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Antibiofilm Power of Cocoa Bean Pod Husk Extract (*Theobroma Cacao*) Against *Entercoccus Faecalis* Bacteria (In Vitro)

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ABSTRACT

Endodontic pathogens which can form biofilms in the root canal. To remove *E. faecalis* biofilms, that must be taken is EPS degradation by oxidizing agents. Cacao bean pod husk (*Theobroma cacao*) is one of the potential herbal plants containing antimicrobial substances. The purpose of this study is to analyze the anti-biofilm activity of cacao bean pod husk extract against *E. faecalis* biofilms. Method: *E. faecalis cells* were cultured in microtiter plates. Cells remaining adhered to the wells were subsequently stained with crystal violet. After 24 hours of incubation, the optical density of each well was measured. Cocoa bean pod husk extract showed a decrease in the OD value at concentrations 100%, 50%, 25%, 12.5%, 6.25%, and 3.12% were lower than the OD values at concentrations of 1.56%, 0.78%, 0.39%, 0.19% as well as in the control groups. In conclusion, the Minimum Biofilm Inhibitory Concentration (MBIC) of cocoa bean pod husk extract is 3.12% concentration.

INTRODUCTION

Root canal treatment plays an important role in the field of dentistry to overcome pulp and periapical diseases. Root canal treatment can be considered to be successful if it is able to eliminate the source of infections by terminating microorganisms in the root canal (Gomes et al., 2003).

The most common microorganism found in the root canal is *Enterococcus faecalis (E. faecalis)* bacteria. *E. faecalis* bacteria are not only considered as the most resistant bacteria in the root canal but also as one of factors triggering the recurrence of the disease that has been treated with root canal treatment (Chavez, 2004).

E. faecalis bacteria, moreover, can invade the dentine tubules to protect themselves from chemo mechanical root canal preparation and intra-canal dressing technique. In a research on cultures of various bacteria inoculated into root canals, it is also known that *E. faecalis* bacteria may have good colonization and can survive in the root canal without any supports from other bacteria (Hojo et al., 2009). The colony of *E. faecalis* bacteria then forms biofilms as their defense. Bacteria, according to a research conducted by Keller & Costeron's (2009), mostly can communicate, perform signaling, as well as form colonization known as biofilms.

In general, there are three basic principles in root canal treatment, known as endodontic triads consisted of biomechanical preparation, sterilization, and obturation. Mechanical preparation should always be followed by root canal irrigation to clean up the remaining pieces of pulp tissue, dentin flakes, bacteria, debris, and necrotic tissue (Shahani & Subba Reddy, 2011).

In the other side, traditional medicine has been chosen to reduce the use of harmful chemicals and synthetic substances. Besides, it is also easy to obtain and affordable. Thus, traditional medicine can accelerate the potential utilization of medicinal plants around us.

Chocolate, for instance, contains flavanols, a unique form of polyphenols. Flavanoids isolated from chocolate have some biological effects, such as anti-inflammatory, that can inhibit bacteria in the mouth and neutralize acid in the mouth (Ooshima et al., 2002).

As a result, it is necessary to conduct a research to determine the anti-biofilm power of the cocoa bean pod husk extract against *E. faecalis* bacteria. The cocoa bean pod husk extract, consequently, is expected to be used as an alternative material of root canal irrigation solution. Therefore, this research aimed to determine the Minimum Biofilm Inhibitory Concentration (MBIC) of cocoa bean pod husk extract in inhibiting the biofilms of *E.faecalis* bacteria.

MATERIALS AND METHOD

This research was a laboratory experimental study. This research was conducted in the Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya. Samples used were *Enterococcus faecalis* ATCC 29212 bacteria. Besides, materials used were cocoa bean pod husk extract obtained from UPT Materia Medika, 70% ethanol, and sterile distilled water.

Moreover, the ability of the cocoa bean pod husk extract to inhibit the formation of *E. faecalis* biofilms was performed with certain procedures (Merritt et al., 2011). First, the cocoa bean pod husk extract (*Theobroma cacao*) were weighed and dissolved using Brain Heart Infusion (BHI) media (e.g concentration of 100%, meaning 1 gram of extract in 1 ml of BHI medium). Next, the suspension of *E. faecalis* bacteria (Mc Farland 0.5 or 1.5 x 108 CFU / ml) was prepared. Third, having obtained the same turbidity, the suspension was diluted up to 1 x 106 CFU / ml (*E. faecalis*). Fourth,

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the bacterial suspension was cultured in each microtiter plate (96-well flat-bottomed plastic tissue culture plates) containing media Brain Heart Infusion with 1% glucose, and then incubated (at 37° C for 3 days) for generating the maximum biofilm production. The total number of wells was adjusted to the number of replicated test materials used and labeled according to the concentration of each test material.

On the 3rd day, biofilm formation was verified with simple staining (crystal violet). Next, 100µl of the cocoa bean pod husk extract was inserted into microtiter columns (based on the extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39% and 0.19%). Microtiter columns containing the bacteria without the cocoa bean pod husk extract were used as positive controls. They then were incubated (at 37 ° C for 48 hours) in an incubator. After 48 hours of contact with the test materials, those microtitter plates were washed with phosphate buffered saline (PBS) four times to remove planktonic bacteria, then dried and stained with 50 mL of 0.1% crystal violet solution. Afterwards, they were incubated (at room temperature for 15 minutes). They then were flushed with sterile distilled water and dried. Next, optical density (OD) of those research groups was measured with a wavelength of 570 nm using a spectrophotometer. Results of the optical density (OD) measurement, furthermore, were classified into three categories (Husain et al., 2013), namely no attachment / no biofilm formation (A570 <0.1), weak attachment / weak biofilm formation (0.2> A570> 0.1), and strong attachment / strong biofilm formation (0.2 <A570).

The procedure then was repeated eight times for generating *Enterococcus faecalis* biofilms. Next, the average values of OD were measured. After that, Kolmogorov Smirnov and One-way ANOVA test was performed to analyze the differences of the OD value between all research groups.

RESULTS

The inhibition of *E. faecalis* biofilm formation by the cocoa bean pod husk extract was tested using microtitter plate assay method. First, the microtitter plates were given the cocoa bean pod husk extract with the concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, and 0.19% to determine the Minimum Biofilm Inhibitory Concentration (MBIC) of the cocoa bean pod husk extract for inhibiting the formation of *E. faecalis* biofilms. This treatment then was repeated eight times.

Next, results of the inhibition test were analyzed using spectrophotometer with the wavelength of 570 nm and expressed in Optical Density (OD). The results showed that there were a decrease in Optical Density (OD) values of E. faecalis biofilms as the concentration of the cocoa bean pod husk extract increased. In the biofilm groups treated with the extract at the concentrations of below 3.12% as well as in the biofilm group without the extract (control), the OD values were high. Meanwhile, in the biofilm groups treated with the extract at the concentrations of up to 3.12%, the OD values were low.

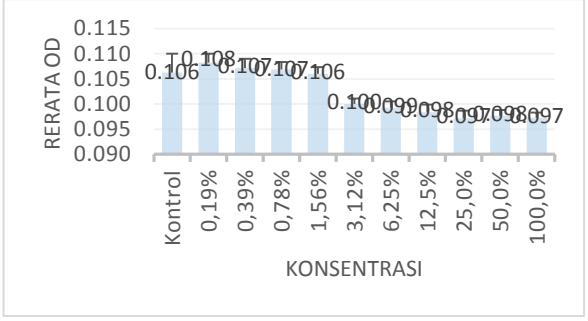

Fig. 1 Graph of Optical Density (OD) values of *Enterococcus Faecalis* biofilms

Before the difference test was conducted, normality and homogeneity tests were performed. For the normality test, One Sample Kolmogorov-Smirnov Test was used. Results of the normality test showed that the data were normally distributed (p> 0.05). For the homogeneity test, Levene test was used and indicated the data were homogeneous (p> 0.05).

Next, to see the significance of the difference in the OD values of *E. faecalis* biofilms among all groups treated with the cocoa bean pod husk extract at some concentrations, One-way Anova test was conducted. Results of the test showed that there was a significant difference in the OD values between the research groups (p <0.05).

Table 1 Homogenous subsets of all research groups

Groups	N	Subset for alpha = 0.05	
		1	2
Concentration of 25%	8	.09712	
Concentration of 100%	8	.09725	
Concentration of 50%	8	.09762	
Concentration of 12.5%	8	.09837	
Concentration of 6.25%	8	.09925	
Concentration of 3.12%	8	.10000	
Concentration of 1.56%	8		.10600
Control	8		.10625
At the concentration of 0.78%	8		.10688
At the concentration of 0.39%	8		.10713
At the concentration of 0.19%	8		.10825

Table 1 illustrated that the control group as well as the biofilm groups treated with the cocoa bean pod husk extract at the concentrations of 0.19%, 0.39%, 0.78%, and 1.56% were categorized into subset 1. It indicated that there was a significant difference in the OD values between those groups. On the other hand, the other biofilm groups treated with the cocoa bean pod husk extract at the concentrations of 3.12%, 6.25%, 12.5%, 25%, 50%, and 100% were categorized into subset 2. Thus, it can be said that there was a significant difference between the subset 1 and the subset 2. The results of the data also showed a significant decline in the OD values from the concentration of 3.12% to 100%.

DISCUSSION

This research aimed to reveal the inhibitory power of cocoa bean pod husk extract (*Theobroma cacao*) against biofilms formed by *Enterococcus faecalis* bacteria as one of the alternative materials for root canal irrigation. Thus, microtitter plate assay method was performed using cocoa bean pod husk extract at concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56% 0.78%, 0.39%, and 0.19% to determine the Minimum Biofilm Inhibitory Concentration (MBIC) of cocoa bean pod husk extract in inhibiting the biofilms of *E.faecalis* bacteria.

Based on the results of the spectrophotometer reading on the biofilms of E. faecalis bacteria at each concentration, there was an average decrease in the Optical Density (OD) value of the biofilm groups treated with cocoa bean pod husk extract at the concentrations of more than 31.2% compared to those OD values of the biofilm groups treated with cocoa bean pod husk extract at the concentrations of less than 31.2% as well as those of the untreated

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(control) biofilm group. This is due to greater anti-biofilm power contained in cocoa bean pod husk extract at the concentration of 100% than those at the concentrations of 50%, 25%, 12.5%, 6.25%, and 3.12%. In other words, the greater the concentration of cocoa bean pod husk extract is, the greater the anti-biofilm power contained is).

The results of the Optical Density (OD) measurement on the biofilms of *E. faecalis* bacteria, moreover, showed that the average value of OD in the biofilm group treated with cocoa bean pod husk extract at the concentration of 100% was 0.097. In general, the average values of OD in the biofilm groups treated with the cocoa bean pod husk extract at the concentrations of more than 3.12% could be categorized into A570 < 0.1. It indicated that there was no biofilm attachment occured. Meanwhile, the average values of OD in the biofilm groups treated with the cocoa bean pod husk extract at the concentrations of below 3.12% were respectively 0.106, 0.106, 0.107, and 0.108. Therefore, the concentrations of 1.56%, 0.78%, 0.39%, and 0.19% were categorized into 0.2> A570> 0.1. It indicated weak biofilm attachment.

The inhibition of *E. faecalis* biofilm formation may be caused by anti-biofilm materials contained in the cocoa bean pod husk extract. The anti-biofilm effect of the cocoa bean pod husk extract is derived from flavonoid. Similarly, Manner et al (2013) state that flavone can act as an inhibitor of biofilm formation. Like Manner, Wai-Leung & Bassler (2009) and Li & Tian (2012) also argue that flavone can interfere with signaling quorum sensing pathway by damaging an interaction between acyl-homoserine lactone (AHL) and its receptor. AHL is a molecular or auto inducer signal used by Gram positive bacteria in the quorum sensing process. AHL is composed of a ring of homoserine lactone (HSL), containing from C3 to C8 acyl chains. Next, LuxR-like proteins act as a receptor responsible for recognizing AHL, which then binds to the specific elemental DNA promoter and subsequently activates the transcriptional regulator of the target gene. This compound, flavone, may interfere with the signaling quorum sensing pathway through the modification or substitution of the C 3 group from the acyl AHL chain with the hydroxyl-O-acyl group into 3-hydroxy AHL. Through the substitution, AHL then cannot bind to the LuxR-like protein, leading to inactivation of the transcriptional regulator of the target gene, so the quorum sensing gene regulation does not occur.

Furthermore, in the groups of *E. faecalis* biofilms treated with the cocoa bean pod husk extract at the concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.12%, the average values of OD were high. The high value of OD in the *E. faecalis* biofilms can be caused by the small degradation of the biofilm layers formed. Similarly, Mathew & Boopathy (2010) argues that *E. faecalis* bacteria can accumulate and form biofilms, which can make them a thousand times more resistant to antimicrobial agents. Thus, *E. faecalis* bacteria, according to Figdor et al (2003), can survive on treated root canals, including resistant to intra-channel medicaments with capabilities of forming biofilms, conducting invasion into dentin tubules, and being persist for long term-nutritional limitations. When bacteria grow as biofilms, the genetic processes and bacterial metabolism will turn into complex matrices to prevent antimicrobial agents to work (George et al., 2005).

In addition, results of this research revealed that the cocoa bean pod husk extract at those concentrations examined had an ability to inhibit biofilms formed by *E. faecalis* bacteria. Nevertheless, there was no significant difference in OD values between those concentrations. It may be due to procedures of absorbance or turbidity calculation. In the calculation of absorbance or turbidity, simple staining using crystal violet is known to be required, but the crystal violet can stain bacteria cells, both living and dead ones, resulting in greater turbidity (Patel et al., 2013) .

Besides, the insignificant difference can also be caused by the great concentration range of the cocoa bean pod husk extract. Consequently, to obtain a significant and appropriate Minimum Biofilm Inhibitory Concentration (MBIC) value, the concentration range of the cocoa bean pod husk extract examined had been reduced into 5% or 10%. The results then indicated that there was a correlation between the concentration of the cocoa bean pod husk extract and the OD value of biofilms formed by *E. faecalis* bacteria. In other words, the higher the concentration of the cocoa bean pod husk extract (*Theobroma cacao*) is, the lower the OD value of biofilms is formed by *E. Faecalis* bacteria. Since the cocoa bean pod husk extract at the low concentration examined could inhibit biofilm formation, the low concentration of the cocoa bean pod husk extract that can inhibit biofilm formation or Minimum Biofilm Inhibitory Concentration (MBIC) for *E. faecalis* bacteria was 3.12%.

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