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ORIGINAL RESEARCH

MSC Secretome's Antibacterial Activity on *P. aeruginosa* Isolated from Diabetic Ulcer Patient

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ABSTRACT

Diabetic ulcers, a common complication of diabetes, are characterized by persistent wounds that are prone to infection, often by bacteria such as *Pseudomonas aeruginosa (P. aeruginosa).* Mesenchymal Stem Cells (MSCs) are recognized for their regenerative properties and are being explored for their antibacterial effects. Recent studies show MSCs can combat various harmful bacteria, with products like the secretome mirroring cell traits. This study aims to investigate how effective the secretome from adipose tissue MSCs is against P. aeruginosa bacteria from diabetic ulcer patients. Bacteria were isolated from specimens obtained from diabetic ulcer patients and identified using MacConkey and Blood Agar media followed by Polymerase Chain Reaction (PCR) molecular identification. Confirmed P. aeruginosa bacteria were used to evaluate the MSC secretome's antibacterial activity via the Kirby-Bauer method. Isolated bacteria grew on MacConkey media as gram-negative, non-lactose-fermenting bacteria, showing hemolytic capabilities on Blood Agar media. PCR identification yielded positive results for P. aeruginosa. In antibacterial activity testing against isolated bacteria, MSC secretome at concentrations of 1.25%, 2.5%, 5%, and 10% exhibited efficacy with average inhibitory zones measuring 8.17 mm, 8.23 mm, 8.52 mm, and 9.30 mm, respectively. The MSC secretome demonstrates antibacterial activity against P. aeruginosa bacteria isolated from diabetic ulcer patients.

Keywords: Antibacterial, MSC secretome, diabetic ulcer, P. aeroginosa.

Pendahuluan

Diabetic ulcers are one of the complications of diabetes mellitus that are currently a public health concern and a leading cause for diabetic patients to be admitted to the hospital, with risks of amputation and even death (Sankar, Moinuddin and Mohan, 2020). As diabetic ulcers develop, they are susceptible to infection due to the loss of innate defense mechanisms (Alavi *et al.*, 2014). The gram-negative pathogenic bacterium *P. aeruginosa* is frequently found in diabetic ulcer infections, following the presence of *S. aureus* (Ertugrul *et al.*, 2017; Sultana *et al.*, 2023). This nosocomial pathogenic bacterium is currently receiving attention in hospital organizations due to high morbidity and mortality rates in patients (Pachori, Gothalwal and Gandhi, 2019). Furthermore, *P.aeruginosa* is one of the gram-negative bacteria with many cases of resistance to various effective antibiotics and has been categorized as multidrug-resistant bacteria (Al-Ahmadi and Roodsari, 2016).

One of the breakthrough biotechnology products that is gaining attention from various medical sectors due to its biological significance and clinical applications is Mesenchymal Stem Cells (MSC). Various studies have demonstrated the antibacterial activity produced by MSC. Some of them associate the antibacterial activity in MSC with the presence of antimicrobial peptides (AMP) LL-37 as an expression of the immune cell's defense against pathogens and an increase in phagocytosis activity (Alcayaga-Miranda, Cuenca and Khoury, 2017). On the other hand, the products of stem cells, such as the secretome, can recapitulate many characteristics the cells possess. The secretome produced by MSC contains various serum proteins, hormones, angiogenic factors, growth factors, cytokines, extracellular matrix proteases,

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extracellular matrix proteins hormones, and even in low abundance, genetic material and lipid mediators released by stem cells into the media (Khanabdali *et al.*, 2016).

Materials and Metode

Materials

The research utilized a range of materials and reagents, including Dulbecco's Modified Eagle Medium (Gibco®, United States), Adipose Tissue Mesenchymal Stem Cells (Merck®, Germany), Fetal Bovine Serum (Gibco®, United States), trypsin enzyme, various agar types such as MacConkey Agar (Becton Dickinson®, United States), Nutrient Agar (Oxoid®, United Kingdom), Blood Agar (Becton Dickinson®, United States), and Mueller Hinton Agar (Himedia®, India). Sterile distilled water (aquadest) and 70% alcohol were used for sterilization, while Nutrient Broth (Oxoid®, United Kingdom) facilitated bacterial culture. Additionally, the research involved ceftriaxone antibiotic (Hexpharm®, Indonesia), sterile 0.9% NaCl (Otsuka®, Japan), McFarland 0.5 solution, a Genomic DNA kit (Promega®, United States), specific oprL primer (Genetika Science®, Indonesia), agarose powder, TBE Buffer, Cybergreen DNA gel stain, loading dye, and a DNA ladder, crystal violet, gram's iodine solution, ethanol, safranin.

Methods

1. **Preparation of Mesenchymal Stem Cell (MSC) Secretome**

In this research, MSCs were obtained from human adipose tissue supplied by PT. Merck Tbk. Typically, MSCs in passage 4 (P4), which are in their fourth growth phase, were used. Subsequently, cell culture was performed, ensuring that the cells reached a confluence of 70-80% as observed under an inverted microscope. The MSC secretome was then collected by filtration using a 0.22 µm filter. The secretome of MSCs was prepared in four different concentrations, namely 1.25%, 2.5%, 5%, and 10%, for antibacterial testing.

2. **Bacterial Isolation**

The test bacteria were isolated from pus specimens obtained from diabetic ulcer patients wounds in the internal ward of RSUP Dr. M Djamil, Padang. Specimens were collected by swabbing the inside of the wound area to gather pus using a swab method. The specimens were placed in sterile transport containers and promptly conveyed to the laboratory. The isolated specimens were cultured on differential media, namely MacConkey and Blood Agar, and then incubated at 37°C for 24 hours. Subsequently, the fermentation profile of lactose by the bacteria growing on MacConkey media and the hemolytic capability of the bacteria on blood agar media were observed.

3. **Identification of *Pseudomonas aeruginosa* using Gram Staining**

Before the Gram staining procedure, a slide of cell sample from the bacterial isolate is created. Subsequently, the staining process is carried out while observing the time for each reagent to be applied. Firstly, crystal violet is applied to the preparation for 60 seconds, then rinsed with water. The second reagent applied is iodine for 60 seconds, followed by rinsing. A decolorizer, ethanol, is added to the preparation for 15 seconds. Finally, safranin stain is applied to the preparation for 60 seconds, followed by rinsing. The preparation is allowed to dry and then observed under a microscope at a magnification 400x.

4. **Identification of *Pseudomonas aeruginosa* using Polymerase Chain Reaction (PCR)**

In the identification of *P. aeruginosa* bacteria using PCR, the Wizard® Genomic DNA Purification Kit (Promega) is utilized to isolate DNA. The procedure is performed according to the manufacturer's kit instructions. The subsequent steps involve amplification using a thermal cycler. In this research, a reaction mixture is used, which consists of a master mix containing DNA template, specific primers oprL, MyTaq Red Mix 2x, and ddH2O. The thermal cycler is run with the following settings: 94°C for 5 minutes, followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, concluding with a final extension at 72°C for 10 minutes. For gel electrophoresis, DNA ladder and the isolated bacterial DNA are loaded into separate wells of an agarose gel pre-soaked with 1X TBE buffer (sample volume = 10 µL/well). The

equipment is run at 80 V for 45 minutes. Subsequently, the visualization of the electrophoresis results is performed using a UV transilluminator.

5. Bacterial Antimicrobial Activity Test

The assessment of antibacterial activity is carried out through the Disc Diffusion method, also known as the Kirby-Bauer Test. To prepare the bacterial suspension, a bacterial culture that has been subcultured is taken from a pure culture, and one loopful is diluted with a 0.9% physiological saline to reach the level of turbidity equivalent to a 0.5 McFarland standard (2x10^8 CFU/L). For the antibacterial test using this method, Mueller Hinton Agar (MHA) with a depth of 6 mm in a petri dish is used. The agar is allowed to solidify for approximately 30 minutes before inoculating the bacterial suspension. Next, the prepared bacterial suspension is streaked onto the surface of the agar using a cotton swab. After allowing the inoculum to dry for 3-5 minutes, disks are placed on the agar surface with gentle pressure to ensure contact. The test includes 6 disks, each of which will be impregnated with different substances: ceftriaxone as a positive control, distilled water as a negative control, and secretome with four concentrations (1.25%, 2.5%, 5%, and 10%) as the samples. Each disk is impregnated with 30 µl using a micropipette. Then, all the Petri dishes with these disks are incubated for 18 hours. The diameter of the inhibition zone (including the 6 mm diameter of the disk) is measured using calipers. This process is repeated three times for accuracy and consistency..

Data Analysis

The results of the inhibitory measurements at each concentration were further analyzed to test data's normality using SPSS. To assess the impact of various secretome concentrations on Pseudomonas aeruginosa, a One-Way Analysis of Variance (ANOVA) test was performed.

Result and Discussion

Bacterial Isolation

P.aeruginosa bacteria were isolated directly from a specimen of diabetic foot ulcer pus. The pus specimen was then inoculated onto differential media such as Blood Agar and MacConkey and incubated for 24 hours at 37°C. MacConkey media is commonly used for bacterial growth due to its selective support for gram-negative bacteria and further differentiation based on their fermentation profiles. MacConkey media contains bile salts and crystal violet, that impede the proliferation of gram-positive bacteria. Additionally, this media contains a pH indicator, neutral red. The detection of organisms capable of fermenting lactose results in a decrease in pH, which is observed as a change in color in the media to pink. Conversely, organisms that do not ferment lactose will not cause a color change to pink in the medium (Allen, 2016)

Figure 1 shows the results of bacterial isolation from the patient's pus specimen. Colonies of bacteria are visible, but there is no pink color change observed in the media. This indicates that the growing bacterial colonies are gram-negative and belong to the non-lactose-fermenting bacteria group. It is known that *P.aeruginosa* is a bacterium classified as gram-negative, and it cannot ferment lactose. Therefore, the isolation results suggest a likelihood of the presence of *P. aeruginosa*.

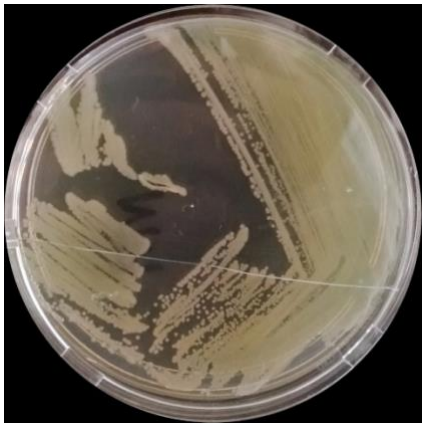


Figure 1. The results of bacterial isolation on MacConkey media

In addition to MacConkey media, blood agar media was also used in this isolation. Blood agar media allows the growth of pathogenic bacteria and determination of bacterial hemolysis patterns. Analysis of the virulence characteristics of a bacterium can be inferred from its ability to lyse erythrocytes. On blood agar media, bacteria capable of producing hemolysin will exhibit a color change in the colony growth area (Buxton, 2016).

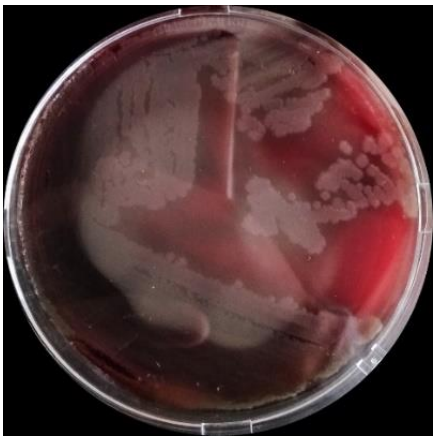


Figure 2. The results of bacterial isolation on Blood Agar media

As a pathogenic bacterium, it is known that *P. aeruginosa* is capable of lysing red blood cells, a phenomenon referred to as hemolysis. As depicted in Figure 2, concerning the hemolytic capability of bacteria growing on the medium, the isolated bacteria can be classified as pathogenic bacteria.

Bacterial Identification

Identification of *Pseudomonas aeruginosa* using Gram Staining

To strengthen the suspicion that the isolated bacterium is *P. aeruginosa*, macroscopic identification was performed using Gram staining. In Figure 3, which represents the results of bacterial identification through Gram staining, it can be observed that the bacterium falls under the category of Gram-negative bacteria. This conclusion is based on the morphology of the bacterium, which appears rod-shaped and takes on a red color after the Gram staining process. Gram-negative bacteria possess a peptidoglycan layer that is thinner when compared to that of Gram-positive bacteria. Their structure is predominantly composed of lipids, so when treated with alcohol, the crystal violet dye leaches out and retains the stain when subsequently exposed to the counterstain, safranin. This is in contrast to Gram-positive bacteria with their thick peptidoglycan layers, which exhibit a stronger affinity for crystal violet. Their ability to form a complex between crystal violet and iodine causes the cell wall pores to shrink during the alcohol wash,

reducing the cell wall's stain absorption capacity. As a result, when stained with safranin, the color remains purple (Smith and Hussey, 2019)

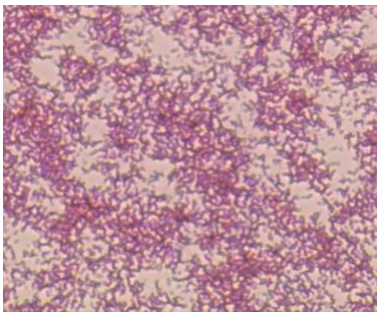


Figure 3. The results of Gram staining on the bacterial isolate

Identification of *Pseudomonas aeruginosa* with Polymerase Chain Reaction (PCR)

In the process of identification using PCR, chromosomal DNA originating from the bacterium itself is required. Therefore, a DNA isolation/extraction process is performed to obtain the DNA genome of *P.aeruginosa*. The obtained DNA isolate is then subjected to amplification. The identification process is continued by visualizing the DNA bands using electrophoresis. The DNA bands formed are observed under ultraviolet (UV) light at 470 nm to determine the position of the bands corresponding to the expected size of the oprL gene. Below are the visualization results obtained from the DNA bands of four samples along with a 100 bp DNA ladder used as a marker.

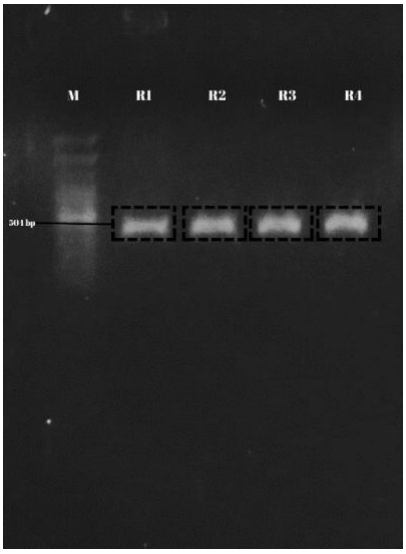


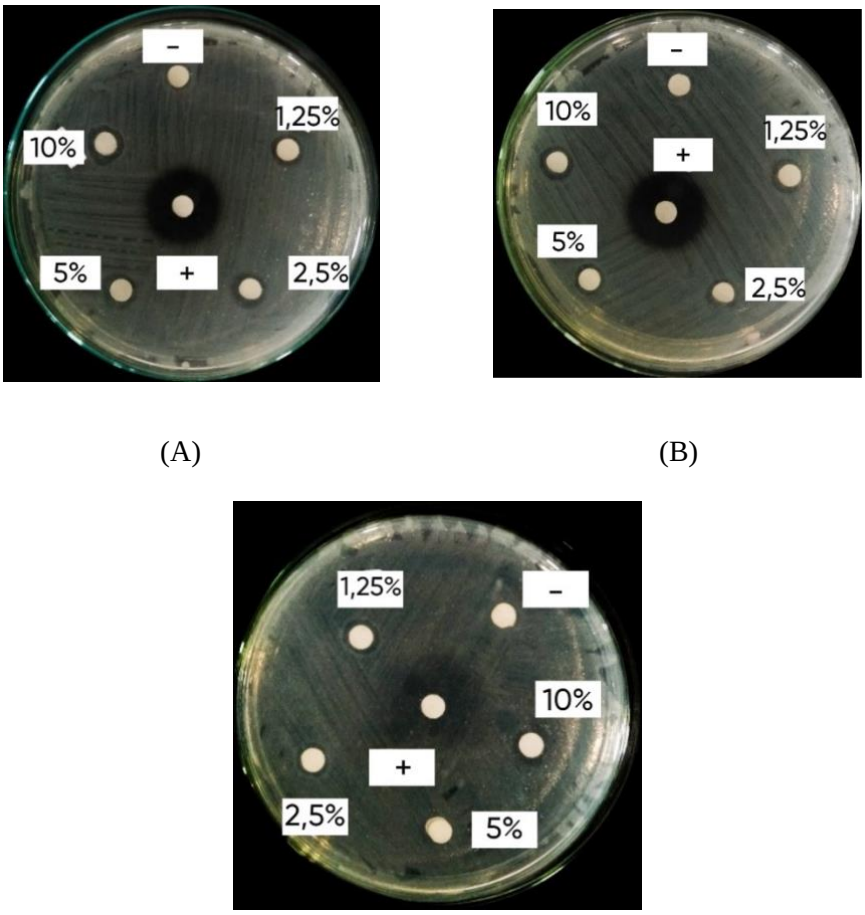
Figure 4. The visualization of DNA bands from bacterial isolates under UV light at 470 nm with a 100 bp ladder marker (M) and four replicates

The image indicates the detection of a bacterial DNA band for the oprL gene at 504 bp. Therefore, it can be confirmed that the bacterium isolated from the diabetic foot ulcer patients is *P.aeruginosa.*

Bacterial Antibacterial Activity Test

The confirmed *P.aeruginosa* isolate was used as the test bacterium to evaluate antibacterial activity using MSC secretome. The method employed was the Kirby-Bauer method. The Kirby-Bauer method falls under the category of diffusion testing, where antibacterial activity is determined based on the diffusion of antibacterial substances on the surface of agar media that had previously been inoculated with the test bacterium. This method utilizes disks/discs as carriers of antibacterial substances, allowing them to diffuse onto the media's surface. the activity assessment is observed based on the diameter of the inhibition zone,

also known as the clear zone (Hudzicki, 2016). Below is an image showing the bacterium's inhibitory effects with three repetitions.



(A) (B)

(C)

Figure 5. (A) Antibacterial Activity First Replication. (B) Antibacterial Activity Second Replication. (C) Antibacterial Activity Third Replication

The measurement of the diameter of inhibition zones formed in each treatment was conducted using a caliper. Below are the calculated results of the inhibitory activity of MSC secretome against *P.aeruginosa* isolated from a diabetic foot ulcer patient.

Table 1. The inhibitory activity of MSC secretome against *P.aeruginosa*

Repetition	Inhibition Zone Diameter (mm)					
	Concentration of MSC Secretome				Control (+)	Control (-)
	1.25%	2.5%	5%	10%		
1	8,55	8,75	8,95	9,35	20,10	0
2	8,40	8,45	8,35	9,10	20,05	0
3	7,55	7,50	8,25	9,45	19,50	0
Mean	8,17	8,23	8,52	9,30	19,88	0
SD	0,54	0,65	0,38	0,18	0,33	0

The results of the inhibitory measurements at each concentration in Table 1 were further analyzed to test the normality of the data used. Based on the SPSS analysis, the data variable was found to be normally

distributed with a significance value of 0.564, where this value is >0.05. Regarding the hypothesis testing for data variance in this study, it was found that the data had equal variances with a significance value of 0.16. With the fulfillment of these conditions, a One-Way Analysis of Variance (ANOVA) test was performed to assess the impact of varying secretome concentrations on the suppression of *P.aeruginosa*. Based on the results of the One Way Anova analysis, the bacterial inhibitory power produced from various concentrations using a 95% confidence level ($\alpha = 0.05$) yields a significance value of 0.064. Therefore, the inhibitory power produced at various concentrations is not significantly different because the significance value is more significant than 0.05.

Among the four concentrations of secretome, there are variations in the inhibitory results produced by each against *P.aeruginosa*. In this experiment, the highest concentration, which is the 10% MSC secretome, yields the largest bacterial inhibition zone compared to the other three concentrations. The average inhibition zone from the three repetitions measures 9.30 mm. Meanwhile, at a lower concentration, specifically the 5% MSC secretome, a smaller inhibition zone is observed with a value of 8.52 mm. In contrast, the 2.5% and 1.25% MSC secretome concentrations provide nearly similar inhibition zones, measuring 8.23 mm and 8.17 mm, respectively. Based on the bacterial inhibition classification by David & Stout (1979), all concentrations of the MSC secretome result in a moderate inhibition zone. Although not significantly different, the magnitude of the inhibition zone produced is directly proportional to the concentration of MSC used. This aligns with a study that suggests that increasing the concentration of antibacterial compounds likely enhances the penetration of these compounds into the interior of bacterial cells to disrupt the cell's metabolic system and induce cell death (Talapko *et al.*, 2022).

Various studies have provided evidence of antibacterial activity produced by MSCs. Some of them have linked the antibacterial activity of MSCs to the presence of the antimicrobial peptide (AMP) LL-37 as an immune cell defense response against pathogens, as well as an increase in phagocytic activity (Alcayaga-Miranda, Cuenca and Khoury, 2017). On the other hand, the secretome, which is essentially the secretion from the cells, requires a direct mechanism from the secreted factors to act as antibacterial agents. As reported, the secretome of MSCs originating from bone marrow exhibits significant antibacterial activity even without pathogenic bacterial stimulation. At the very least, this research indicates that factors presumed to be responsible for the antibacterial activity of the MSC secretome are constitutively produced for release into conditioned media. Through microdilution methods, this study demonstrates that the MSC secretome, without pathogenic bacterial stimulation, can reduce the turbidity level of V. cholerae bacterial suspension from 10^8 CFU/ml to 10^7 CFU/ml (Bahroudi *et al.*, 2020).

In this particular study, the MSC secretome from adipose tissue was tested using a different approach to assess its antibacterial activity compared to previous research. The antibacterial activity was evaluated using the Kirby-Bauer method, based on the inhibition zone produced by the MSC secretome against *P.aeruginosa* bacteria isolated from diabetic ulcer patients. The antibacterial activity exhibited by the MSC secretome at various concentrations was relatively weak and categorized as moderate. It is worth noting that the *P.aeruginosa* bacteria used in this study were directly isolated from diabetic ulcer patients who had received prior antibiotic therapy. Therefore, the previous antibiotic exposure will likely have an impact on the sensitivity of *P. aeruginosa* bacteria (Kunz Coyne *et al.*, 2022).

Conclusion

The secretome of MSC (Mesenchymal Stem Cells) exhibits antibacterial activity against *P. aeruginosa* isolated from diabetic foot ulcer patients. Successively, the concentrations of MSC secretome at 1.25%, 2.5%, 5%, and 10% demonstrated average bacterial inhibition zones measuring 8.17 mm, 8.23 mm, 8.52 mm, and 9.30 mm, respectively. The antibacterial activity produced by all concentrations is categorized as moderate. Furthermore, the concentration does not significantly influence the inhibitory effect of MSC secretome on Pseudomonas aeruginosa isolated from diabetic foot ulcer patients.

Acknowledgement

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