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Research Report

Minimum inhibitory concentration of cocoa pod husk extract in *Enterococcus faecalis* extracellular polymeric substance biofilm thickness

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ABSTRACT

Background: *Root canal treatment constitutes a treatment sequence for infected pulp to eliminate the etiological factors of pulp necrosis and periapical lesion. Enterococcus faecalis (E. faecalis) is an organism commonly found in a high proportion of root canal failure because of its ability to form biofilm. Degradation of extracellular polymeric substance (EPS) by oxidizing agents such as sodium hypochlorite is the first step in removing biofilm. However, the toxicity of sodium hypochlorite constitutes the main concern and, therefore, the safest alternative irrigants possible are required. The use of fruits, herbs and plants is widespread, especially in the fields of medicine and dentistry. Food crops are known to be rich in bioactive compounds, especially polyphenols, which have antioxidant and antimicrobial properties. Cocoa pod husk extract can, therefore, represent an alternative irrigant.* ***Purpose:*** *This study aimed to determine the minimum inhibitory concentration of cocoa pod husk extract in relation to the thickness of E. faecalis EPS biofilm.* ***Methods:*** *Four groups of E. faecalis cultured biofilm samples were analysed: group one contained E. faecalis without cocoa pod husk as a positive control; group two contained E. faecalis with 1.56% cocoa pod husk extract; group 3 contained E. faecalis with 3.125% cocoa pod husk extract; and group 4 contained E. faecalis with 6.25% cocoa pod husk extract. The biofilm thickness of all groups was measured by confocal laser scanning microscopy with statistical analysis subsequently undertaken by means of a post hoc test and Tukey HSD.* ***Results:*** *The average values of EPS biofilm thickness were as follows: group 1: 9500 nm; group 2: 8125 nm; group 3: 8000 nm; and group 4: 6375 nm. A post hoc Tukey HSD test indicated a significant difference between group 1 and group 4, while in group 2 and group 3 compared to group 1, there were no significant differences with the values of each being p = 0.340 and p = 0.267 (p>0.05).* ***Conclusion:*** *6.25% cocoa pod husk extract reduces E. faecalis EPS biofilm thickness.*

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

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INTRODUCTION

The pathology of pulp tissue and periapical tissue is directly or indirectly related to microorganisms. Microbes can be removed and minimized by root canal treatment whose success is influenced by several interrelated factors[1], including; effective diagnosis, aseptic action, knowledge of dental anatomy, chemical-mechanical preparation, three-dimensional obturation and the use of root canal dressing. All of these factors relate to one key point, specifically; root canal decontamination.[1]

The effectiveness of root canal preparation can be increased by the use of irrigation solutions such as sodium hypochlorite (NaOCl), chlorhexidine, and ethylenediaminetetraacetic acid (EDTA). NaOCl represents the gold standard for root canal irrigation solutions because, at present, no other solution has demonstrated similar effectiveness. However, the drawback of NaOCl is its

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cytotoxicity which can cause acute injury if it infiltrates the periapical region. If NaOCl comes into contact with tissue, it rapidly oxidizes the surrounding living tissue and triggers rapid hemolysis, inhibits neutrophil migration, and damages endothelial and fibroblast cells.[2] The higher the concentration of NaOCl, the higher the anti-bacterial effect and tissue dissolution, and also the higher the toxicity.[3] Moreover, the very low concentrations of NaOCl (>0.01%) present in in vitro cell culture cause the death of human fibroblast cells.[4]

Root canal treatment can fail due to the absence of an effective coronal seal, microleakage, failure of chemical-mechanical preparation, or poor quality root canal filling with the result that certain microorganisms survive or reinfection ensues. Of the microorganisms associated with the failure of root canal treatment, one of the most common is *Enterococcus faecalis (E. faecalis).*[5] This is due to its ability to survive in environmental conditions low in nutrition and to form biofilms which renders *E. faecalis* 1000 times more resistant to phagocytic cells, antibodies and antimicrobials compared to those organisms unable to manufacture biofilm.[1,6]

Biofilms are defined as multicellular microbial communities characterized by cells that attach strongly to the surface and produce a matrix extracellular polymeric substance (EPS).[7] EPS consists of bacterial proteins, nucleic acids, polysaccharides and fats. Microbes that form biofilms are thought to be the cause of 80% of infections.[8]

Herbs, fruits and plants are widely used, especially in the fields of medicine and dentistry. Food crops are known to be rich in bioactive compounds, especially polyphenols, which possess antioxidant and antimicrobial properties. One of the food plants rich in antioxidants whose pod husk has antimicrobial properties is cocoa.[9–12] The cocoa pod husk contains unsaturated fatty acids and epakitin polymers which promote antibacterial and anti-glucosyltransferase activity, whereas the coco pods consist mainly of polysaccharides (cellulose and hemicellulose), lignin and small quantities of phenolic compounds, tannin, purine alkaloids and cocoa butter.[10] The minimum concentration that can inhibit *E. faecalis* biofilm formation is one of 3.125%.[13]

To the best of the authors' knowledge, no studies evaluating the effect of cocoa pod husk on the thickness of *E. faecalis* EPS biofilm have, to date, been conducted. Therefore, the purpose of this research 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 consisted of Forastero type cocoa fruit (*Theobroma cacao L.*) extract at concentrations of 1.56%, 3.125%, and 6.25%. The cocoa pods obtained from the Coffee Research Center and Cocoa Jember were of the Forestero type with a yellow mark when picked which had been cooked. Before processing, the picked pods were left for approximately five days to facilitate the release of their entire contents, including the seeds, from the cocoa husk. This is a process known as maceration.[13]

The 6 kg of fresh cocoa pod husk used in this study was cut and aerated. Half-dried pod husk was further dehydrated in an oven at a temperature of 50° C, producing 1 kg of desiccated husk which was subsequently milled, macerated with 70% ethanol for 24 hours and filtered. This process produced filtrate and dregs which were soaked and filtered a second time. The maceration and filtration processes were repeated until a clear filtrate had been obtained. At this point, ethanol evaporation was conducted by means of a rotary evaporator at a temperature of 50° C to obtain cocoa pod husk extract thick in texture. Five liters of ethanol were required during the solvation process which produced 134 grams of cocoa pod husk extract.

This study used 32 samples divided into four treatment groups, namely; group 1 (control group) consisting of *E. faecalis* without cocoa pods husk extract; group 2 containing *E. faecalis* bacterial culture with 1.56% cocoa pod husk extract; group 3 containing *E. faecalis* bacterial culture with 3.125% cocoa pod husk extract; and group 4 containing *E. faecalis* bacterial culture with 6.25% cocoa pod husk extract.

E. faecalis bacteria stock was diluted in accordance with standard McFarland 0.5 or 1.5 x 10 CFU/ml to obtain a density of 106 CFU/ml. The stock was then cultured in TSB media in a flat button 24 well microtiter plate before being incubated for 3 x 24 hours at 35ºC.[13,14]

After the biofilm formation process, cocoa pod husk extract was applied to each titer at concentrations of 1.56%, 3.125%, and 6.25% and then incubated again at 35º C for 24 hours. At this point, the contents of each microtiter plate were aspirated, washed four times with 0.2 ml of phosphate-buffered saline (pH 7.3), cleared of planktonic bacteria by means of a pipette and, finally, dried.

The biofilms attached to the microtiter plate were stained with 1ml of Alexa Dextran (Thermo Fisher Scientific, Singapore) stored in dark conditions for thirty minutes and rinsed with aquadest to remove any dyestuffs present. Following the staining procedure, the appropriate specimens were immediately examined with a confocal laser scanning microscope (CLSM) at 400x magnification (Olympus, Tokyo, Japan). Preliminary research was conducted to obtain the minimum concentration using the calculation of bacterial density in biofilms conducted by an optical density (OD) unit incorporating an ELISA reader.

The difference between the treatment group and the control group was determined by completion of a post hoc test ($p = 0.05$). A Tukey HSD was used to assess the significance of the differences between the treatment groups.

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RESULTS

Figure 1 shows the intensity values of EPS biofilms reviewed through 3D slices. The brighter color of the graph indicates the remaining EPS biofilms with dyes that remain attached to this EPS and their higher intensity values. From the image, it is evident that there is a difference in intensity value between the EPS biofilm of the control group (group 1) and that of the treatment groups (group 2, group 3 and group 4). The 6.25% cocoa pod husk has the lowest intensity, while the control group has the highest intensity.

The mean and standard deviation of each sample group used to quantify the value of *E. faecalis* EPS biofilm thickness is shown in the Table 1. The results of the post hoc Tukey HSD test (Table 2) confirmed a significant difference between group 1 and group 4. In contrast, when group 2 and group 3 are compared to group 1, there were no significant differences between the values of each, viz; p=0.340 and p=0.267 (p>0.05).

DISCUSSION

This study aims to establish the concentration of inhibitory formation *E. faecalis* EPS biofilm following exposure to cocoa extract (*Theobroma cacao*) which represents a potential alternative material for root canal irrigation. This study used cocoa pod husk extract at concentrations of 1.56%, 3.125% and 6.25%.

In this study there was a significant difference between the control group (group 1) and the 6.25% cocoa pod husk extract (group 4), whereas in the 1.56% cocoa pod husk extract group (group 2) and the 3.125% cocoa pod husk extract group (group 3) no significant difference was evident when compared to the control group (group 1). It can be seen that the level of concentration of cocoa pod husk extract influences the inhibition of *E. faecalis* EPS biofilm formation which reached the minimum inhibitory concentration of 6.25%. This is due to the fact that cocoa pod husk contains alkaloid, flavonoid, tannin and saponin all of which possess antibacterial properties.[14]

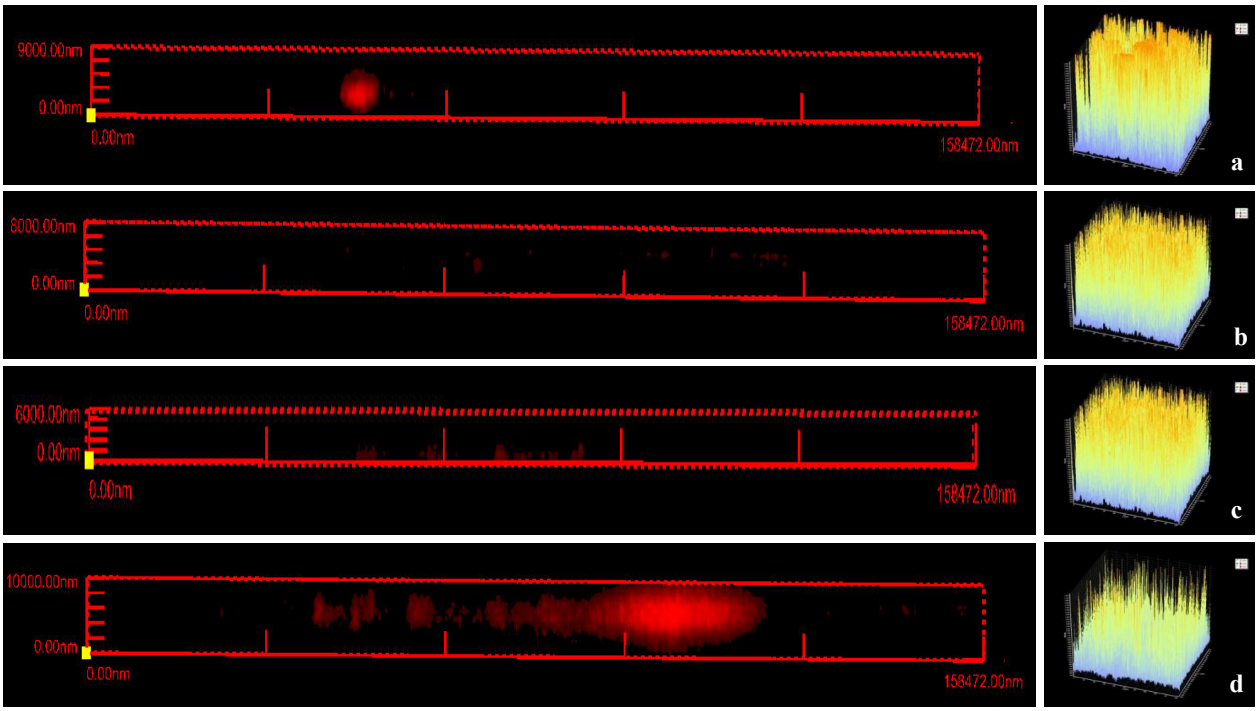

Figure 1. Fluorescence color intensity chart and EPS thickness. (a) Group 1 (b) Group 2 (c) Group 3 (d) Group 4.

Table 1. Mean and standard deviation of *E. faecalis* EPS biofilm thickness

Group	N	Mean(nm)	SD (nm)
Group 1	8	9500.00	1195.23
Group 2	8	8125.00	1727.89
Group 3	8	8000.00	2000.00
Group 4	8	6375.00	1408.00

Note: N = number of samples; Mean = average; SD = standard deviation

Table 2. Difference test between treatment groups (Tukey HSD test)

	Group 1	Group 2	Group 3	Group 4
Group 1		0.340	0.267	0.03*
Group 2			0.999	0.156
Group 3				0.206
Group 4				

Note: * there is a significant difference (p<0.05)

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The mechanism of tannin inhibiting the formation of EPS biofilms is that of binding and precipitating proteins in EPS. In addition, tannins are able to bind to carbohydrates where the greater the molecular weight, the stronger the interaction. Tannin is also a chelating agent because it is able to form bonds with iron ions which will result in a rupturing of the EPS matrix bond.[15]

The mechanism of saponin as antibiofilm involves reducing the bacterial extracellular DNA component resulting 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.[16] From the explanation 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 at a concentration that can reduce the thickness of *E. faecalis* EPS biofilm.

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