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Research Report

The efficacy of sarang semut extract (*Myrmecodia pendens Merr & Perry*) in inhibiting *Porphyromonas gingivalis* biofilm formation

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ABSTRACT

Background: *Porphyromonas gingivalis (P. gingivalis) is a pathogenic bacteria present in the oral cavity involved in the pathogenesis of chronic periodontitis and biofilm. This mass of microorganisms represents one of the virulent factors of P. gingivalis which plays an important role as an attachment initiator in host cells. Sarang semut is a natural material possessing the ability to inhibit the growth of P. gingivalis.* ***Purpose:*** *This study aims to analyze the effect of sarang semut extract on the formation of P. gingivalis biofilm.* ***Methods:*** *The study used methanol sarang semut extract and P. gingivalis ATCC 33277 and phosphomycin as a positive control. Treatment was initiated by means of culturing. Biofilm test and P. gingivalis biofilm formation observation were subsequently performed by means of a light microscope at a magnification of 400x.* ***Results:*** *The formation of P. gingivalis biofilms tended to increase at 3, 6, and 9 hours. Results of the violet crystal test showed that concentrations of 100% and 75% of the sarang semut extract successfully inhibited the formation of P. gingivalis biofilm according to the incubation time. Meanwhile, the sarang semut extracts at concentrations of 50%, 25%, 12.5%, and 6.125% resulted in weak inhibition of the formation of P. gingivalis biofilm. The biofilm mass profile observed by a microscope tended to decrease as an indicator of the effects of the sarang semut extract.* ***Conclusion:*** *Sarang semut extract can inhibit the formation of P. gingivalis biofilm, especially at concentrations of 100% and 75%. Nevertheless, phosphomycin has stronger anti-biofilm of P. gingivalis effects than those of the sarang semut extract at all of the concentrations listed above.*

Keywords: *Porphyromonas gingivalis; biofilm; Sarang Semut extract, phosphomycin*

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INTRODUCTION

In Indonesia, the prevalence of periodontal disease across all age groups has been estimated at 96.58%.[1] The main cause are gram-negative bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), also known as biofilm-forming bacteria, which demonstrate the ability to grow into a biofilm mass.[2] This is positively correlated with their phenotypic characteristics as a cause of periodontitis.[3] These anaerobic bacteria have been identified as the main orbital microbiota of biofilm formation in sub-gingiva, and contribute to the pathogenesis of root canal infection together with other bacteria facilitated by co-aggregation proteins.[4] Interactions of both proteins derived from these different pathogens with type 1 collagen of the host cell are considered to stimulate the pathogens to adhere, invade, and infect.[5]

There are a number of different antibiotics that have been used to eliminate the development of pathogens associated with periodontal disease, including that caused

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by *P. gingivalis*. Ripamfisin and phosphomycin are the most commonly used antibiotics to prevent infection of anaerobic group bacteria. However, the use of synthetic drugs and antibiotics tends to increase host antibody immune-tolerance against pathogens.[6] Unfortunately, the long-term use of these drugs may result in bacteria becoming resistant. Their consequent ability to grow within the mass of biofilms can protect these bacteria from detection by the body's defense system, both specific and adaptive.[7]

On the other hand, polyphenol plants containing several active components (flavonoids, tannins, anthocyanins, phenolic acids, stilbenes, coumarins, lignans, and lignins) as antibacterial and antioxidant agents are greatly preferred as anti-bacterial materials by some pharmacologists. In addition to their antibacterial properties, polyphenols also act as antioxidants protecting a host's defense system against pathogens, as well as non-toxic active components for mammalian cells to protect them from oxidative stress.[8] Moreover, the content of prenyl flavonoids may also inhibit expression or function of gingipains adherence, thus preventing *P. gingivalis* biofilm formation.[9] Our previous research even indicates that sarang semut extract proves highly effective in inhibiting the growth of *Enteroccus faecalis* ATCC 29212.[10]

Unfortunately, the application of sarang semut extract on *P. gingivalis* biofilm formation has not been found in any of the references consulted. Therefore, the study reported here aimed to determine the effectiveness of sarang semut extract against *P. gingivalis* biofilm ormation through comparison with phosphomycin.

MATERIALS AND METHODS

This research was approved by the Ethics Review Committee of the Faculty of Medicine, Universitas Padjadjaran, Bandung, No. 557/UN6.C1.3.2/KEPK/PN/2016. Methanol sarang semut extract was obtained from the Natural Organic Chemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia. *P. gingivalis* ATCC 33277 bacteria were drawn from the stock in the laboratory. The sarang semut extract was subsequently tested for its anti-biofilm potential against *P. gingivalis*, while phosphomycin (Meiji, Japan) was used as a positive control.

Sarang semut extract was analyzed in order to predict its bioactive component by means of a prediction of activity spectra for substances (PASS) (Pharmaexpert, Moscow, Russia) approach with a positive PASS value ≥ 0.70.[11] In general, the sarang semut extract had a PASS value in excess of 0.70, confirming that flavonoids contained in the sarang semut both possessed a complexity value and met the standard value of phytochemical analysis.[12]

P. gingivalis ATCC 33277 bacteria taken from the stock of glycerol-800C were, thereafter, cultured within a Mueller-Hinton Agar (MHA) medium (Thermo Fisher Scientific Inc, Oxoid, UK) and incubated in an anaerobic atmosphere at 37 °C for 48 hours using an anaerocult® gaspack (Merck, Darmstadt, Germany) aerobic jar. A colony of *P. gingivalis* bacteria was re-cultured in 5 ml of Mueller-Hinton Broth (MHB) medium (Thermo Fisher Scientific Inc, Oxoid, UK) at an anaerobic temperature of 37 °C, for 48 hours. *P. gingivalis* bacteria grown in the aqueous medium were further compared with McFarland 0.5 (-1 x 108 CFU/ml) and used for biofilm testing.

At that point, a biofilm test was performed on the basis of Peeters' working principle, modified by the use of violet crystals at several stages.[13,14] Each well on the micro plate (96-well plate) was coated with 100 µl of MHB for 15 minutes before being re-suspended. 100 µl of *P. gingivalis* bacteria was added and incubated for 5 minutes at room temperature. The supernatant then having been removed, 100 µl of the test material was added to each well on the micro plate at concentrations of 100%, 75%, 50%, 25%, 12.5%, and 6.125% (µg/ml). They were further cultured in an anaerobic atmosphere with incubation time periods of 3, 6, and 9 hours using an anaerocult® gaspack (Merck, Darmstadt, Germany). 100 µl of phosphate buffer saline (PBS) (Merck, Darmstadt, Germany) was added to each well twice and then agitated for 10 minutes at 300 rpm. 100 µl of 2% violet crystals was introduced into each well and then incubated for 10 min at 250 rpm. The crystals were washed with PBS twice for 10 minutes above the sheker. The extraction of violet crystals from microorganisms was effected by adding 100 µl of 98% ethanol prior to agitating the solution for 5 min at 300 rpm.[15] Optical density (OD) of serial duplo biofilm was then measured with an Elisa Reader (Bio-Rad Laboratories Inc., CA, USA) using a wavelength of 590 nm.

The resultant biofilm mass of *P. gingivalis* bacteria formed on each well base after interaction with the sarang semut extract was prepared with 100 µl of glycerol for 24 hours to maintain its moisture level. Biofilm mass visualization was then performed by adding 10 µl of emersile oil (Thermo Fisher Scientific Inc, Oxoid, UK) to each cell well plate before observation was carried out under a light microscope (Olympus, Shinjuku, Tokyo, Japan) at a magnification of 400x.[16]

RESULTS

P. gingivalis has been ability a biofilm formation that is strongly in 9 hours and will be decreased in 6 hours (Figure 1). While, the extract of sarang semut has the capability to inhibitory the biofilm formation of *P. gingivalis* in 9 hours compared 3 hours and 6 hours, specifically in concentration of 100%, 75%, and 50% (µg/ml), nonetheless the fosfomycin has been strongest biofilm inhibit of E-faecalis compared sarang semut extract (Figure 2). Figure 3 had shown the mass of biofilm formation of *P. gingivalis*

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on the micro-plate 96-well before and after treated by the material of assay.

DISCUSSION

P. gingivalis bacteria represent anaerobic gram-negative bacteria contributing to the pathogenesis of periodontitis with biofilm as one virulent factor determinant.[2] Anaerobic bacteria contribute to colonization, adhesion, and penetration activities in host cells.[17] The formation of *P. gingivalis* biofilm in this research was evaluated by the use of violet crystals.[18]

The results of the biofilm test, illustrated in Figures 1 and 2 showing *P. gingivalis* biofilms which had formed, were observed at the end of 3, 6, and 9-hours incubation periods and analyzed on the basis of an absorbance value

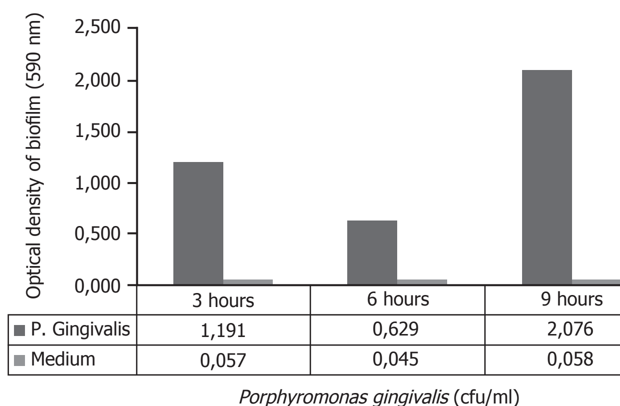

Figure 1. The biofilm formation activity of *P gingivalis* based on the incubation periods of 3, 6, and 9 hours.

at a wavelength of 590 nm. As shown in Figure 2, the inhibitory effects of the biofilm formation in all three-treatment groups were equally effective. During the initial 6-hours incubation period, the biofilm mass of *P. gingivalis* diminished, although it increased during the following incubation period lasting 9 hours (Figure 1). Similarly, the inhibitory effects of the biofilm formation in the sarang semut extract at the highest to lowest concentration tended to increase after the 9-hours incubation period. Fluctuation in such effects is closely related to the biofilm formation phases (initiation, adhesion, and maturation) which indicate gradation in the intensity of the biofilm formation.[19] The high or low expression of biofilm proteins by each pathogen is highly dependent upon environmental influences and communal formation intensity with other bacteria.[20] Although this research evaluated only the formation of *P. gingivalis* biofilm as monospesies, a decrease in potential biofilm formation of *P. gingivalis* was assumed to be related to environmental changes (in wells), such as changes in the pH of the medium due to the introduction of test material that may have affected the metabolism of *P. gingivalis* biofilm mass.[21]

Environmental factors, such as pH, temperature, cytokines, hormones, and oxidative stress have an effect on the formation of bacterial biofilms, including *P. gingivalis,* in the pathogenesis of periodontal infection. Specifically, changes in temperature could improve attachment, co-aggregation, and production of protease.[22] Alkaline pH (8.2) may increase hydrophobicity potentially inducing co-adhesion and biofilm formation of *P. gingivalis*.[23] From the results of this research (Figure 1), it could be assumed that during this 6-hours period bacteria would pass through the adaptation phase (the first maturation stage) with the

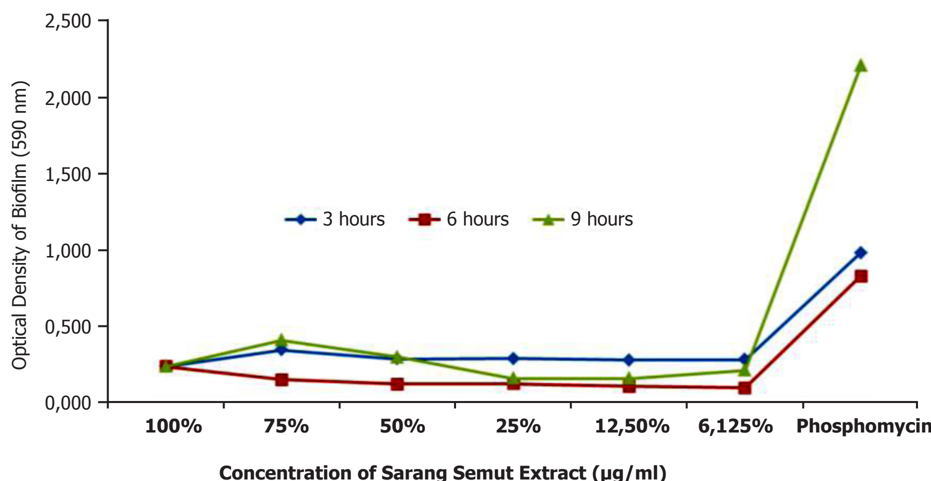

Figure 2. The inhibitory effect of the sarang semut extract on *P. gingivalis* biofilm formation compared to that of phosphomycin.

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environment so that the biofilm protein expression activity might be interrupted before entering the second maturation phase.[24]

The time frame used to observe the tendency of biofilm formation activity is related to the initiation, adaptation, and maturation phases.[25] The results showed that during the initiation phase (the first observation period of 3 hours) OD value increased, indicating the presence of *P. gingivalis* activity associated with potential biofilm formation. On the elapsing of the second observation period of 6 hours (the adaptation phase), the OD value had decreased, although the intensity then increased again after the biofilm had reached the maturation phase (the third observation period of 9 hours). The adaptation phase is included in the initial facula of biofilm formation by a number of pathogens or bacteria.[26] The decrease in *P. gingivalis* biofilm formation in this phase is related to the change in properties to interact with the target (host cell) or communication properties with other pathogens to form co-adhesion and co-aggregation.[27] As a result, it is possible that the production activity of the biofilm protein ceases before passing through the maturation phase. Therefore, the increased biofilm formation in the maturation phase (9 hours) can potentially be triggered by the increase in co-adhesion and co-aggregation activities of bacteria which is in line with the augmented biofilm formation as a link between the two activities.[28,29]

Unlike these previous researchs, research conducted by Davey *et al*. used the period of 48 hours as the maximum indicator of *P. gingivalis* biofilm formation.[25] Similarly, research conducted by Martin observed *P. gingivalis* biofilm formation over varying time periods of 3, 24, 48, and 72 hours. The study identified an increasing trend in the biofilm formation of *P. gingivalis* between 3 hours to 20 hours, followed by a decrease after 24, 48, and 72 hours.[30] Meanwhile, research conducted by Yamamoto used longer periods (3, 6, 9 and 14 days) to model visually observed biofilm formation of *P. gingivalis* with a confocal laser scanning microscopy (CLSM). This study revealed that the maximum activity of biofilm formation occurred at 14 days, confirming that time becomes a determinant factor in the development of *P. gingivalis* biofilm formation.[3]

Based on Figures 1 and 2, *P. gingivalis* bacteria were shown to be capable of forming a stronger biofilm. However, the formation of this bacterial biofilm was inhibited by about 14% after the administration of the sarang semut extract at a concentration of 100% during an incubation period lasting nine hours. In general, Figure 2 shows that phosphomycin had a considerably stronger inhibitory effect on P. gingivalis biofilm formation than the sarang semut extract at all concentrations. However, compared to concentrations of 50%, 25%, 12.5% and 6.125%, the sarang semut extract at concentrations of 75% and 100% produced better inhibitory effects, especially at 9 hours. It can be argued that the active component of the sarang semut extract (flavonoid) interacts with nitrogen-fixing bacteria facilitated by plant bind. Nodd protein also binds to nod-factor receptors (NR), thus damaging the bacterial cell flagella[31] where several biofilm proteins and gram-positive and negative bacterial adhesion proteins are located. Meanwhile, phosphomycin is able to destroy the bacterial cell surface proteins and also prevents the interaction between bacterial cell fimbriae proteins and extra cellular cell matrix proteins.[32]

The advantage of phosphomycin is that it disrupts cytoplasmic activity in peptidoglycan biosynthesis as well as inhibits the synthesis of the MurA enzyme that attaches to host cells.[33] Therefore, phosphomycin may act as a potent anti-biofilm to the micro plate since they are

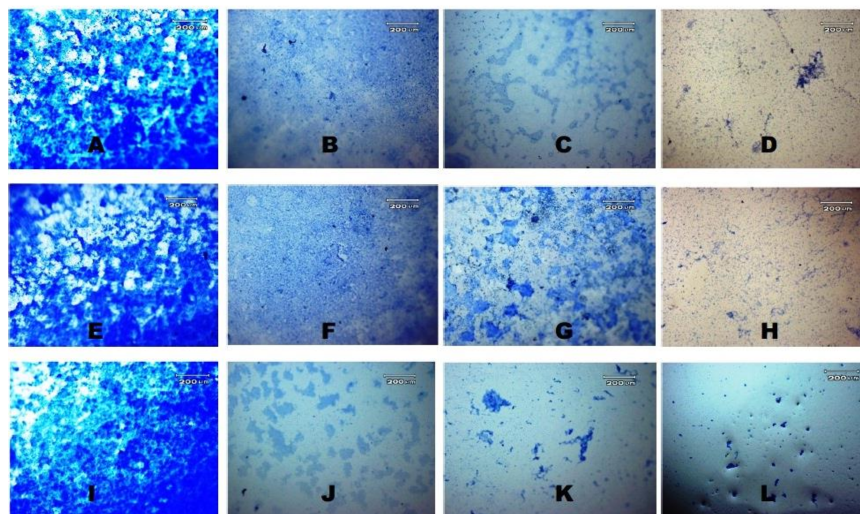

Figure 3. *P. gingivalis* biofilm mass without the administration of the sarang semut extract as control groups (on paths A, E, I). *P. gingivalis* biofilm mass with the administration of the sarang semut extract on paths B, F, J (with a 3-hours incubation period), on paths C, G, K (with a 6-hoursincubation period), and on paths D, H, L (with a 9-hours incubation period). In this research, the sarang semut extract was at concentrations of 100% (on path B, C, D) and 75% (on path F, G, H, I). Meanwhile, phosphomycin was used on path J, K, L.

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capable of forming a covalent bond to activate cysteine residue of bacterial cells which, in turn, activate UDP-N-acetyl glucosamine to form hydrogen bonds to inhibit peptidoglycan synthesis constituting an antibacterial defense site.[34]

Figure 2 shows that the ability of the sarang semut extract as an anti-biofilm of *P. gingivalis* remained limited. Moreover, as shown in Figure 3, the sarang semut extract at the two highest concentrations (100% and 75%) was not yet effective in inhibiting the biofilm mass on the base of the micro plate. This indicates that the sarang semut extract components were still less effective as an anti-biofilm of *P. gingivalis*. It may also be assumed that bacteria (*P. gingivalis*) growing in the mass of biofilm demonstrate metabolic activity triggering resistance to antibiofilm[35] which, in case of this research, is the sarang semut extract. In other words, this extract was not able to penetrate the mass of biofilms while continuously forming during the 3, 6, and 9-hour incubation periods.

In general, the results showed that sarang semut extract possessed the ability to inhibit *P. gingivalis* biofilm formation since some of its active components, such as flavonoids and bioflavonoid, are assumed to be able to inhibit bacterial cell protein synthesis. Secreted cysteine proteases and some proteins classified into the gingipains protein group,[3] for example, fimA protein facilitate bonding with epithelial cells through interactions with extra cellular matrix proteins such as fibrinogen, fibronectin, and collagen type I.[36] Finally, it can be concluded that sarang semut extract (Myrmecodia pendens Merr & Perry) exerts an inhibitory effect on *P. gingivalis* biofilm formation, especially at high concentrations (100% and 75%), such effect remains far lower compared to that of phosphomycin.

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