# Antibacterial Efficacy of Secang Heartwood (*Caesalpinia sappan* L.) Extract Solutions Against *Enterococcus faecalis* Biofilm Obtained from Clinical Isolates

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**Abstract**

*Enterococcus faecalis* is the common bacteria causing persistent root canal infections, and its virulence is 1000 times stronger in biofilms than planktons. Chlorhexidin is a golden standard irrigation material in eliminating *E. faecalis* in endodontic treatment, however it has cytotoxicity, which can increase the number of free radicals with risk of cell death. Due to some side effect of chemical irrigation solutions used during root canal preparation, it is useful to seek for herbal alternatives with better profile of side effects and equivalent efficacy. Thus, this study aimed to analyze the efficacy of secang heartwood extract in eliminating *E. faecalis* biofilm.

The study sample was the patient with a diagnosis of chronic apical periodontitis and chronic apical abscess. Bacterial growth of *E. faecalis* clinical isolates bacteria was seen from 4 patients (total 7 patients) and confirmed through bacterial DNA with conventional PCR and electrophoresis tests. Biofilms were exposed to various treatments: secang heartwood in four different concentrations (625, 1,250, 2,500, and 5,000 µg/mL) and 2% CHX (standard irrigation medium). The antibacterial effects were assessed based on the optical density (OD) value obtained using an ELISA reader which reflects the amounts of bacterial cells.

The mean OD value from five concentrations: 625, 1,250, 2,500, 5,000 µg/mL and 2% CHX were identified (0,037, 0,084, 0,126, 2,007 and 0,037) $p > 0.05$.

Secang heartwood has an antibacterial property that is effective in eliminating *E. faecalis* biofilm. Secang heartwood concentration of 625 µg/mL had the same antibacterial efficacy as 2% CHX solution, whereas a concentration of 5,000 µg/mL had the lowest efficacy. The results of this study are expected to add new insight and knowledge in the field of endodontics with the use of natural ingredients that are not toxic but have antibacterial efficacy against *E.faecalis* biofilms.

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## Introduction

*Enterococcus faecalis* is the most common bacterial species found in infected root canals after endodontic treatment, root canals after failed endodontic treatments, and persistent infections in root canals.[1] Athanassiadis *et al*. (2007) have found that *E. faecalis* causes 4%–40% of primary endodontic infections.[2] Kauffman *et al*. (2005) have shown that *E. faecalis* is associated with 29%–77% of obturated root canal cases.[3] Meanwhile, other studies have found that *E. faecalis* accounts for 90% of root canal infections after root canal treatment.[4]

The main cause of an unsuccessful root canal is the presence of microorganisms in the apical part of the root.[5] Based on the research of Peters *et al*. (2001), mechanical preparation with various techniques will leave more than 35% of the surface of the untreated root canal, and as a result, bacteria in the root canal are not completely eliminated.[6] A previous research has reported that mechanical root canal preparation with the additional use of irrigation materials can reduce the number of bacteria up to 40% - 60%.[7] Therefore, antibacterial substances in the form of irrigation and medicaments can be used to eliminate the remaining bacteria in the root canal.

There are various types of antibacterial substances that have antibacterial properties

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used in the endodontic field. CHX 2% has become golden standard in eliminating *E. faecalis*.[8] However, CHX has proven to have cytotoxicity that will disrupt the stability of redox cells so it can increase the amount of free radicals with risk of cell death. Increasing the CHX dose increases the potential of cytotoxicity.[9]

*Caesalpinia sappan* L., also known as secang, is one of the Indonesian herbs that have antibacterial effects against *E. faecalis*.[10,11] It is a herbaceous plant that has long been used as a traditional medicine, as it possesses anticonvulsant, anti-inflammatory, antiproliferative, anticoagulant, antiviral, immunostimulant, antioxidant, and antimicrobial properties.[12]

Until now, the efficacy of secang heartwood extract solution in eliminating *E. faecalis* biofilm obtained from endodontic clinical isolates has not been examined. Therefore, this research will investigate the concentration of secang heartwood extract solution that has the optimum antibacterial effect against *E. faecalis* biofilm obtained from clinical isolates and conduct an antibacterial test to compare the efficacy of secang heartwood extract solution with that of 2% chlorhexidine (CHX) solution.

## Materials and methods

### Secang heartwood extract preparation

Secang heartwood powder weighed as much as 2500 grams and extracted by maceration method using 96% ethanol solvent until submerged and shaken for three days. Soaking and filtering extract solution is done 3 times. Evaporated with rotavapour until the viscous extract was obtained.[12]

Chemical compound analyses were performed with high-performance liquid chromatography (HPLC). HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities.[13] Secang extracts were diluted with 10% dimethyl sulfoxide (DMSO) to obtain the following concentrations: 625, 1,250, 2,500, and 5,000 µg/mL. The concentration of 5,000 µg/mL was 5 mg extract solution in 1 mL of DMSO 10%. Concentration made by diluting the extract solution in DMSO because it is an amphilic compound, which is a compound that has hydrophilic characteristics or hydrophobic. DMSO is also neutral and has no antibacterial effect so it does not affect the results of antibacterial tests.[14] This study was approved by ethic board.

### Bacterial sample preparation

The study sample (*E. faecalis*) was the patients with chronic apical periodontitis and apical abscess who came to the Dental Conservation Clinic of RSGMP FKG UI for endodontic treatment. Before the sample was taken, the teeth were isolated with rubber dam. Work area is sterilized with $H_2O_2$ solution 30%, disinfectant and cleaned with saline solution. Then the access preparation is done with sterile bur without water cooling. Furthermore, the root canal is explored without irrigation to K-file # 20 and sampling with K-file # 20. Files are inserted with filing motion up to 1 mm less than apex based electronic apex locator and radiographic photo. If the root canal is dry, give it a little saline solution. Then 2 sterile paper points are inserted into the root canal, with same working length, for 1 minute. Files and both paper points were placed in a sterile tube containing 1 ml PBS (Buffered Saline phosphate). Samples are immediately stored at 4 $^{o}$C before being cultured.

### Bacterial culture of clinical isolates in Chrom Agar

Samples were obtained from the patients and then cultured in ChromAgar medium. ChromAgar was chosen because it provides presumptive identification of. *E. faecalis*, direct from clinical samples. The ChromAgar plates was incubated at 37 ºC for 24 hours. It appears as bluish colonies. Bacterial growth of *E. faecalis* clinical isolates bacteria was seen from 4 patients (total 7 patients).

### DNA identification from clinical isolates

DNA extraction and amplification of *E. faecalis* were conducted with conventional polymerase chain reaction (PCR), followed by electrophoresis process for DNA identification. The electrophoresis test results showed a white band, which is parallel to the primer of *E. faecalis* at 138 bp, and this result was similar to that of previous research.[15]

**Disc diffusion assay**

The disc diffusion assay was performed to determine the optimum dose of secang heartwood extract. The bacterial suspension is applied evenly over the agar surface and allowed to dry for 4-5 minutes. Put 4 soaked discs with 20 µl samples of each concentration, then incubated for 18 - 24 hours at 37 ºC. In this research, minimum bactericidal consentration (MBC) was determined with disc diffusion assay. Inhibition zone formed according to the consentration of the secang heartwood solution. The diameter of inhibition zone formed clear region around the disc was measured using a caliper.

***E. faecalis* biofilm exposure to various treatments**

The biofilm of *E. faecalis* was made at 96 well plates and incubated at 37 ºC for 24 hours. The supernatant was removed and washed with PBS. The study continued with conducting further research by exposing biofilms with various treatments: secang heartwood extracts in four different concentrations and 2% CHX (Gluco-Chex) were added and incubated for 15 minutes at 37 °C. There were no negative control. Then, 200 µl of 0.1% crystal violet solution was added and incubation was carried out again for 15 min. The crystal violet solution was removed from each well plate and washed with PBS. Next, 200 µl of 95% ethanol was added to each well plate. Optical density (OD) value was assessed using an ELISA reader machine. The higher OD value the more bacteria there are in the biofilm, so that it means that the test material (extract solutions) is not effective as an antibacterial solution.

**Data analysis**

Data were tabulated and analyzed using the Statistical Package for the Social Sciences software version 20.0. The *E. faecalis* biofilm growth after exposure from test material group was tested for normality and homogeneity of the data. If the variation of data is normal and homogeneous, then the statistical test is using one-way ANOVA. If the data distribution is not normal or the data is not homogeneous, then statistical analysis is done by non-parametric test of Kruskall-Wallis with significance level ($p < 0.05$).

**Results**

Bacterial growth was observed in bluish-green bacterial colonies in the four ChromAgar from 7 ChromAgar (57% of all bacterial samples) of *E. faecalis* clinical isolates. *E. faecalis* clinical isolates were confirmed through bacterial DNA extraction with conventional PCR and electrophoresis tests (Figure 1).

Approximately 0.33% of brazilin were present in the secang heartwood viscous extract solution, which was determined using HPLC.

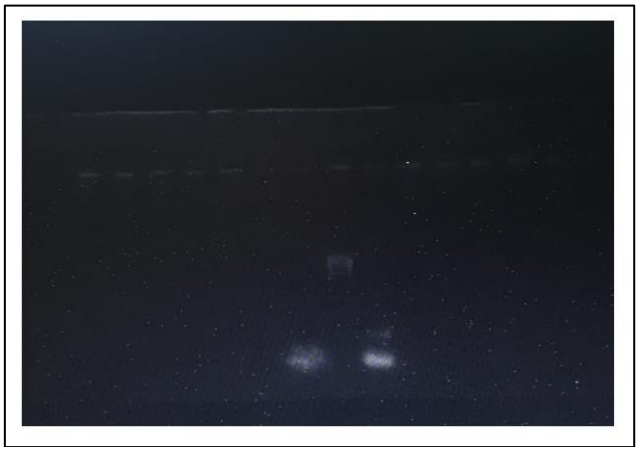

**Figure 1.** PCR Banding Patterns of *Enterococcus Faecalis* Clinical Isolate and Primer that is Aligned at 138 bp, which is Considered Identical.

In this research, the minimum bactericidal concentration was determined with disc diffusion assay. The inhibition zone was formed according to the concentration of the secang heartwood solution. Table 1 shows that the concentration of secang heartwood extract solution of 5,000 µg/mL yielded the largest inhibitory diameter with a mean of 17.25 mm, which indicates that this concentration is most effective in eliminating *E. faecalis*. There were significant differences in the inhibition zone diameter between the study groups ($p < 0.05$). Post-hoc tests revealed significant differences between the four concentrations of secang heartwood extract (Table 2). The 5,000 µg/mL had the strongest inhibitory effect on bacteria.

| Test samples (secang solution, µg/mL) | N* | Mean ± S.D. | 95% confidence interval of the mean | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| 625 | 4 | 0.50 ± 0.29 | -0.42 | 1.42 |
| 1,250 | 4 | 7.00 ± 0.41 | 5.70 | 8.30 |
| 2,500 | 4 | 11.50 ± 0.65 | 9.45 | 13.55 |
| 5,000 | 4 | 17.25 ± 0.48** | 15.73 | 18.77 |

*N: number of samples. ** the biggest mean inhibition zone diameter.

**Table 1.** Mean Inhibition Zone Diameters for *Enterococcus Faecalis* Obtained using the Disc Diffusion Assay.

The study continued testing the efficacy of secang heartwood solution and 2% CHX in eliminating *E. faecalis* biofilm using crystal violet assay with an ELISA reader. In this research, the mean OD value (Table 3) of secang heartwood solution at concentrations of 625 µg/mL was the lowest (0.037), while that for a concentration of 5,000 µg/mL was the highest (2.007). When the OD value was higher, more bacteria were observed in the biofilm. Thus, the 5,000 µg/mL concentration was not an effective antibacterial solution. However, concentrations of 625 µg/mL and 2% CHX solution have the same OD value (0.037 mm). This indicates that the solution has the same antibacterial effect as 2% CHX solution ($p \leq 0.05$).

| Test samples (secang solution, µg/mL) | Test samples (secang solution, µg/mL) | | | |
|---|---|---|---|---|
| | 625 | 1,250 | 2,500 | 5,000 |
| 625 | | 0.00* | 0.00* | 0.00* |
| 1,250 | | | 0.00* | 0.00* |
| 2,500 | | | | 0.00* |
| 5,000 | | | | |

* Test: one-way ANOVA. The mean difference was significant ($p \leq 0.05$)

**Table 2.** Significant Difference in the Zone Inhibitions Between the Test Samples.

Table 4 shows that the OD value of secang heartwood extract solution at varying concentrations of 1,250 µg/mL, 2,500 µg/mL, and 5,000 µg/mL is significantly different from that of secang heartwood extract solution with a concentration of 625 µg/mL. A concentration of 625 µg/mL has the most effective antibacterial effect compared to other concentrations. There were no differences in OD value between concentrations 1,250 µg/mL and 2,500 µg/mL ($p>0.05$). Thus, both concentrations had the same antibacterial effect. Statistically, except at a concentration of 5,000 µg/mL, the effect of all the concentrations did not significantly differ to that of 2% CHX solution.

| Test samples | N* | Mean ± S.D. | 95% confidence interval for the mean | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| 625 µg/mL of secang heartwood solution | 6 | **0.037 ± 0.00**** | 0.026 | 0.047 |
| 1,250 µg/mL of secang heartwood solution | 6 | 0.084 ± 0.005 | 0.070 | 0.098 |
| 2,500 µg/mL of secang heartwood solution | 6 | 0.126 ± 0.017 | 0.080 | 0.171 |
| 5,000 µg/mL of secang heartwood solution | 6 | **2.007 ± 0.155***** | 1.608 | 2.405 |
| 2% CHX solution | 6 | 0.037 ± 0.023 | -0.022 | 0.096 |

*N: number of samples. **the smallest mean OD value. ***the biggest mean OD value.

**Table 3.** Mean Optical Density Value of the *E. Faecalis* Biofilm Obtained using Crystal Violet Assay with ELISA Reader.

| Test samples | Test samples | | | | |
|---|---|---|---|---|---|
| | 625 µg/mL | 1,250 µg/mL | 2,500 µg/mL | 5,000 µg/mL | 2% CHX solution |
| 625 µg/mL of secang heartwood solution | | 0.01* | 0.033* | 0.001* | 1.000 |
| 1,250 µg/mL of secang heartwood solution | | | 0.490 | 0.001* | 0.646 |
| 2,500 µg/mL of secang heartwood solution | | | | 0.001* | 0.123 |
| 5,000 µg/mL of secang heartwood solution | | | | | 0.000* |
| 2% CHX solution | | | | | |

* Test: One-way ANOVA. The mean difference was significant ($p \leq 0.05$).

**Table 4.** Significance of differences in the optical density value between the test samples.

## Discussion

This is a preliminary study that aimed to determine the antibacterial effect of secang heartwood solution against *E. faecalis* biofilm obtained from endodontic clinical isolates. *E. faecalis* was found in 57% of all bacterial samples in this study. Thus, *E. faecalis* is primarily found in endodontic infections. This result is in accordance with the research

conducted by Athanassiadis *et al*. (2007) showing that *E. faecalis* can be found in as much as 4% – 40% of primary endodontic infection cases [[2]]. The survival and virulence factors possessed by *E. faecalis* including its ability to compete with other microorganisms, invade dentinal tubulus and resist nutritional deprivation.[16] *E. faecalis* can also be found as a single species in obturated root canals associated with obdurate periradicular lesions, one may ween that this species is able to maintain or generate periradicular inflammation by itself and hence can be an endodontic pathogen and the major causative factor associated with post-treatment periradicular lesions.[17]

A phytochemical analysis of secang heartwood extract was conducted with HPLC because gas chromatography-mass spectrometry (GCMS) cannot detect brazilin at extremely small quantity. HPLC has advantages over GCMS.[18] That is, it is capable of detecting brazilin because of its high sensitivity, it is effective in separating sample components, and it can analyze samples at small quantity.[13] We found that the secang heartwood viscous extract only contains 0.33% brazilin, which is similar to the results from previous research.[19]

Currently, the optimum dosage standard for secang heartwood solution is not yet identified. In this study, various concentrations of secang heartwood solution were tested. The concentration was selected based on a previous study by Yim *et al*. who found that secang heartwood concentrations of 625, 1,250, 2,500, and 5,000 µg/mL can eliminate *E. faecalis* (KCTC3206).[10] However, the previous study tested *E. faecalis* in the form of planktonic KCTC3206 isolate. Meanwhile, in this study, the antibacterial effect of the secang heartwood extract was tested using the biofilm of *E. faecalis* obtained from clinical isolates. *E. faecalis* bacteria in the form of biofilms have different properties with bacteria in planktonic form. Biofilm can tolerate antimicrobials up to 1000 times more than bacteria planktonic The ability of bacteria *E. faecalis* to form biofilms is one of the causes of bacteria *E. faecalis* is difficult to eliminate.[20]

The results from the disc diffusion assay showed that there was a significant difference in the antibacterial effect between the varying concentrations of secang heartwood extract solution. That is, the greater the concentration, the greater the inhibition zone produced, which indicates that the antibacterial properties were stronger. This result is in accordance with that of previous research showing that a higher concentration of extract provided a greater inhibition zone diameter.[21]

To confirm the result of the disc diffusion assay, the antibacterial effect of secang heartwood solution at various concentrations and 2% CHX solution was examined using the biofilm of *E. faecalis.* Table 3 shows that the secang heartwood extract solution has an antibacterial effect against the biofilm of *E. faecalis*.

The OD values of the secang heartwood solution with concentrations of 1,250, 2,500, and 5,000 µg/mL were significantly higher from that of the solution with a concentration of 625 µg/mL. Thus, a concentration of 625 µg/mL is more effective than the other three concentrations. Meanwhile, no significant difference was observed between the concentrations of 1.250 and 2500 µg/mL; therefore, both had the same antibacterial effect. A significant difference was observed between the OD values for 5,000 µg/mL of secang heartwood solution and 2% CHX solution, which indicates that a concentration of 5,000 µg/mL is not as effective as 2% CHX solution (Table 4). However, varying concentrations of 625, 1,250, and 2,500 µg/mL and 2% CHX solution were not significantly different; thus, the concentrations had the same efficacy as 2% CHX solution. Hence, a difference was observed in terms of the antibacterial efficacy of secang heartwood solution and 2% CHX solution against the biofilm of *E. faecalis*.

The difference of the research results between the disc diffusion assay and the OD test on *E. faecalis* biofilm is probably caused by the mechanism of the active ingredient of secang heartwood solution. Unlike chemical drugs that only have one active ingredient, plant extracts have more than one active ingredient or multiple compounds. There are multicompounds that work synergistically or antagonistic.[22] When the compounds act antagonistically, an extract solution with a higher concentration may have an increasingly antagonizing active ingredient. There was no clinical signs of toxicity and no mortality even at a dose level of 100-2,000 mg/kg in 14 days observation period.[23] Secang heartwood extract solution is not toxic and safe, so it has the potential to be used in the food, beverage, cosmetics and pharmacy.[12] It is

advisable to separate brazilin from secang heartwood solution using the fractionation method to identify more optimal antibacterial effects.

The violet crystal test was used based on a previous study that has shown that the examination of bacterial biofilm formation can be conducted by measuring stains on the biofilm.[24] However, in this study, the biofilm test results were different from the disc diffusion assay results probably because of the characteristics of the secang heartwood solution, which may have affected the results. The violet crystal test used purple crystal violet dye. *E. faecalis* is a gram-positive bacteria that has a plasma membrane coated by peptidoglycan, which instantly binds to the violet crystal to retain the color. The secang heartwood extract has the characteristic of a dye and it binds similarly to the violet crystal dye. Thus, it can affect the OD value because these values use the light-bending principle by bacteria on biofilms stained using violet crystals.[25] If any residual extract of the secang heartwood solution binds to the violet crystals, it can affect the ELISA reader and the results will be false positive.

Thus, for further research, different methods, namely, methylthiazol tetrazolium (MTT) assay must be used to avoid affecting the OD values. This method measures cell proliferation and counts living cells using the ELISA reader. The value obtained is the OD value of MTT to a purple formazan after exposure to the reductase enzyme produced by the mitochondria. This method has a faster and more accurate examination time.[26]

## Conclussion

Secang heartwood solution has an antibacterial property that is effective in eliminating the biofilm of *E. faecalis*. Varying concentrations of secang heartwood solution at 625, 1,250, and 2,500 µg/mL have an antibacterial efficacy similar to that of 2% CHX solution, whereas a concentration of 5,000 µg/mL has the lowest antibacterial efficacy compared with the three other concentrations and 2% CHX solution. The results of this study are expected to add new insight of natural ingredients that can be used in the field of dentistry, as well as to make a concept of endodontic treatment with the use of natural ingredients which is not toxic but still has antibacterial efficacy against *E. faecalis* biofilms.

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