ORIGINAL ARTICLE

Effectiveness of *Graptophyllum pictum (L.) Griff* Leaves Extract Toward *Porphyromonas gingivalis* Adhesion to Neutrophils

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ABSTRACT

Introduction: *Porphyromonas gingivalis* adhesion to neutrophils is an important initial process in periodontal disease. The process of bacterial adhesion must be inhibited so that periodontal disease does not develop into severe. Graptophyllum pictum contains several active substances that can affect the function of neutrophils. Aim of the research to find out the inhibition of *Graptophyllum pictum* leaf extracts (GLE) in various concentrations against the adhesion of *Porphyromonas gingivalis* to neutrophils. **Methods:** The method used is experimental laboratory using the post test only control group design. This research used a sample of isolate neutrophils taken from the blood of subjects with inclusion criteria. The sample was divided into 5 groups: the control group (without GLE), GLE 3.125%, GLE 6.25%, GLE 12.5%, and GLE 25%.The neutrophils isolate was incubated with GLE for 3 hours, then exposed to *Porphyromonas gingivalis* for 8 hours. The adhesion index is calculated by the average number of *Porphyromonas gingivalis* attached to 100 neutrophils. **Results:** Based on these results can be interpreted that GLE 3.125%, GLE 6.25% and GLE 12.5% showed no significant difference, in the mean those concentrations doesn't have ability to inhibit the adhesion of *Porphyromonas gingivalis* in neutrophils. So GLE 25% only which is able to inhibit the adhesion of *Porphyromonas gingivalis* in neutrophils. **Conclusion:** GLE can inhibit the adhesion of *Porphyromonas gingivalis* to neutrophils by GLE 25% effectively only.

Keywords: Adhesion, *Graptophyllum pictum*, *Porphyromonas gingivalis*, neutrophils

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INTRODUCTION

Periodontal disease is a multifactorial disease that is common in developing countries (13). In Indonesia, the prevalence of periodontal disease is high relatively. Based on Health Research (RISKESDAS) in 2013 showed that the prevalence of periodontal disease Indonesian population by 23.4% in 2008 and increased to 25.9% in 2013 (1). Periodontal disease is an infection of the tissues supporting the teeth, namely the gingival, periodontal ligament, cementum and alveolar bone (10). The main cause of periodontal disease is caused by bacteria in plaque (14). Plaque is formed from soft deposits that form a biofilm layer and adhere closely to the tooth surface, gums and other hard surfaces in the oral cavity (24). Plaque contains pathogenic colonies such as P*orphyromonas gingivalis, Treponema forsythia, Aggregatibacter Actinobacillus, Treponema denticola, Prevotella intermedia* (14). *Porphyromonas gingivalis* is one of the bacteria that causes pathological changes of periodontal tissue (15). Among other periodontal pathogens, *Porphyromonas gingivalis* was one of "Key pathogen" in periodontal disease (14). Bacteria *Porphyromonas gingivalis* has infected about 40-100% of patients with chronic periodontitis. *Porphyromonas gingivalis* bacteria was found in 85.75% of subgingival plaque in chronic periodontitis patients (7). Before colonization, invasion and the onset of an infection, the adhesion of bacteria on the tissue initially. Adhesion is a process that adherens to the surface with the host plasma membrane, which can occur related to two components, namely receptors and adhesin. Receptors are components in the form of peptide residues and specific carbohydrates in the host, while adhesin is a macromolecular components found on the bacterial cell surface (24). When bacteria get into the body of the host, there is competition between infection by bacteria or elimination of bacteria by the host (27). One of the leukocyte cells that have important roles in the body's defense system of the host and first appeared when there is a bacterial invasion is neutrophils (19). Neutrophils are the most leukocyte cells in periodontal tissues, in the blood the neutrophil count reaches 62% (5). *Porphyromonas gingivalis* can viably invade into the blood circulation, so that can directly

contact with neutrophils inflammatory agents and can bind via specific receptors. This event will initiate *Porphyromonas gingivalis* induction on the increase of degradative enzymes in neutrophils, as well as being able to interrupt neutrophil activity (12). Bacteria that survive intra cellularly in neutrophils can use these cells to spread through the blood circulation system (27). Therefore, the adhesion of *Porphyromonas gingivalis* in neutrophils need to be inhibited, so that the early stages of infection can be prevented and does not interfere with the working of neutrophils. *Graptophyllum pictum* is one of the thirteen commodities developed by the Directorate General of Drug and Food Control (DITJEN POM) as a superior medicinal plant (2). This plant is widely used by people as hemorrhoid / hemorrhoids, constipation, urine laxatives, facilitating menstruation, boils, and several conditions such as anti-fungal, anti-inflammatory and anti-plaque. *Graptophyllum pictum* can also be used for the treatment of wounds, swelling, ulcers, boils, skin diseases, and experimentally *Graptophyllum pictum* extracts inhibit the swelling and decrease the permeability of the membrane (22). The composition of *Graptophyllum pictum* leaf content is alkaloids, pectin, formic acid, glycosides, steroids, saponins, tannins, flavonoids and alcohol. In vitro, *Graptophyllum pictum* are known to have anti-microbial activity against Staphylococcus aureus. The existence of these compounds, especially flavonoid compounds, has been known to inhibit the adhesion of Staphylococcus aureus bacterial cells, both the attachment of bacteria to the surface of the substrate and the attachment between bacteria (11). Research on the inhibitory power of the *Graptophyllum pictum* extract against adhesion of *Porphyromonas gingivalis* bacterial to neutrophils has never been done. According Indriana et al. (2017) in her research report that *Graptophyllum pictum* extract can inhibit bacterial growth of root canals with a minimum concentration which is 12.5% (8). Research associated with Streptococcus mutanss bacteria adhesion barrier has been done by Kurniawati et al (2019). Reported that *Graptophyllum pictum* have the effect of inhibiting the attachment of the bacteria in neutrophil cell membrane receptors to reduce the function of the cell membrane hydrophoblastis attachment process by hydrophobic interactions (18). Declared that the concentration of *Graptophyllum pictum* 3.125%, 6.25%, 12.5% and 25% can inhibit the adhesion of Sreptococcus mutans, but it is not known how effective concentrations in inhibiting the adhesion of these bacteria (*Porphyromonas gingivalis*) to neutrophils. Based on that research above, it is determined that the concentration of GLE 3.125%, 6.25%, 12.5% and 25% are chosen in this study.

MATERIALS AND METHODS

Samples

This research is an experimental laboratory in vitro. The research design used is the post-test only control group design to determine the differences between treatment and control groups. Based on the design of research in this study, the sample size is determined by referring to the Federer formula, where the number of samples is at least 4 more so it is rounded up to 5. This research used a sample of isolate neutrophils taken from the blood of subjects with inclusion criteria. Adult males criteria, no history of systemic disease, blood disorders and smoking habits, and be willing to fill out an informed consent as a sign of agreement that the blood drawn will be made into a research sample. The reason for selecting samples with these criteria is aimed at homogeneity of the sample and the same normal condition. Men are chosen because they are relatively not affected by hormonal changes, adulthood because the number of neutrophils varies according to age, systemic diseases, blood disorders and bad habits such as smoking affect the number and function of neutrophils.

Isolates of neutrophils and adhesion test performed in Laboratorium Bioscience Hospital Dental Faculty of Dentistry, University of Jember. The research was conducted in January to February 2018 with amount 20 samples devided into 5 groups, namely the control (without GLE) group and the group of *Graptophyllum pictum* leaf extract (GLE) as GLE 3.125%, GLE 6,25%, GLE12,5%, and GLE 25% groups. There's no control positive because the aim of the research to find out the inhibition of *Graptophyllum pictum* leaf extracts in various concentrations against the adhesion of *Porphyromonas gingivalis* to neutrophils and inhibition differences in various concentrations,not testing the ability of *Graptophyllum pictum* leaves extract (GLE) in treating periodontitis so that is does not need to be compared with positive control. This research has met eligibility requirements by the Health Research Ethics Committee of the Faculty of Dentistry, University of Jember letter No. 035 / UN25.8 / KEPK / DL / 2018.

Graptophyllum pictum leave extract (GLE) preparation

Plant identification *Graptophyllum pictum* conducted in UPT Plant Conservation Center Purwodadi-Pasuruan and has identified as Grapthophyllum pictum L. Griff with letter No.105/IPH.06/HM/I/2018. Preparation of the extract conducted at the Laboratory of Biology Faculty of Pharmacy, University of Jember. *Graptophyllum pictum* leaves criteria used, fresh leaves free from pest contamination, not younger and not older and leaves are picked at the same time that is late afternoon because the photosynthesis process is complete so that the rich chemical compounds contained. Leaves were taken from the medicinal plant garden of the Faculty of Pharmacy at Jember University and it was identified as *Graptophyllum pictum*. GLE made from *Graptophyllum pictum* with fresh state, free of pest contamination. *Graptophyllum pictum* as much as 700 grams washed with running water, cut into small pieces and dried. *Graptophyllum pictum* dried and then blended and sieved to obtain the fine powder of 200 grams. *Graptophyllum pictum* powder added ethanol

96% with a ratio of 1:7. 5 times the bulbs is 1500 ml and macerated for 24 hours in the macerator for 3 days. After 3 days, maceration filtered out of the waste by using filter paper. Maceration then evaporated with a rotary evaporator for 45 minutes at a temperature of 45o–50oC to obtain concentrated preparation (concentration of 100%). From the concentrated preparation, it is then diluted with distilled water to obtain concentrations of 3.125%, 6.25%, 12.5% and 25%

Making sub culture and identification of *Porphyromonas gingivalis*

The research was conducted at the Laboratory of Microbiology, Faculty of Dentistry, University of Jember for bacterial identification. The first making of BHI-A solid media was carried out by means of 0.37 grams of BHI-A and 10 cc of aquadest mixed in an erlemeyer tube then stirred for homogeneity. After being homogeneous, the tube is covered with cotton and then sterilized on an autoclave at 121 ° C for 15 minutes. Subsequently added with 1 µl of vitamin K, 5 µl of hemin, and 50 µl of yeast extract which is then homogenized. Perform a BHI-A media sterilization test by inserting it into an incubator at 37 ° C for 24 hours. BHI-A media are considered to be sterile if they are not contaminated with fungus and bacteria and are clear in color, poured on unclosed petri dish and then wait until solid. Furthermore, *Porphyromonas gingivalis* was planted on the media, and incubated at 37 ° C for 2x24 hours, then the *Porphyromonas gingivalis* colony was harvested. After that it is opened to make a bacterial removal preparation and carried out Gram stainning. *Porphyromonas gingivalis* identified in study was ATTC 33277 strain.

Isolation of neutrophils.

The subject was taken as much as 6 cc of blood from a peripheral vein and mixed with anticoagulant (heparin) in heparin tubes. The blood was divided into two tubes with an amount of 3 cc each. Setting up 3 ml of Histopaque 1119 in a falcon tube, then add 3 ml of Limphoprep 1077 solution. Centrifuge with a speed of 900 g for 30 minutes. PMN layer is taken and added HBSS with a ratio of 1: 2. Centrifugation with a speed of 600 g for 10 minutes, so that will be formed 6 layer in the falcon tube taht composed from top to bottom is a plasma, monocytes, Ficoll solution, granulocytes, Histopaque solution, and erythrocytes. Granulocyte layer is taken and added to 1000 mL of HBSS. Antifungal added, namely fungizone 5 µl and penicillin-streptomycin solution is stabilized as much as 20 mL to 1000 mL cell media solution in order to avoid contamination of microorganisms.

Adhesion test

Coverslip as many as 20 pieces sterilized given neutrophils were 100 µl and then incubated in the incubator shaker for 20 minutes, temperature of 37°C for neutrophils attach to the coverslip. After that, re suspended with 1000 µl of RPMI and added 20 µl penicillin-streptomycin and 5 µl Fungizone, then do pipetting. Neutrophils were incubated for 30 minutes at 37 ° C. RPMI is taken, then replaced by complete medium (M199) 1000 µl. If there is no contamination added *Graptophyllum pictum* extract with concentration of 3.12%, 6, 25%, 12.5%, and 25% of 100 µl and do pipetting until completely homogenized using a shaker incubator and incubated for 3 hours at 37 °C to see the absorption of the extracts on neutrophils. The incubation media are discarded, and replaced with M199 of 1000 µl. Each of them was added a *Porphyromonas gingivalis* suspension of 100 µl, incubated for 8 hours at 37 °C, 5% CO2. Wash with HBSS then fixed with absolute methanol for 3 minutes. Do staining with Giemsa and observed under inverted microscope with 400x magnification to calculate the adhesion index. Calculations performed on all samples by calculating a median of bacteria attached to each of 100 neutrophils.

Statistical Analysis

Statistical analysis of the data used is the normality test using the Shapiro-Wilk test with a significance value of $p>0.05$ which shows the data are normally distributed. Furthermore, parametric statistical tests, one way ANOVA, are aimed at identifying differences between groups and least significance different (LSD) test with a significance level of 95% ($\alpha = 0.05$).

RESULTS

Neutrophil Isolation

Preparations resulting from neutrophil isolation by Giemsa staining which were examined using an inverted microscope are shown in Figure 1A. Neutrophil isolates. In the picture shows neutrophils are not contaminated with other blood cells and describe the normal neutrophils. Neutrophils have a nucleus with two to five lobes that are purplish pink (arrows). Observation using an inverted microscope (400x magnification). Fig 1B. The yellow arrows indicate the neutrophil lysis. Lysis cells are seen in the form of incomplete cells, crenated nucleus and sometimes destroyed, formless.

Adhesion Test

The results showed that the average index of the highest adhesion in the control group and the lowest in the group GLE25%. Diagram Figure of the average adhesion index of *Porphyromonas gingivalis* to neutrophils can be seen in Fig 2. The results of the analysis with the Shapiro-Wilk test showed that the data were normally distributed ($p> 0.05$). The results of data analysis showed that the data is normally distributed. Therefore, parametric test is One Way Anova. Statistical analysis showed that the significant value of 0.000 ($p <0.05$) means that there are significant differences in the entire research group. Next, the Least Significant Different (LSD) test is performed to determine significant differences between each group. Based on the LSD test, showed significant difference

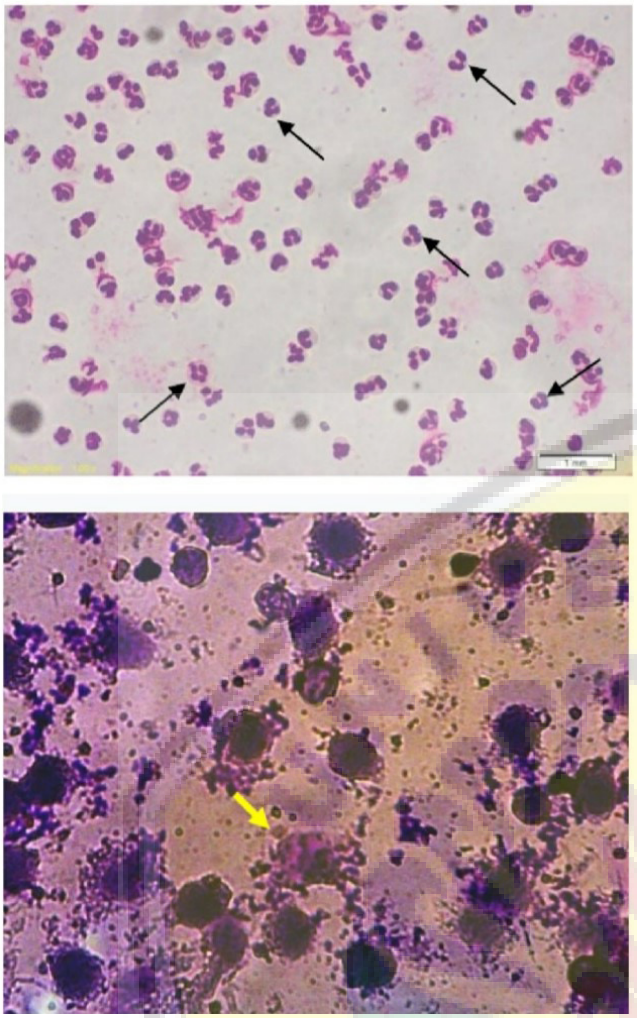

Figure 1: Neutrophil isolates. (A): In the picture shows neutrophils are not contaminated with other blood cells and describe the normal neutrophils. Neutrophils have a nucleus with two to five lobes that are purplish pink (arrows). Observation using an inverted microscope (400x magnification). (B): The yellow arrows indicate the neutrophil lysis. Lysis cells are seen in the form of incomplete cells, crenated nucleus and sometimes destroyed, formless. The neutrophils incubation of the Graptophyllum pictum leaf extract (GLE 50%) showed excessive adhesion processes so that many neutrophils were lysis.

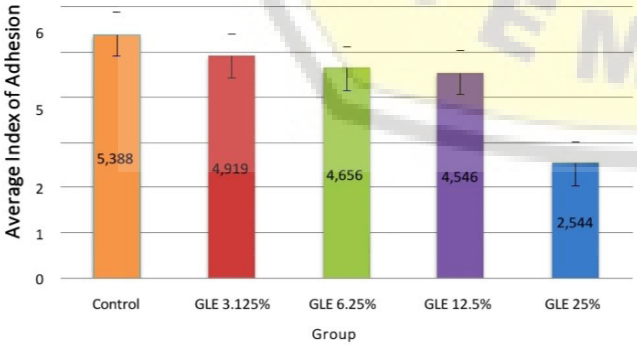

Figure 2: Bar chart of the average adhesion index of Porphyromonas gingivalis to neutrophils. Control: without GLE, GLE 3,125%: Concentration Graptophyllum pictum leaf extracts 3,125%, GLE 6,25%: Concentration Graptophyllum pictum leaf extracts 6,25%, GLE 12,5%: Concentration Graptophyllum pictum leaf extracts 12,5%, and GLE 25%: Concentration Graptophyllum pictum leaf extracts 25%.

with the control extract *Graptophyllum pictum* only at a concentration of 25%. Where the adhesion index is smaller than the control. Based on these results can be interpreted that GLE 25% only which is able to inhibit the adhesion of bacteria *Porphyromonas gingivalis* in neutrophils. While GLE 3.125%, GLE 6.25% and GLE 12.5% showed no significant difference, in the mean the third concentration does not have the ability to inhibit the adhesion of *Porphyromonas gingivalis* in neutrophils. The results of research in the form of adhesion of *Porphyromonas gingivalis* in neutrophils for each group can be seen in Fig 3. In the GLE incubated group, a neutrophil size larger than the control group (without GLE) was seen. In addition, the higher the concentration of GLE, the less bacteria are attached to neutrophils.

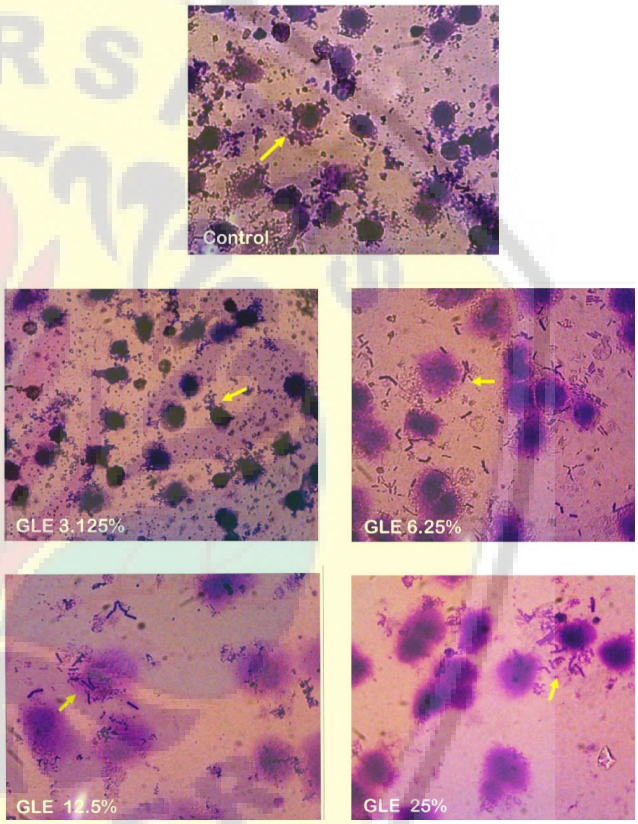

Figure 3: Observations of *Porphyromonas gingivalis* adhesion to neutrophils (Giemsa staining, magnification 1000x). Control: without Graptophyllum pictum leaf extracts (GLE), GLE 3,125%: Concentration Graptophyllum pictum leaf extracts 3,125%, GLE 6,25%: Concentration Graptophyllum pictum leaf extracts 6,25%, GLE 12,5%: Concentration Graptophyllum pictum leaf extracts 12,5%, and GLE 25%: Concentration Graptophyllum pictum leaf extracts 25%.

DISCUSSION

Plant identification *Graptophyllum pictum* has identified as Grapthophyllum pictum L. Griff with letter No.105/IPH.06/HM/I/2018. The results of identification stating that the leaves are used as a sample in this study included the the caricature-plant varieties which according to the type of *Graptophyllum pictum* are often used for treatment (4). These research concentrations are chosen

based serial dilution method, this method is usually used to estimate the substance concentration and minimum inhibitory concentration, concentration elections starting from a high concentration and then divided by 2 repeatedly (5). Besides that, concentration is determined based on the results of a viability test that has been done as a preliminary study in which the concentration of 100%, 75%, and 50% neutrophil cells die because it is suspected that the extract of the *Graptophyllum pictum* leaf is too thick, So it is toxic and causes neutropyl cells to become lysis (fig. 3B). In addition to the research based on Kurniawati et al. (2019), that the concentration of GLE 3.125%, 6.25%, 12.5% and 25% can inhibit the adhesion of Sreptococcus mutans, but it is not known how effective concentrations in inhibiting the adhesion of *Porphyromonas gingivalis* to neutrophils. Based on that research above, it is determined that the concentration of GLE 3.125%, 6.25%, 12.5% and 25% are chosen in this study.

The results of this study showed that there is a difference in the ability of GLE to inhibit bacterial adhesion to neutrophils according to the level of extract concentration. The higher the concentration of GLE given, the fewer bacteria attached to neutrophils due to a decrease in the bacterial adhesion index compared to the control group (without GLE) In the treatment group GLE 3.12%, GLE 6.25% and 12.5% (fig. 2) it appears that Porpyromonas gingivalis is able to adhere to neutrophils cells but the results are not statistically significant. This means that the active compound contained in the GLE concentration is not effective enough in reducing the bacterial adhesion index against neutrophils (18)
The group that was given GLE extract on microscopic examination showed larger neutrophils compared with the control group who were not incubated GLE (fig.2). This is presumably due to the GLE that lining the neutrophil membrane, so the neutrophil size appears larger. The presence of these layers is thought to isolate neutrophil receptors so that the bacterial adhesin of *Porphyromonas gingivalis* cannot bind to the receptors which ultimately causes the adhesion of *Porphyromonas gingivalis* bacteria to neutrophils to be inhibited (17). Direct contact between *Porphyromonas gingivalis* and host cells process begins with the adhesion process. Adhesion of bacteria on the surface of cells or tissues requires the role of two factors, namely receptors and adhesin. Receptors are peptide residues and specific carbohydrates on the surface of host cells (22, 26). While the bacterial adhesin is a molecule found on the surface of bacterial cells that serves to attach the surface of host cells (25). Neutrophil cells will respond to the presence of invading bacteria as the active neutrophil cells at the beginning of the inflammatory reaction that begins with the attachment process. This attachment process due to the interaction between the components of the bacteria and the host cell surface (8). Adhesion of bacteria on the surface of cells can be inhibited by the enzymes and chemicals that specifically destroy or isolate the bacterial adhesin and host cell receptors. Enzymes and chemicals allegedly also contained in the *Graptophyllum pictum* leave extract (22, 26). *Graptophyllum pictum* leave extract contains several chemical compounds, among others: triterpenoids / free steroids, alkaloids, flavonoids, glycosides, saponins, and tannins (17). Adhesion of bacteria on the surface of cells can be inhibited by specific chemicals that destroy or isolate the bacterial adhesion and the host cell receptor (21). This chemical is also found in the *Graptophyllum pictum*. The test results Thin Layer Chromatography (TLC), which has been done in the previous investigators *Graptophyllum pictum* showed positive results in flavonoids and alkaloids (9) which is a chemical compound that has the ability to inhibit the adhesion of *Porphyromonas gingivalis* in neutrophils. Alkaloids were able to inhibit the adhesion of bacteria to alter the tertiary structure of proteins on the surface of the bacteria. The protein or compound interjects on the hydrophobic side of the bacterial protein, resulting in decreased hydrophobicity of bacterial cells that interact with the fimbriae and result in clumping of proteins on the surface of bacteria. As a result this protein loses its hydrophobic structure and results in decreased bacterial hydrophobicity. This decrease in hydrophobicity will prevent the hydrophobic interaction of the bacterial surface components with host cells thereby inhibiting bacterial adhesion to host cells (16). Alkaloids also can interfere with the formation of a constituent component of peptidoglycan in bacteria, so that the cell wall layers are not fully formed and cause death in bacteria (20). According to Harborne and Williams (2000), which are lipophilic flavonoids, would damage the membrane, so that the permeability increases and disrupt bacterial metabolism (6). Flavonoid in *Graptophyllum pictum* allegedly has antioxidant activity capable of protecting the membrane lipids of neutrophils from damaging oxidation reactions, thus maintaining the integrity of neutrophils (19). Due to the flavonoids, alleged that the adhesin of *Porphyromonas gingivalis* can not do its part to perform neutrophil adhesion receptors.

In the control group, neutrophils exposed to *Porphyromonas gingivalis* without incubation of the GLE showed excessive bacterial adhesion processes so that many neutrophils were lysis (Fig 2). Neutrophils become lysis because neutrophils have the ability to phagocytosis 3 to 20 microorganisms. After phagocytosis of microorganisms, neutrophil cells becomes inactive and lysis (5).

Another reason that causes lysis of neutrophils that is the *Porphyromonas gingivalis* virulence factors that are destructive host one of which is a proteolytic enzyme. The main proteolytic enzyme produced by *Porphyromonas gingivalis* is gingipain. Gingipain (extracellular proteases) in black-pigmented bacteria Gram-negative anaerobes are used to evade the host immune response by breaking molecules of bacteria on

the host identifier, so that the bacteria can survive in the periodontal tissues. In addition, gingipain also can modulate the immune system and interfere with the inflammatory response, inactivates TNF-α and increases cytokine secretion through PARs (Protease-activated receptors). It makes gingipain potential to increase tissue damage (14) The proteolytic enzyme can break down the protein in the host cell membrane such as neutrophils, causing neutrophils into lysis (27). The outer membrane of *Porphyromonas gingivalis* bacteria is composed of lipopolysaccharides (LPS). LPS can induce the production and release of inflammatory cells, such as reactive oxygen species (ROS), which can cause a chain reaction and generate free radicals just in large numbers, which is highly toxic and can cause oxidative damage from the level of cells to the organs (3). The presence of virulence factors that proteolytic enzymes and LPS cause host cells (neutrophils) to lysis. The *Graptophyllum pictum* leaf extract (GLE) have studied its benefits in influencing phagocytic function and ability in the formation of TNF-α in mice (28). *Graptophyllum pictum* leaves containing flavonoid. Flavonoids have been identified from the *Graptophyllum pictum* leaf extract (GLE) that flavonol quercetin, kaempherol and myrecetin. Flavonols kaempherol able to bind to estrogen receptors (ER) which is found on the surface of neutrophils and macrophages. So with giving GLE, is expected to occur bond between flavonol with TLR-2 receptor on the cell surface of neutrophils (29). Those ties will result in signal transduction conductivity. It is known that the flavonol quercetin may activate TLR-2 and NF-kB (30). Signal transduction is initiated by the increased activity of IKB bound to NF-kB. This results in increased degradation and phosphorylation of IkB and increased translocation of NF-kB into the nucleus. Increased activation of NF-kB will also increase TNF-α and IFN-γ. Increased expression of cytokines TNF-α and IFN-γ will activate neutrophils so that the function of phagocytosis is increased, consequently the amount of *Porphyromonas gingivalis* colonies decreases. Pro inflammatory cytokines TNF-α and IFN-γ can activate neutrophils back. Activated neutrophils will issue antibacterial molecules such as Reactive Oxygen Intermediate and Reactive Nitrogen Intermediate (ROI and RNI). ROI generated by the phagocyte oxidase (Phox) and inducible NO synthase (iNOS). iNOS will issue NO. iNOS and NO release stimulated by IFN-γ would lead to the killing of *Porphyromonas gingivalis*. iNOS expression will also trigger inflammation through the formation of TNF-α, which stimulates the migration of immune cells to the site of infection. The migration of immune cells begins with the aggregation of macrophages, neutrophils, followed by proliferation and exudation on the tissue.

CONCLUSION

Based on the research that has been done can be concluded that *Graptophyllum pictum* leaf extract (GLE) can inhibit bacterial adhesion of *Porphyromonas gingivalis* in neutrophils at a concentration of 25% only.

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