

RESEARCH ARTICLE

The Effect of *Pluchea indica* Less Leaves Extract Against Biofilm of *Enterococcus faecalis* and *Fusobacterium nucleatum* In Vitro

(Efek Ekstrak Daun *Pluchea indica* Less Terhadap Biofilm
Enterococcus faecalis dan *Fusobacterium nucleatum* In Vitro)

Agni Febrina Pargaputri*, Elly Munadzirah**, Retno Indrawati***

* Oral Biology, Faculty of Dentistry, Hang Tuah University Surabaya

** Dental Material and Technology, Faculty of Dentistry, Airlangga University

*** Oral Biology, Faculty of Dentistry, Airlangga University Surabaya

ABSTRACT

Background: *Enterococcus faecalis* and *Fusobacterium nucleatum* are the most common bacteria found in infected root canal teeth and most of them often caused failure in endodontic treatments. These bacteria can form biofilm which makes them more resistant against antibacterial agents. Biofilm formation also causes a decrease in antibiotics and antimicrobials sensitivity. *Pluchea indica* Less leaves is a species of plants that has several chemical properties. It consists of flavonoids and polyphenols which have benefits to inhibit biofilm formation. Because of its benefits, the extract of *Pluchea indica* Less leaves can be potentially developed as one of sterilization dressing in root canal teeth. **Purpose:** The aim of this study was to determine biofilm formation inhibition of *Pluchea indica* Less leaves extract against *Enterococcus faecalis* and *Fusobacterium nucleatum*. **Materials and Methods:** The dilution method was done first to show the Minimum Inhibitory Concentration (MIC) of the extract. The inhibition biofilm formation was tested using microtiter plate assay by measuring the bacterial biofilm Optical Density (OD) from ELISA reader's results and using autoaggregation assay to show the inhibition of adherence bacteria. The *Pluchea indica* Less leaves extract concentration used for inhibition of biofilm formation were 100%, 50%, 25%, 12,5%, and 6,25%. **Results:** The result of biofilm formation inhibition showed that *Pluchea indica* Less leaves extract were able to inhibit *Enterococcus faecalis* and *Fusobacterium nucleatum* biofilm formation with strong moderate effect. The autoaggregation assay showed a decrease in autoaggregation percentation of *Enterococcus faecalis* and *Fusobacterium nucleatum*. **Conclusions:** *Pluchea indica* Less leaves extract has effect to inhibit biofilm formation of *Enterococcus faecalis* and *Fusobacterium nucleatum*.

Keywords: *Pluchea indica* Less leaves extract, *Enterococcus faecalis*, *Fusobacterium nucleatum*, biofilm.

Correspondence: Agni Febrina Pargaputri, Department of Oral Biology, Faculty of Dentistry, Hang Tuah University, Arif Rahman Hakim 150, Surabaya, Phone.031-5912191, Email: agni_febrina@yahoo.com

ABSTRAK

Latar belakang: *Enterococcus faecalis* dan *Fusobacterium nucleatum* merupakan mikroorganisme yang sering ditemukan pada gigi dengan infeksi saluran akar, dan sebagian besar dari mikroorganisme tersebut merupakan penyebab kegagalan dalam perawatan saluran akar. Mikroorganisme tersebut dapat membentuk biofilm yang membuatnya lebih resisten terhadap bahan antibakteri. Biofilm juga dapat menurunkan sensitifitas bahan antibakteri dan antibiotik. Daun beluntas (*Pluchea indica* Less) merupakan salah satu tanaman yang memiliki kandungan senyawa kimia yaitu flavonoid dan polifenol yang telah dilaporkan memiliki fungsi sebagai antibakteri dan antibiofilm. Karena kandungan tersebut, ekstrak daun beluntas dapat dikembangkan sebagai salah satu alternatif bahan sterilisasi saluran akar. **Tujuan:** Tujuan dari penelitian ini adalah untuk membuktikan hambatan pembentukan biofilm dari ekstrak daun beluntas (*Pluchea indica* Less) terhadap bakteri *Enterococcus faecalis* dan *Fusobacterium nucleatum*. **Bahan dan Metode:** Uji dilusi/penipisan seri dilakukan terlebih dahulu untuk mengetahui nilai Konsentrasi Hambat Minimal dari ekstrak daun beluntas. Uji hambatan pembentukan biofilm dilakukan menggunakan microtiter plate assay dengan mengukur nilai Optical Density (OD) dan uji autoagregasi untuk mengetahui hambatan perlekatan bakteri. **Hasil:** Uji hambatan pembentukan biofilm menunjukkan bahwa ekstrak daun beluntas mampu menghambat pembentukan biofilm bakteri *Enterococcus faecalis* dan *Fusobacterium nucleatum* dengan kemampuan kuat sedarf. Hasil uji autoagregasi menunjukkan penurunan prosentase autoagregasi bakteri *Enterococcus faecalis* dan *Fusobacterium nucleatum*. **Simpulan:** Ekstrak daun beluntas memiliki efek dalam menghambat pembentukan biofilm bakteri *Enterococcus faecalis* dan *Fusobacterium nucleatum*.

Kata kunci: Ekstrak daun beluntas, *Enterococcus faecalis*, *Fusobacterium nucleatum*, biofilm.

Korespondensi: Agni Febrina Pargaputri, Departemen Biologi Oral, Fakultas Kedokteran Gigi Universitas Hang Tuah, Arif Rahman Hakim 150, Surabaya, Telepon 031-5912191, Email: agni_febrina@yahoo.com

BACKGROUND

The role of microorganisms as the cause of inflammation in pulp and periapical tissues have long been proven. Several studies have reported the existence of microorganisms in infected pulp and root canals, 90% are facultative anaerobic and species of Gram-positive bacteria, followed by Gram-negative bacteria and some groups of yeast.^{1,2} *Enterococcus faecalis* has been discovered in the failure of root canal treatments, with a prevalence 67% to 77% of cases accompanied by persistence endodontic infections. In addition to *Enterococcus faecalis*, bacteria with the greatest frequency obtained in

necrosis root canals is *Fusobacterium nucleatum*, found as many as 60% to 70% of cases in teeth with periodontal lesions.¹

Root canal treatment will be successful when it can cast off all sources of infection through the actions of preparation, sterilization, and filling. But sometimes there are some failures in root canal treatments. One of the causes in failure of root canal treatments is the presence of microorganisms that can survive both in root canal and tooth apical region. In some studies, it has been known that microorganisms able to form biofilm as a significant mechanism to increase the virulence of the pathogen, avoid host defense system, and make the

microorganisms more resistant to antimicrobial agents.⁴

Enterococcus faecalis can attach to the root canal walls, accumulate, and form biofilms, which can make them more resistant to phagocytosis, antibody, and antimicrobial agents, compared with microorganisms which do not produce biofilm. Bacterial biofilm that makes the microorganisms resistant to the antimicrobial agents, due to the protective barrier derived from the extracellular polymeric matrix.⁵ *Fusobacterium nucleatum* also have been identified within the biofilm extra radicular associated with apical periodontitis. *Fusobacterium nucleatum* able to form biofilm in root canal due to several reasons, among others, for their ecological needs and the advantages in terms of nutrition. However, interactions which is caused by the presence of adhesin, also plays an important role of bacterial pathogens in forming biofilm.⁶

Many researchs which were use biofilm's bacteria as models to determine the effectiveness of antibacterial agents for root canal treatments are still being developed. Results of several studies have shown the ability and inability of the antibacterial agents as a root canal dressings in eliminating or reducing biofilm bacteria.⁷ Therefore some alternate agents derived from plants extract are become an interesting thing to be the one of choice as antimicrobial agents, because they have natural effects and lower side effects than the chemical drugs. One among plants that have antibacterial ability is *Pluchea indica Less. Pluchea indica L.* is a plant that has long been known by the people of Indonesia. These plants are often used as hedge plant, and traditionally efficacious as fever, appetite enhancer,

and sweat bullets. *Pluchea indica L.* leaves contain chemical properties such as tannins, flavonoids, polifenolat, and essential oils that are known to have antibacterial effect and can inhibit biofilm formation of bacteria.^{8,9}

Based on the background above, *Pluchea indica L.* leaves have antibacterial capabilities, but there is no research on *Pluchea indica L.* leaves extract in inhibiting biofilm of *Enterococcus faecalis* and *Fusobacterium nucleatum* which become the cause of failure in root canal treatments. Therefore, research using *Pluchea indica L.* leaves extract in inhibiting the biofilm formation of *Enterococcus faecalis* and *Fusobacterium nucleatum* biofilm is expected to be developed as an alternative to root canal sterilization dressing.

MATERIAL AND METHOD

The type of this research was true experimental research with post test only control group design. The sample size in this study were 42 wells in microtiter plate divided into six groups.

The tools used in this study were blender, funnel cups, stirrers, measuring cups 500 ml, beaker glass 600 ml, erlenmeyer tube 500 ml, rotary evaporator, filter paper, analytical balance (Analytical Balance CPA 423S Sartorius), oese, spiritus burner, test tubes (BD Falcon), micropipette (Eppendorf), microtiter plate (96-well flat-bottomed plastic tissue culture plate), inverted microscope (IX 51 Olympus), autoclave (SX 700), incubator (500 Memmer), anaerobic jar, petridish, mixer vortex, ELISA Reader (Thermo Scientific Multiskan Fc), ultrasonic (VirSonic 50 VirTis

USA), and spectrophotometer (Shimadzu Japan).

The materials used in this study include *Pluchea indica* L. leaves fresh obtained from UPT Materia Medika Batu, ethanol 80%, sterile distilled water, Brain Heart Infusion (BHI) medium, stock of *Enterococcus faecalis*, stock of *Fusobacterium nucleatum*, glucose 1%, phosphate-buffered saline solution (pH 7.3), *Pluchea indica* L. leaves extract with concentration of 100%, 50%, 25%, 12.5%, and 6.25%, sterile distilled water, NaOCl 2.5%, 0.2 ml of crystal violet, 200 mL of isopropanol, potassium phosphate buffer, 80% ethanol and 10% CO₂.

Plants extraction. *Pluchea indica* L. leaves extract obtained from the leaves dried then mashed in a blender and sieved to obtain *Pluchea indica* L. leaves powder, then macerated with 80% ethanol, protected from sunlight, and allowed to stand for overnight. After that, the filtering done to obtain a filtrate which is separated from the dregs. Dregs from first filtering then macerated again with 80% ethanol and then allowed to stand overnight and filtered to obtain a second filtrate and dreg. The second dregs then macerated with 80% ethanol, allowed to stand overnight, and filtered to obtain third filtrate and dregs. The first, second, and the third filtrate then mixed and concentrated by rotary evaporator for one hour. Once concentrated, the extract is heated with a water bath at a temperature of 60°C to obtain thick extracts.¹⁰

Bacteria culture. The use of bacteria in this research by creating a suspension of bacterial colonies of *Enterococcus faecalis* and *Fusobacterium nucleatum* with Brain

UV-visible (UV-1601

Heart Infusion Broth (BHIB) media in a test tube, which was then incubated at 37°C for 48 hours anaerobically. Turbidity suspension of bacteria *Enterococcus faecalis* and *Fusobacterium nucleatum* equated with Mc Farland 0.5 which was equivalent to 1.5x10⁸ CFU/ml. Having obtained the same turbidity, the suspension was diluted up to an infectious dose of bacteria as much as 1x10⁶ CFU/ml (*Enterococcus faecalis*) and 1x10⁸ CFU/ml (*Fusobacterium nucleatum*)^{11,12}.

Inhibition of biofilm formation.

The test of inhibition biofilm formation was done using microtiter plate assay. Suspension of bacteria then cultured in each microtiter plate (96- well flat-bottomed plastic tissue culture plate) containing Brain Heart Infusion media with 1% glucose, incubated at 37°C for 6 days to obtain the maximum biofilm production. Number of well for the replication of the test were adjusted and labeled according to concentrations of each extract. On the 6th day verification of biofilm formation, provided by a simple staining using crystal violet and viewed on an inverted microscope. *Pluchea indica* L. leaves extract as much as 100µl then inserted into each microtiter column that has been provided (according to the extract concentration of 100%, 50%, 25%, 12.5%, and 6.25%). Microtiter column that contains only bacteria without given the extract as a positive control. Microtiter column that contains only the media without bacteria and without the extract as a negative control. The entire microtiter plate then incubated at 37°C for 24 hours in anaerobic incubator. After 24 hours, microtiter plate was washed with phosphate buffered saline (PBS) four times, then dried and staining with 50 mL of 0.1%

solution of crystal violet. Furthermore incubated in room temperature for 15 minutes. Flushing was done using sterile distilled water and dried. Then measuring the Optical Density (OD) at a wavelength of 570 nm using ELISA Reader.¹⁴ Results of the measurement of OD was classified as follow:¹⁵

$OD \leq OD_c$: no adherent
 $OD_c < OD \leq 2 \times OD_c$: weak adherent
 $2 \times OD_c < OD \leq 4 \times OD_c$: moderate adherent
 $4 \times OD_c < OD$: strong adherent
 OD_c (Cut off OD) =
 Mean OD negative control + (3xSD)

Seven replicates were made for each concentration of the extract.

Inhibition adhesion of bacteria.

Test for inhibition adhesion of bacteria carried by autoaggregation assay. The suspension of *Enterococcus faecalis* and *Fusobacterium nucleatum* was created equivalent to the turbidity of Mc Farland 0.5 or comparable to 1.5×10^8 CFU/ml. Having obtained the same turbidity, the suspension was diluted up to an infectious dose of bacteria as much as 1×10^6 CFU/ml (*Enterococcus faecalis*) and 1×10^8 CFU/ml (*Fusobacterium nucleatum*).^{12,15} Suspension of bacteria then cultured in tubes containing Brain Heart Infusion media with 1% glucose. *Pluchea indica* L. leaves extract as much as 100µl then inserted into each tube that has been provided (according to the extract concentration of 100%, 50%, 25%, 12.5%, and 6.25%). Tube that contains only bacterial culture without extract as a positive control, whereas a tube containing only BHI

media as a negative control. The entire tube was incubated at 37°C for 24 hours. After incubation, each tube was vibrated using a vortex mixer for a few minutes, and measured spectrophotometrically at 570nm (OD_0). Furthermore, media samples removed from the tube using a pipette, and bacterial culture was mixed with potassium phosphate buffer, followed by centrifugation. Cells that had been centrifuged resuspended using ultrasonic VirSonic 50 at maximum amplitude for 10 seconds and then measured spectrophotometrically at 570nm (OD_s).¹⁶ Seven replicates were made for each concentration of the test extracts. Determine the amount of bacterial adhesions using the formula below:¹⁷

$$\% \text{ autoaggregation} = (OD_0 - OD_s) \times 100 / OD_0$$

OD_0 = initial bacterial cells

OD_s = supernatant cells

Statistical analyses of data were performed by One Way Anova test to assess significant differences, and LSD test to verify significant differences among each concentration. The level of significance was 5%.

RESULTS

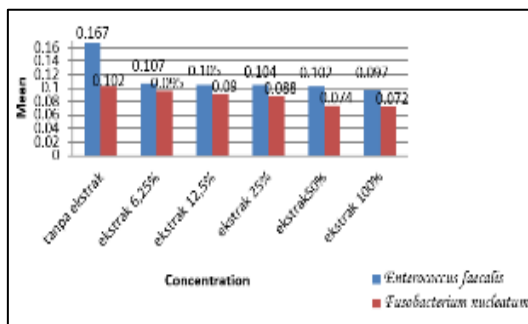
The test of inhibition biofilm formation against *Enterococcus faecalis* and *Fusobacterium nucleatum* was conducted using microtiter plate assay and expressed in Optical Density (OD) obtained the following results:

Table 3. The average value of Optical Density (OD) of bacterial biofilms of *Enterococcus faecalis* and *Fusobacterium nucleatum* in each group with microtiter plate assay.

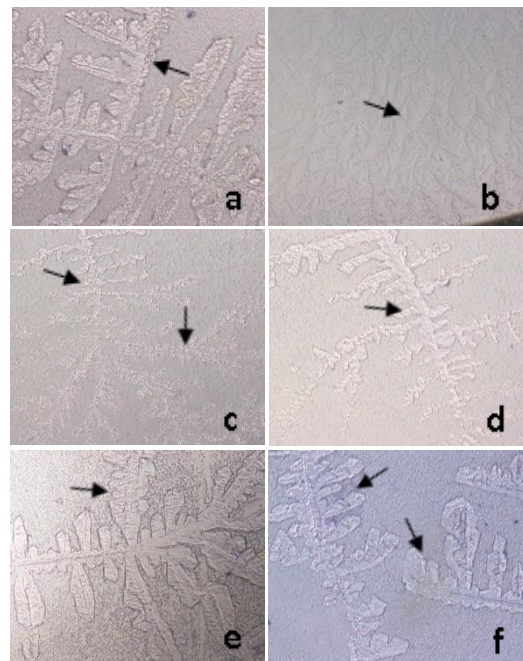
Bacteria	Mean					
	Control (+)	Concentration 6,25%	Concentration 12,5%	Concentration 25%	Concentration 50%	Concentration 100%
<i>Enterococcus faecalis</i>	0.167 (SA)	0.107 (SA)	0.105 (SA)	0.104 (SA)	0.102 (SA)	0.097 (MA)
<i>Fusobacterium nucleatum</i>	0.102 (MA)	0.095 (MA)	0.090 (MA)	0.088 (MA)	0.074 (MA)	0.072 (MA)

MA: Moderate Adherent, SA: Strong Adherent

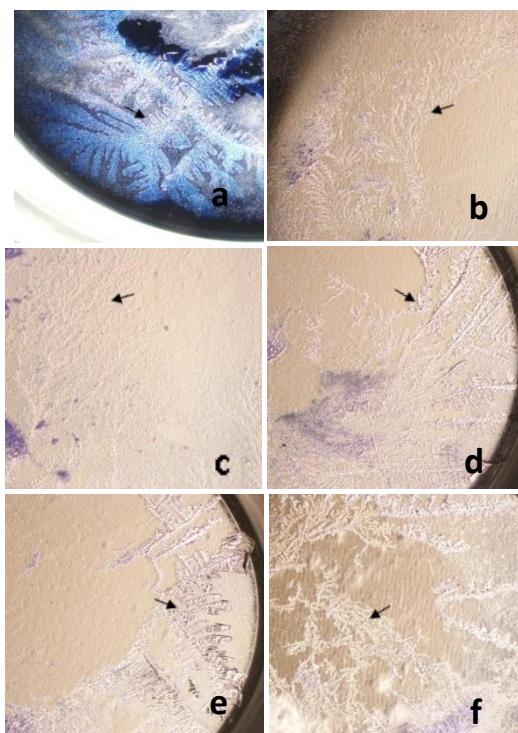
Table 3 showed a decrease in Optical Density (OD) of biofilm *Enterococcus faecalis* and *Fusobacterium nucleatum* according to the increase of extract concentration. Based on the classification of OD value in *Enterococcus faecalis* group, it showed a strong adherent biofilm on extract concentration 6,25%, 12,5%, 25%, and 50%. While on the extract concentration 100% showed a moderately adherent biofilm. On the *Fusobacterium nucleatum* group which was treated using extract with concentration 6,25%, 12,5%, 25%, 50%, and 100% showed a moderately adherent biofilm.



Graphic 1. Measurement of biofilm Optical Density (OD) *Enterococcus faecalis* and *Fusobacterium nucleatum*.



Picture 1. Microscopic views of *Enterococcus faecalis*' biofilm using microtiter plate assay (inverted microscope x400). Black arrow showed biofilm that attached on plate wall. (a) Without extract/control, (b) Extract concentration 100%, (c) Extract concentration 50%, (d) Extract concentration 25%, (e) Extract concentration 12,5%, (f) Extract concentration 6,25%.



Picture 2. Microscopic views of *Fusobacterium nucleatum* biofilm using microtiter plate assay (inverted microscope x400). Black arrow showed biofilm that attached on plate wall. (a) Without extract/control, (b) Extract concentration 100%, (c) Extract concentration 50%, (d) Extract concentration 25%, (e) Extract concentration 12,5%, (f) Extract concentration 6,25%.

To see the difference in Optical Density (OD) values between treatment

groups, we used One way Anova statistical test, and showed the significance value (p) <0.05 , which means that between treatment group there were significant differences. Then to see the real differences between each group of extract concentration, we used LSD test. LSD results of *Enterococcus faecalis* showed significance value (p) <0.05 in extract concentration 100% when compared with the group without extract (control). It can be said that there was significant differences between extract concentration 100% when compared to the group without extract. LSD results of *Fusobacterium nucleatum* between each extract concentration showed significance value (p) > 0.05 . It can be said that the average value of OD biofilm of *Fusobacterium nucleatum* between each extract concentration showed no significant difference.

Test for inhibition adhesion of *Enterococcus faecalis* and *Fusobacterium nucleatum* was carried by autoaggregation assay, which was viewed by the magnitude of adhesion between cells of bacteria (autoaggregation), the results were as follows:

Table 4. The percentage of autoaggregation of bacterial cells *Enterococcus faecalis* and *Fusobacterium nucleatum* in each study group.

Bacteria	Percentage of autoaggregation					
	Control (+)	Concentration 6,25%	Concentration 12,5%	Concentration 25%	Concentration 50%	Concentration 100%
<i>Enterococcus faecalis</i>	77,2%	67,5%	63%	61,5%	59,2%	46,1%
<i>Fusobacterium nucleatum</i>	53,1%	46,7%	40,7%	37,5%	34,6%	31%

DISCUSSION

Minimum Inhibitory Concentration (MIC) of the extract conducted by dilution method against *Enterococcus faecalis* was obtained at 12.5%, while against *Fusobacterium nucleatum* obtained at a concentration of 50%. Then we specified concentrations above and below the MIC (100%, 50%, 25%, 12.5%, and 6.25%), which was then used in the test of biofilm formation inhibition of *Enterococcus faecalis* and *Fusobacterium nucleatum*.

The inhibition of biofilm formation of *Enterococcus faecalis* and *Fusobacterium nucleatum* was carried by microtiter plate assay. In microtiter plate assay, either on biofilm *Enterococcus faecalis* or *Fusobacterium nucleatum*, showed a decrease in the average value of Optical Density (OD). *Pluchea indica* L. leaves extract contains chemical compounds that can act as antibiofilm. The antibiofilm's effects of *Pluchea indica* L. leaves extract derived from flavonoids and polyphenols. Flavonoids in the *Pluchea indica* L. leaves composed by some active substances, such as flavone and naringenin. Flavone has been shown to act as inhibitors of biofilm formation. These compounds can interfere with quorum sensing signaling pathway by disrupting the interaction between the acyl-homoserine lactone (AHL) and its receptor. AHL is an autoinducer or signaling molecules of Gram-negative bacteria that is used in the process of quorum sensing²⁶. Naringenin also play a role in inhibiting biofilm formation by its activities as a quorum sensing inhibitor. Action of naringenin in inhibiting the quorum sensing system is probably caused by the combination

and reduction of molecules AHL and Lux-R type transcription factor which is then followed by a reduction in the expression of quorum sensing-related gene²⁷. Quorum sensing is one of the regulation of extracellular polymeric substance (EPS), or commonly called polysaccharides, and play a role in the formation of bacterial biofilm. If the quorum sensing pathway is inhibited, there will be inhibition of EPS formation, resulting in the inhibition of bacterial biofilm formation.²⁸

In addition to flavonoids, *Pluchea indica* L. leaves also contains a compound called polyphenol. According to research by Borges et al²⁰, polyphenols can inhibit biofilm formation because it contains anti-adhesive properties. The results of the studies suggest that the bacterial adhesion is one of the factors that play a role in the colonization of bacteria in root canal teeth or periapex, and also in the formation of biofilm. Bacteria able to adhere to host cells in the presence of adhesin. Adhesin and polysaccharide matrix play a role in holding the biofilm matrix to adhere on substrat surface, then into three-dimensional structures and continues until maturation period. Polyphenols will bind to the collagen binding protein, which can suppress the function of some genes in bacteria. Rupture of collagen binding protein will cause an inhibition in binding bacteria to extracellular matrix proteins, resulting in inhibition of biofilm formation.^{20,29}

Based on the results, there was a difference in the average value of Optical Density (OD) in *Enterococcus faecalis*' biofilm and *Fusobacterium nucleatum*' biofilm. *Enterococcus faecalis*' biofilm that had been treated by *Pluchea indica* L. leaves extract with a concentration of 100%, 50%, 25%,

12.5%, and 6.25% showed an average OD higher than the average OD of *Fusobacterium nucleatum* that was treated by extract with the same concentration. From the results of the study also showed that in the group of *Enterococcus faecalis*, at concentrations of 50%, 25%, 12.5%, and 6.25% showed strong adherent of biofilm formation, whereas at concentration of 100% shows moderately adherent. In the group of *Fusobacterium nucleatum*, in the extract concentration of 100%, 50%, 25%, 12.5%, and 6.25% showed moderate adherent of biofilm formation.

The average values of OD that high in the group of *Enterococcus faecalis* was caused by small degradation in biofilm formation. It can be caused by the ability of the resistance possessed by *Enterococcus faecalis* against antibacterial or antibiofilm agents. *Enterococcus faecalis* can accumulate and form biofilms, which can make them a thousand times more resistant to antibacterial agents.³⁰ *Enterococcus faecalis* can survive in post root canal treatment, invasion into the dentinal tubules, and survive in the long term of limitation nutrition.^{31,32} When bacteria grows as biofilm, the bacterial genetics and metabolism will be transformed into a complex matrix to prevent them from antimicrobial agents.³³

From these results, the average value of Optical Density (OD) of *Fusobacterium nucleatum* which treated with *Pluchea indica* L. leaves extract concentration 6.25% to 100% showed the ability to inhibit biofilm formation with moderate effect. It maybe because in this study was performed as in vitro study and only use monospecies of *Fusobacterium*

nucleatum. It is known that *Fusobacterium nucleatum* has a vital function in formation of biofilm due to the ability of these bacteria as a bridge (bridging organism) that can help coaggregation other bacteria in the process of biofilm formation. Bacteria that serve as a bridge (bridging organism) will show strong coaggregation when converge with different species of bacteria. When inter-species of bacteria that form biofilm coaggregate each other, it will make them more resistant to antibacterial or antibiofilm agents.^{6,17} Because in this study we used monospecies of *Fusobacterium nucleatum*, then the ability of bacterial aggregation was not as strong as when the bacteria were in an environment that containing multispecies bacteria.

To support the results in inhibition of biofilm formation based on microtiter plate assay, then the tested of inhibition in bacterial adhesions was carried out to see the percentage of adhesion between cells of bacteria (autoaggregation). This test was done without staining procedure with crystal violet. As it's known that in the calculation of absorbance or turbidity requires simple staining using crystal violet, but the crystal violet can color the alive cells or died cells of bacteria, causing the bigger in turbidity level and affect the great value of Optical Density.³⁴ The results of inhibition in bacterial adhesion showed a decrease in percentage of adhesions between bacterial cells (autoaggregation) with increasing concentration of the extract.

CONCLUSION

Based on this study, it can concluded that *Pluchea indica* L. leaves extract has the ability to inhibit

bacterial biofilms of *Enterococcus faecalis* and *Fusobacterium nucleatum* with strong moderate effect.

REFERENCE

1. Bolstad A. Isine, Harald B. Jensen, and V. Bakken. Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clinical Microbiology Review*. Vol.9(1):55; 1996.
2. Ercan E., M. Dalli, İ. Yavuz, and T. Özekinci. Investigation Of Microorganism in Infected Dental Root Canals. *Biotechnol. & Biotechnol*; 2006.
3. Pinheiro ET., Gomes BPFA., Ferraz CCR., Teixeira FB., Zaia AA., and Souza Filho FJ. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. *Oral Microbiology and Immunology*. Vol. 18: 100–3; 2003.
4. Walton and Torabinejad.. *Prinsip dan Praktik Ilmu Endodonsi*, Edisi kedua. Alih Bahasa: drg. Narlan Sumawinata. Penerbit Buku Kedokteran EGC: Jakarta. 1994; pp: 361-64.
5. Mathew Sabeena, and Boopathy T. *Enterococcus faecalis* -An Endodontic Challenge. *Review Article*. 2010; pp:33-37.
6. Metzger Zvi, Jaron Blasbalg, Miri Dotan BA., and Ervin I. Weiss. Enhanced Attachment of *Porphyromonas gingivalis* to Human Fibroblasts Mediated by *Fusobacterium nucleatum*. *Journal Of Endodontics*. Vol.35(1): 82-5;2009.
7. Spratt DA., Pratten J., Wilson M., and Gulabivala K. An in vitro of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *Int Endod Journal*. Vol. 34: 300-7; 2001.
8. Dalimartha, Setiawan.. *Atlas Tumbuhan Obat Indonesia*. Trubus Agriwidya: Jakarta. 1999.
9. Andarwulan Nuri, Batari Ratna., Sandrasari Dini Agustini, Bolling Bradley, and Wijaya Hanny. Flavonoid Content and Antioxidant Activity of Vegetables from Indonesia. *Journal of Food Chemistry*. 2010; pp:121.
10. Depkes RI. *Ekstra Farmakope Indonesia*. Lembaga Farmasi Nasional. Jakarta;1974.
11. Saman Soleimanpour, Fereshteh Sadat Sedighinia, Akbar Safipour Afshar, Reza Zarif , Javad Asili, and Kiarash Ghazvini. Synergistic Antibacterial Activity of *Capsella bursa-pastoris* and *Glycyrrhiza glabra* Against Oral Pathogens. *Jundishapur J Microbiol*. October, 2012;Vol. 6(8):7262.
12. Abbas Bahador, Azad Khaledi, and Roghayeh Ghorbanzadeh. Evaluation of antibacterial properties of nano silver Iranian MTA against *Fusobacterium nucleatum*. *European Journal of Experimental Biology*. Vol.3(6): 2013; pp: 88-94.
13. Anita Arbet, Siti Khotimah, dan Ari Hepi Yanti. Aktivitas Antibakteri Ekstrak Daun Benalu Jambu Air (*Dendrophoe pentandra* (L.) Miq) Terhadap Pertumbuhan *Salmonella typhi*. *Jurnal Protobiont*. Vol. 3(2): 2014;pp:268-272.
14. Merritt Judith H., Daniel E. Kadouri, and George A. O'Toole. Growing and Analyzing Static Biofilms. *Current Protocols in Microbiology*. John Wiley & Sons, Inc: 1B.1.1-1B.1.18; 2011.
15. Donelli Gianfranco, Claudia Vuotto, Rita Cardines, and Paola Mastrantonio. Biofilm-growing Intestinal Anaerobic Bacteria. *Immunology Medical and Microbiology*. Vol. 65: 2012;pp:318-325.
16. Duarte Simone, Pedro L. Rosalen, Mitsue F. Hayacibara, Jaime A. Cury, William H. Bowen, R.E. Marquis, Vera L.G. Rehder, Aldison Sartoratto, Masaharu Ikegaki, and Hyun Koo. The Influence of a Novel Propolis on Mutan *Streptococci* Biofilms and Caries Developments in Rats. *Archieves of Oral Biology*. Vol. 51: 2006; pp:15-22;
17. Mirriam E. Nyenje, Ezekiel Green, and Roland N. Ndip. Evaluation of the Effect of Different Growth Media and Temperature on the Suitability of Biofilm Formation by *Enterobacter cloacae* Strains Isolated from Food Samples in South Africa. *Molecules*. Vol.18: 2013;pp:9582-9593.
18. Pelczar MJ dan Chan ES. *Dasar-Dasar Mikrobiologi*, Jilid 1. Penerjemah Ratna Sari Hadioetomo. UI Press:Jakarta;2005.
19. Susanti, Ari. Daya Antibakteri Ekstrak Etanol Daun Beluntas (*Pluchea indica* Less) terhadap *Eschericia coli* Secara In Vitro. *Fakultas Kedokteran Hewan Universitas Airlangga: Surabaya*. 2007;pp:5.
20. Borges A., C. Abreul, J. Malheiro1, M. J. Saavedra, and M. Simões. Biofilm prevention and control by dietary phytochemicals. *Microbial pathogens and strategies for combating them: science, technology and education*. 2013;pp:32-37.

21. Cowan MM.. Clinical Microbiology Reviews-Plant Products as Antimicrobial agents, 1999. Available at : <http://cmr.asm.org/cgi/reprint/12/4/564>, diakses tanggal 29 Desember 2012.
22. Jawetz B. Melnick and Adelberg's. Mikrobiologi Kedokteran. Penerjemah: Bagian Mikrobiologi Fakultas Kedokteran Universitas Airlangga. Salemba Medika: Jakarta. 2001;pp: 327-335.
23. Wahyuningtyas E. Pengaruh Minyak Atsiri Zingiber purpurea Terhadap Pertumbuhan Candida albicans Serta Kekuatan Transversa Plat Dasar Gigi Tiruan Resin Visible Light Cured dan Resin Akrilik. Karya Tulis Ilmiah Yogyakarta. Program Pendidikan Dokter Gigi Spesialis Fakultas Kedokteran Gigi Universitas Gadjah Mada: Vol.36:7; 1998.
24. Hudzicki, Jan. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. United States of America: University of Kansas Medical Center; 2013.
25. Konate Kiessoun, Adama Hilou, Jacques F. Mavoungou, Alexis N. Lepengue, Alain Souza, Nicolas Barro, Jacques Y. Datte, Bertrand M'Batchi, and Odile G. Nacoulma. Antimicrobial Activity of Polyphenol-Rich Fractions from Sida alba L. (Malvaceae) Against Cotrimazol-Resistant Bacteria Strains. Annals of Clinical Microbiology and Antimicrobials. 11:5; 2012.
26. Manner Suvi, Malena Skogman, Daria Goeres, Pia Vuorela, and Adyary Fallarero. Systematic Exploration of Natural and Synthetic Flavonoids for the Inhibition of Staphylococcus aureus Biofilms. Int. J. Mol. Sci. Vol 14, p: 19434-19451; 2013.
27. Vandeputte Olivier M., Martin Kiendrebeogo, Tsiry Rasamiravaka, Caroline Ste' vigny, Pierre Duez, Sanda Rajaonson, Billo Diallo, Adeline Mol, Mondher El Jaziri, and Marie Baucher. The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in Pseudomonas aeruginosa PAO1. Microbiology. Vol 157: 2120-32; 2011.
28. Barbara Vu, Miao Chen, Russel J. Crawford, and Elena P. Ivanova. Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. Molecules. Vol 14: 2535-2554; 2009.
29. Kayaoglu Guven and Orstavik Dag. Virulence factors of Enterococcus faecalis: relationship to endodontic disease. Crit Rev Oral Biol Med. Vol. 15(5): 308-20; 2004.
30. Mathew Sabeena, and Boopathy T. Enterococcus faecalis -An Endodontic Challenge. Review Article; 2010; pp: 33-37.
31. Distel W. John, Hatton F. Jhon, and Gillespie M. Jane. Biofilm formation in medicated root canals. Journal of Endodontics, vol. 28(10), October 2002.
32. Figdor D., Davies JK., and Sundquist G. Starvation Survival, Growth, and Recovery of Enterococcus faecalis in Human Serum. Oral Microbiol Immunology, vol 18(4): 2003;pp:234-239.
33. George S., Kishen A., and Strong KP. The Role of Environmental Changes on Monospecies Biofilm Formation on Root Canal Wall by Enterococcus faecalis. Journal Of Endodontics, vol. 31(12): 867-72; 2005.
34. Patel Ina, Vaibhavi Patel, Asha Thakkar, and Vijay Kothari. Microbiol Biofilms: Microbes in Social Mode. International Journal of Biotechnology Research and Practice, vol. 1(1): 2013;pp:19-34.