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Ambroxol Therapy as an Antibiofilm Candidate in Diabetic Ulcer Patients

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ABSTRACT

Patients with diabetic ulcers are susceptible to infection because the tissue in their extremities is constantly exposed to various pathogenic bacteria, so biofilm formation plays an important role in developing the disease. Based on previous studies, ambroxol can inhibit the formation of biofilm bacteria. The purpose of this study was to identify biofilm-forming bacteria, prove that ambroxol has the potential to inhibit and eradicate biofilms formed by bacteria in diabetic ulcers and analyse the potential of ambroxol in eradicating biofilms formed by bacteria in diabetic ulcer patients based on PEDIS and SEM scores. The method used was a 96-well microtiter plate assay (MtPA) with crystal violet and MTT staining techniques, a test using human samples with a quasi-experimental method with sampling carried out by nonprobability sampling. The samples used were diabetic ulcer patients with culture results forming biofilms. The results obtained identified 3 clinical isolates that formed biofilms with 100% Gram-negative bacteria, including two isolates of *Pseudomonas aeruginosa* and one isolate of *Escherichia coli* with the ability to form biofilms are moderate. Ambroxol effectively inhibits and eradicates 50% of biofilms formed by diabetic ulcer bacteria at a minimum concentration of 1 mg/ml. Based on the PEDIS score and SEM images of diabetic ulcer patients, there was a decrease in the PEDIS score in each patient, with an average score of 2. Several studies have demonstrated that combining Ambroxol with antimicrobials can synergistically enhance the antibiotic's efficacy against biofilms. The co-administration of Ambroxol with other antimicrobial agents represents a promising approach to improving antibiotic effectiveness. Nevertheless, careful consideration must be given to the concentration of Ambroxol used.

Keywords: Diabetic Ulcer, Antibiofilm, Ambroxol, MtPA, PEDIS, SEM

INTRODUCTION

Diabetic foot ulcers are a chronic complication of diabetes mellitus, characterized by wounds on the surface of the foot, accompanied by internal tissue damage or tissue necrosis, with or without infection. This condition is associated with neuropathy and/or peripheral arterial disease in diabetic patients. The lifetime incidence rate of diabetes-related foot ulceration is 19-34%, with a yearly incidence rate of 2%. After successful healing, the recurrence rate of diabetes-related foot ulceration is 40% within a year and 65% within 3 years. Therefore, the prevention of diabetes-related foot ulceration is paramount to reduce the risk to the patient and the resultant economic and social burden on society (Nicolaas *et al*, 2023).

Patients with diabetic ulcer are highly susceptible to infections due to constant exposure of their extremities to various pathogenic bacteria. Biofilm formation plays a significant role in disease progression and contributes to antibiotic resistance in pathogens found in foot infections. Biofilm formation is considered one of the most important virulence factors in foot infections, as it protects bacteria from phagocytosis and facilitates antibiotic resistance. Biofilms act as a barrier, reducing the diffusion of antibiotics, antimicrobial proteins, lysozymes, and small-molecule antimicrobial agents such as defensins (Pugazhendhi & Dorairaj, 2018).

Biofilms are complex microbial communities of microbial cells embedded within a self-produced extracellular polymeric substance (EPS) matrix. This matrix comprises proteins, lipids, nucleic acids, polysaccharides, and other components that allow

bacteria to adhere to biotic and abiotic surfaces (Banerjee *et al.*, 2019). Around 65% of all bacterial infections in humans are estimated to be associated with biofilms (Balaure & Grumezescu, 2020). The presence of biofilms in diabetic foot wounds necessitates precise strategies for biofilm detection and appropriate therapy selection to achieve the desired clinical outcomes.

Ambroxol HCl, one of the most used microkinetic and expectorant drugs, is believed to have antibiofilm properties. Previous studies have demonstrated that Ambroxol significantly inhibits biofilm formation by *Proteus mirabilis* in a concentration-dependent manner, disrupts the structural integrity of *Pseudomonas aeruginosa* biofilms—making them thinner and more fragmented—and interferes with the reversible attachment, irreversible attachment, and maturation stages of biofilm formation (Abbas, 2013; Wang *et al.*, 2016). This study aims to identify the bacterial profile and biofilm-forming ability of bacteria in diabetic ulcers and determine Ambroxol's effectiveness in inhibiting and eradicating biofilms formed by diabetic ulcer bacteria analyse the potential of ambroxol in eradicating biofilms formed by bacteria in diabetic ulcer patients based on PEDIS and SEM scores.

RESEARCH METHODS

This study is an experimental laboratory study using diabetic ulcer patients whose culture results form biofilms. The method of sampling ulcer patients using a purposive sampling technique with the criteria of ulcer age of more than 3 weeks (indicating infection) and receiving antibiotic therapy. Ambroxol was then tested in vitro and applied directly to diabetic ulcer patients as a preliminary test. The potential of Ambroxol as an antibiofilm was assessed based on the parameters of the decrease in PEDIS scores before and after therapy ≥2 and SEM description. This study obtained ethical clearance from the FKKMK UGM Ethics Committee with reference number KE/FK 1056/EC/2022.

Tools

The tools used in this research are Autoclave Hirayama HVE-50®, Biosafety Cabinet Healforce 900LC®, Centrifuge Biocen 22 merk Orto Alresa, Elisa reader SMART Reader MR- 900®, CO_2/O_2 Incubator IN 55 MEMMERT®, Scanning Electron Microscope (SEM) JEOL JSM IT200®, Vortex DLAB MX – S®.

Materials

The tools used in this research are Isopropanol Acid, Ambroxol standard, Clinical isolates from diabetic ulcers, TSB (Tryptic Soy Broth) Merck®, PBS (Phosphate Buffered Saline) Merck®, NaCl, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Merck®, MacConkey agar Merck®, Crystal violet, Sterile containers, 96-well microplate Biologix®, 12-well microplate Biologix®.

Biofilm Formation Assay

Biofilm-forming bacteria were cultured by preparing a bacterial suspension (clinical isolates) by inoculating TSB with a bacterial loop and incubating for 24 hours. The overnight culture was checked to ensure turbid microbial growth. The overnight culture was centrifuged for 5 minutes at 3000 rpm and washed with 2 mL PBS. The pellet was resuspended with 3 mL NaCl and adjusted to a 0.5 McFarland standard to obtain a concentration of 10^8 CFU/mL, either by visual comparison.

A total of 100 µL of TSB was added to each well of a 96-well microtiter plate, followed by 10 µL of bacterial suspension (10^8 CFU/mL), maintaining a culture-to-medium ratio of 1:10. A plate containing only the medium was used as a negative control. The plate was sealed with parafilm and incubated at 37°C for 24 hours.

After incubation, the wells were carefully emptied using a multichannel micropipette without touching the walls or bottom of the wells. The wells were washed twice with 200 µL PBS. Then, 125 µL of 0.1% w/v crystal violet solution was added to each well and incubated at room temperature for 10-15 minutes. The plate was washed 3-4 times by submerging it in a water-filled container. The plate was inverted and placed on tissue paper to dry completely.

To dissolve the crystal violet stain, 125 µL of 96% ethanol was added to each well. The plate was incubated at room temperature for 10-15 minutes, and absorbance was measured using a plate reader at 595 nm. Biofilm formation was interpreted based on the OD values obtained, as shown in the table below (Singh *et al*, 2017).

Table 1. Interpretation of Biofilm Formation Test

No.	Average OD Value	Biofilm Production
1	≤ ODc / ODc < ~ ≤ 2x ODc	Non-producing/weak producing Biofilm
2	2x ODc < ~ ≤ 4x ODc	Moderate
3	> 4x ODc	Strong

Note: ODc: mean OD negative control + 3 x SD negative control SD: standard deviation

Biofilm Inhibition Assay

Ambroxol solutions were prepared at concentrations of 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml dan 5mg/ml. Ambroxol solution (100 µL) was added to the wells in column 1 along with 90 µL of liquid TSB medium at a final concentration of 5mg/ml. In column 2, 110 µL of TSB and 80 µL of Ambroxol solution were added to achieve a concentration of 4mg/ml.

Column 3 contained 130 µL of TSB and 60 µL of Ambroxol solution for a concentration of 3 mg/ml. Column 4 contained 140 µL of TSB and 40 µL of Ambroxol solution 2mg/ml, while column 5 had 160 µL of TSB and 20 µL of Ambroxol solution 1mg/ml.

Test bacteria (10 µL) were added to each column. Several control wells were prepared: Negative control (growth control, 100% growth): 90 µL liquid medium + 10 µL overnight culture**,** Solvent control: 100 µL Ambroxol solution (propylene glycol + distilled water)**,** Media control: 100 µL TSB**,** Blank control: Empty well.

The microtiter plate was incubated at 37°C for 24 hours. After incubation, the medium was carefully discarded, and wells were washed three times with 200 µL PBS. Then, 100 µL of MTT solution was added to each well containing biofilm cells. The plate was sealed and incubated at 37°C for 2 hours. After incubation, the lid was removed, the solution was discarded, and 100 µL of 5% isopropanol acid was added. The plate was incubated at room temperature for 1 hour. Absorbance was measured using an ELISA reader at 590 nm. The percentage of biofilm inhibition was calculated using the following formula (Kwasny & Opperman, 2010).

$$\% \text{ Inhibition} = \frac{\text{OD Average growth control-OD test sample average}}{\text{OD average growth control}} \times 100$$

Biofilm Eradication Assay

Bacterial isolates were grown in a 96-well microtiter plate with TSB medium and incubated at 37°C for 24 hours. After incubation, the supernatant and planktonic cells were carefully removed, and the wells were washed three times with 200 µL PBS. A serial dilution of Ambroxol concentrations was prepared in a separate 96-well plate. A total of 100 µL of Ambroxol solution + liquid medium was added to the wells containing biofilm. The plate was incubated at 37°C for 24 hours. After incubation, the medium was discarded, and the wells were washed three times with 200 µL PBS.

A total of 100 µL of MTT solution was added to each well containing biofilm cells. The plate was sealed and incubated at 37°C for 2 hours. The lid was then removed, the solution was discarded, and 100 µL of 5% isopropanol acid was added. The plate was incubated at room temperature for 1 hour. Absorbance was measured using an ELISA reader at 590 nm. The sample concentration that degraded at least 50% of biofilm formation was considered the MBEC50 (Minimal Biofilm Eradication Concentration), determined using the following formula (Kwasny & Opperman, 2010).

$$\% \text{ Eradication} = \frac{\text{OD Average growth control-OD test sample average}}{\text{OD average growth control}} \times 100$$

Testing on Diabetic Ulcer Patients

The patient identification process was carried out with utmost respect for the patient's privacy and dignity. The patient or the patient's family was fully informed about the study. If they were willing to participate, they were given further information and asked to sign an informed consent. Patients willing to participate in the study were further recorded for their identities and other related data such as comorbidities, history of drug use, clinical outcomes achieved, and other therapies currently being received, acknowledging their significant role in the study.

After the patient's wound was cleaned, regular and systematic observations were made to assess the initial PEDIS score, and a biopsy was performed using a scalpel.

The ulcer tissue was then prepared before being observed using SEM. The cleaned ulcer was then treated with an ambroxol solution. Therapy with ambroxol was carried out consistently every time the patient changed the bandage. After one month of ambroxol therapy, the PEDIS score was re- evaluated, and a biopsy was performed using SEM, maintaining the regularity of our follow- up assessments.

PEDIS Score Assessment

PEDIS Score is A scoring system used to assess the severity of infection in diabetic ulcer patients. Internal medicine specialists performed it at dr. Sardjito General Hospital. Several components assessed are: (Chuan *et al*., 2015).

Ulcer Tissue Preparation

The ulcer tissue was fixed using a 3% glutaraldehyde solution for 30 minutes. After that, it was meticulously rinsed using sterile PBS pH 7.4 thrice for 10 minutes each, ensuring no blood remained on the tissue. The dehydration process was then carried out using graded ethanol: 50% ethanol for 10 minutes, 70% ethanol for 10 minutes, and 96% ethanol for 20 minutes. The ulcer tissue was transferred to a clean container and dried for at least 1x24 hours.

Tabel 2. Skor Skala PEDIS

No.	Description	Value	Interpretation
1	Perfusion	0	No PAD
		1	PAD, No CLI
		2	CLI
2	Extent/size in mm3	0	Skin Intact
		1	<1 cm2
		2	1 – 3 cm2
		3	>3 cm2
3	Depth/tissue loss	0	Skin Intact
		1	Superfacial
		2	Fascia, muscle, tendon
		3	Bone or Joint
4	Infection	0	None
		1	Surface
		2	Abses, fascitis or septic arthritis
		3	SIRS
5	Sensation	0	No Loss
		1	Loss

Scanning Electron Microscope (SEM) Analysis

The prepared dry ulcer tissue was attached to an aluminium stub using conductive adhesive (carbon tape). Then, the dried ulcer tissue was sputter coated using gold (au) for 30 seconds at 10mA. After that, the stub sample was inserted into the SEM vacuum chamber. Imaging and analysis were carried out with SEM parameters of acceleration voltage, namely 15 kV, and magnification of 1000x, 3000x, and 5000x.

RESULTS AND DISCUSSION

Bacterial Profile and Biofilm Strength in Diabetic Ulcers

A total of 3 isolates were obtained with moderate biofilm-forming abilities. This is presented in Table 3 below.

Table 3. Biofilm Formation Ability Test

No.	Sample Code	Biofilm-Forming Bacteria	Biofilm Strength
1	2202 SAM	*Pseudomonas aeruginosa*	Moderate
2	2321 WAS	*Escherichia coli*	Moderate
3	2320 HAR	*Pseudomonas aeruginosa*	Moderate

The prevalence of pathogenic microorganisms in diabetic ulcers can vary depending on the duration, previous antibiotic use, geographic association, and nosocomial infections (Drago *et al*., 2019). Based on Table 3, it can be seen that the pathogenic bacteria that form biofilms in diabetic ulcers are gram-negative bacteria with a percentage of 100% (3 samples) with details, namely *Pseudomonas aeruginosa* (66,7%) with the ability moderate biofilms, *E. coli* (33,3%) forming moderate biofilms. The Enterobacter group (20%) consists of *Enterobacter aerogenes* and *Enterobacter cloacae* with strong biofilms.

The results obtained are like the research conducted by (Chai *et al*., 2021). Their research explained that gram- negative bacteria were relatively large based on the distribution of bacteria, as many as 54.9% of all isolated strains, which showed an increasing trend from 2016 to 2019. Research conducted by (Goh *et al*., 2020) also provided results, namely that the most common gram- negative bacteria found in diabetic ulcers were Pseudomonas aeruginosa, as much as 35%. Meanwhile, the latest research (Maulidiah, 2024) concluded that the pattern of bacteria that infect diabetic ulcers is gram-negative bacteria at 68.81% and gram-positive bacteria at 31.81%.

One of the key mechanisms bacteria use in biofilm formation is quorum sensing (QS). QS is a signalling process where bacterial cells secrete signalling molecules to communicate with other cells in a population-density-dependent manner. Bacterial Communication is not limited to interactions within the same species but can also occur between different species, allowing bacteria to modify behaviours for communal benefits or competitive advantages (Doğaner *et al*., 2016).

In Table 3, *Escherichia coli* (33,3%) was one of the Gram-negative bacteria detected in diabetic ulcers with moderate biofilm-forming capability. *E. coli* is a typical intestinal microorganism in humans and

animals. While typically a harmless commensal, it can acquire mobile genetic elements encoding virulence factors, making it capable of causing a broad spectrum of intestinal and extraintestinal diseases (Pakbin *et al*, 2021).

An in vitro study by (Kamelija *et al*., 2018) found that insulin may contribute to the spread of *E. coli* in diabetic patients by increasing the expression of aspartyl proteinase, a virulence factor and autoinducer that facilitates quorum sensing. This aligns with the present study, as all diabetic ulcer patients included were undergoing insulin therapy. *E. coli* can adapt to wound environments by expressing virulence genes such as fimH, colicin genes, OmpT, cnf1, and hlyA (Pakbin *et al*, 2021).

Effect of Ambroxol on Inhibition and Eradication of Biofilm Bacteria

Figure 1 shows the percentage results of inhibition and eradication of ambroxol samples with various concentrations.

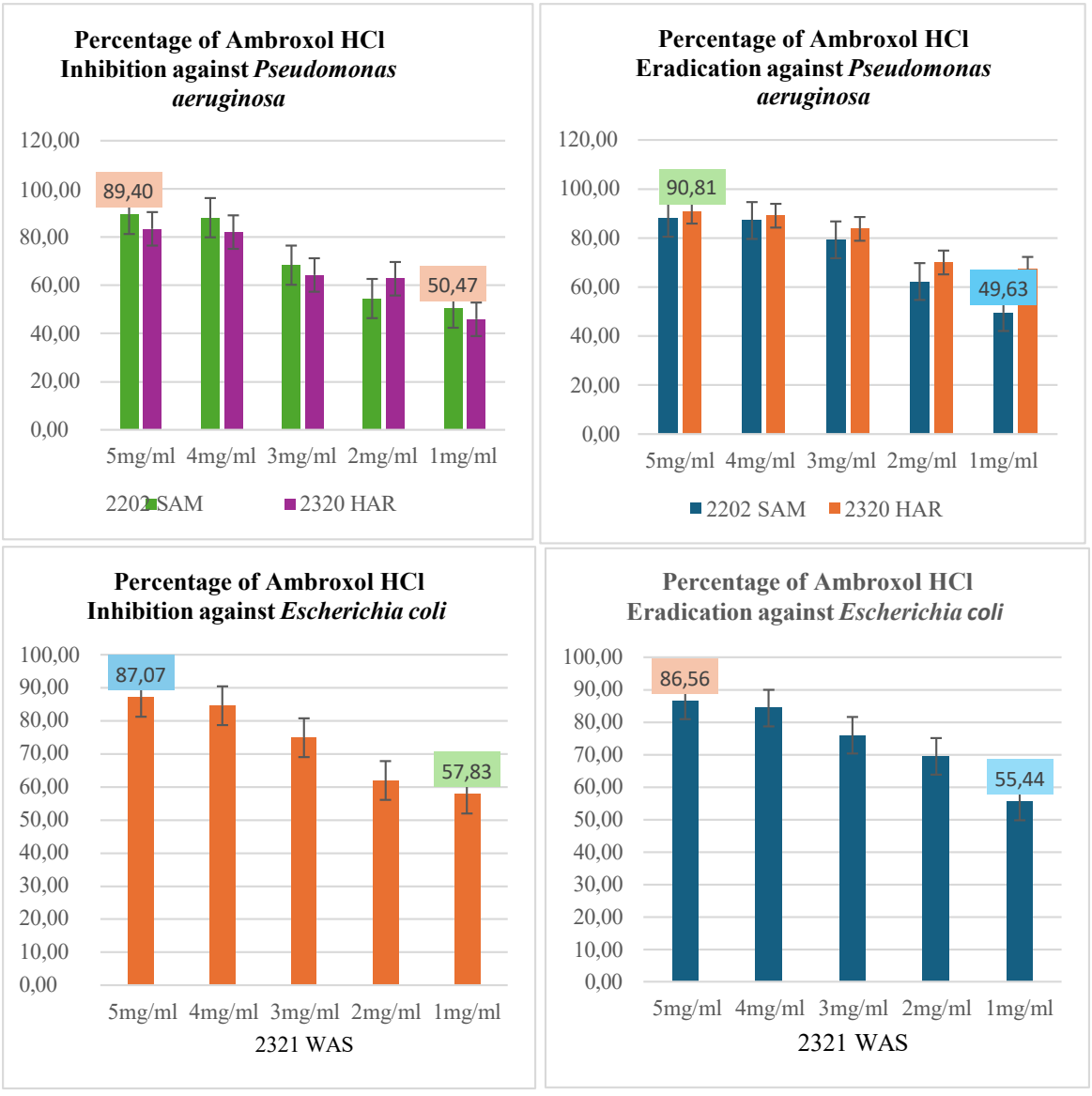

Figure 1. Percentage of Inhibition and Eradication of Biofilm-Forming Clinical Isolates with Ambroxol Samples

Based on Figure 1, ambroxol can inhibit 50% of all gram-negative biofilm bacteria at an average percentage of at least 59.27% at a concentration of 1mg/ml the highest at an average percentage of 94.04% with a concentration of 5mg/ml. For eradication, ambroxol can eradicate 50% of all gram-negative bacteria at an average percentage of at least 52,72% with a concentration of 1mg/ml and the highest average percentage of 88.69% with a concentration of 5mg/ml. The results obtained vary considerably, considering that the isolates used were clinical isolates of patients with different patient characteristics.

The differences in inhibition and eradication of each isolate are likely due to variations in strains and sensitivity patterns of bacteria. The results of the

research that has been carried out are in line with research conducted by (González *et al.*, 2024) that ambroxol significantly reduced the biofilm biomass after 24 and 48 h incubation of the four strains tested compared with the control without ambroxol. *E. coli* biofilm was reduced by 99.5% at 24 h and 98.1% at 48 h (*p*-value 0.005 and 0.003). Research on ambroxol's inhibition and eradication of biofilms is still very limited, but the research that has been carried out can add information and references to research on ambroxol's action on *E. coli* biofilms. As we already know, certain strains of *E. coli* are majorly responsible for morbidity and mortality, as in the cases of various medical device-associated infections such as urethral and intravascular catheters, prosthetic joints, and shunts and prosthetic grafts (Sharma *et al.*, 2016). Escherichia coli biofilms are found to be the primary causative agent of many intestinal infections. The dense bacterial cells in biofilm communicate with each other via the chemical signalling pathway known as quorum sensing (QS).

Now, *Escherichia coli* biofilm is resistant to several antibiotics, mostly due to putative multidrug resistance pumps. The development of the extracellular matrix and the observed increased resistance to common antibiotics create a challenge to controlling infections caused by *E. coli* biofilms. Recent advances have been in exploring and developing new approaches and therapeutic methods to cure *E. coli* biofilm-related infections (Sharma *et al.*, 2016).

The existence of reseacrh on ambroxol in inhibiting and eradicating E.coli biofilm is an initial step for further research on E.coli biofilm bacteria, especially those obtained from medical devices. Several studies have been conducted to test ambroxol as an antibiofilm, including research conducted by (Cataldi *et al.*, 2014) that ambroxol can be used as a prophylaxis and help treat respiratory tract infections caused by biofilm bacteria. A study conducted by (Lu *et al.*, 2010) to explore the hypothesis that ambroxol interferes with the quorum sensing of *Pseudomonas aeruginosa* will result in reduced biofilm formation.

The concentrations of ambroxol used were 1.875 mg/ml and 3.75 mg/ml. The results showed that ambroxol affects the quorum-sensing circuit of *Pseudomonas aeruginosa*. This observation demonstrates that the anti-quorum sensing properties of ambroxol will expand its potential use against organisms associated with chronic infections. In addition, ambroxol has the potential to be used in the treatment of patients with cystic fibrosis or the prevention of biofilms on devices. Furthermore, the potential of ambroxol to disrupt the integrity of the biofilm structure is intriguing and of interest.

Several studies on the synergistic effect of ambroxol with antibiotics in eradicating bacterial biofilms, namely research conducted by (Y. Zhang *et al.*, 2015) obtained the results that ambroxol exhibits significant efficacy to potentiate the bactericidal effect of vancomycin on *S. epidermidis* biofilm both in vitro and in vivo. The antibiotic lock therapy using a combination of ambroxol and vancomycin reveals a high ability to eradicate *S. epidermidis* biofilms in vivo.

These results provide the basis for a proper anti-infection strategy for treating the commonest cause of catheter-related bloodstream infections (CRBSIs). In addition, research conducted (Cheng *et al.*, 2015) was to test the effect of combined treatment (Ciprofloxacin plus erythromycin or ambroxol) on acute lung infection caused by *Pseudomonas aeruginosa* with biofilm formation in a rat model of endotracheal intubation and to compare the efficacy of the two combination treatments.

The results showed that erythromycin or ambroxol, in combination with ciprofloxacin, could eliminate *P. aeruginosa* biofilms. When combined with ciprofloxacin, ambroxol outperformed erythromycin in eradicating *P. aeruginosa* biofilm Li *et al* (Li *et al.*, 2017) researched the synergistic effects of ambroxol. They found that ambroxol hydrochloride (128μg/mL) exhibits synergistic antifungal effects in combination with fluconazole (2μg/mL) against resistant planktonic *Candida albicans* (*C. albicans*) cells. According to the microdilution method, this combination also showed synergistic effects against resistant *C. albicans* biofilms in different stages (4, 8, and 12 h). *In vitro* data were further confirmed by the success of this combination in treating *Galleria mellonella* infected by resistant *C. albicans*. Concerning the synergistic mechanism, our result revealed that ambroxol hydrochloride affects the drug transporters of resistant *C. albicans*, increasing the uptake and decreasing the efflux of rhodamine 6G, a fluorescent alternate of fluconazole.

This is the first study to investigate the *in vitro* and *in vivo* antifungal effects and the possible synergistic mechanism of ambroxol hydrochloride in combination with fluconazole against resistant *C. albicans*. The results show the potential role of this drug combination as a therapeutic alternative to treat resistant *C. albicans* and provide insights into the development of antifungal targets and new antifungal agents.

Based on the research results and Figure 1, it can be concluded that there is a correlation between the concentration of ambroxol and the percentage of inhibition and eradication; namely, the higher the

concentration of ambroxol, the higher the percentage of inhibition and eradication of the biofilm bacteria formed.

Ambroxol Test Results on Diabetic Ulcer Patients

This research, which conducted a preliminary test on 3 diabetic ulcer patients who received ambroxol solution therapy with different ulcer conditions, has the potential to significantly impact diabetic ulcer treatments. After administration of ambroxol solution therapy, the patient's ulcer conditions markedly improved. The image below presents the patient's clinical condition parameters, PEDIS score, and SEM observations, all of which point to a promising future for diabetic ulcer treatments.

Tabel 4. Diabetic Ulcer Patient Profile

No.	Sampel Code	Bacteria	Biofilm Strengh	Wound Healing Process	PEDIS Score
1	2202 SAM	*Pseudomonas aeruginosa*	Moderate	Improving	7◎5
2	2321 WAS	*Escherichia coli*	Moderate	Improving	7◎5
3	2320 HAR	*Pseudomonas aeruginosa*	Moderate	Improving	8◎6

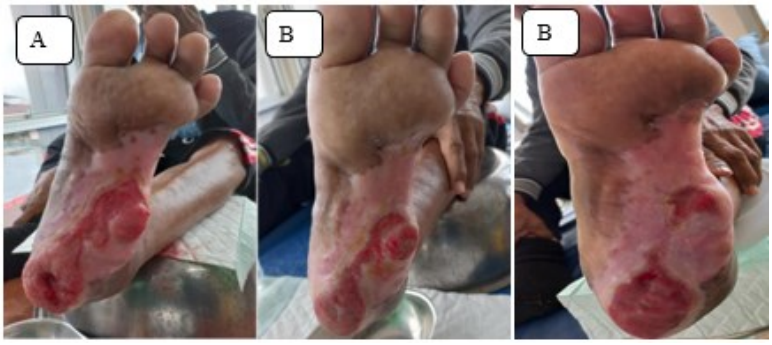

Figure 2. Sample PX 1. Code A patient's wound condition before administration of ambroxol solution. Code B1 patient's wound condition 1 month after administration of ambroxol solution. Code B2 patient's wound condition 2 months after administration of ambroxol solution. The pre- and post-images show a significant change in the patient's wound condition, with the PEDIS score decreasing from 7 to 5 over the course of our treatment timeline, demonstrating the progression of the treatment

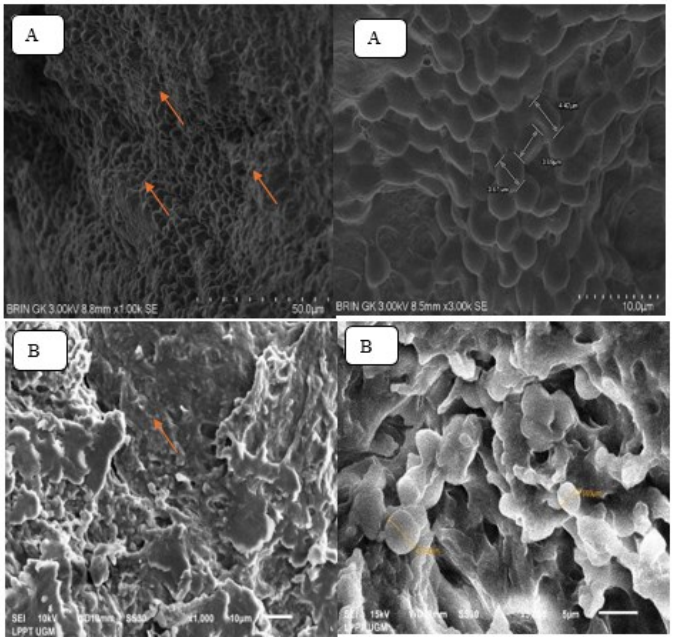

Figure 3. SEM image of ulcer tissue of patient PX1, Code A, before being given ambroxol solution with 1000x and 3000x magnification. The arrow is a thick layer of EPS matrix with bacterial sizes of 3.67 μm, 3.69 μm and 4.42 μm, indicating the presence of gram-negative bacteria, a concerning aspect of the ulcer condition. Code B is an SEM image of ulcer tissue after 1 month of ambroxol solution therapy with 100x and 3000x magnification. The arrow is a small layer of EPS matrix with bacterial sizes of 2.593 μm and 4.302 μm, which are also gram- negative bacteria

Sample PX1 is a patient who received ambroxol solution therapy. Based on Figure 2, the patient's ulcer condition is at a PEDIS score of 7, and 1 month after ambroxol solution therapy, the PEDIS score becomes 5. The results obtained are significant for the development of ulcers in sample PX1. In SEM observations (Figure 3), before therapy, a thick matrix layer was seen, and based on the size of the bacteria, the results obtained were gram-negative bacteria, namely *Pseudomonas aeruginosa* with sizes of 3.67 µm, 3.69 µm, and 4.42 µm. Furthermore, the SEM results of 1 month of ambroxol therapy showed that the remaining EPS matrix layer was small, and the size of the bacteria was 2.593 µm and 4.302 µm, which detected *Pseudomonas aeruginosa* bacteria.

In this PX1 sample, ambroxol solution effectively degrades the biofilm layer formed by bacteria. Ambroxol has been shown to inhibit biofilm formation in several microbial species, including *Pseudomonas aeruginosa* and *Candida albicans*. It disrupts quorum sensing, a key regulatory mechanism in biofilm formation, leading to reduced biofilm thickness and increased porosity (Lu *et al.*, 2010 ; Wahyuddin *et al.*, 2024) Besides that, ambroxol not only prevents biofilm formation but also disrupts pre-existing biofilms. This has been demonstrated in *Pseudomonas aeruginosa*, where ambroxol HCl treatment altered biofilm structure and significantly reduced bacterial counts (F. Li *et al.*, 2008, 2011).

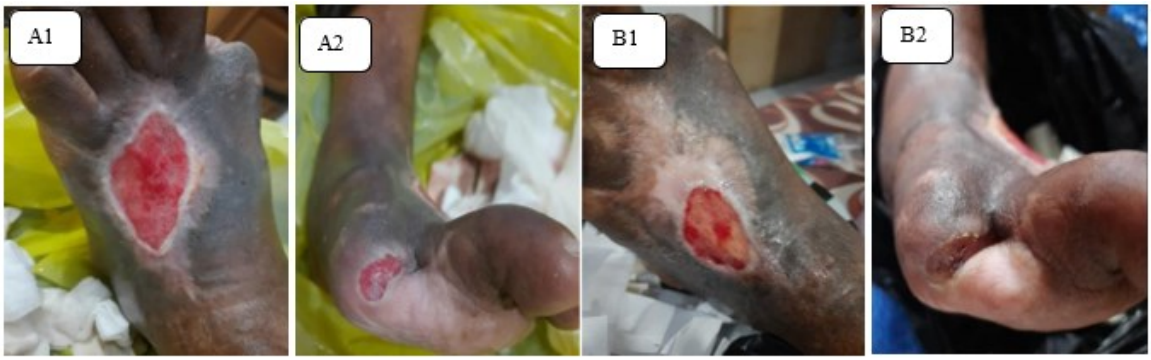

Figure 4. Sample PX 2. Code A is the patient's wound condition before administration of ambroxol solution. Code B is the patient's wound condition 1 month after administration of ambroxol solution. The pre- and post-images show a significant change in the patient's wound condition from the initial PEDIS score of 7 to a PEDIS score of 5

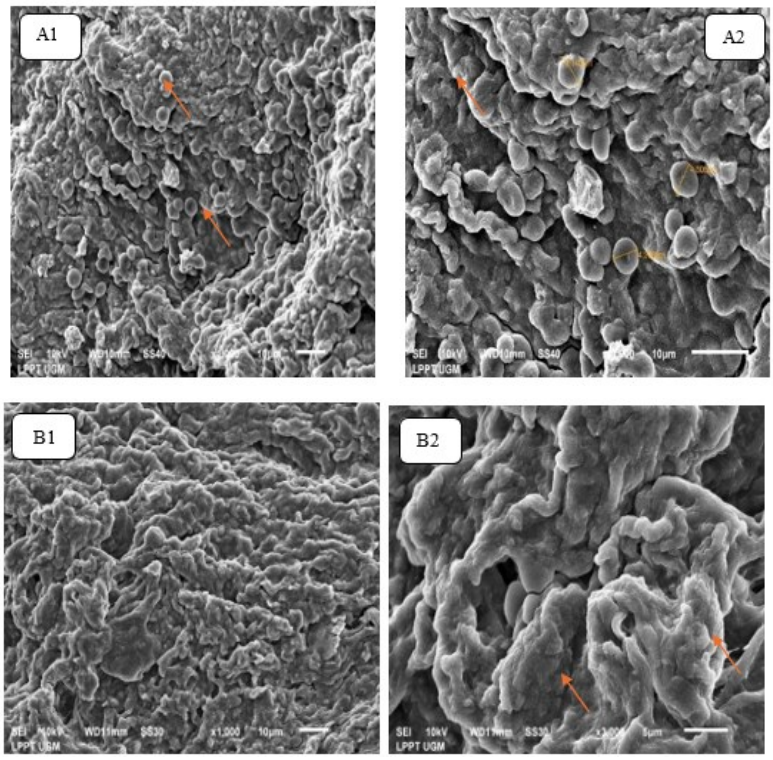

Figure 5. SEM image of ulcer tissue of patient PX2, Code A, before being given ambroxol solution with a magnification of 1000x, 3000x and 5000x. The arrow is a thick EPS matrix layer. The size of the bacteria is around 4µm, which is gram-negative bacteria. Code B is an SEM image of ulcer tissue after 1 month of therapy with magnification of 1000x, 3000x and 5000x. The arrow is a thin EPS matrix layer, a significant reduction from the initial thickness, with no visible bacterial growth

PX2 with *Escherichia coli* infection, it can be seen in Figure 4 that the initial condition of the patient's ulcer was at a PEDIS score of 7 to 5 after 1 month of ambroxol solution therapy. Figure 5 shows an SEM image of PX2 before therapy; a thick EPS matrix layer with a bacterial size of around 4 µm indicates gram-negative bacteria. After 1 month of therapy, a thin EPS matrix layer is visible, and the bacteria are not identified. Several previous studies have shown no data showing ambroxol's effect in inhibiting *Escherichia coli* biofilms.

However, the in vitro results obtained in this study showed that the effect of ambroxol could inhibit and eradicate biofilms produced by *Escherichia coli*. This research could potentially inspire new treatment strategies for *Escherichia coli* infections. In general, the mechanism of action of ambroxol is a surfactant that works by reducing surface tension so that it reduces the ability of bacteria to adhere to each other's surfaces, which inhibits the formation of more substantial and stable biofilms.

Disrupted adhesion causes bacterial cells to be more easily detached from the biofilm structure and return to planktonic form. Planktonic bacteria are more susceptible to antibiotic attacks and the body's immune response than bacteria hiding in biofilms (Lu *et al.*, 2010).

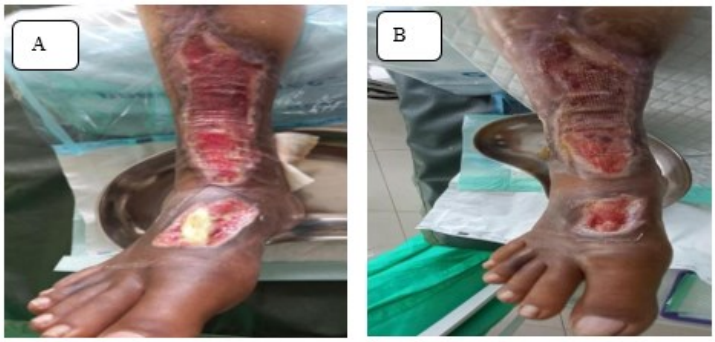

Figure 6. Sample PX3. Code A is the patient's wound condition before administration of ambroxol solution. Code B is the patients wound condition 1 month after administration of ambroxol solution. The pre-and post-images show a change in the patient's wound condition, as indicated by the reliable PEDIS score, which decreased from 8 to 6

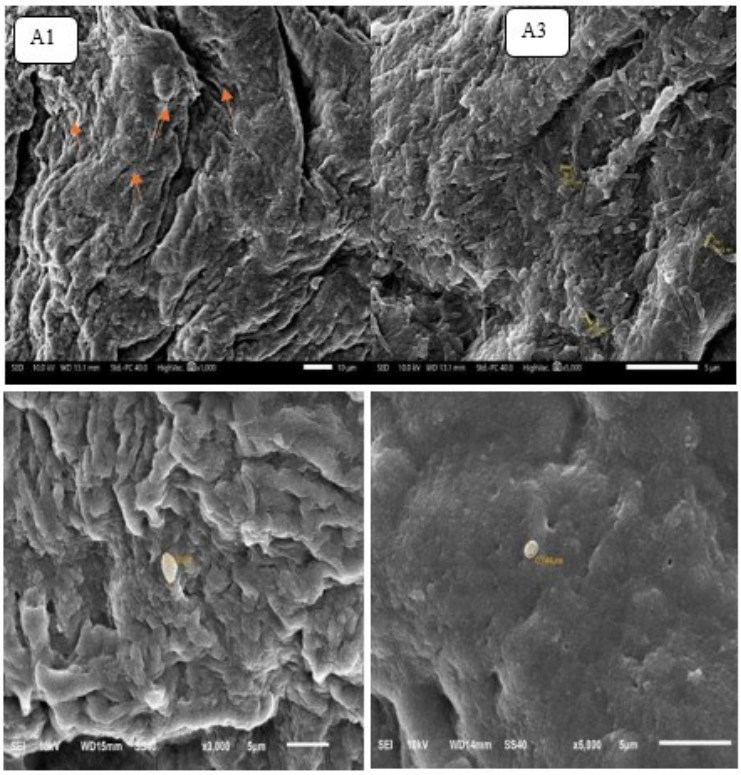

Figure 7. SEM image of ulcer tissue of patient PX3, Code A, before being given ambroxol solution with a magnification of 1000x, 3000x and 5000x. The arrow is a small layer of EPS matrix with bacterial sizes of 0.6893µm, 0.6174µm and 0.7635µm, indicating the presence of gram-negative bacteria. Code B is an SEM image of ulcer tissue after 1 month of ambroxol therapy with magnification of 3000x and 5000x. There is no EPS matrix layer with bacterial sizes of 0.744 µm, 2.485 µm and 2.510 µm.

The ulcer condition of the patient sample PX3 (figure 6) before receiving ambroxol solution therapy was at a PEDIS score of 8, and after 1 month of therapy, the PEDIS score became 6. In SEM observations before therapy (figure 7), a small layer of EPS matrix was seen with bacterial sizes of 0.6893 μm, 0.6174 μm and 0.763 μm, indicating gram-negative bacteria. After 1 month of therapy, the most intriguing observation was the complete disappearance of the EPS matrix layer, with bacterial sizes of 0.744 μm, 2.485 μm and 2.510 μm.

According to previous studies, ambroxol can influence bacterial cell death within biofilms through its surfactant properties via several mechanisms disruption of the Extracellular Polymeric Substance (EPS) Matrix; biofilms are protected by an extracellular matrix (EPS) rich in polysaccharides, proteins, and extracellular DNA, which shields bacteria within. Ambroxol reduces surface tension around the biofilm as a surfactant, weakening the bonds between EPS components.

This reduction in surface tension helps break down the EPS structure, making the biofilm more permeable to antimicrobial agents and allowing antibiotics or immune cells to penetrate the biofilm more effectively. As a result, bacteria within the biofilm become more vulnerable and are more likely to die (Lu *et al*., 2010 ; Zhang *et al.*,2013).

moreover ambroxol promotes the release or dispersal of bacterial cells from the biofilm by reducing surface tension. These detached bacterial cells often experience environmental stress and lose the protective advantages of the biofilm, making them more likely to undergo cell death due to direct exposure to antibacterial agents or external conditions (Cataldi *et al.*, 2014).

Research conducted by (Y. Zhang *et al*., 2015) also concluded that ambroxol enhances antibiotic penetration into biofilms by reducing biofilm viscosity and cohesion, with improved antibiotic penetration, bacteria hidden within the deeper layers of the biofilm become more exposed and vulnerable to eradication. This synergistic effect strengthens the antibacterial action of antibiotics, ultimately leading to increased bacterial cell death within the biofilm (Zhang *et al*., 2015). Although this research has many limitations, it is expected to be a basis and reference for the idea that ambroxol has the potential to be used as an antibiofilm in patients with diabetic ulcers.

CONCLUSION

Based on the research conducted, it can be concluded that diabetic ulcers in this study were characterized by a 100% prevalence of Gram-negative bacteria with moderate biofilm- forming capacity. Ambroxol demonstrated effective anti-biofilm activity, inhibiting and eradicating 50% of bacterial biofilms at a minimum 1 mg/ml concentration. Additionally, clinical observations using PEDIS scores and SEM imaging further support ambroxol's potential as a promising anti-biofilm agent for treating diabetic ulcer infections with an average decrease in pedis score of 2.

These findings highlight its therapeutic value in managing biofilm- associated complications in diabetic wound care. Several studies have demonstrated that combining Ambroxol with antimicrobials can synergistically enhance the antibiotic's efficacy against biofilms. The co-administration of Ambroxol with other antimicrobial agents represents a promising approach to improving antibiotic effectiveness. Nevertheless, careful consideration must be given to the concentration of Ambroxol used.

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