

Antibacterial Potency of Sarang Semut (*MyrmecodiaPendans* Merr. & Perry) to Prevent Adhesion and Growth of *Enterococcus faecalis*

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Abstract: Introduction: *Enterococcus faecalis* is one of the anaerobic facultative bacteria that causes root canal infections. The growth intensity and adhesion strength of *E. faecalis* against the host cell makes it difficult to eliminate. Previous studies have reported that extract of Sarang Semut (*Myrmecodia pendans* Merr. & Perry) has a promising sensitivity to *E. faecalis*, especially at high concentrations. Objective: The purpose of this study was to analyze the potential of extracts of Sarang Semut in preventing adhesion and growth of *E. faecalis* based on the time of incubation. Method: *E. faecalis* ATCC 29212. *E. faecalis* is equalized with Mc. Farlan 0,5 (-1x10⁸). Anti-adhesion activity of extract of Sarang Semut on *E. faecalis* ATCC 29212 was analyzed by spectrophotometry at wavelength 590 nm and the growth inhibitory activity was analyzed by using culture approach. Results were analyzed by One Way Anova test ($p < 0,05$). Result: Based on the incubation times (3, 6, 9, 12, 16, and 24 hours), the activity of the extract of Sarang Semut significantly showed the ability of *E. faecalis* to prevent adhesion. This result also correlated with decreased growth of *E. faecalis* colonies, particularly in the concentration of 100% ($\mu\text{g/ml}$) as the highest concentration. Conclusion. Based on the incubation time, the extract of Sarang Semut has ability to prevent both adhesion and growth of *E. faecalis*, mainly in highest concentration of Sarang Semut extracts.

Keywords: Growth, adhesion, Sarang Semut Extract, *E. faecalis*

1. Introduction

Enterococcus faecalis is an anaerobic facultative bacteria that is often reported in post-endodontic treatment as opportunistic bacteria in tooth root canals [1] Post-treatment of the root canal, this bacteria is persistent which can cause healing of the apical region inhibited, consequently, this condition can increase root canal infections.[2]

As an anaerobic bacteria, *E. faecalis* is more difficult to remove than other anaerobic obligate bacteria. Compared with other microorganisms in the dentin tubule, *E. faecalis* has the ability to correlate with its competitive properties to survive in disadvantage conditions such as low nutrients condition.[3] This activity is related to the ability of *E. faecalis* to secrete proteinases as a source of nutrients as well as proteins that facilitate adhesion to host cells.[4] *Enterococcus faecalis* is also able to adapt to unfavorable conditions such as hyperosmolarity, heat, acid, and alkaline. This capability is in line with the intensity of growth, growth, and adhesion of host cells in dentinal tubulus.[5] Komiyama (2006) has reported that the ability to increase growth and adhesion are two important virulent factors of *E. faecalis* that increase the frequency of invasion and infection of host cells.[6] Chaharom (2014) has reported that the top spread phase of the infection of *E. faecalis* in the root canal, occurs in the adhesion and growth phases that predominantly damage the intercellular defense system and dentin tubule tissue.[7] Based on problems above, *E. faecalis*

becomes very difficult to remove, even though the dentist has performed endodontic treatment.[8]

One of antibiotics that has been widely used to eliminate *E. faecalis* in the root canal is phosphomycin. This drug known have good ability to inhibit protein synthesis and destroying surface proteins to disrupt of bacterial cell membrane permeability which ultimately aims to prevent the interaction of bacterial cell fimbriae to host cells.[9]. The virtues of phosphomycin are also known to interfere with cytoplasmic activity in the process of peptidoglycan biosynthesis and inhibit the synthesis of the MurA enzyme from *E. faecalis* that attaches to the host cells.[10], because these pathogens are able to adapt to the immune system that affects anaphylactic response and stimulate of cytotoxicity and genotoxicity.

The application of antibiotics has a tendency that can improve immunotolerance of host antibody to pathogen as a trigger of resistance,[11] because these pathogens are able to adapt to the immune system that affects anaphylactic response and stimulate of cytotoxicity and genotoxicity.[12] The active components of a natural substance such as flavonoids, tannins, anthocyanins, phenolic acids, stilbenes, coumarins, lignans, and lignins can be an alternative choice for pharmacologists as antibacterial and antioxidant agents. Beside, the active component of herbal plants claimed has safety issue to cytotoxicity and cell genotoxicity as well as able to prevent oxidative stress.[13] Soraya also reported that the extract of Sarang Semut has a high sensitivity in inhibiting the growth of *Enterococcus faecalis*. [14]

Volume 6 Issue 5, May 2017

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2. Material and Method

This research has been licensed by ethical clearance letter from Research Ethics Commission Faculty of Medicine University of Padjadjaran, Bandung Indonesia No. 482UN6.C1.3.2/ KEPKN/ 2016. The research material consisted of extract of methanol of SarangSemut, *E. faecalis* ATCC 29212, and Phosphomycin (Meiji, Japan) as control group. The extract of SarangSemut is obtained from Laboratory of Organic Chemistry Department of Chemistry-Faculty of Mathematics and Natural Sciences, Padjadjaran University-Bandung, Indonesia. All materials standardized by bioactive component analysis based on Prediction of Activity Spectra for Substances approach with the indicator of value is 0.70.[15]

Enterococcus faecalis inoculated in 20 mL Mueller-Hinton Broth (MHB) (Thermo Fisher Scientific Inc, Oxoid, UK) at 37°C for 24 hours and synchronized to McFarland 0.5 (1 x 10⁸ CFU/mL). Then divided all extract of SarangSemut into six different concentrations (µg/ml): 100%, 75%, 50%, 25%, 12.5%, and 6.125%. The adhesion activity was analyzed by Gram staining approach,[16] while growth activity was analyzed by Total Plate Count approach.[17] Both concentration, protein (*E. faecalis*) and active components (SarangSemut) were analyzed by Bradford method (Bio-Rad) using Bovine Serum Albumin (BSA) as standard which is analyzed by spectrophotometer at 596 nm.[18, 19]

Duple series of 96-well microplate coated with 50µl Mueller-Hinton Broth (MHB) (Thermo Fisher Scientific Inc., Oxoid, UK) for 15 minutes. After that added 100µl of *E. faecalis* into separated well based on concentrations, then incubated for 3 hours. Retract 70µl of supernatant of each well, then prepared to the extract of Sarang Semut based on concentrations, then incubated six different times' 3, 6, 9, 12, 16, and 24 hours under anaerobic conditions using an anaerocult gaspack ® (Merck, Darmstadt, Germany).

The growth activity of *Enterococcus faecalis* analyzed using culture principle. Then 50 µl of *E. faecalis*+ extract of SarangSemut placed into microplate and cultured in Mueller-Hinton Agar (MHA) medium (Thermo Fisher Scientific Inc, Oxoid, UK), then incubated for 48 hours in anaerobic condition using anaerocult® gaspack (Merck, Darmstadt, Germany). Calculations of the number of colonies using colony counter.

The adhesion method using incubation time on a 96-well microplate based on modification of Gamble[20] by using violet crystals and safranin. The adhesion activity analyzed by spectrophotometer rapidly after retraction all residual solutions (50 µl, *E. faecalis*+medium) from microplate and remained for 10 minutes in room temperature. Each microplates applied with 50 µl of violet crystals 2% for 5 minutes and then washed with PBS (Phosphate Buffer Saline) for two times then placed lugol solution for 1 minute and washed once more with PBS. The rest of the cell that identified not a bacterial cell was dissolved with 96% alcohol for 20 seconds until effect of dye is disappeared. Furthermore, placed 50 µl safranin solution for 2 min and washed once more with PBS.[21] The adhesion activity of *E. faecalis* cells on the basis of microplate was analyzed by

Spectrophotometer Elisa reader (Bio-Radd Laboratories, Hercules, CA) at 590 nm.[18] Serial duplo of optical density of anti adhesion of Sarang Semut to *E. faecalis* calculated focus on mean and standard deviation value. The statistical significance determined by One Way Anova test (p <0.05), while colony data of *E. faecalis* growth analyzed descriptively.

3. Results

Table 1: Effect of Sarang Semut to colony of *E. faecalis*

Concentration (%)	3 H (cfu/µl)	6 H (cfu/µl)	9 H (cfu/µl)	12 H (cfu/µl)	16 H (cfu/µl)	24 H (cfu/µl)
6,125	423	390	295	245	230	229
12,5	375	345	256	286	258	192
25	178	171	125	124	112	85
50	123	97	72	54	21	13
100	32	21	3	1	0	0
Control	1	0	0	0	0	0

Note; Control is phosphomycin

Both incubation time of extract of Sarang Semut concentration in 100% (µg / ml) 16 and 24 hours showed the bacteriocidal effect, while other concentration on bacteriostatic level.

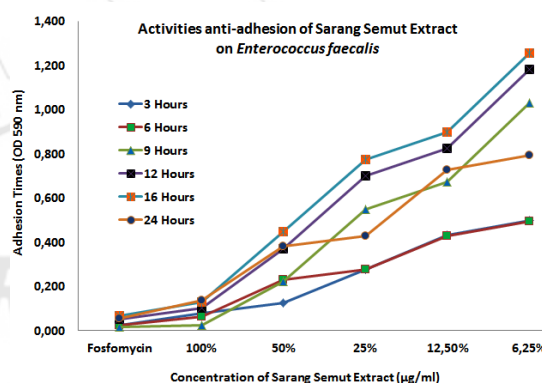


Figure 1: Anti-adhesion activity of Sarang Semut on *E. faecalis*.

The concentration 100% (µg / ml) showed better capability as anti-adhesion than other concentration. Phosphomycin showed a better capability as a positive control of *E. faecalis* anti adhesion at all treatment times.

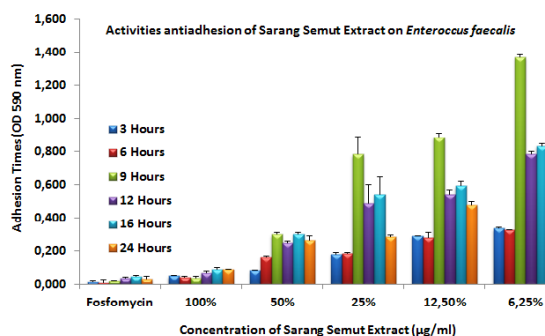


Figure 2: Effect of Sarang Semut as anti adhesion of *E. faecalis*

The mean values showed, time is important factor to determinate adhesion change after Sarang Semut application. While, standard deviation (error bar) on all concentrations

showed variation of data with the assumption of changes in antidepressant activity of Sarang Semut as expected to this study.

Table 2: One Way Anova of antiadhesion of extract of Sarang Semut to *E. faecalis*

Concentration of Sarang Semut	n	Mean±SD	p
100%	36	0,064±0,004	p<0,05
50%	36	0,226±0,010	
25%	36	0,411±0,055	
12,5%	36	0,512±0,010	
6,25%	36	0,696±0,005	

Table 2 above showed the result of one way ANOVA which explains that the concentration of extract of Sarang Semut has a significant effect ($p < 0,005$) to *E. faecalis*.

4. Discussion

Enterococcus faecalis has a number of surface proteins that act like proteases, serine proteases, gelatinases, and collagen-binding proteins (Ace) that have contributed to adhesion to the root canals of teeth.[22] This indicates that surface proteins have an important role in the host cell infection phase.[23] Furthermore, both pH and temperature are the supporting factors for the development of *E. faecalis*. In addition, *E. faecalis* also has the ability to grow in anaerobic environment make this bacterium as dominant to be studied in root canal infection. A number of these aspects are important to be tested with antibacterials derived from plants, one of which is the extract of Sarang Semut using time approach as an aspect of the sensitivity of anti-adhesion.

Sarang Semut has two potencies to *E. faecalis*: bacteriostatic and bacteriocide. According to Table 1, both of these potencies starting from 12 hours to 24 hours, especially at concentrations of 100% ($\mu\text{g/ml}$). Even though concentrations of 12.5% (9-24 hours), 25%, and 50% confirm that Sarang Semut has potency to inhibit of *E. faecalis* growth to below 300 colonies (cfu/ml) but this result study also confirm the potency of to inhibit of *E. faecalis* growth is still in minimum category. In other word, except concentration 100% of ant nets, the potency of active components has not been able to eliminate the growth of *E. faecalis*. This result match with previous research that reported the standard value number of *E. faecalis* colonies which has been sensitive to Sarang Semut is 300 colonies (cfu/ml) which is concentration of 100% ($\mu\text{g/ml}$) showed highest effect.[14] This ability can be assumed that extracts of the Sarang Semut can inhibit the activity of *E. faecalis* surface proteins such as aggregation substance (Agg) and Enterococcal surface proteins (Esp) which is known act as proteins that stimulate growth improvement to host cells.[24] This result similar to Apriyanti that isolated and identified three of flavonoids sub-compound in Sarang Semut (*M. Pendans*) properties such as butein, 3-methoxy-epikatekin-3-O-epikatekin, and dibenzop-dioxin-2,8-dicarboxylic acid. These three flavonoid sub-compounds has antibacterial bacteria including *E. faecalis*. [15]

Based on optical value of density, Sarang Semut has anti-adhesion properties against *E. faecalis*, especially at high

concentration. Result also confirm phosphomycin as a broad-spectrum antibiotic that particularly suitable for eliminating *E. Faecalis*. Olesen SH stated, phosphomycin can interrupt the cytoplasm in biosynthesis of peptidoglycan and inhibit the synthesis of the MurA enzyme that plays a role in attachment to host cells.[10] The association is, phosphomycin can act as a strong anti-adhesion on microplate, because phosphomycin forming hydrogen bonds that consequently inhibit synthesis of peptidoglycan that known as system of anti-bacterial defense.[25] Otherwise, the ability of anti-bacterial in this study, confirmed Sarang Semut as amount of quantity of flavonoids repeatedly.[26, 27] As we know, flavonoid antibacterial activity has the ability to activate enzymes, transport of proteins and prevent of adhesion and also break bacterial cell membranes.[28] As an anti-adhesion material, the activity of Sarang Semut is related to the sensitivity perception system of plants to bacterial flagellin. As we know, flagellin is an important aspect of virulence factor for bacteria to infect host cells.[29] Thus, as in-vitro modeling, this study presence an active component such as flavonoids may interfere the adhesion activity of *E. faecalis* cells as confirmed on the bottom of well-96 microplate well in this study. Vinothkumar (2013) stated that in-vivo application of Neem extract is very effective in prevent of bioactivated adhesion and colonization of *E. faecalis* in dentine.[30]

Temperature and pH are important factors that increasing of adhesion both aerobic and anaerobic bacteria, including *E. faecalis*. Change of temperature can improve attachment, coaggregation, and protease production.[31] This result also matched with Zilm that stated alkaline condition (8.2) can increase hydrophobicity which can induce co-adhesion and biofilm formation of *P. gingivalis*. [32] The results of this study can be assumed that anti-adhesion activity of Sarang Semut is able to stabilize pH (6-7) and temperature (30-35°C) to decrease virulence of bacteria including intensity of adhesion.

The application of incubation time in this study could an alternative reference to observe the development of anti-adhesion of Sarang Semut, which showed that fit incubation range time is 12-24 hours. This capability is similar to Mobili (2016) that reported incubation time and the pH change of the extract solution significantly ($P \leq 0.01$). [33]

Meanwhile, Natarajan and Schmittel showed that temperature is an important variable against the anti-bacterial activity of a number of plant extracts such as leaves of *Camellia sinensis*. [34] The results of this study are in line with the results of statistical analysis of this study ($p < 0.05$) meaning that the anti adhesion activity of the Sarang Semut extract shows the difference ability on the various concentrations. While Shah's study (2016) using Soluneem extract (Azadirachtin) showed that concentration became the determinant factor in antibacterial. [35]

Figure 2 showed that the standard deviation (bar error) has a variant, which explains that anti-adhesion activity of Sarang Semut is related to concentration and incubation times. Rosaline (2013) has reported based on one-way ANOVA analysis with multi-comparison of the anti-adhesion of Neem extract which showed significant ($p < 0.05$) could decrease

amount of *E. faecalis* in dentin (9.30%), making it clear that the Neem extract is very effective in preventing adhesion *E. faecalis*. [36] This decrease in specific adhesion activity occurs maximum at 24 hours. [37] Goy's (2009) has reported that the most significant antibacterial activity variables occur initiate at 12, 24, and 36 hours. [38] While Samat (2016) also reported that at 24 hours of Gambier extract can inhibit the growth of *E. faecalis* ($p < 0.05$). [39] Similar to Morandi (2005) who reported that *E. faecalis* has high sensitivity after adapted in pH 5.0 for 10 h at 37°C. [40] This result similar to anti-adhesion activity of Sarang Semut to *E. faecalis* which is presented in Tables 1 and 2, Figures 1 and 2. Based on the time of incubation and concentration of extract of Sarang Semut, it can be concluded that extract of Sarang Semut can prevent adhesion of *E. faecalis* as well as inhibit its growth, especially at high concentration ($p < 0,05$).

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