to compare the antifungal effects of the active ingredient of secang heartwood extract solution with 2.5% NaOCl against biofilms of *C. albicans* ATCC 10231.

METHODS

Secang heartwood preparation was done by macerated 2.5 kg of secang heartwood in 500 ml 96% ethanol for 3 h×24 h. The concentration process is carried out with rotary evaporator equipment at 80°C and continued to crystallization phase until thick extract solution of 100% secang heartwood is obtained. The solution was diluted using 10% dimethyl sulfoxide to obtain the desired concentrations, i.e., 25%, 33%, and 50%, and stored at 4°C. The active ingredient brazilin was tested using high-performance liquid chromatography (HPLC). HPLC analysis was carried out using a binary HPLC pump with a photodiode array detector and an autosampler. The calibration curve of brazilin was established using authentic Brazilin at the concentration ranges of between 15.6 and 250 µg/ml. The system used was as follows: A gradient program for 45 min from 5% to 100% methanol in 0.05% aqueous trifluoroacetic acid at a flow rate of 10 ml/min. This preparation step gave crude brazilin, protosappanin A, and sappanone B.

C. albicans ATCC 10231 was cultured for 24 h at 37°C in Sabouraud Dextrose Broth (SDB), and the inoculum density results were calculated to obtain a suspension containing 1×10^6 cells/mL.

Before biofilm examination, an inhibition zone test was performed to determine the optimal concentration of the secang heartwood extract that was required to eliminate *C. albicans* in planktonic cells. Determination of the optimum concentration of secang heartwood extract solution against *C. albicans* was performed by measuring the diameter of the inhibition zone using the concentrations of 25%, 33%, and 50% secang heartwood, NaOCl 2.5%, and control group. After the optimum dosage was obtained, then we continue to analyze the effect on *C. albicans* biofilm.

Antifungal activity against *C. albicans* biofilm was determined by adding 200 μ L of SDB solution containing *C. albicans* ATCC 10231 to a 96-well plate and incubating at 37°C for 90 min to allow the cells to attach. The cells were then rinsed with 100 μ L of sterile phosphate-buffered saline (PBS), and then, 100 μ L of extract was added to the 96-well plates except the control group based on the design that was made before. Biofilm production was detected by measuring optical density (OD) in microtiter plate with ELISA reader. The *Candida* that is grown in Sabouraud's agar is added into 5mL sterile saline with 0,85% concentration to density of a 0.5 McFarland nephelometer standard tube no 3 was matched with the growth of *Candida* in test tube which is approximately 107 cells / ml. Followed by a 1:20 dilution in Sabouraud's broth with a final concentration of 8% glucose. Then, 100 μ L of suspension was incubated at 37°C overnight in flat-bottomed 96-well microtiter plate for biofilm production. The microtiter plate was washed with PBS, and microtitre plate was stained with 110 μ L of 0.4% aqueous crystal violet solution for 45 min. Afterward, each well was washed and destained with 200 μ L of 98% ethanol. After 45 min of destaining, the colorimetric change reading was taken in a microtiter plate reader at 492 nm using microtiter plate ELISA reader production (Lablife ER 2007 Microplate washer, DIAGNOVA).

The normality and homogeneity of the data were then analyzed using SPSS 22.0. The normality test using Shapiro-Wilk test because the sample is <50. If the data were normally distributed and homogeneous, a one-way ANOVA statistical test was performed if there was a significant difference ($p < 0.05$) and it was followed by Bonferroni *post hoc* test. One-way ANOVA was used to compare OD values in various treatments of *C. albicans* biofilm. The significance level considered in this study is 0.05.

RESULTS

Measurement of antifungal activity of secang heartwood extract solution against *C. albicans* biofilm was performed by examining the OD value using microtiter ELISA reader; a low OD value represented low levels of fungi. Before biofilm examination, an inhibition zone

test was performed to determine the optimal concentration of the secang heartwood extract that was required to eliminate *C. albicans* in planktonic cells (free-floating cells).

Table 1 shows that the mean inhibition zone diameter in the 50% concentration group was the greatest, with a value of 10.75 mm. One-way ANOVA results showed a significant ($p < 0.05$) difference in the inhibition zone diameter in all study groups. Next, Bonferroni test was used to determine the effect of each concentration because the Levene test resulted in homogeneity of the studied variables. There was no significant difference between the 25% and 33% or the 33% and 50% secang extract solutions, whereas there was a significant difference between the 25% and 50% solutions ($p = 0.001$). Since there is no significant difference in all three concentrations, all the concentrations were used in this study to determine the antifungal activity of secang heartwood against *C. albicans* biofilm.

After exposing various treatment groups to *C. albicans* ATCC 10231 biofilm, the OD values obtained were normally distributed (Table 2). The lowest OD value was observed with 2.5% NaOCl and the highest OD value was observed in the biofilms of the control group. The OD values at concentrations of 25%, 33%, and 50% were lower than those for the untreated group, demonstrating the antifungal effects of secang heartwood extract on *C. albicans* biofilm.

Higher concentrations of secang heartwood extract solution showed higher OD values, demonstrating lower antifungal activity of the solution (Table 2); therefore, the concentration of secang heartwood extract solution was not directly proportional to the antifungal effect on *C. albicans* biofilm.

One-way ANOVA showed that there was a significant difference in all treatment groups. A Bonferroni test is used to examine the relationship between each treatment group (Table 3). *Post hoc* Bonferroni is used because the Levene test showed that the data are homogenous. However, there was no significant difference between the antifungal effectiveness of 33% and 50% solutions against *C. albicans* biofilm.

DISCUSSION

The present study aimed to determine the antifungal effectiveness of secang heartwood extract solution on *C. albicans* ATCC 10231 biofilm. Microbiological studies of chronic apical periodontitis showed that *C. albicans* is the most common fungus found in around 7–18% of all infections. While the prevalence of fungi is relatively low, its presence is symbiotic with *E. faecalis*, which plays a role in the formation of persistent infection, especially in teeth that have undergone root canal

Table 1: Mean value of inhibition zone diameter (mm) after exposing *C. albicans* ATCC 10231 to various concentrations of secang heartwood extract solution

Treatment group	n (sample)	Mean \pm SD	95% CI		p value
			Upper border	Lower border	
Secang heartwood extract, 25%	4	6.75 \pm 0.5	5.9544	7.5456	*0.001
Secang heartwood extract, 33%	4	7.5 \pm 0.57	6.58	8.4	
Secang heartwood extract, 50%	4	10.75 \pm 2.6	6.56	14.9	

*One-way ANOVA, $p < 0.05$, *C. albicans*: *Candida albicans*, SD: Standard deviation, CI: Confidence interval

Table 2: Mean OD values after exposing various treatment groups to *C. albicans* ATCC 10231 biofilm

Treatment groups	n	Mean \pm SD	95% CI		p value
			Upper border	Lower border	
Secang heartwood extract, 25%	6	0.02133 \pm 0.005447	0.015	0.27	*0.001
Secang heartwood extract, 33%	6	0.06108 \pm 0.0149	0.045	0.076	
Secang heartwood extract, 50%	6	0.8533 \pm 0.00689	0.085	0.092	
NaOCl, 2.5%	6	0.0045 \pm 0.001	0.003	0.005	
Control	6	0.2065 \pm 0.001	0.20545	0.20755	

*One-way ANOVA, $p < 0.05$, NaOCl: Sodium hypochlorite, OD: Optical density, *C. albicans*: *Candida albicans*, SD: Standard deviation, CI: Confidence interval

Table 3: Significance of difference in OD between treatment groups against biofilm of *C. albicans* ATCC 10231

Treatment groups	Secang heartwood extract, 25%	Secang heartwood extract, 33%	Secang heartwood extract, 50%	NaOCl 2.5%	Biofilm without treatment
Secang heartwood extract, 25%	-	0.007	0.000	0.005	0.000
Secang heartwood extract, 33%		-	0.082	0.002	0.000
Secang heartwood extract, 50%			-	0.000	0.000
NaOCl, 2.5%				-	0.000
Biofilm without treatment					-

*Post-hoc Mann-Whitney U-test, $p \leq 0.05$, NaOCl: Sodium hypochlorite, OD: Optical density, *C. albicans*: *Candida albicans*

treatment [13]. The success of antimicrobial agents in eliminating *E. faecalis* should also consider their ability to eliminate *C. albicans*. Successful elimination of *E. faecalis* accelerates the morphogenesis of *C. albicans* hyphae and increases the virulence of *C. albicans*, resulting in persistent endodontic infections that become increasingly difficult to eliminate [14,15].

Secang heartwood is a herbal product known to contain brazilin compounds that can kill microorganisms [9]. Hangoluan also claimed the pharmacological activity of brazilin, including its antimicrobial activity [16].

The active ingredients in secang heartwood are extracted using ethanol as a solvent. Ethanol is considered a suitable solvent for extracting polyphenols and optimum levels of active compounds deemed safe for human consumption [17].

Batubara *et al.* previously reported the isolation of the active compound from secang heartwood using HPLC [18]. The present study was conducted *in vitro* using *C. albicans* ATCC 10231 samples capable of forming biofilms. The inhibition zone test in our study aimed to determine the most effective concentration in inhibiting the growth of *C. albicans* ATCC 10231 using secang heartwood at concentrations of 25%, 33%, and 50%, in accordance with a previous study by Kusmiati and Priadi [12].

In Table 1, the inhibitory zone test shows that higher concentrations of secang solution (the bold font) resulted in a larger diameter of inhibition zone. This is in accordance with the results reported by Kusmiati and Priadi, who stated that higher concentrations of extracts of secang showed a greater diameter of growth inhibition against microorganisms, indicating a higher efficacy of secang heartwood extract solution as an antimicrobial agent [12].

Table 2 shows that the lowest concentration of secang extract revealed the lowest OD value, demonstrating that the lower the concentration, the greater the antifungal ability of secang heartwood solution against *C. albicans* biofilm.

However, the difference between the results of the inhibition zone and OD value tests on *C. albicans* biofilm could be due to differences in the diffusion ability of each concentration of extract into the *C. albicans* membrane; the lower the concentration, the lower the viscosity of the extract solution, resulting in easier penetration of the solution into the fungal membrane leading to interference with the metabolic processes of *C. albicans*. Another possibility is that the greater the concentration, the more dense coloring of the solution of secang heartwood solution. The crystal violet coloration used in the OD value test not only showed color changes occurring in the cell but also the secang solutions color attached to the surface of the plate; therefore, it may have overestimated the number of microorganism cells attached [7,14,15].

In the present study, 2.5% NaOCl showed greater antifungal ability than secang heartwood solutions from the three concentrations studied through the antimicrobial effect of chlorine-inhibiting enzymes in the fungus, leading to oxidation of sulfhydryl group in the fungal enzyme. Hypochlorite acid and hypochlorite ions result in the degradation of amino acids and hydrolysis [16]. This is in line with the findings of

Spencer *et al.*, which showed that 2.5% NaOCl as a root canal irrigant had better antifungal potential against *C. albicans* than chlorhexidine, MTAD, and EDTA 17% [6,13].

The antifungal mechanism of Secang wood is derived from Brazilin, the main active ingredient [19]. Brazilin is a derivative of flavonoids belonging to the phenol class as a secondary metabolite of secang and belongs to a 3-benzylchroman derivative [20]. The antifungal mechanisms of brazilin have not only been clearly defined but also are thought to be similar to those of phenols, which penetrate into the cell nucleus after denaturing protein bonds and lysing the cell membrane. Phenols bind through their hydroxyl group to the sulfhydryl group of fungal proteins to alter the cell membrane protein and inhibit fungal enzymes such as cellulase, pectinase, and lactase. They also cause a deficiency of nutrient substrates, such as metal complex and insoluble protein, and inhibit oxidative phosphorylation [17]. Based on toxicological studies of *C. sappan* by Athinarayananana *et al.*, there were no death rats recorder as a result of oral treatment to the test plant sample and none of the test dye samples exhibited any sign of toxicity/adverse reactions in the animal models used in the study [21].

The unique structure of brazilin, such as the hydroxyl group at the seventh position (7- oxygenation), the hydroxyl substituent at C4, and the double bond at C2-C3, plays the roles in its effectiveness as an antifungal agent [3,18].

CONCLUSION

Secang heartwood has antifungal effects on biofilms of *C. albicans* ATCC 10231. Increasing the concentration of secang heartwood extract solution does not lead to a directly proportional antifungal effect on *C. albicans* biofilm. The antifungal effect of 25% secang heartwood extract solution was the most effective concentration of *C. albicans* biofilm compared with other concentrations of secang heartwood extract solution; however, 2.5% NaOCl showed the best antifungal effects among the studied treatments against biofilm *C. albicans*. The use of secang heartwood extract solution as an antifungal of endodontic irrigant can be helpful since it is biocompatible to surrounding tissues and lack of serious risk compared to NaOCl. Pre-clinical and clinical trials are recommended for evaluating its safety and biocompatibility before its use as an intracanal irrigating solution in the clinical setting.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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