Minimum inhibitory concentration of cocoa pod husk extract on Enterococcus faecalis extracellular polymeric substance biofilm thickness By Tamara Yuanita

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ABSTRACT

Background: Root canal treatment is a treatment sequence for the infected pulp to eliminate these etiological factor of pulp necrosis and periapical lesion. Enterococcus faecalis (E. faecalis) is an organism that commonly found in high percentage of root canal failure because its ability to form biofilm. Degradation of extracellular polymeric substance (EPS) by oxidizing agent such as sodium hypochlorite is the first step to remove biofilm. However sodium hypochlorite toxicity is the main concern, so the safest alternative irrigants needed. The development of herbal uses, especially in the fields of medicine and dentistry, fruit and plants are widely used. Food crops are known to be rich in bioactive compounds, especially polyphenols, which have properties as antioxidants and antimicrobials. Cocoa pod husk extract can be alternative choice. Purpose: The study aimed to determine the minimum inhibitory concentration of cocoa pod husk extract on E. faecalis EPS biofilm thickness.

Methods: Four groups sample of E. faecalis cultured biofilm; group one is E. faecalis without cocoa pod husk as positive control, group two for E. faecalis with 1.56 % cocoa pod husk extract, group three for E. faecalis with 3.125% cocoa pod husk extract and group four for E. faecalis with 6.25% cocoa pod husk extract. All groups will be measured biofilm thickness with confocal laser scanning microscopy and then statistical analysis is taken by post hoc test and Tukey HSD. Results: The average value of EPS biofilm thickness, group 1: 9500 nm, group 2: 8125 nm, group 3: 8000 nm, and group 4: 6375 nm. Post Hoc Tukey HSD test showed a significant difference between group 1 and group 4. While in group 1 and group 2 compared to the group 3, there were no significant differences with the values of each p = 0.340 and p = 0.267 (p>0.05). Conclusion: 6.25% cocoa pod husk extract reduce E. faecalis EPS biofilm thickness.

Keywords: cocoa pod husk extract; endodontic; Enterococcus faecalis; extracellular polymeric substance biofilm INTRODUCTION

Pathology of pulp tissue and periapical tissue directly or indirectly related to microorganisms. The microbas can be removed and minimized by root canal treatment. The success of root canal treatment is influenced by several factors that are interrelated with one another.1 These factors include proper diagnosis, aseptic action, knowledge of dental anatomy, chemical-mechanical
preparation, three-dimensional obturation and use of root canal dressing. All of these factors are based on one point, namely root canal decontamination. The effectiveness of root canal preparation can be increased by the use of irrigation solutions such as sodium hypochlorite (NaOCl), chlorhexidine, ethylenediaminetetraacetic acid (EDTA). NaOCl is the golden standart for root canal irrigation solutions, because until now, no other solution has been similar effectiveness with NaOCl, however the disadvantage of NaOCl is its cytotoxic activity which can cause acute injury if it reaches the periapical region. If NaOCl is in contact with tissue, it rapidly oxidizes the surrounding living tissue and triggers rapid hemolysis, inhibits the neutrophil migration, damages endothelial and fibroblast cells. The higher concentration of NaOCl the higher anti-bacterial effect and tissue dissolution, but the higher toxicity. In addition, in vitro cell culture, very low concentration of NaOCl (<0.01%) caused death of human fibroblast cell. Root canal treatment can fail due to the absence of a good coronal seal, microleakage, failure in chemical-mechanical preparation, and poor quality root canal filling so that there are microorganisms that still survive or reinfection also occurs. Some microorganisms are associated with failure of root canal treatment. One of the most common microorganisms is Enterococcus faecalis (E. faecalis). This is due to the ability to survive E. faecalis in environmental conditions that are low in nutrition and its ability to form biofilms so that 1000 times more resistant to phagocytic cells, antibodies and antimicrobials compared to organisms that are unable to make biofilm. Biofilms are defined as multicellular microbial communities characterized by cells that attach strongly to the surface and produce matrix extracellular polymeric substance (EPS). EPS consists of bacterial proteins, nucleic acids, polysaccharides and fats. Microbes that form biofilms are thought to be the cause of 80% of infections. The development of herbal uses, especially in the fields of medicine and dentistry, fruit and plants are widely used. Food crops are known to be rich in bioactive compounds, especially polyphenols, which have properties as antioxidants and antimicrobials. One of the food plants that are rich in antioxidants and has an antimicrobial effect is cocoa. The cocoa pod husk contains unsaturated fatty acids and epikarin polymers which have antibacterial and antiglucocyl transferase activity, whereas the coco pods consist mainly of polysaccharides (cellulose and hemicellulose), lignin and a small portion of phenolic compounds, tannin, purine alkaloids and cocoa butter. The minimum concentration that can inhibit E. faecalis biofilm formation is equal to 3.125%. To the best of our knowledge, there have been no studies to date evaluating the effect of cocoa pod husk on the thickness of E. faecalis EPS biofilm. Thus, the purpose of this study was to determine the minimum inhibitory concentration of cocoa pod husk extract on E. faecalis EPS biofilm thickness. MATERIALS AND METHODS The ingredients used in this study were Forastero type cocoa fruit (Theobroma cacao L.) extract with concentrations of 1.56%, 3.125% and 6.25%. The cocoa pods used for Forastero type is obtained from the Coffee Research Center and Cocoa Jember, the cocoa pods taken is the one that has been cooked with the yellowing mark when picked. Before processing, the pods that have been picked is left for about 5 days to facilitate the release of seeds from the cocoa husk. The cocoa pods husk in fresh form is separated by seeds or the entire contents of the pods. The extraction process of cocoa pods husk is done by maceration. The cocoa pods husk used in this study is 6 kg fresh then cut and aerated. When half-dried pods husk is then put into an oven with a temperature of 50°C. After drying, 1 kg of cocoa pods husk is obtained. Cocoa pods (1 kg) was milled then macerated with 70% ethanol for 24 hours then filtered. After filtering cocoa pods, filtrate and dregs were obtained. The dregs is then soaked after it is filtered again. The process of maceration and filtration occurs repeatedly until a clear filtrate is obtained. After obtaining a clear filtrate, ethanol evaporation was carried out by using a rotary evaporator with a temperature of 50°C to obtain cocoa pods husk extract with a thick texture. During the solvation process, 5 liters of ethanol are needed. Cocoa pods husk after extraction has a weight of 134 grams. This study used 32 samples divided into 4 treatment groups, namely group 1 is E. faecalis without giving cocoa pods husk extract (control group), group 2 is E. faecalis bacterial culture with 1.56% cocoa pods husk extract, group 3 is E. faecalis bacterial culture with 3.125% cocoa pods husk extract and group 4 is E. faecalis bacterial culture with 6.25% cocoa pods husk extract. Stock of E. faecalis bacteria according to standard Mc. Farland 0.5 or 1.5 x 10 CFU/ml was diluted with dilution
method to reach a density of 106 CFU/ml, then culture on TSB media in a microtiter plate flat button 24 well then incubated for 3 x 24 hours at 35º C.13,14 Cocoa pods husk extract was applied to each titer with concentrations of 1.56%, 3.125%, and 6.25% after the biofilm formation process. Then incubated again at 35º C for 24 hours. Then the contents of each microtiter plate were aspirated and washed 4 times with 0.2 ml of phosphate-buffered saline (pH 7.3) using a pipette to remove planktonic bacteria and then dried. Biofilms attached to the microtiter plate were stained with 1 ml Alexa Dextran (Thermo Fisher Scientific, Singapore) under dark conditions for 30 minutes and finally rinsed with aquadest to remove dyestuffs. After the staining procedure, the appropriate specimens were immediately examined with a confocal laser scanning microscope (CLSM) under 400X magnification (Olympus, Tokyo, Japan). The preliminary research has carried out to get the minimum concentration using the calculation of bacterial density in biofilms as measured by the bacterial optical density (OD) unit using ELISA reader. The difference between the treatment group and the control group were determined with Post Hoc Test (p = 0.05). Tukey HSD was used to test the significance of the differences between treatment groups. RESULTS The figure 1 shows the intensity values on EPS biofilms which are reviewed through 3D slices. The brighter color of the graph shows the remaining EPS biofilms with dyes that are still attached to this EPS and the higher intensity value. From the picture shows that there is a difference of intensity value in the EPS biofilm of the control group (Group 1) and the treatment group (Group 2, Group 3 and Group 4). The 6.25% cocoa pod husk has the lowest intensity and the control group has the highest intensity. Post Hoc Tukey HSD test (Table 2) showed a significant difference between Group 1 and Group 4. While in Group 1 and Group 2 compared to the Group 1, there were no significant differences with the values of each p=0. 340 and p=0. 267 (p>0.05). DISCUSSION This study aims to obtain the concentration of inhibitory formation E. faecalis EPS biofilm due to exposure to the extract of cocoa (Theobroma cacao) which is expected to be used as an alternative material for root canal irrigation. This study used cocoa pod husk extract with a concentration of 1.56%, 3.125% and 6.25%. Post Hoc Tukey HSD test showed a significant difference between the control group and the concentration of 6.25%. While cocoa pods husk extract with a concentration of 1.56% and a concentration of 3.125%, there was no significant difference of the formation of E. faecalis EPS biofilm. It can be shown that the amount of concentration in cocoa pod husk extract affects the inhibition of the E. faecalis EPS biofilm formation, which obtained the minimum inhibitory concentration is 6.25%. This is due to the fact that there is an alkaloid, flavonoid, tannin and saponin content in the husk of cocoa pods husk that has antibacterial properties. 14 The mechanism of tannin inhibition against the formation of EPS biofilms is by binding and precipitating proteins on EPS. In addition, tannins are also able to bind to carbohydrates, where the greater the molecular weight the stronger the interaction with tannin. Tannin is also a chelating agent because it is able to form bonds with iron ions which will result in breaking of the EPS matrix bond.25 Mechanism of saponin as antibiofilm by decreasing bacterial extracellular DNA component, so that it will result in decreased biofilm formation. Bioactive fractions that are rich in saponins can also inhibit the formation of biofilms by preventing the initial cell-surface attachment of bacteria.26 From the description above, it can be concluded that the presence of compounds found in the extract of cocoa pod husk can inhibit the formation of E. faecalis EPS biofilms. The extract of 6.25% cocoa pod husk is a concentration that can reduce the thickness of E. faecalis EPS biofilm. REFERENCES 1. Hargreaves KM, Berman LH. Cohen’s Pathways of The Pulp. 11th Edit. Elsevier Health Science. USA. 2016; p. 621-5 2. Guivarch M, Ordioni U, Ahmed HM, Cohen S, Catherine JH, Bukiet F. Sodium hypochlorite accident: a systematic review. Journal of endodontics. 2017 Jan 1;43(1):16-24. 3. Spencer HR, Ike V, Brennan PA. The use of sodium hypochlorite in endodontics—potential complications and their management. British dental journal. 2007 May;202(9):555. 4. 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