In Vitro Study of Reduction of Oral *Enterococcus faecalis* Biofilm on Application of Combination of *Chrysomya megacephala* Maggot Extract and Sodium Hypochlorite

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Abstract: The tooth's infected root canal relates to the bacteria invasion, such as Enterococcus faecalis. The eradication of the bacteria using single root canal irrigants becomes difficult because of the formed biofilm. We aimed to investigate the effectiveness of a combination of C. megacephala maggot extract with the common irrigant, sodium hypochlorite 3%, on the biofilm reduction of E. faecalis. The C. megacephala maggot extract was tested at concentrations 25%, 50%, and 100%, and the combination of each extract concentration with sodium hypochlorite in a volume ratio of 1:1; 1:2, 1:3, 2:1, and 3:1. All treatments were performed three times of replication with incubation time for 1 hour and 3 hours. Antibiofilm effect was measured with crystal violet staining and the optical density reading. Data was analyzed with the Statistical Package for the Social Sciences Statistic Version 22. The least biofilm formation was observed in combination of maggot extract 25% with sodium hypochlorite (2:1) for 1 hour incubation (p=0,05) and combination of maggot extract 25% (1:1) for 3 hours incubation (p=0,000). This combination effectively inhibits the biofilm of E. faecalis. This study identified the protease enzymes in C. megacephala maggot extract and investigated C. megacephala maggot extract's antibiofilm effect combine with the other root canal irrigant.

1 INTRODUCTION

One of the bacteria found in the root canal of the infected tooth is Enterococcus faecalis. These bacteria are persistent and can form biofilms. Therefore they are difficult to be removed. The bacteria in the root canal treatment are eradicated by applying root canal irrigant. The agent commonly used as root canal irrigant is sodium hypochlorite (NaOCl), with 1,25-5% (Mulyawati, 2011). NaOCl effectively removes planktonic bacteria, but it is less effective in reducing biofilms produced by bacteria (Dunavant et al., 2006). NaOCl is usually combined with other irrigation solution such as chlorhexidine (CHX) to increase the antibacterial and antibiofilm effects.

Several researchers have also carried out the combination of root canal irrigant with natural agents. Geethapriya et al. (2016) combined chitosan with EDTA against E. faecalis biofilms with a ratio of 1: 1 in their study, which showed effective results in inhibiting E. faecalis biofilm formation (Geethapriya et al., 2016). Other studies combined chitosan with chlorhexidine (CHX), whose results showed the same effect with 5% NaOCl in inhibiting E. faecalis biofilm (Jaiswal et al., 2017). Other studies regarding the combination of NaOCl with natural agents have never been done.

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Maggot extract is material from natural sources that have not been studied as root canal irrigant. Some maggots, such as maggot Lucilia sericata contain proteolytic enzyme components that can degrade bacterial biofilms' extracellular matrix (Chen et al., 2012). Chymotrypsin in L. sericata secretion can affect bacterial biofilms' adhesion (Harris et al., 2013). Other types of maggots, such as Chloroprocta sp. contain protease enzymes that can reduce the extracellular biofilm matrix in Staphylococcus epidermidis (Anjarwati et al., 2017). Another maggot extract, such as Chrysomya megacephala maggot extract, has an excretory and secretory. This product contains serine (trypsin and chymotrypsin), an antibacterial effect on Escherichia coli, Staphylococcus aureus, and Bacillus subtilis (El-Ebiarie et al., 2012; Mohamed 2015a, 2015b).

In this study, the maggot was taken from the maggot Chrysomya megacephala, a local maggot that is quite common in Indonesia (Putri, 2018). Combination of maggot extract C. megacephala with 3% NaOCl as root canal irrigant is expected to increase the reduction of E. faecalis biofilm.

2 METHODS

2.1 Material

This experimental laboratory study conducted the E. faecalis biofilm test. The materials used were C. megacephala maggot extract with a concentration of 25%, 50%, 100% and a combination of each concentration of maggot extract with 3% NaOCl with a volume ratio of 1: 1, 1: 2, 1: 3, 2: 1 and 3: 1, 3% NaOCl as a positive control, tryptic soy broth (TSB) and sterile phosphate-buffered saline (PBS) as negative controls. Each treatment was replicated three times. Biofilm reduction was measured by added 1% crystal violet with optical density measured at a wavelength of 595 nm (OD595) using a microtiter plate reader at the Cancer and Stem Cell Research Center Laboratory, Muhammadiyah University, Purwokerto.

2.2 Bacterial Strains

The sample used in this study is that the colony is E. faecalis ATCC 29212, a moderate biofilm-producing bacteria. The colony has been isolated and cultured with Mueller Hinton Agar (MHA) media in the Microbiology Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto. The powder of MHA with 12 grams was solved with 240 mL of aquadest and poured into the culture tray (20 mL/tray). The media was sterilized in an autoclave for 15 minutes (2 atm, 121oC), then put in an incubator for 24 hours to check if there is contamination or not. The E. faecalis were cultured in sterile media with an anaerobic environment. The bacteria's growth colony was taken and diluted with NaCl 0,9% until the concentration equal to 106 CFU/mL (CFU: Colony Forming Unit) or 0,5 Mc Farland Standard (Howarto et al., 2015).

2.3 Rearing Maggot and Collecting Maggot Extract

The process of collecting C. megacephala maggot extract was carried out at the Faculty of Medicine, Universitas Jenderal Soedirman. The rearing of the maggot C. megacephala was done by installing traps of flies that have been given raw fish (fish waste). After one day, the fly eggs that have been collected in fish waste were transferred to fresh raw fish and left to grow into a maggot. After growing into a maggot, the maggot at the end of the second instar and the beginning of the third instar was collected in a vessel and washed using ethanol and distilled water three times (Arora et al., 2010).

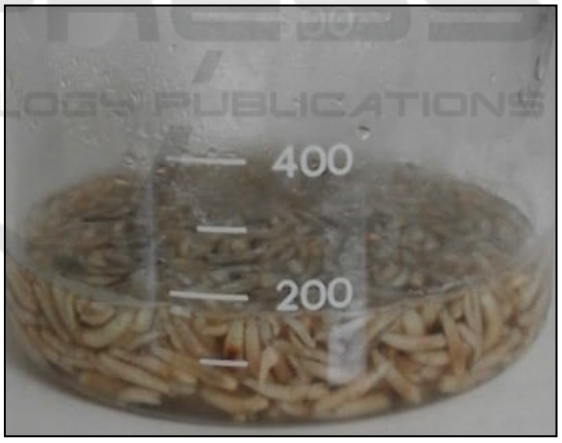

Figure 1: Soaking *C. megacephala* maggot in Phosphate Buffered Saline (PBS).

Every 1 gram of maggot was immersed in 1 ml of sterile PBS solution for 1 hour at room temperature in a dark room (Figure 1). Continued Soaking was for 24 hours (moved to room light). Then maggot in PBS was incubated at 37 °C for 48 hours. Then the maggot and liquid were separated, then the liquid was centrifuged at 25°C, with 10,000 rpm for 15 minutes.

Table 1: The result of optical density reading in 1 hour and 3 hours incubation.

No.	Treatment	Mean of absorbance	
		1 hour	3 hours
1.	TSB with *E. faecalis* (control of bacteria)	0,851	3,064
2.	*C. megacephala* maggot extract 25%	0,253	2,966
3.	*C. megacephala* maggot extract 50%	0,319	2,902
4.	*C. megacephala* maggot extract 100%	0,424	2,927
5.	Combination of *C. megacephala* maggot extract 25%, and NaOCl 3% (1:1)	0,214	0,176
6.	Combination of *C. megacephala* maggot extract 50%, and NaOCl 3% (1:1)	0,756	2,809
7.	Combination of *C. megacephala* maggot extract 100%, and NaOCl 3% (1:1)	0,619	2,915
8.	Combination of *C. megacephala* maggot extract 25%, and NaOCl 3% (1:2)	0,268	0,294
9.	Combination of *C. megacephala* maggot extract 50%, and NaOCl 3% (1:2)	0,501	0,211
10.	Combination of *C. megacephala* maggot extract 100%, and NaOCl 3% (1:2)	0,190	2,998
11.	Combination of *C. megacephala* maggot extract 25%, and NaOCl 3% (1:3)	0,209	0,212
12.	Combination of *C. megacephala* maggot extract 50%, and NaOCl 3% (1:3)	0,232	0,216
13.	Combination of *C. megacephala* maggot extract 100%, and NaOCl 3% (1:3)	0,176	2,830
14.	Combination of *C. megacephala* maggot extract 25%, and NaOCl 3% (2:1)	0,155	0,294
15.	Combination of *C. megacephala* maggot extract 50%, and NaOCl 3% (2:1)	0,498	2,925
16.	Combination of *C. megacephala* maggot extract 100%, and NaOCl 3% (2:1)	0,664	2,273
17.	Combination of *C. megacephala* maggot extract 25%, and NaOCl 3% (3:1)	0,343	2,918
18.	Combination of *C. megacephala* maggot extract 50%, and NaOCl 3% (3:1)	0,273	2,962
19.	Combination of *C. megacephala* maggot extract 100%, and NaOCl 3% (3:1)	1,395	2,920
20.	PBS with *E. faecalis*	0,317	2,982
21.	NaOCl 3% with *E. faecalis*	0,157	0,194

Table 2: The calculating of the value of $MBRC_{50}$ and $MBRC_{80}$ in biofilm test with 1 hour and 3 hours incubation.

Time of incubation	OD of Bacterial control	OD of Blank	$MBRC_{50}$ (OD of bacterial control –OD of blank) x 50%	$MBRC_{80}$ (OD of bacterial control –OD of blank) x 20%
1 hour	0,851	0,097	0,394	0,151
3 hours	3,064	0,109	1,477	0,591

The supernatant obtained from the centrifuge process was collected and sterilized with a 0.2 µm membrane filter. Maggot extract obtained from this process was stored at -20 ºC (Honda et al., 2011).

2.4 Biofilm Reduction Measurement by Administering Chrysomya megacephala Maggot Extract and Its Combination with Sodium Hypochlorite

The biofilm of E. faecalis (ATCC 29212) was measured using 96-well microtiter plates. The bacteria were transferred from Mueller Hinton Agar (MHA) media into Tryptic Soy Broth (TSB) and incubated for 24 hours under anaerobic conditions, which remained at 37 oC. The culture was diluted with 1: 100 on the medium. Then 20 µl of bacterial culture was inoculated with 200 µl TSB in each well, dispensed into 96-well microtiter plates with a flat bottom. After anaerobic incubation at 37 °C for 24 hours, the planktonic bacteria from each well were disposed of carefully by using a micropipette slowly until the bottom of the well looked clear. Each well was washed with 300 µl phosphate-buffered saline (PBS) 2 times slowly.

As many as 100 µL of maggot extract filled into each well of the 96-well microtiter plate was filled with at different concentrations (25%, 50%, 100%) and a combination of maggot extract and 3% NaOCl with a ratio of 1: 1, 1: 2, 2: 1, 1: 3, and 3: 1. The 96-well microtiter plate that has been inserted maggot extract and a combination of maggot extract and NaOCl are then incubated for 1 hour and 3 hours. After incubation, the biofilm is examined by giving a dye of 200 µL of 1% crystal violet solution in water for 30 minutes and washed with distilled water. Wells are reversed and dried on paper towels and dry air. Then, 200 µL of 5% acid isopropanol was added to each well to remove biofilm colour. The optical density was measured at 595 nm (OD595) using a microtiter plate reader (Pierce et al., 2010).

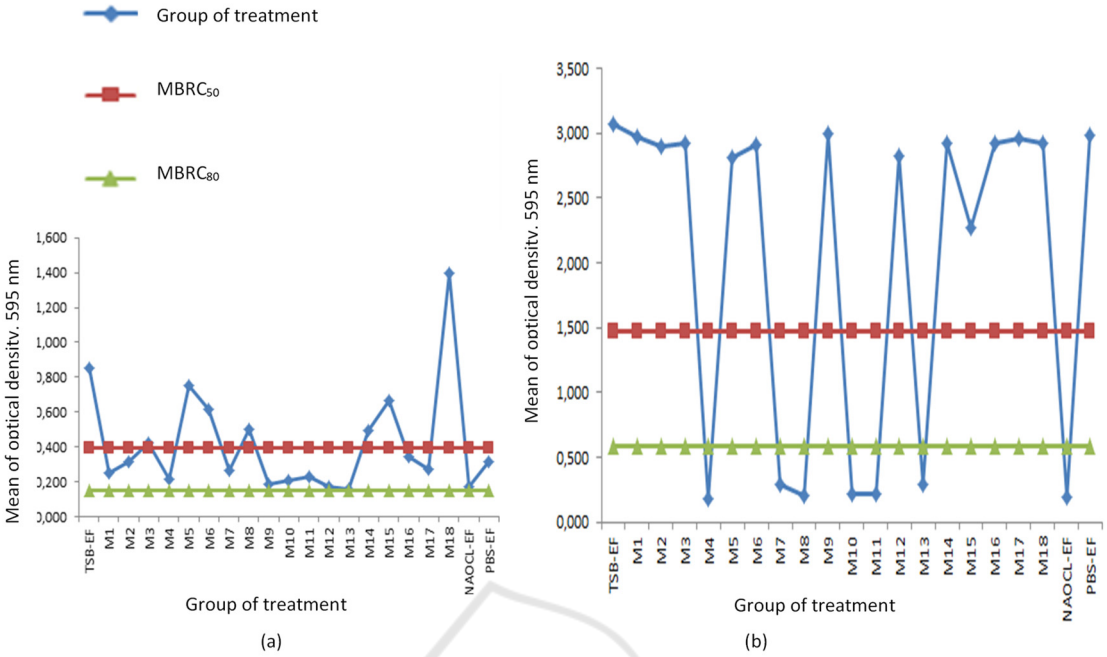

Figure 2. The effect of Chrysomya megacephala maggot extract on biofilm reduction of Enterococcus faecalis ATCC 29212 for 1-hour incubation (a) and 3 hours incubation (b)

2.5 Statistical Analysis

The effects of various maggot extract concentrations and their combinations with sodium hypochlorite in reducing biofilms were analyzed using one-way ANOVA and Post hoc LSD (Least Significant Difference) tests by using The Statistical Package for the Social Sciences Statistic Version 22.

3 RESULTS

From all treatment groups with 1-hour incubation, the most extensive treatment group in reducing E. faecalis biofilm was a combination of 25% maggot extract and sodium hypochlorite with a ratio of 2: 1 (reducing 81.75% of biofilm production produced from the bacterial control, $p < 0,05$)). The combination of maggot extract (25%) and sodium hypochlorite (2:1) reduced biofilm better than 3% sodium hypochlorite only. The Post hoc LSD test showed that the combination of 25% maggot extract and sodium hypochlorite with ratio 2:1 does not have a different effect than 3% sodium hypochlorite in reducing the biofilm of E. faecalis ($p>0,05$).

In 3 hours of incubation, the most extensive treatment group in reducing E. faecalis biofilm was a combination of 25% maggot extract and sodium hypochlorite with a ratio of 1: 1 (reducing 94.25% of biofilm production produced from bacterial control, $p <0.001$) (Table 1). The 25% maggot extract and sodium hypochlorite application (ratio 1:1) showed more effect than 3% sodium hypochlorite only. The Post hoc LSD test also showed that the combination of 25% maggot extract and sodium hypochlorite with ratio 1:1 does not have a different effect than 3% sodium hypochlorite in reducing the biofilm of E. faecalis ($p>0,05$).

In 1 hour incubation, the value of MBRC50 in this research is 0,394 and MBRC80 is 0,151 (Table 2). In 1 hour incubation, MBRC50 could be found in maggot extract 25%, 50%, combination of maggot extract 25% (1:1), 25% (1:2), 100% (1:2), 25% (1:3), 50% (1:3), 25% (2:1), 25% (3:1), and50% (3:1). Meanwhile, the value of MBRC80 could not be found in all the treatment in 1 hour incubation (Figure 1). In 3 hours incubation, the value of MBRC50 is 1,477 and the value of MBRC80 is 0,591 (Table 2). In 3 hours incubation, MBRC50 and MBRC80 could be found in the same treatment which are the combination of maggot extract 25% (1:1), 25% (1:2), 50% (1:2), 25% (1:3), 50% (1:3), and 25% (2:1) (Figure 2).

Figure 2 showed that in 1-hour incubation, MBRC50 could be reached of maggot extract application (concentration 25%, 50%, the

combination of 25% maggot extract (1:1), 25% maggot extract (1:2), 100% maggot extract (1:2), 25% maggot extract (1:3), 50% maggot extract (1:3), 25% maggot extract (2:1), 25% maggot extract (3:1), and 50% maggot extract (3:1)). Meanwhile, MBRC80 could not be found in 1-hour of incubation.

4 DISCUSSION

Compared to the results of biofilm reduction at 1-hour incubation (maggot extract 25% (2:1)), there was a decrease in the concentration of maggot extract needed to reduce biofilms for a longer time (3 hours), namely with extract concentration of 25% (1:1). Due to a decrease in maggot extract protease activity, the possibility is as time increases, therefore at 3 hours incubation. The possibility of more significant antibiofilm activity is due to the combination of 3% sodium hypochlorite and maggot extract (Łaba et al., 2010).

Compared with the effect of giving 3% sodium hypochlorite, the combination of 25% (1:1) maggot extract (1 hour) and a combination of 25% (2: 1) (3 hours) maggot extract had no different effects on Enterococcus faecalis biofilm reduction ($p>0.05$). This result shows that an evaluation in the research process is needed to increase the antibiofilm effect to be more maximal. In PBS solvent with E. faecalis, compared with a combination of 25% 2: 1 maggot extract (1-hour incubation) and 25% (1:1) (3 hours incubation) combination of maggot extract, there were no significant differences in antibiofilm effects. This result showed that the presence of PBS as a solvent in making maggot extract is not a factor that influences the reduction of bacterial biofilms. However, the antibiofilm effect produced comes from the extract of the maggot.

This study also showed that the C. megacephala maggot extract's antibiofilm effect was more effective at 3 hours incubation ($p <0.001$). In other studies, incubation time also significantly affected the reduction of extracellular biofilm matrix after maggot extract. This is thought to be due to proteases requiring time to break down proteins into dissolved proteins in exopolysaccharide (EPS) on bacterial walls. Protease is one type of enzyme contained in maggot extract. The effect of enzyme damage is directly proportional to the length of time the interaction of environmental exposure to the enzyme. The longer the exposure to the environment will damage the enzyme's structure, so that a decrease in enzyme activity Łaba et al., 2010)

5 CONCLUSIONS

C. megacephala maggot extract and its combination with sodium hypochlorite affect the reduction of biofilms produced by E. faecalis. Further research is needed to develop C. megacephala maggot extract, such as identifying other protease enzymes in C. megacephala maggot extract to reduce other bacterial biofilms and determine the antibacterial and antibiofilm effects of C. megacephala maggot extract with a combination of other root canal irrigation materials.

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