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Research Report

Effects of sarang semut (*Myrmecodia Pendens* Merr. & Perry) extracts on *Enterococcus faecalis* sensitivity

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ABSTRACT

Background: *Enterococcus faecalis (E. faecalis) is a gram positive oral pathogen that reported at the main agent infection of endodontic treatment. Its activities are influenced by the virulence factors facilitating the interaction process between agents with host cells. Like aggregation substance, cytolysin, extracellular superoxide, gelatinase, hyaluronidase, sex pheromones, and surface adhesions molecules. Plant extracts are reported as the material antibacterial as well as E. faecalis in pathogenesis of endodontic infections.* ***Purpose:*** *Purpose of this study was to analyse of sarang semut extracts (Myrmecodia Pendens Merr. & Perry) towards sensitivity of E. faecalis.* ***Method:*** *This research used the methanol extract of sarang semut, E. faecalis ATCC 29212, and fosfomycin also chlorhexidine as the positive controls. Whereas, Bradford protein method was measured the concentration of the surface protein of E. faecalis and active component of the sarang semut extract.* ***Result:*** *Generally, the sarang semut extract possessed low sensitivity toward E. faecalis (≤ 13 mm), but on the concentrations of 100 µg/ml and 75 µg/ml better than inhibition of other concentrations, round 10.6-11.6 (mm). Specifically, on 100 µg/ml has indicator the minimal bactericidal concentration (MBC) on E. faecalis. Whereas minimal inhibition concentration (MIC) on the concentration of 3,125 µg/ml.* ***Conclusion:*** *Based on MBC and MIC assay, the extract of sarang semut has potential effects to adherence growth of E. faecalis, mainly on the highest concentration 100 µg/ml also MIC on 3,125 µg/ ml.*

Keywords: *Enterococcus faecalis; extracts of sarang semut; sensitivity; minimum bactericidal concentration; minimum inhibitory concentration*

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INTRODUCTION

Enterococcus faecalis (*E. faecalis*) bacteria are the most common pathogens isolated in root canal, especially after endodontic treatment. *E. faecalis* bacteria have properties to withstand a variety of conditions in the root canal. Thus, despite eliminating them with various medication materials, their existence still can threaten tooth root tissue repair since they are able to survive in an acidic environment, even under conditions of nutrient deficiency and drug influence. The prevalence of *E. faecalis* bacteria in the case of endodontic infections reached 24 to 77%, in which the presence of these bacteria in the root canal is often associated with chronic apical periodontitis.[2]

The pathogenesis of *E. faecalis* bacterial infection begins with the formation of biofilms on the root canal tissue. This capability is facilitated by a number of other oral pathogens, then colonizing together in the root canal. *E. faecalis* bacteria decay a number of proteins to form acidic conditions, in which those pathogens facilitating colonization will die because of the increasing intensity of acidity in the root canal.[3] One of virulence factors expressed by *E. faecalis* bacteria in the pathogenesis of their infection is liphoteichoic acid (LTA), serving to contaminate the root canal and form colonies on the dentine surface, while surface proteins like collagen binding protein will interact with dentin collagen that can support the colonization of *E. faecalis* bacteria in the root canals.[4] Other virulent *E.*

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faecalis proteins that inhibit other pathogens are cytolysin, aggregation substance (AS-48) and bacteriosin sex pheromones, extraceluller superoxide production (ESP), gelatinase lytic enzyme, hyalurodinase, as well as cytolysin toxin. In addition, carbohydrates or glycoconjugates are also categorized as virulence factors in pathogenesis of enterococcal infections.[5,6]

The prevention concept of *E. faecalis* infection is based on the sensitivity level of the anti-bacterial materials considered as irrigation materials.[7] Chlorhexidine (CHX) is reported as very good irrigation material since it has higher sensitivity than *calcium hydroxide*.[8] Nevertheless, CHX has limitations due to its insensitiveness against cell damage although it is able to prevent the formation of *E. faecalis* bacterial biofilms for five minutes.[9] Similarly, fosfomysin is reported to have anti *E. faecalis* bacteria by inhibiting phosphoenolpyruvate synthetase, but it can also trigger some negative effects, such as metabolic disorders in urinary system and kidney disfunction.[10]

Therefore, using herbs is considered as an alternative effort to prevent bacterial infections, including *E. faecalis* infection.[11] Each plant actually has an excellent system of sensitivity perception, especially against bacteria, one of important virulence factors in the pathogenesis of bacterial infection.[12] One of the important effects of herb extract as an anti-bacterial material is an ability to damage cells of pathogens (citotoxicity) by disrupting the membranes of the surface proteins, such as polysaccharide layer, fatty acids, and phospholipids, which eventually can degrade the structure of the cell membrane, thereby reducing the potential Ca^{++}.[13]

Based on previous research, sarang semut has chemical compounds of flavonoid and terpenoids, playing a role as an anti-bacterial compound, unfortunately, whether flavonoids and terpenoids, active compounds, in the sarang semut can potentially inhibit *E. faecalis* has not clearly been known yet.[14] Thus, this research aimed to analyze effects of sarang semut extract on the sensitivity of *E. faecalis*.

MATERIALS AND METHOD

Anti-bacterial potency of sarang semut methanol extract was tested on *E. faecalis* (ATCC 29212, Tech atcc, Manassas, USA). The sensitivity of the bacteria was analyzed using disc method, minimum inhibitory test, and minimum bactericidal test. The sensitivity of *E. faecalis* to *fosfomycin* (Meiji Inc, Japan) and CHX was also used as positive controls, while the medium without *E. faecalis* was used as a negative control. To determine the effectiveness of the methanol extract of the sarang semut to the development of *E. faecalis,* several steps were conducted.

The sarang semut extract was obtained from the Research Laboratory of Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia. In addition to phytochemical test, a predictive analysis had been conducted for determining the bioactive compounds in the sarang semut extract based on prediction of activity spectra for substances (PASS) approach with an indicator PASS (Pharmaexpert, Moscow, Rusia) value of ≥0.70. Results of this analysis showed that the sarang semut had a value of more than 0.70. It means that flavonoids contained in the sarang semut have a complectivity value required based on the standard value of phytochemical analysis.[15]

E. faecalis ATCC 29 212 bacteria were inoculated in 20 mL of Mueller-Hinton Broth (MHB) (Thermo Fisher Scientific Inc, Oxoid, UK) at 37°C for 24 hours, and then synchronized with the 0.5 McFarland Standard (1 x 108 CFU/ mL). Multilevel dilution from 10^{-1} to 10^{-8} MHA was performed, and then they were cultured in MHA medium at 37° C for 24 hours. Colonies growing as much as 30-300 CFU/ ml were used as references for inoculum candidate against the sensitivity of *E. faecalis* using the minimum inhibitory test and minimum bactericidal test.[16] This research, 10^{-4} was used as a reference of dilution, then used as a reference of evaluation with an average colony of 53 CFU/ ml. [17]

Prior to the minimum inhibitory concentration and the minimum bactericidal concentration tests of the sarang semut extract against *E. faeccalis*, the concentrations of both the proteins of *E. faecalis* and the active compounds of the sarang semut extract were measured using Bradford method (Bio-Rad). The proteins of *E. faecalis* were extracted with lysozyme extract (Bioseutica B.V, Zeewolde, Netherlands).[18] Meanwhile, the active compounds of the sarang semut extract were extracted using HCl principle approach.[19] Moreover, to conduct this Bradford test, bovine serum albumin (BSA) (Polysciences Inc, Warrington, PA-USA) was used to obtain a standard protein concentration, ranging from 62.5 to 500 (pm/ ml). The *E. faecalis* proteins and the ant-nets extract were respectively put into Elisa plate well as much as 160 µl (10 µl sample + 150 µl phosphate buffer saline (PBS)). Another Elisa plate well was given BSA as a standard protein as much as 160 µl (10 µl BSA + 150 µl PBS). Afterwards, both the samples and BSA were added with 40 µl of protein assay (Bradford), and then by using a multi-channel pipette they were re-suspended and incubated at a room temperature for 1 hour. The concentration of the proteins then was measured using Elisa reader based on Optical Density at a wavelength of 595 nm (Bio-Rad Laboratories Inc, CA, USA).

Furthermore, a sensitivity or susceptibility test was conducted on *E. faecalis* bacteria using diffusion method based on the Clinical and Laboratory Standards Institute (CLSI), the standard for fosfomysin and chlorhexidine applications against *E. faecalis* bacteria with three categories, namely resistant if ≤13mm, intermediate if 14-16 mm, and susceptible if ≥17.[20] Experiments were performed in duplicate with repetition as much as 2 times. 1 ml of *E. faecalis* inoculum was spread in the MHA medium. Control and treatment discs were inserted. Paper discs were dipped in fosfomysin and CHX with a concentration of 25 mg/ 6.25 ml of 0.9% NaCl, and then settled for 15 minutes.

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The positive controls were put in the Muller Hilton agar (MHA) (Thermo Fisher Scientific Inc, Oxoid, UK) medium with tweezers. The sample discs were dipped into the 0.1 ml of the inoculum in 1 ml of the extract, and then settled for 15 minutes. The extract at the concentration of 100% (500 ug/ ml) was diluted for further concentrations, from 75, 50, 25, 12.5, 6.25, to 3.125%, and then incubated in an incubator at a temperature of 37°C. After the 24 hour incubation, the light zone (zone of inhibition) was measured by using a calipers (Automation and Metrology Inc, OH, USA). The value (mm) of the diameter zone then was used as an indicator of the sensitivity of the sarang semut extract against *E. faecalis*.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were conducted to determine the effects of the sarang semut extract on the development of *E. faecalis*. The *E. faecalis* ATCC 29 212 bacteria in Nutrient Broth Mc Farland 0.5 (equivalent to 1.5 x 108 CFU/ ml) was diluted with serial techniques, and then the number of *E. faecalis,* 10^{-4}, was diluted in order to be used as a reference to the MIC and MBC tests0.1 ml of *E. faecalis* inoculum was respectively put into 1 ml of the sarang semut extract, the positive controls (fosfomycin and chlorhexidine), and the negative control (physiological saline). Each sample was settled for 10 minutes, and then cultured on MHA medium under an-aerobic atmosphere for 48 hours at a temperature of 37°C. *E. faecalis* colonies that grew were used as a reference to the ability of the sarang semut extract in inhibiting and killing *E. faecalis* with the positive controls and the negative control as references for assessment.[21,22]

RESULTS

The concentrations of both the cell wall proteins of *E. faecalis* and the active compounds of the sarang semut extract

Bradford protein test using bovine serum albumin as the standard was performed to determine the quantity of both the cell wall proteins of *E. faecalis* and the active compounds of the sarang semut extracts, so the reactivity or interaction value of both would meet the analysis standard.[23,24] Both the cell wall proteins of *E. faecalis* and the active compounds of the sarang semut extracts have a threshold concentration, approaching the bovine serum albumin's concentration of 500%.

DISCUSSION

The results of the phytochemical test showed that the sarang semut extracts positively contains flavonoids, tannins, saponins, and alkaloids. Flavonoids and saponins are known to act as anti-bacteria.[15] The other active compounds can also play a role as anti-bacteria and anti-oxidant, and even saponins in particular have antibacterial properties with a wide spectrum.[26] In addition, as shown in Figure 1, the sarang semut extracts had active compounds sufficient to be used as anti-bacteria after calibrated with bovine albumin serum (BSA), as done by Yesilada,[27] using BSA to predict the active compounds of *Sambucus ebulus L.* extract as an anti-bacterial material. In other words, the two samples equally possessed good sensitivity when interacted on the anti-bacterial test.

Moreover, Pessione[28] argues that in each test on an interaction between pathogens and anti-pathogens, the concentration of proteins should be measured before, especially proteins on cell wall of pathogens or bacteria using a recommended Bradford assay method. For instance, Bohle[29] used Bradford method to measure protein concentration of *E. faecalis* in order to examine the expression of proteins associated with response of stress. This is in line with Schneewind[30] explaining that the peptidoglycan of gram-positive bacteria serves as surface organelles to interact with the environment, particularly on the tissue of the host infected, and the concentration of the proteins contained of the cell walls determines the level of interaction with the host cell infected.

Based on the analysis of PASS, furthermore, the sarang semut extract had a value of Pa. If a compound

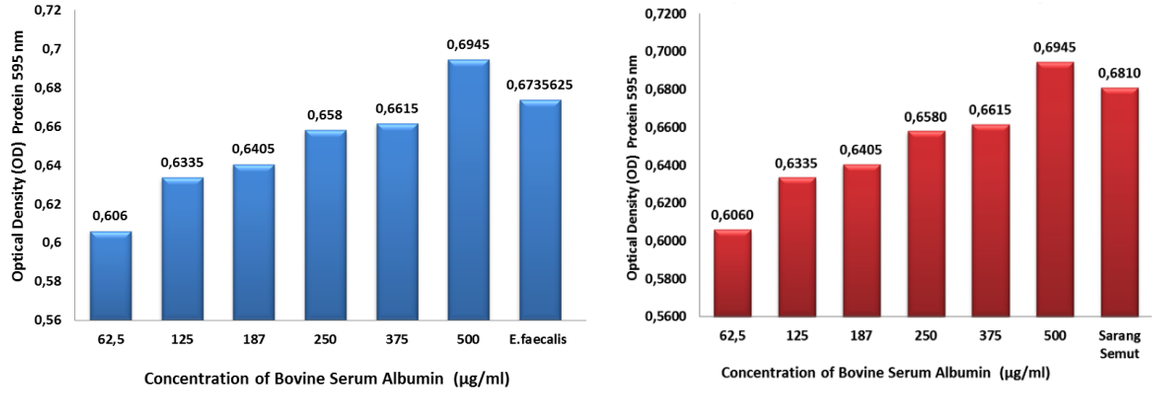

Figure 1. Profiles of both the cell wall proteins of *E. faecalis* (left) and the active compounds of the sarang semut extracts (right).

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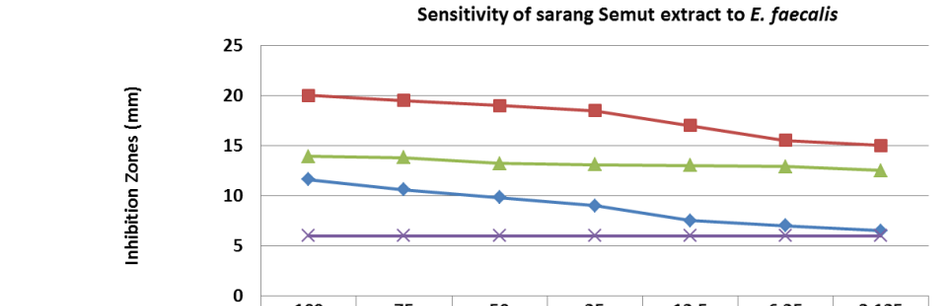
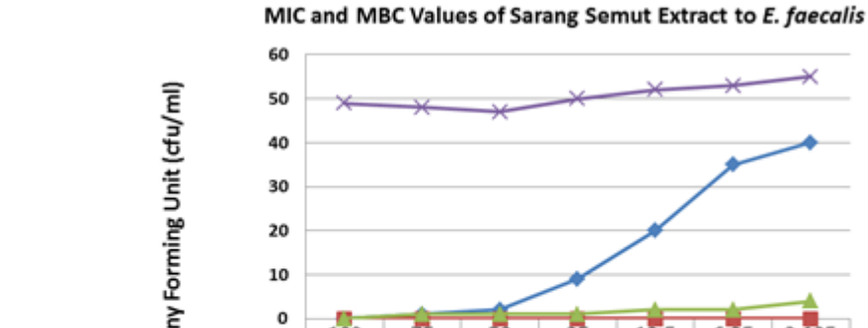
Results of the potency test of the sarang semut extracts on the sensitivity of *E. faecalis*



	100	75	50	25	12,5	6,25	3,125
Extract+E. faecalis	11,6	10,6	9,8	9	7,5	7	6,5
Fosfomycin+E. faecalis	20	19,5	19	18,5	17	15,5	15
chlorhexidine + E. faecalis	13,9	13,8	13,2	13,1	13	12,9	12,5
Medium + E. faecalis	6	6	6	6	6	6	6

Figure 2. Sensitivity of sarang semut extracts to *E. faecalis*. It shows that based on the Clinical and Laboratory Standards Institute (CLSI), the sarang semut extracts at all concentrations had low sensitivity against *E. faecalis* (≤13mm). It can be said that the extracts can be categorized into resistance to *E. faecalis*. Unlike the sarang semut extract, fosfomysin had a susceptible sensitivity at the concentration of 100% up to 12.5% (≥17), while at the concentrations of 6.25% and 3.125% (14-16 mm), it can be categorized into an intermediate level. At the concentrations of 100% and 75%, on the other hand, chlorhexidine had a potential anti-bacteria of *E. faecalis,* while at the concentrations of 50% to 3.125%, it can be categorized as an intermediate level.[20, 25]

Results of MIC and MBC tests of the sarang semut extracts on the development of *E. faecalis*



	100	75	50	25	12,5	6,25	3,125
Extract+E. faecalis	0	1	2	9	20	35	40
Fosfomycin+E. faecalis	0	0	0	0	0	0	0
chlorhexidine + E. faecalis	0	1	1	1	2	2	4
Medium + E. faecalis	49	48	47	50	52	53	55

Figure 3. Minimal inhibition concentration value of sarang semut extracts to *E. faecalis*. It shows that the sarang semut extracts had a MIC value at the concentration of 3.125%, while a MBC value at the concentration of 100%. Unlike the sarang semut extracts, fosfomysin had a MBC value at all concentrations. Meanwhile, CHX had a MBC value at the concentration of 100%. Similar to the sarang semut extracts. However, CHX as the positive control had higher MBC value than the sarang semut extracts and the negative control.

has a value of Pa, greater than 0.7, it means that the compound has a specific biological activity.[15] According to Lagunin,[31] PASS and Pharma Expert methods can be used to evaluate biological activity of a natural product, including marine sponge alkaloids as well as triterpenoids and their derivatives, wherein the methods computationally demonstrate the ability to evaluate multiple targets from the effects of natural products, both additive/ synergetic and antagonistic. Another assumption of this information is that the sarang semut extract tested for its effectiveness against *E. faecalis* can be classified into synergetic and antagonistic groups, and have properties of adhesion, bio-tolerance, and bio-resistance against bacteria.[32]

Figure 1 shows that *E. faecalis,* after the levels of their protein was measured using Bradford method, had protein expression profiles proportional to bovine serum albumin

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at a concentration of 500%. Dominica[33] reported that the quantity and quality of the anti-bacterial activities of *Enterococcus spp.* bacteria, such as Arabian pea protein (Glycate pea protein), are always determined by the protein concentration of the pathogens determined using Bradford method with bovine serum albumin as the standard.

Figure 2 shows that the sarang semut extract had a low sensitivity (≤ 13mm). This value, based on the Clinical and Laboratory Standard Institute (CLSI), is classified as resistance.[22] Bacteria can possibly become resistant to drugs since some bacteria are not capable of destroying expressions of β-lactamases produced by pathogens when interacting with drugs or medicinal plant extracts, as a result, the bacterial cell membrane is able to avoid the damage of the bioactive compounds contained in the drugs or medicinal plant extracts.[34] Besides, the resistance value is also related to the genitive property of gram-positive bacteria, expressing a gene behavior in the form of N-acyl homoserine lactone (AHL) signal when interacting with the environment. The principle of the AHL signal is to prevent interaction with bioactive components of plants as well as pathogenic, symbiotic, and saprophytic bacteria. A number of plant extracts, such as *exudates pea* (*Pisum sativum*) have properties imitating bacterial AHL signal, consequently, it can affect bacterial adaptation to anti-bacteria.[35]

In addition, Figure 3 shows that the MIC value of the sarang semut extract was obtained at the concentration of 3.125%, while the MBC value was at the concentration of 100%. The abilities are related to the role of active flavonoid and tannin compounds to inhibit the growth of *E. faecalis.*[15] Similarly, garlic extract can inhibit trypsin-like enzyme and total protease activities of *P. gingivalis* at concentrations of 92.7% and 94.88%. It indicates that both the sarang semut extract and the garlic extract can inhibit the growth of oral pathogens, both in endodontic and periodontal therapies.[36] The MIC value of *A. nilotica* extract ranges from 4.9 to 313 ug/ ml. The MIC value of the *A. nilotica* extract against *E. faecalis* is 9.75 ug/ ml, while the MBC value is 78 ug/ ml.[37] This indicates that *E. faecalis* ATCC 29 212 used as the subjects in this research had good sensitivity to anti-bacteria. Another assumption is that the cellular and molecular role of flavonoids and tannins contained in the sarang semut extract is very important to inhibit DNA synthesis, cytoplasmic membrane function, and energy metabolism.[38] In conclusion, based on MBC and MIC assay, the extract of sarang semut has potential effects to adherence growth of *E. faecalis*, mainly on the highest concentration 100 µg/ml also MIC on 3,125 µg/ ml.

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