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Research Report

A comparative study of the *E. faecalis* antibiofilm efficacy of photoactivated curcumin, chlorophyll and riboflavin

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ABSTRACT

Background: *Photosensitisers play a vital role for reactive oxygen species (ROS) production in diode laser 405 nm therapy. Curcumin, chlorophyll and riboflavin have been used and viewed in several studies as effective photosensitisers for the elimination of Enterococcus faecalis (E. faecalis), a persistent microorganism that may cause endodontic failure. While each has given valuable and promising results as an alternative endodontic irrigant, no study has compared the efficacy of these three natural dyes.* ***Purpose:*** *To prove that the photosensitiser curcumin in diode laser 405 nm therapy is more effective for E. faecalis biofilm degradation than chlorophyll and riboflavin, and that a duration of irradiation of 90 seconds is more effective than 60 seconds.* ***Methods:*** *The biofilm monospecies E. faecalis was divided into two microplates with 96-well according to the irradiation periods: 60 seconds (Group 1) and 90 seconds (Group 2). Each group contained one control (without a photosensitiser) and three treatments were carried out by adding the photosensitisers curcumin, chlorophyll and riboflavin, where each treatment contained eleven samples. After irradiation for 60 seconds and 90 seconds, a crystal violet assay was carried out for each group.* ***Results:*** *The one-way ANOVA test showed a significant difference between groups based on the irradiation period. Tukey's test showed each treatment in each group also showed a significant difference ($p<0.05$) with curcumin as the best substance to cause biofilm degradation in both groups. The duration of the irradiation showed that the longer the biofilm was illuminated, the lower the absorbance value or optical density (OD).* ***Conclusion:*** *Curcumin irradiated for 90 seconds gives better biofilm degradation on E. faecalis due to its natural properties and molecular structure, highlighting its efficacy in photodynamic therapy.*

Keywords: *chlorophyll; curcumin; Enterococcus faecalis; photosensitiser; riboflavin*

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INTRODUCTION

The success of a root canal treatment is mainly related to the process of cleaning and shaping. The use of chemicals during root canal treatment is necessary to eliminate the bacteria that colonise the dentine tubules.[1] Of the several chemical agents used as root canal irrigation, Sodium Hypochlorite (NaOCl) has a non-specific ability as a strong proteolytic agent in disrupting and damaging biofilms. It can also dissolve necrotic tissue. Nonetheless, NaOCl has disadvantages. It has a bad odour, it can cause tooth discoloration when it reacts with certain antibiotics such as tetracycline or when combined with other irrigation solutions such as chlorhexidine (CHX), it is corrosive to metal instruments and it can cause irritation when pushed into periapical.[2]

An alternative that is being developed is photodynamic therapy using laser light. The process of photodynamic therapy requires three essential components, namely a photosensitiser (PS), a light source and oxygen. A PS is a molecule that is local to the target cell or tissue and can only be activated by light.[3] Behind its contribution in the treatment of pathogenic microorganisms in the field of medicine and dentistry, some synthetic chemical PSs such as toluidine blue (TB) have weaknesses. Research says that although it can inhibit bacterial adhesion and

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biofilm formation, irradiated Ortho-toluidine (TB-O) has toxic effects on fibroblast cells.[4] Therefore, natural PSs are now widely used in photodynamic therapy. A PS made from natural materials is claimed to have almost the same potential and function as chemically synthesised photodynamic agents, even exceeding them. In addition, safety must be considered when choosing natural PSs, because these ingredients are not toxic and are safe for consumption. The product of the solution is therefore safe and environmentally friendly.[5] Some natural ingredients used as PSs in photodynamic therapy include curcumin (CUR), chlorophyll (CHL) and riboflavin (RFV).[6] These three ingredients, according to the literature, are non-toxic and safe materials because they do not cause adverse effects in normal cells.[7–9]

In this study, monospecies biofilms containing *Enterococcus faecalis* (*E. faecalis*) bacteria were used. *E. faecalis* was chosen because a number of studies have been conducted showing that this bacterium is associated with secondary infections in the failed treatment of root canals. This species is also found in primary intraradicular infections, that is when the pulp is open because of caries, trauma or other factors that cause the integrity of the hard tooth tissue to be disrupted.[10] The superior effect of curcumin as a PS in photodynamic therapy on the *E. faecalis* biofilm has been shown in several previous studies.[11,12] However studies comparing curcumin with PS from other natural ingredients have not been conducted. The natural PS ingredients used as a comparison in this study were chlorophyll and riboflavin. Chlorophyll can eliminate *E. faecalis* better than irrigation material CHX, and ethylenediaminetetraacetic acid (EDTA).[13] Riboflavin also provides good antibacterial power against Gram-positive and Gram-negative bacteria and fungi, including *E. faecalis*.[14]

Curcumin, chlorophyll and riboflavin were chosen as PS in this study because all three of these materials have been used in many photodynamic therapy studies mainly to overcome chronic and recurrent infections caused by pathogenic microorganisms. Curcumin, chlorophyll and riboflavin are natural compounds that when irradiated with light will produce reactive oxygen species (ROS), which are effective in causing damage to cell structures and bacterial biofilms. The purpose of this study is to prove that PS curcumin with 405 nm diode laser irradiation has a better biofilm degradation ability against *E. faecalis* than PS chlorophyll and riboflavin.

MATERIALS AND METHODS

This study was approved by the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, with number 022/HRECC.FODM/I/2020. This type of research is a purely experimental laboratory research post test control group design. The sample in this study was the *E. faecalis* biofilm which was cultured from the ATCC 29212 bacterial stock by making an *E. faecalis* colony suspension in the Brain Heart Infusion Broth (BHIB) medium. The sample size was obtained by the Federer formula, and each treatment group had a sample size of 11.

The formation of the *in vitro* biofilm *E. faecalis* was carried out by culturing bacterial cells in 4 ml of BHIB media for 24 hours at 37°C. These cells were then harvested by centrifugation at 16,000 rpm for 5 minutes at 4°C and washed twice with sterile BHIB. A suspension of bacterial cells was obtained with a density of 1.5 108 cells/mL (0.5 McFarland standard), then as much as 200 ml of sediment was taken with a pipette and put on 96-well polystyrene microtiter plate. The plate was then covered with parafilm and incubated for 48 hours at 37°C without agitation.

Curcumin PS material was prepared by dissolving standard curcumin with a molecular weight of 368.38 g/mol (C1386, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in a 5% polyethylene glycol solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), until it reached a concentration of 2.5 mg/mL. It was maintained at pH 6.[12] Meanwhile, the chlorophyll PS comes from alfalfa, *Medicago sativa L.* (K-Link liquid chlorophyll, South Jakarta, Indonesia), with a concentration of 1.6mg/mL in a normal saline solution.[14] The riboflavin PS was made by dissolving 25 grams of standard riboflavin, with a molecular weight of 376.36 g/mol (47861, Supelco, Merck KGaA, Darmstadt, Germany), in a 25 ml (1g/mL) sterile phosphate buffer saline (PBS) solution. It was then stored in a dark room.[15]

After incubating each 1.5 10^8 CFU/mL suspension on a sterile microplate at 37ºC for 48 hours, the well microplate was filled with each of the following materials: a) the control group (+): 20µl *E. faecalis* biofilm, 90µl BHIB; b) treatment group I: 20µl *E. faecalis* biofilm, 90µl BHIB, 90µl PS curcumin; c) treatment group II: 20µl *E. faecalis* biofilm, 90µl BHIB, 90µl PS chlorophyll; and d) treatment group III: 20µl *E. faecalis* biofilm, 90µl BHIB, 90µl riboflavin PS. The microplate was labelled for 60 seconds (group I) and 90 seconds (group II). Each treatment group was numbered to 11 so that each microplate had 44 wells filled. Before irradiation, the two microplates were incubated in a dark room for 5 minutes at room temperature (25 ± 2ºC). The microplate containing the control group (+) and treatments I, II, and III was then inserted into a laser diode computer numerical control (CNC Biophysics Laboratory, Faculty of Science and Technology, Airlangga University, Surabaya) for irradiation with a wavelength of 405 nm. The instrument was set at a 10 mm irradiation distance, a 60 second irradiation duration for microplate 1 and a 90 second duration for microplate 2. After the irradiation, the microplate was incubated in an anaerobic jar for 48 hours.

The crystal violet (CV) test began with media removal and the gentle rinsing of the plate with 200µl PBS. Then, staining with 0.1% CV 50µl took place in order to dye the bacterial biofilm at room temperature for 15 minutes. After that, the CV was discarded and rinsed with PBS

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200µl. The plates were added with 96% 50µl ethanol and left for 10 minutes at room temperature to dry. Then, each microplate well was filled with 33% 150µl acetic acid. The plate was then inserted in the microplate reader to determine the degradation of the biofilm. This was measured by calculating the absorbance of the solution on the microplate reader with a wavelength of 570nm.

The absorbance value produced by the microplate reader (Epoch, Biotek Instruments, Inc., USA) showed the absorption of light received by the biofilm formed on the well wall of the microplate with optical density (OD) units. The results of the study were then analysed using the Statistical Package for the Social Sciences software (SPSS Statistics 17.0, IBM, USA) by conducting normality, homogeneity and one-way ANOVA tests.

RESULTS

The results of this study is absorbance values or optical density (OD) and biofilms formation (Figure 1). The data that shows the description and the analysis of the results in this study can be seen in Table 1.

The one-way ANOVA test was carried out to compare between the irradiation duration groups and all treatments in each group. There were significant differences between the irradiation length groups, which were shown through the one-way ANOVA test with $p = 0.000$. In the treatment group with a 60-second irradiation period, because the variance with the Levene test showed an inhomogeneous result, $p = 0.008$ ($p < 0.05$), a non-parametric (Kruskal–*Wallis*) test was carried out and obtained a significant

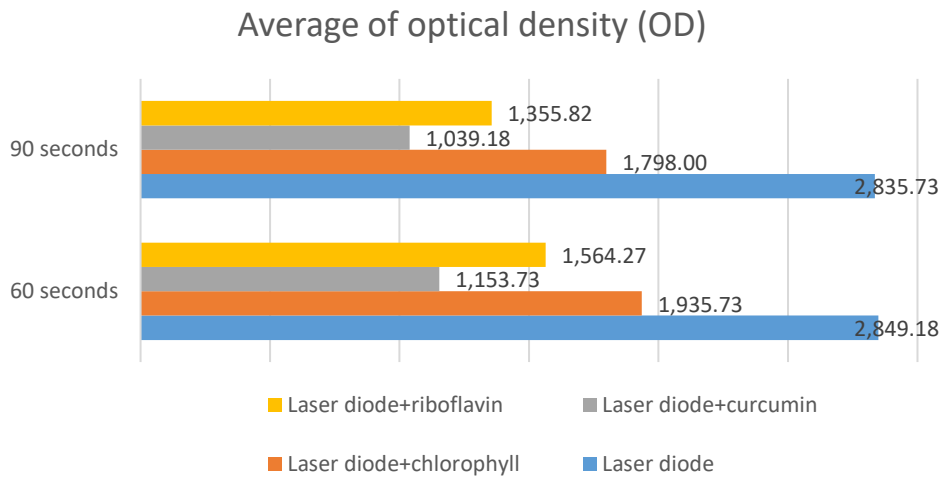

Figure 1. A diagram of the mean optical density (OD) of each PS with a long irradiation of 60 seconds and 90 seconds.

Table 1. Description and data analysis of the results of a comparative study of PS type and exposure time

Type of Treatment		Mean ± Std. Deviation	Comparison between groups	Comparison of treatments between groups	Comparison between treatments in each group
Group 1 (irradiation of 60 sec)	Laser diode	2,849.18 ± 0.020663	*	**	***
	Laser diode + curcumin	1,153.73 ± 0.035429			
	Laser diode + chlorophyll	1,935.73 ± 0.067088			
	Laser diode + riboflavin	1,564.27 ± 0.027821			
Group 2 (irradiation of 90 sec)	Laser diode	2,835.73 ± 0.037990	*	*	***
	Laser diode + curcumin	1,039.18 ± 0.034289			
	Laser diode + chlorophyll	1,798.00 ± 0.063786			
	Laser diode + riboflavin	1,355.82 ± 0.048410			

Note: Significance values for comparing between groups were obtained through the one-way ANOVA test with $p < 0.05$*; intergroup 1 treatment was obtained through the Kruskal–Wallis test with $p < 0.05$**, and intergroup 2 treatment was obtained through the one-way ANOVA test with $p < 0.05$. While the comparison between treatments in each group was obtained through the Tukey/HSD test with $p < 0.05$***.

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result p = 0.000. In the treatment group with a 90 second irradiation time, the ANOVA test results showed significance, which was p = 0.000.

Furthermore, the Tukey test in each time group showed a significant difference in each treatment, p = 0.000. PS curcumin had the best antibiofilm effect, followed by riboflavin and chlorophyll ($p < 0.05$).

DISCUSSION

The results of this study indicate that curcumin has the best antibiofilm effect, which is indicated by the lowest average optical dentisy (OD), followed by riboflavin and chlorophyll. This is because curcumin has the lowest molecular weight, which is 368.38 g/mol so it has a better diffusion ability. Consequently, in the same incubation period, curcumin can be present in biofilms in greater amounts than riboflavin and chlorophyll with molecular weights of 376.36 g/mol and 893.51 g/mol[16] respectively.

To carry out its function as an antibiofilm, PS in photodynamic therapy can work in two ways: destroying the biofilm matrix, or entering further past extracellular polymeric substance (EPS) and damaging the microbial cells in it. Penetration of PS material further into the area will produce massive damage.[17] Therefore, diffusion of PS, its localisation and the duration of irradiation are all important in photodynamic therapy. Diffusion is the movement of a substance from a high concentration to a low concentration. PS diffusion is related to the molecular weight of the material. The higher the molecular weight of a PS, the slower it diffuses into cells/tissues.[18,19] This may be the reason why curcumin is more effective in causing damage to the *E. faecalis* biofilm structure than chlorophyll and riboflavin.

The duration of irradiation and the characteristics of the PS become important factors that influence the outcome of photodynamic therapy. Based on previous studies, the irradiation duration that was effective in causing damage to the *E. faecalis* cell wall was 60 seconds and 90 seconds.[13] In this study, there were significant differences between the time groups of 60 seconds and 90 seconds. Each PS showed a decrease in *E. faecalis* biofilm density with an increasing duration of irradiation. The penetration of PS material in order to induce intracellular damage depends on the length of the incubation before the irradiation, which gives the PS time to interact with the cells. Therefore, PS material must be left at least 5 minutes to allow time for PS material to penetrate the cell/tissue so that the photodynamic process can work optimally. However, in this study, each PS has not reached the maximum inhibitory power against *E. faecalis* biofilm. This is because the biofilm structure is more complex than the planktonic cells and thus requires a longer incubation time for the PS material penetration. Diogo *et al.*[13] recommends allowing an incubation period of 15 minutes before irradiation.

The superior ability of curcumin to damage biofilms is in line with several previous studies. Neelakantan *et al.*[11] concluded in their study that light-activated curcumin provides an excellent antibiofilm effect, even more effective in killing bacteria in the root canal, including *E. faecalis*, than light-activated NaOCl. Other studies suggest that light-activated curcumin has a very good antibiofilm effect on *E. faecalis* when compared with some commonly used intracanal medicaments such as antibiotic paste (trimix), 2% chlorhexidine, and calcium hydroxide.[12] Masoule *et al.*[20] investigated the antibacterial and antibiofilm effects of curcumin on two endopathogenic species *E. faecalis* and Pseudomonas aeruginosa, both in the form of planktonic bacteria and biofilms. The results when doing antibacterial photodynamic therapy (aPDT) with PS curcumin showed a significant decrease in cell viability and OD biofilm when compared with the administration of curcumin or irradiation with a light-emitting diode (LED) alone.

The advantage of curcumin as a PS is the presence of three reactive functional groups, a ketone and two phenol groups that play a role in the phototherapeutic activity. The phenol group is an essential factor in the interaction of the curcumin and the biological membrane where the attachment of the hydrogen group plays an important role.[21] Biological chemical reactions that occur are as follows: hydrogen donations that cause oxidation, the addition of reversible and irreversible nucleophilic (Michael reaction), hydrolysis, degradation, and enzymatic reactions.[22] Research using curcumin as a PS for root canal disinfection was first reported to show that the canal curcumin base in the presence of two conjugated electron systems made curcumin an effective PS.[11]

Riboflavin, vitamin B2 and flavin (as part and product of degradation) are PSs that are efficient in inducing oxidative damage to tissue exposed to light. Phenolic, N-heterolytic amino acids and their peptides and proteins deactivate triplet-excited riboflavin states in controlled diffusion processes, which efficiently compete with deactivation by oxygen, resulting in the degradation of direct proteins (referred to as type I) through electron transfer or proton-electron pair transfer.[23]

Chlorophyll is an important pigment that plays a role in the process of photosynthesis. Chlorophyll is very efficient at absorbing light and sending excitation energy to the photosystem reaction centre before a photosynthetic electron transport occurs. Excited chlorophyll will produce singlet oxygen, which can cause severe cellular damage. The chlorine portion obtained in Chl-a is a conjugated ^{1}-tetrapyrrole group that acts as a potential phototoxic chromophore.[24] Moreover, PSs with tetrapyrrole groups (except bacteriochlorin) tend to produce oxygen singlet (type II), which is more dominant than ROS (type I), which is usually produced by PSs with other structures, in photodynamic therapy.[6]

The three PS materials used in this study can produce an effective ROS in causing biofilm damage, which is characterised by a lower optical density (OD) value than

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the control group. The penetration of PS material into the structure of biofilms, matrices and the microbial cells in it, is not a major factor. Wherever the PS is located, ROS will be generated during the irradiation to initiate the second step, namely multitarget oxidative damage. The abundant production of ROS will weaken the antioxidant defences of microbial cells. As soon as ROS is produced, it will attack many related cell molecules, including targets in the biofilm matrix (polysaccharides), on the cell surface (lipids) and in the cells (proteins and DNA), which results in the collapse of the biofilm matrix and the disintegration of microbial cells.[17] It can be concluded that curcumin as a PS in photodynamic therapy gives a better antibiofilm effect on *E. faecalis* compared to chlorophyll and riboflavin. In addition, the 90-second irradiation duration of all PSs was shown to be more effective in the degradation of *E. faecalis* biofilms. This relates to the nature of curcumin and its molecular structure, which enhance its function in photodynamic therapy. However, further research needs to be carried out in order to identify and compare the production of ROS from each PS.

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