Secang Heartwood Extract in Serial Dilution as Antibacterial Agent Against *Biofilm E. faecalis* Clinical Isolate

Sasi Suci Ramadhani[1], Ratna Meidyawati[2]*, Dewa Ayu NPA[2]

1. Postgraduate student, Conservative Dentistry Department, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia
2. Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia

Abstract

E. faecalis bacteria are frequently encountered in persistent and resistant root canal infections. Their ability to form biofilms contributes to its persistence. Importantly, chemical-based irrigation usage is linked to side effects. Caesalpinia Sappan Linn, known as Secang heartwood, has been traditionally used for a variety of medical purposes, including antimicrobial. We aimed analyze the antibacterial effects of Secang heartwood extract solution on *E. faecalis* biofilms. *E. faecalis* biofilms from clinical isolates were grown on microtiter well plates, incubated for 24 h and subjected to various concentrations of Secang heartwood extract solution: 312 µg/ml, 625 µg/ml, 1250 µg/ml, 2500 µg/ml, 5000 µg/ml (and CHX 2% as control). Following a 15 min incubation, wells were rinsed with PBS and scrapped off. Biofilms were diluted, spread on a solid medium and incubated for 24 h. Subsequently, viable grown colonies were counted.

The concentration of Secang heartwood extract solution that produced the least inhibition of *E. faecalis* bacteria growth was 5000 µg/ml. At a concentration as low as 625 µg/ml, we observed no colony growth. Secang heartwood extract solution has an antibacterial effect on biofilm of *E. faecalis* bacteria. The concentration of aqueous extract of 625 µg/ml has an antibacterial power equivalent to CHX 2%, representing the optimal antibacterial concentration.

Experimental article (J Int Dent Med Res 2019; 12(2): 383-388)

Keywords: *Caesalpinia sappan L*, *E. faecalis biofilm*, Antibacterial effects.

Received date: 10 February 2019 **Accept date:** 15 April 2019

Introduction

Root canal infections are generally caused by microorganisms' invasion into the pulp and dentin tubules and failure of endodontic treatment.[1, 2] *E. faecalis* bacteria are often found in root canals in absence of a procedure, following a failed endodontic treatment, with persistent infections.[3] *E. faecalis*'s ability to form biofilms contributes to infection during root canal treatment.[4] Biofilms form following the aggregation of bacteria, and are attached to the surface forming a closed community rich in extracellular polymer matrix. Bacteria living in biofilms are highly resistant to antimicrobials.[5]

Chronic infections allow bacteria to make their way in the entire root canal system through ramifications, isthmus or dentinal tubules. In such locations microbes can live despite mechanical preparation.[2] Specifically, root canal preparation can remove bacteria along with infected pulp tissue, however studies have shown that despite complete preparations, bacteria and toxins may be left behind.[6] Therefore, in order to reach an optimal root canal preparation, an antibacterial irrigation solution and medicaments are required.[6]

Chemical based fluids, such as NaOCl and CHX 2%, have long been used for irrigation. In a study performed by Ma et al. (2015), it was shown that following NaOCl irrigation *E. faecalis* biofilm can still regenerate to form a new biofilm, while following CHX irrigation *E. faecalis* did not grow a new biofilm.[7] However, such irrigations often cause side effects such as bacterial resistance and toxicity. Therefore, an alternative irrigation material that is more affordable, nontoxic, effective, and made from herbal plants is needed.[8]

Caesalpinia sapan Linn (C. sappan) known as Secang heartwood, is part of the Caesalpiniaceae family. It is traditionally used for

Corresponding author:
Ratna Meidyawati
Department of Conservative Dentistry,
Faculty of Dentistry, Universitas Indonesia,
jakarta, Indonesia.
E-mail: meidyawati58@gmail.com

medications with a variety of medical properties such as anticonvulsive, anti-inflammatory, antiproliferative, anticoagulant, antiviral, immunostimulant, antioxidant, and antimicrobial.[9] Based on research conducted by Keramat et al. (2014), the aqueous extract of Secang heartwood solution has an effective antibacterial effect against *E. faecalis, S. salivarius, A. Viscosus, and S. sanguis.*[10] Several research such as Keramat and Yim have reported that Secang has an antibacterial effect on *E. faecalis*.[10, 11] However, to date its antibacterial effects on *E. faecalis*'s biofilm are unknown. The aim of this study was to analyze the antibacterial effect of Secang heartwood extract solution on *E. faecalis*'s biofilm.

Materials and methods

The study conducted at the Oral Biology Laboratory of the Oral Department of Biology, Faculty of Dentistry, University of Indonesia, in May-June 2018. *E. faecalis* bacterial biofilms originated from clinical isolates extracted from non-vital root canals. Inclusion criteria consisted in: adult patients who come to a Conservative Dentistry clinic at RSGMP FKG UI; diagnosis of chronic apical periodontitis and chronic apical abscess; presence of teeth pre- and post-endodontic treatment. Exclusion criteria were: the tooth cannot be installed a rubber dam; teeth have periodontal; tooth disorders with root canal obliteration. Samples were cultured for 24 h at 37 °C

We performed *E. faecalis* DNA extraction followed by PCR amplification and used *E. faecalis* forward and reverse as primer. Samples subsequently underwent electrophoresis to identify the presence *E. faecalis* bacteria. *E. faecalis* biofilm was prepared by swapping of BHI media, then incubated for 24 h at 37 °C. Following the 24 h incubation period, *E. faecalis* bacteria were collected with the use of an ose needle until 1 full loop, then inserted into a tube containing 10 mL of BHI solution and subsequently incubated for 24 h at 37 °C. For the biofilm preparation, 10 µl of *E. faecalis* bacteria were obtained for each well according to well design, to which we added 10 µl BHI broth, followed by a 24 h incubation at 37 °C.

We prepared the Secang heartwood extract by maceration methods. Subsequently, in order to identify the amount of brazilin components in the extract, we analyzed it by performing a chromatogram profile test using the HPLC (High Performance Liquid Chromatography) method. We then diluted the Secang heartwood extract into several dilutions 312, 625, 1250, 2500, and 5000 µg/ml with dimethyl sulfoxide (DMSO) 10%.

The biofilms were then exposed to the various dilutions Secang heartwood extract solution (312 µg/ml, 625 µg/ml, 1250 µg/ml, 2500 µg/ml, 5000 µg/ml) and CHX 2% as positive control. Following a 24 h incubation in 96-well microtiter plates, they were gently washed with PBS to remove planktonic cells. The biofilms were subsequently scrapped off the bottom of each well and mixed by vortexing with 200 µl of PBS. The biofilm suspension was diluted $1{:}10^2$ with PBS and incubated on BHI solid medium at 37 °C anaerobically with 5% CO_2 for 24 h. Colonies were counted following incubation, by Colony-Forming Unit Counts (CFU). One-way Analysis of variance (ANOVA) was used to analyze viable cell counts. Statistical analysis was performed using the SPSS 20.0 software. A $P<.05$ was considered to be statistically significant.

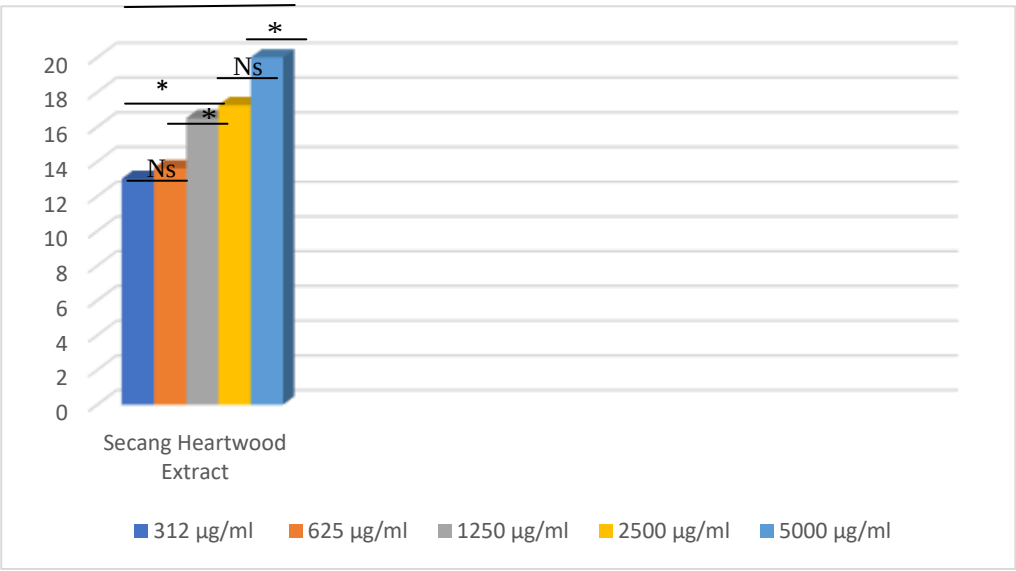
Results

We observed that the Minimum Inhibitory Concentration (MIC) of Secang heartwood extract solution indicates that this solution has antibacterial effects. Specifically, the greatest antibacterial effect lies in Secang heartwood extract solution with a concentration of 5000 µg/ml with an average diameter of 20 mm clear zone (Table 1). This means that the most effective dose for *E. faecalis* bacteria elimination is a concentration of 5000 µg/ml. Figure 1 shows the inhibition zones of Secang heartwood, at different concentration.

Test Material (Secang heartwood extract solution in µg/ml)	N	Mean ± SD	95% Confidence Interval		P Value
			Lower bound	Upper bound	
312	4	13.00 (0.40)	12.35	13.64	**0.00**
625	4	13.62 (0.47)	12.86	14.38	
1250	4	16.50 (0.40)	15.85	17.14	
2500	4	17.25 (0.64)	16.22	18.27	
5000	4	**20.00 (0.40)**	19.35	20.64	

Table 1. The average value of inhibition zone diameter of *E. faecalis* bacteria growth after exposure to various Secang heartwood test materials.

The difference of significance in each group was tested by One-way Anova, following a normality test with Shapiro Wilk, which showed normal data. The Anova test results showed a p value = 0.00 (P≤.05). These results indicate the presence of significant differences in the entire group of test materials. Figure 1 also showed that the Secang heartwood extract solution with concentration of 312 µg/ml did not significantly differ with the concentration of 625 µg/ml, and the Secang heartwood extract solution concentration of 1250 µg/ml did not significantly differ with the concentration of 2500 µg/ml.



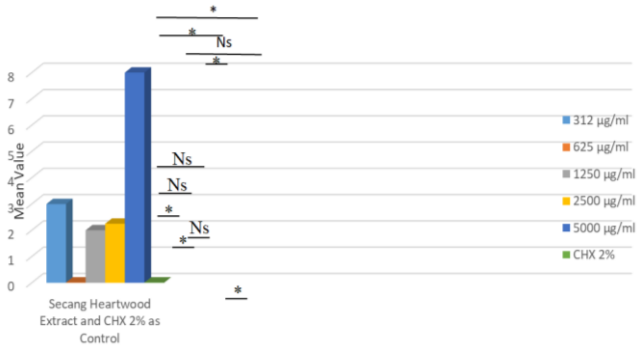
*Anova significance test between groups with P ≤ 0.05
Ns : Not significance

Figure 1. Mean value of inhibitory zone diameter of *E. faecalis* bacteria growth after exposure to Secang heartwood extract solution test material

Test Material	N	Mean ± SD	95% Confidence Interval		P Value
			Lower bound	Upper bound	
[a]312 µg/ml	4	3.00 (0.81)	1.70	4.30	**0.00**
[a]625 µg/ml	4	**0**	-	-	
[a]1250 µg/ml	4	2.00 (0.81)	0.70	3.30	
[a]2500 µg/ml	4	2.25 (0.95)	0.73	3.77	
[a]5000 µg/ml	4	**7.25 (0.95)**	5.73	8.77	
CHX 2%	4	**0**	-	-	

[a] Secang heartwood extract solution

Table 2. The average value of *E. faecalis* bacteria colony growth in biofilm following exposure to the test material



* Anova significance test between groups with P ≤ 0.05
Ns : Not significance

Figure 2. The significance value of *E. faecalis* biofilm colonies following exposure to different concentrations of Secang heartwood extract solution and CHX 2%

Evaluation of the antibacterial power of Secang heartwood extract solution on *E. faecalis* bacteria's biofilm was performed using the colony count method. The mean value of colony counts for each treatment was obtained with a concentration of extract of Secang wood of 625 µg/ml and CHX 2%. Neither showed *E. faecalis* bacterial colony growth. Our observations demonstrate that a solution of Secang heartwood extract with a concentration of 625 µg/ml has the same antibacterial power as a 2% CHX solution. Importantly, the extract solution with a concentration of 5000 µg/ml showed the highest colony growth, equivalent to 7,25. This finding suggests that this concentration of Secang heartwood extract solution has no antibacterial power. The average growth rate of *E. faecalis* bacteria colonies is shown in Table 2.

Figure 2 shows the statistical significance of colony growth following exposure of biofilms to different concentrations. Specifically, concentrations of 1250 µg/ml and 2500 µg/ml did not have a statistically significant difference from the concentration of 312 µg/ml, and the concentration of 1250 µg/ml did not have a statistically significant difference from the concentration of 2500 µg/ml. In addition, the three antibacterial effects are the same. Importantly, result in Figure 2 also showed that the concentration of 625 µg/ml had no significant difference compared to CHX 2%, suggesting that this concentration has the same antibacterial power as CHX 2%.

Discussion

The aim of the present study was to analyze the antibacterial effect of Secang heartwood extract solution on biofilm *E. faecalis* clinical isolates. *E. faecalis* bacteria was chosen because they are often found: in root canals where no endodontic treatments have been performed; following failed endodontic treatments; on root canals with persistent infections.[3] *E. faecalis* bacteria's ability to form biofilms is a contributing factor during root canal treatment.[4] Biofilms are well-organized microbial communities, consisting of bacterial clumps that form like fungus and surrounded by an extracellular polymeric substation matrix (EPS), and exhibit different phenotypic growths.[5, 12, 13] Biofilm bacteria are highly resistant to antibodies and antibiotics and 1000 times stronger to phagocytosis than in planktonic condition.[5] Consterton (2002) suggests that the *E. faecalis* bacteria biofilm may have a phenotype which causes the bacteria to be resistant to antibiotics. Specifically, the barrier ability of the EPS matrix present in the biofilm matrix will inhibit penetration and inactivate antibiotics by adsorbing them into the EPS and dissolving them before they reach the bacterial cells in the biofilm. This makes the biofilm more resistant to antibiotics.[5]

Secang heartwood (Caesalpinia sappan L) is a plant often used as a traditional medicine. It is characterized by having a variety of medicinal properties such as anticonvulsive, anti-inflammatory, antiproliferative, anticoagulant, antiviral, immunostimulant, antioxidant, and

antimicrobial.[9] Secang wood is often used in traditional treatment because it contains tannin, brasilin, brasilein, alkaloids, flavonoids, saponins, phenyl propane, terpenoids, and essential oils.[14] Brazilin is the main homoisoflavonoid contained in Secang wood, and it is known to have antibacterial and bacteriostatic activities.[15] Brazilin is a phenolic compound that can kill microorganisms by enzyme inhibition, oxidize compounds by reaction of sulfidril group or induce non-specific protein interactions.[16] In their studies Xu and Lee (2004), claimed that brazilin has antibacterial functions due to its ability to inhibit bacterial DNA and protein synthesis. However, brazilin's exact antibacterial mechanism of action remains unclear.[17] The flavonoid antibacterial activity is linked to its ability to bind complexly to the bacterial walls.[16, 18] Specifically, flavonoids form complex compounds against extracellular proteins therefore inhibiting the integrity of bacterial cells cytoplasmic membranes.[18] As a consequence, the cytoplasmic membranes are damaged so that H + ions of flavonoid compounds will attack the polar group (phosphate group) and the phospholipid molecule will break down into glycerol, carboxylic acid, and phosphoric acid. This causes the phosolipid to not be able to maintain the cytoplasmic membrane's shape, and as a result the membrane leaks, causing bacterial growth restriction until death.[19]

Similarly to results shown by Balawala (2012), inhibition zone observed in the present study (Table 1) demonstrated that the higher the concentration of the Secang heartwood extract solution used, the higher the inhibition zone formed.[20] The smallest inhibitory zone obtained by Secang heartwood extract solution was seen with a concentration of 312 µg/ml. This result was not significantly different than the concentration of 625 µg/ml. The concentration of the Secang heartwood extract solution of 1250 µg/ml did not significantly differ compared to the concentration of 2500 µg/ml. However, these two concentrations still have a larger inhibition zones with respect to the concentrations of 312 µg/ml and 625 µg/ml. The solution of Secang heartwood extract with a concentration of 5000 µg/ml had the largest inhibition zone equivalent to 20,00. These results are in line with research conducted by Keramat et al (2014). Specifically, in his research on the effectiveness of antimicrobial Secang wood against *E. faecalis, S. salivarius, A. Viscosus and S. sangui* bacteria states that the concentration of 5000 µg/ml is a concentration that has the biggest inhibition zone.[10]

We performed colony count of *E. faecalis* bacteria biofilm to evaluate the antibacterial effects of different concentrations of the Secang heartwood extract solution on the biofilms. Based on previous research conducted by Gomes et al (2001), we exposed *E. faecalis* bacteria's biofilms to various concentrations of Secang heartwood extract solution (and control) for 15 min.[21] Specifically, previous studies showed that optimal exposure time to natural antimicrobial agents, in order to eliminate bacteria was 15 min. Similarly, to the research conducted by Liu et al. (2017), the calculation of bacterial biofilm can be done by counting the visible colonies of biofilm growing on BHI solid medium. Such method has the advantage of being easy, cheap and accurate because it is not affected by coloring and subsequently to the calculation of the bacterial colony it can still be used for additional research.[22,23] Results of the present study showed that the average colony count at a concentration of 5000 µg/ml showed the highest growth of colonies. Specifically, it was equal to 7.25 (Table 3). Such findings indicate that at this concentration the solution of wood extract Secang has no antibacterial power. On the contrary, we observed that colony growth was very low at a concentration of 625 µg/ml, specifically 0 or no colony growth. Importantly, in Table 4 we show that the concentration of 625 µg/ml has no significant difference with CHX 2%. Therefore, we can say that the concentration of 625 µg/ml has the same antibacterial power as CHX2%. Additionally, it represents the concentration that has the most optimal antibacterial power vs. other concentrations. It can be concluded that the most optimal concentration in inhibiting *E. faecalis* bacteria' biofilm is 625 µg/ml.

Our results on the antibacterial effect of Secang heartwood extract solution on biofilm of *E. faecalis* bacteria differ from the results of the inhibition zone which states that the higher concentration of Secang wood extract solution used, the higher the inhibition zone produced. These results can be explained by the fact that the research on inhibition zone was performed on *E. faecalis* bacteria clinical isolates in planktonic conditions. According to Donland and Consterton

(2002), isolated bacteria in biofilms have a higher protection and will be harder to eliminate with respect to the planktonic condition.[5] In addition, it is thought that the chemical compounds contained in the Secang extract are very diverse, so interactions between compounds that have both synergistic and antagonistic effects are not possible.

Conclusions

The Secang wood extract solution has an antibacterial effect on *E. faecalis* bacteria's biofilms. A concentration of 625 µg/ml of the Secang wood extract solution has an antibacterial power equal to CHX 2%. Therefore, we conclude that the optimum concentration of Secang wood extract solution with antibacterial effects is 625 µg/ml.

Declaration of Interest

The authors report no conflict of interest.

References

1. Suchitra U, Kundabala M. Enterococcus faecalis: An endodontic pathogen. Available from: medind. nic. in/eaa/t06/i2/eaat06i2p11. pdf 2006. date : May, 18th 2018
2. Torabinejad M, E WR. Endodontic Principle and Practice. Missaouri: Saunders; 2015.
3. K.M H, Louis B. Chapter 6 Cleaning and Shaping the Root Canal System.pdf. Cohen's Pathway to The Pulp Eleventh Edition. Missouri; 2016.
4. Jenkinson HF, Lappin-Scott HM. Biofilms adhere to stay. TRENDS in Microbiology 2001;9(1):9-10.
5. Donlan RM, Costerton JW. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clinical Microbiology Reviews 2002;15(2):167-93.
6. Byström A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. European Journal of Oral Sciences 1981;89(4):321-28.
7. Ma J, Tong Z, Ling J, Liu H, Wei X. The effects of sodium hypochlorite and chlorhexidine irrigants on the antibacterial activities of alkaline media against Enterococcus faecalis. Arch Oral Biol 2015;60(7):1075-81.
8. Jain P, Ranjan M. Role of herbs in root canal irrigation-A review. IOSR Journal of Pharmacy and Biological Sciences 2014;9(2):06-10.
9. Kusmiati, Dameria, Dodi P. Analisa senyawa aktif ekstrak kayu secang (caesalpinia sappan L) yang berpotensi sebagai antimikrobial. Paper presented at: Seminar Nasional Teknologi Industri Hijau, 2014.
10. Keramat H, Moaddabilb A, Ranjbari A. In Vitro Antimicrobial Effects of Aqueous Extract of Caesalpinia Sappan Linn Derivatives Against Oral Pathogens. Indian J.Sci.Res 2014;7:342-47.
11. Yim NH, Jung YP, Cho WK, et al. Screening of aqueous extracts of medicinal herbs for antimicrobial activity against oral bacteria. Integr Med Res 2013;2(1):18-24.
12. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeymen AL. Comparative evaluation of endodontic irrigants againts enterococcus faecalis biofilms. Journal of endodontics 2006;32.
13. De Paz C. Gram-positive organisms in endodontic infections. 2004.
14. Hidayat MS. Ramuan tradisional ala 12 etnis Indonesia: Penebar Swadaya; 2005.
15. Kumala S, Devana D, Tulus D. Aktivitas Antibakteri Rebusan Secang (Caesalpinia sappan L.) terhadap Salmonella thypii Secara In Vivo. Agritech;33(1).
16. Cowan M. Plant products as antimicrobial agents Clinical Microbiology Reviews, vol. 12. No. 4: 564 1999;582.
17. Xu HX, Lee SF. The antibacterial principle of Caesalpina sappan. Phytotherapy Research 2004;18(8):647-51.
18. Juliantina F, Citra D, Nirwani B, Nurmasitoh, T. B, ET. Manfaat sirih merah (Piper crocatum) sebagai agen antibakterial terhadap bakteri gram positif dan gram negatif. Jurnal kedokteran dan kesehatan Indonesia 2009;1(1):12-20.
19. Retnowati Y, Bialangi N, Posangi NW. Pertumbuhan-Bakteri-Staphylococcus-Aureus-Pada-Media-Yang-Diekspos-Dengan-Infus-Daun-Sambiloto-Andrographis-Paniculata.pdf. 2011.
20. Balawala BG. Aktivitas Antibakteri Ekstrak Etanol Kayu Secang (Caesalpinia sappan L.) Terhadap Staphylococcus epidermidis, Pseudomonas aeruginosa ATCC 10145, dan Klebsiella pneumonia ATCC 10031. Fakultas Farmasi. Universitas Muhammadiyah Surakarta: Universitas Muhammadiyah Surakarta; 2012.
21. Gomes B, Ferraz C, Vianna M, et al. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. International endodontic journal 2001;34(6):424-28.
22. Brugger SD, Baumberger C, Jost M, et al. Automated counting of bacterial colony forming units on agar plates. PLoS One 2012;7(3):e33695.
23. Sieuwerts S, de Bok FA, Mols E, de vos WM, Vlieg JE. A simple and fast method for determining colony forming units. Lett Appl Microbiol 2008;47(4):275-8.