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Article

Identification of Klebsiella pneumoniae and the Inhibitory Effect of Soursop Leaves (Annona muricata L.) on Swab Samples from Diabetes Patients

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Yulia Ratna Dewi[1]*, Emma Ismawatie[1], Yulita Maulani[1]

[1]Bachelor of Applied Science in Medical Laboratory Technology, Politeknik Indonusa Surakarta, Surakarta, Indonesia

Abstract. Diabetes mellitus is a chronic metabolic disorder that increases susceptibility to bacterial infections, particularly in chronic wounds. This study aims to identify Klebsiella pneumoniae in wound swab samples from diabetic patients and evaluate the antibacterial activity of soursop leaf (Annona muricata L.) extract. Wound swabs were collected aseptically and analyzed through Gram staining, biochemical testing, and selective media to confirm the presence of Klebsiella pneumoniae. The antibacterial effect of the extract was tested at concentrations of 20%, 40%, and 60% using the well diffusion method, with gentamicin and chloramphenicol as positive controls. Phytochemical screening revealed the presence of secondary metabolites such as alkaloids, flavonoids, tannins, saponins, and steroids. Results demonstrated a concentration-dependent inhibition of Klebsiella pneumoniae, with the highest inhibition zone observed at 60%, though smaller than the standard antibiotics. This study highlights the potential of Annona muricata extract as a natural antibacterial agent for diabetic wound infections and supports its role in developing alternative therapies. Further studies are recommended to optimize its efficacy and explore clinical applications.

Corresponding Author :
Yulia Ratna Dewi
Bachelor of Applied Science in Medical Laboratory Technology, Politeknik Indonusa Surakarta, Surakarta, Indonesia
Email : yuliaratnadewi@poltekindonusa.ac.id

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia due to impaired insulin secretion or action. This condition is a leading cause of various serious complications in vital organs such as the heart, kidneys, and nerves. According to the 2021 report by the International Diabetes Federation (IDF), 537 million adults worldwide are living with diabetes, and this number is projected to rise to 643 million by 2030 [1-2]. Diabetes is among the top 10 global causes of death, accounting for 1.6 million deaths in 2021, nearly half of which occurred before the age of 70. Elevated blood glucose levels also contribute to 11% of deaths caused by cardiovascular diseases. The prevalence of diabetes continues to rise, particularly in low- and middle-income countries [3-4].

Various complications associated with diabetes mellitus contribute to an increased risk of bacterial infections, exacerbated by factors such as hyperglycemia, dysfunction of innate immune cells, and infections caused by antibiotic-resistant bacterial strains [5]. One of the key characteristics of diabetes is chronic hyperglycemia, which leads to elevated glucose concentrations in blood and tissues [6]. Glucose, as the primary carbon source, supports the growth and virulence of various bacterial pathogens, ultimately worsening infections in diabetic patients [5].

One of the bacterial pathogens frequently associated with diabetes-related infections is *Klebsiella pneumoniae*, a Gram-negative bacterium known as a leading cause of nosocomial infections. Serious complications in diabetic foot ulcers (DFUs) are often caused by polymicrobial infections, including aerobic Gram-negative bacilli such as *Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa*, as well as Gram-positive cocci such as *Staphylococcus aureus* [7-8]. *Klebsiella pneumoniae* is among the bacteria commonly involved in infections in diabetic wounds, particularly in DFUs. These infections can lead to severe complications, including gangrene, which may worsen the condition of diabetic patients [9]. The most common cause of DFUs (reported by 52.5% of respondents) is infection. Recurrent infections significantly complicate treatment and increase healthcare costs [10].

Klebsiella pneumoniae has the ability to cause various serious infections, including pneumonia, urinary tract infections, and wound infections, particularly in immunocompromised patients. Antibiotic resistance in this bacterium, especially against beta-lactams, further complicates the treatment of infections it causes [11]. This resistance is attributed to the ability of Klebsiella pneumoniae to produce beta-lactamase enzymes, which degrade the structure of beta-lactam antibiotics and reduce their effectiveness. Additionally, this bacterium possesses virulence genes such as MagA, which play a role in the production of exotoxins. These exotoxins not only increase the risk of sepsis but also cause tissue necrosis, making wounds difficult to heal [9].

In the search for alternative antibacterial therapies, studies on medicinal plants such as Annona muricata (soursop) have shown promising results. Extracts of A. muricata exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria compared to the standard antibiotic streptomycin. Other studies report that the bioactive compounds in A. muricata, such as alkaloids (annonaine, asimilobine, liriodenine, nornuciferine, etc.), target bacterial membranes (plasma and outer membranes), thereby producing broad-spectrum antibacterial activity [12-13].

Further research indicates that A. muricata leaf extracts demonstrate significant activity against various bacteria responsible for common infections. Extracts using n-hexane as a solvent exhibit strong inhibitory action against both Gram-positive and Gram-negative bacteria. The in vitro antibacterial activity of A. muricata Linn. leaf extracts has been proven effective against various pathogenic bacteria, including *Escherichia coli, Staphylococcus aureus, Acinetobacter baumannii, Moraxella catarrhalis, and Enterococcus faecium* [14]. Additionally, A. muricata extracts show significant antibacterial activity against Staphylococcus aureus and Escherichia coli, with minimum inhibitory concentration (MIC) values ranging from 156 µg/mL to 1,024 µg/mL. These findings highlight the potential of A. muricata as a source of novel phytochemical compounds to combat pathogenic microorganisms, particularly in the development of alternative therapies [15].

Based on this background, this study aims to evaluate the antibacterial activity of Annona muricata leaf extract against Klebsiella pneumoniae, one of the primary pathogens commonly found in wounds of diabetes mellitus patients. The findings of this study are expected to contribute to the development of alternative therapies based on natural compounds as effective antibacterial agents to address infections in diabetic wounds.

2. Experimental Section

2.1. Bacterial Identification

Bacteria were obtained from wound swabs of diabetes patients and placed in transport media. The swabs were then inoculated into Brain Heart Infusion (BHI) broth as a bacterial growth medium and incubated at 37°C for 24 hours. Subsequently, the BHI cultures were inoculated onto Mannitol Salt Agar (MSA) and Nutrient Agar (NA) and incubated for 48 hours. Bacterial identification was performed using Gram staining and biochemical tests.

2.2 Preparation of McFarland 0.5 Standard Solution

A bacterial suspension was prepared by taking one loop of pure bacterial culture and introducing it into a test tube containing 10 mL of 0.9% physiological NaCl solution. The solution was mixed until homogeneous, and the turbidity was adjusted to match the McFarland 0.5 standard.

2.3 Preparation of Bacterial Suspension

A bacterial suspension was prepared by taking one loop of bacterial culture and placing it into a test tube containing 10 mL of 0.9% physiological NaCl solution. The pure culture in the test tube was mixed until homogeneous and adjusted to the McFarland standard.

2.4 Preparation of Soursop Leaf Simplisia

Soursop leaves (Annona muricata L.) used for simplisia preparation were obtained from 1000 grams of fresh green soursop leaves. The leaves were separated from the stems, washed, and air-dried. The leaves were then dried in an oven at 60°C for three days. The moisture content of the dried leaves was measured using a moisture analyzer.

2.5 Preparation of Soursop Leaf Extract

The dried leaves were ground using a mixer and sieved to obtain a fine powder. The powdered leaves were macerated with 70% ethanol for three days. The maceration product was then evaporated for one day to obtain a thick extract. This extract was subsequently diluted to concentrations of 60%, 40%, and 20%.

2.6 Phytochemical Tests

2.6.1 Phenolic Test

A total of 1 mg of extract was added with two drops of 1% $FeCl_3$ solution. A color change to bluish-green indicated the presence of phenolic compounds.

2.6.2 Flavonoid Test

Two grams of the extract were mixed with 5 mL of ethanol and heated for five minutes. Concentrated HCl and 0.2 g of magnesium powder were then added. The appearance of a red color within three minutes indicated a positive result for flavonoids.

2.6.3. Tannin Test
One milligram of extract was boiled with 10 mL of water for 5–10 minutes, filtered, and the filtrate was treated with $FeCl_3$ solution. The appearance of dark blue or greenish-black coloration indicated the presence of tannins.

2.6.4. Steroid Test
Two grams of the extract sample were soaked in acetic anhydride on a drop plate for 15 minutes. Next, 2–3 drops of concentrated sulfuric acid were added. A blue color indicated the presence of steroids.

2.6.5. Saponin Test
Two grams of extract were boiled in distilled water for 2–3 minutes, cooled, and vigorously shaken. The formation of stable foam indicated the presence of saponins.

2.6.6. Alkaloid Test
Two grams of soursop leaf powder were mixed with a small amount of chloroform and ground into a paste. Subsequently, 10 mL of 0.05 N chloroform-ammonia was added, stirred, and the liquid extract was pipetted through a cotton filter. The extract was placed into a test tube and mixed with 5 mL of 2 N sulfuric acid, shaken vigorously to form two layers. The sulfuric acid layer was separated using a pipette and placed in a small test tube. Alkaloid presence was tested using Dragendorff's reagent, with a positive result indicated by the formation of an orange precipitate.

2.7. Antibacterial Testing Using the Well Diffusion Method
Soursop leaf extract was tested at concentrations of 60%, 40%, and 20%, with positive controls of Gentamicin and Chloramphenicol and a negative control of distilled water. Filter paper discs were soaked in the extract for 24 hours. Bacterial suspensions, standardized to 0.5 McFarland, were spread over agar media using sterile swabs. The soaked filter discs were placed on the agar media using sterile tweezers in the desired positions. The petri dishes were incubated at 37°C for 24 hours. After incubation, the inhibition zones around the discs were observed and measured to evaluate the antibacterial activity of the soursop leaf extract. The sample size for each treatment was calculated using the Federer formula:

$$(n-1)(t-1) \geq 15$$

where t the number of treatments, and n is the number of repetitions. In this study, there were four treatments (60%, 40%, 20%, and controls: Gentamicin, Chloramphenicol, and distilled water), with four repetitions per treatment, resulting in a total of 24 treatments.

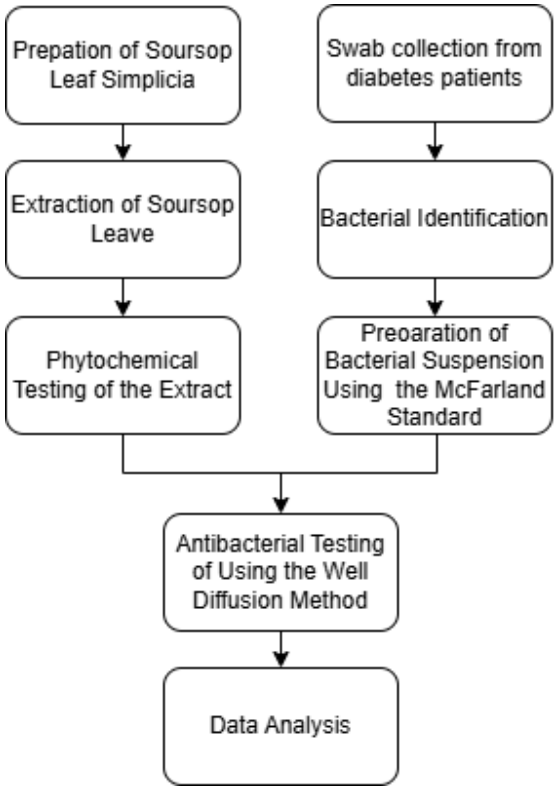

Figure 1. The experiment procedures

3. Results and Discussion

3.1 Phytochemical Testing of the Extract

The results of the phytochemical screening test on soursop leaf extract (Annona muricata L.) are presented in Table 1. Phytochemical screening is an initial step to detect the types of secondary metabolites contained in natural products.

Table 1. Qualitative phytochemical screening results of ethanol extract of soursop leaves

No	Fitokimia Test	Reactor	Observation	Results*
1.	Phenolic Test	$FeCl_3$ 1%	Formation of bluish-green color	+
2.	Flavonoid Test	Mg-HCl Concentrated	Formation of reddish color	+
3.	Tannin Test	$FeCl_3$	Formation of bluish-black color	+
4.	Steroid Test	Anhydrous acetic acid+ H_2SO_4	Formation of green color	+
5.	Saponin Test	H_2O	Formation of foam	+
6.	Alkaloid Test	Dragendorff	Formation of orange precipitate	+

*(+) Identified ; (-) Not Identified

Based on Table 1, the phytochemical screening results show that the ethanol extract of soursop leaves contains phenolics, flavonoids, tannins, steroids, saponins, and alkaloids [16]. These findings are consistent with previous studies that reported the presence of flavonoids, tannins, saponins, and terpenoids in the ethanol extract of soursop leaves. This study reinforces the understanding that soursop leaves contain bioactive compounds relevant to various pharmacological applications [17].

In general, soursop leaves are known as medicinal plants with anti-diabetic, anti-inflammatory, insecticidal, antimalarial, anticancer, antibacterial, and antioxidant activities [18]. The measurement of flavonoid content revealed that 1 gram of ethanol extract from soursop leaves contains 8.32 mg quercetin equivalent. The antioxidant activity of this extract is demonstrated by its ability to scavenge DPPH free radicals, with an IC50 value of 56.73 ppm [19]. Previous studies have also noted that quercetin in flavonoids can disrupt the integrity of bacterial cell membranes, inhibiting the growth of both Gram-positive and Gram-negative bacteria [20-21]. Phenolics, together with flavonoids, play a significant role in antimicrobial activity, which is particularly relevant for clinical applications in treating diabetic foot wounds [22].

Another study reported that bioactive compounds in A. muricata, such as alkaloids (annonaine, asimilobine, liriodenine, nornuciferine, etc.), attack bacterial membranes (plasma and outer membranes), resulting in broad-spectrum antibacterial activity [12]. Bioactive compounds in Annona muricata, such as annonaceous acetogenins, exhibit antimicrobial activity through mechanisms such as cell membrane disruption, enzyme inhibition, interference with DNA replication, and protein synthesis. Its high phytochemical content also inhibits ATP production and mitochondrial function, while synergistic effects among the compounds enhance antimicrobial activity against various microorganisms [23].

Flavonoids exert antibacterial effects by disrupting bacterial cell membranes, inhibiting protein synthesis, and interfering with metabolic pathways [24]. Flavonoids damage cell structures, causing cell lysis, and may also inhibit the synthesis of essential enzymes and affect the expression of genes associated with virulence and biofilm formation [25].

The extract of Annona muricata (soursop) leaves exhibits antimicrobial bioactivity, influenced by the presence of flavonoids, steroids, and alkaloids. These compounds work synergistically to inhibit the growth of microorganisms. Alkaloids in the extract are known to bind to microbial DNA, inhibiting RNA synthesis and glycosidase activity [27]. Flavonoids, such as quercetin, inhibit cytoplasmic membrane function and DNA synthesis by binding to the GyrB subunit of E. coli DNA gyrase and inhibiting ATPase enzymes [28]. Additionally, phenylphenol compounds play a role in binding to microbial membrane proteins, disrupting their functions, and affecting vital enzyme activity [27].

The antibacterial mechanism of polyphenols includes cell membrane disruption, inhibition of protein synthesis through ribosome binding, and the suppression of critical metabolic enzymes. Polyphenols also reduce virulence gene expression, inhibit biofilm formation, and enhance bacterial sensitivity to antibiotics [29]. This study provides scientific evidence supporting the potential use of soursop leaves as a natural alternative in adjuvant therapy, particularly for combating antibiotic-resistant bacterial infections [30].

3.2 Bacterial Identification

The bacterial identification process was performed on wound swab samples from diabetes mellitus patients. Samples were incubated using BHA (Blood Heart Agar) and NA (Nutrient Agar) media to achieve optimal bacterial growth [31]. After incubation, Gram staining was conducted to identify the bacterial characteristics. The staining results revealed that the isolated bacteria were Gram-negative, characterized by a thin cell wall with minimal peptidoglycan layers. In Gram staining, the bacteria appeared rod-shaped, red-colored, and exhibited a dispersed arrangement.

Cultivation on selective MacConkey Agar (MCA) resulted in round, small, pink-colored colonies with a mucoid and smooth texture. To further support the identification, a series of biochemical tests were performed, including Methyl Red-Voges Proskauer (MR-VP), Citrate, Urea, SIM, Triple Sugar Iron Agar (TSIA), and lactose and sucrose fermentation, as summarized in Table 2.

Table 2. Results of identification on biochemical media

Bacteria	Sampel	Biochemical Testing											
		MR	VR	Citrat	Urea	SIM			TSIA			Carbohydrate Fermentation	
						Indol	Motil	H2S	FERM	H_2S	Gas	Laktosa	Sukrosa
Klebsiella pneumoniae	Sampel 1	-	-	–	+	-	-	-	AC/AC	+	+	+	+
Klebsiella pneumoniae	Sampel 2	-	-	–	+	-	-	-	AC/AC	+	+	+	+
Klebsiella pneumoniae	Sampel 3	-	-	–	+	-	-	-	AC/AC	+	+	+	+
Klebsiella pneumoniae	Sampel 4	-	-	–	+	-	-	-	AC/AC	+	+	+	+

In the MR-VP test, a positive result in the Methyl Red test indicates that the bacteria can produce acid during metabolism. The SIM test confirms the production of indole and bacterial motility. The Urea test yields positive results, indicating the ability of the bacteria to break down urea into ammonia. In the TSIA test, glucose fermentation and gas production are clearly identified. Furthermore, a positive result in the Citrate test demonstrates the bacteria's ability to use citrate as a carbon source [32]. Based on all the biochemical test results, the isolated bacteria were identified as Klebsiella pneumoniae.

This identification aligns with the characteristics of Klebsiella pneumoniae as a Gram-negative, encapsulated, and non-motile bacterium. This bacterium is also known for its ability to ferment lactose, which is a key feature in its identification [33]. Previous studies have shown that gangrene, a severe complication involving tissue necrosis in diabetes mellitus (DM) patients, is often caused by bacterial infections such as Klebsiella pneumoniae [34]. Zuliana et al. reported that Klebsiella pneumoniae is the most dominant bacterium found in diabetic wounds, with a prevalence of 15% [35]. Diabetes mellitus significantly increases the risk of invasive Klebsiella pneumoniae syndrome (KPIS) due to impaired innate immunity against virulent strains of K. pneumoniae [36].

Research by Kesuma et al. also identified Klebsiella pneumoniae as one of the primary causes of pus in wounds of diabetic patients [37]. This bacterium is frequently found in diabetic foot ulcers, especially in patients with poor glycemic control [7]. The presence of a polysaccharide capsule in Klebsiella pneumoniae enhances its virulence by protecting it from phagocytosis. Additionally, this bacterium tends to develop resistance to various antibiotics, making infection treatment more challenging [38].

Klebsiella pneumoniae exploits a weakened immune system and wound conditions that favor its growth, thereby worsening the state of uncontrolled diabetic patients [39].Protease and lipase enzymes

produced by Klebsiella pneumoniae play a crucial role in tissue damage in diabetic wounds. This process not only exacerbates the patient's condition but also increases the risk of serious complications. These enzymes facilitate the release of nutrients necessary for bacterial growth [40-41]. Diabetic patients often experience immune system dysfunction, further aggravated by high blood glucose levels. This impairs the function of immune cells, allowing Klebsiella pneumoniae to proliferate rapidly [42]. Moreover, Klebsiella pneumoniae has the ability to form biofilms—organized microbial cell clusters embedded in extracellular polymeric substances (EPS) [43].

The ability to form biofilms is an important virulence trait of several microorganisms, including Klebsiella pneumoniae—a Gram-negative, encapsulated bacterium frequently associated with nosocomial infections [44]. These biofilms enhance bacterial resistance to antibiotics and shield them from immune system attacks. This ability makes Klebsiella pneumoniae one of the most challenging pathogens to manage, particularly in diabetic foot ulcers [45-46]. The findings of this study identified that wound infections in diabetic patients can be caused by one of the bacteria, Klebsiella pneumoniae, which is commonly found in the foot wounds of diabetic patients.

3.3 Antibacterial Testing Using the Well Diffusion Method

The results of antibacterial testing using the well diffusion method showed varying sizes of inhibition zones for each treatment, as presented in Table 3.

Table 3. Diameter of the inhibition zone of the fermentation product extract of Annona muricata L. with different ethanol concentrations

No	Extract Treatment (B)	Total	Average
1	Gentamicin	11	2.75
2	Chloramphenicol	13.15	3.2875
3	60%	10.3	2.575
4	40%	8.7	2.175
5	20%	7.3	1.825
6	Control -	0	0

The data shows a positive correlation between the extract concentration and its antibacterial potential, with higher concentrations resulting in larger inhibition zone diameters. However, the antibacterial effectiveness of Annona muricata L. leaf extract remains lower than that of the positive control. Statistical tests, including normality, homogeneity, and ANOVA analysis, were conducted to evaluate the obtained data. Table 4 presents the results of the normality and homogeneity tests, as well as the significance based on a One-Way ANOVA.

Table 4. Results of normality and homogeneity testing

Treatment	Normalitas P-Value (>0,05) *Saphiro-Wilk*	Homogenitas *P-value(>0,05) Levene Test*	*P-Value One Way* ANOVA
Gentamicin	0.151	0.133	0.0000
Chloramphenicol	0.298		
60%	0.283		
40%	0.441		
20%	0.298		
Control -	0		

The results of the study indicate that the ethanol extract concentration of Annona muricata L. leaves significantly affects antibacterial activity. The inhibition zone diameter increased with higher extract concentrations, as observed at concentrations of 60% (2.575 cm), 40% (2.175 cm), and 20% (1.825 cm). However, the effectiveness of this extract remains lower compared to positive controls, Gentamicin (2.75 cm) and Chloramphenicol (3.2875 cm).

Gentamicin and Chloramphenicol were used as standards in this study due to their well-established effectiveness as antibiotics [47]. The antibacterial activity exhibited by the Annona muricata L. leaf extract is likely attributed to its bioactive compounds, such as acetogenins, alkaloids, flavonoids, saponins, and tannins [48-49]. These compounds have mechanisms that can damage bacterial cell membranes, inhibit protein synthesis, or disrupt bacterial metabolism, ultimately inhibiting the growth of Klebsiella pneumoniae [48][50-52]. For instance, flavonoids are known to reduce free radicals, which can damage the bacterial cell structure, while acetogenins act by inhibiting key enzymes in bacterial respiration [53].

The statistical analysis conducted showed that the data met the assumptions of normality and homogeneity for Gentamicin and the ethanol extract of Annona muricata L. leaves. A one-way ANOVA analysis yielded a p-value < 0.05, indicating a significant difference in antibacterial activity among the treatment groups. An increase in extract concentration corresponded to an enhancement in antibacterial efficacy, suggesting a positive correlation between the concentration of bioactive compounds and their antibacterial activity [54-55].

The inhibitory effect produced by the extract of Annona muricata L. leaves, although lower compared to standard antibiotics, provides an initial indication that this extract has potential as an alternative antibacterial agent. Based on the classification of inhibitory effects according to Davis and Stout, the Annona muricata L. leaf extract at concentrations of 60% and 40% falls into the category of very strong inhibition ($\geqslant$20 mm), while at a concentration of 20%, it is categorized as strong inhibition (10 – 20 mm). This indicates that a higher concentration of bioactive compounds results in a more significant inhibitory effect against Klebsiella pneumoniae [56]. Nevertheless, the antibacterial efficacy of the soursop leaf extract is not yet optimal compared to Gentamicin and Chloramphenicol. Therefore, further development is required to enhance the extract's efficacy. One potential approach is to optimize the extraction method to increase the concentration of bioactive compounds [57]. For instance, using solvents that are more specific to certain compounds, employing ultrasound-based extraction methods, or utilizing liquid-liquid extraction techniques to improve extraction efficiency. Additionally, formulating the extract into pharmaceutical forms such as nanoparticles or emulsions could also influence its antibacterial activity [58].

The combination of Annona muricata L. leaf extract with other antibacterial agents, whether natural or synthetic, can also be explored to produce a stronger synergistic effect. The stability of the extract during storage, its toxicity to human tissues, and its efficacy in clinical applications are important aspects that need to be further evaluated to ensure the safety and effectiveness of this extract.Overall, this study supports the potential of Annona muricata L. leaves as an alternative antibacterial agent. Although its inhibitory effect is not yet equivalent to standard antibiotics, the bioactive compounds in the extract demonstrate significant antibacterial effects. With further development, soursop leaves have the potential to become an innovative, nature-based solution to combat bacterial infections, particularly in diabetes patients who are prone to infections such as Klebsiella pneumoniae.

4. Conclusion

This study successfully identified Klebsiella pneumoniae from wound swab samples of diabetic patients, confirming its role as a common pathogen in diabetic wound infections. The antibacterial activity of Annona muricata leaf extract demonstrated a concentration-dependent inhibition of Klebsiella pneumoniae, with the 60% concentration showing the highest inhibitory effect.

Phytochemical analysis revealed the presence of bioactive compounds, including alkaloids, flavonoids, tannins, saponins, and steroids, which contribute to its antibacterial properties. Although the inhibition zones of the extract were smaller compared to standard antibiotics such as gentamicin and chloramphenicol, the findings highlight the potential of Annona muricata as a natural antibacterial agent. Further research is needed to optimize extraction methods, enhance the bioactive compound concentrations, and evaluate the clinical application of the extract for diabetic wound infections. These results support the development of alternative therapies based on natural products to address bacterial resistance and improve patient outcomes.

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