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Nanogel of Lollipop Leave Extract: A Promising Antibiofilm Agent for Diabetic Ulcer Infections

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Abstract

Treatment of diabetic ulcers presents a considerable healthcare obstacle due to their susceptibility to infection by antibiotic-resistant bacteria that may develop a long-lasting biofilm. The development of bacterial resistance to antimicrobial drugs significantly impacts the process of wound healing and poses a potential risk to both human life and the economy. To overcome this challenge, the integration of the antibacterial properties of lollipop leaf extract with a nanogel delivery technology has the potential to provide a new treatment strategy for addressing biofilm-related infection in diabetic ulcers. Therefore, this study aims to assess the capacity of lollipop leaf extract nanogel to expedite the process of wound healing in diabetic ulcers. The materials used were first subjected to a 5-day extraction process using 96% ethanol. To evaluate the antibacterial effectiveness, microdilution and well methods were used for the mid-phase, maturation phase, and eradication stages. Antibiofilm analysis was then conducted in vivo using adult mice that were induced with type II diabetes. The results showed that at a concentration of 30% w/v, lolipop-derived nanogel extract (LDNE) exhibited potent antibiofilm activity against *Staphylococcus aureus* (SA) in the mid-phase, maturation, and eradication phase. This indicated that LDNE had a concentration-dependent suppressive impact on the proliferation of *Staphylococcus aureus* biofilm

Keywords: Biofilm; Lolipop Extract; Nanogel; Diabetic Ulcer.

1. Introduction

Diabetes mellitus is a persistent disease that has become more prevalent globally [1] . A common complication of diabetes, known as diabetic ulcers, frequently causes persistent infection and poses risks in the healing process [2] . These ulcers are prone to infection by antibiotic-resistant bacteria capable of forming persistent biofilm [3] . Several studies have shown that antimicrobial hydrogels have the potential to manage infection, offering the ability to enable tailored release of antimicrobials [4] . According to previous data, 19 to 34% of diabetes patients experience urinary kidney disease (UKD) in their lives, which increases the risk of mortality by 50 to 68%. This indicates the importance of prevention and effective management, specifically in glycaemic control and foot wound care [5].

The occurrence of infection in UKD is a crucial complication that necessitates prompt medical intervention due to its heightened potential for amputation [6] . In addition, bacterial resistance to antimicrobial agents is a major factor that influences wound healing and is a potential threat to human life as well as the economy. Considering the increase in bacterial resistance to antibiotics and wounds, novel regime strategies are essential to prevent biofilm and its associated infection [7]. Biofilm is a group of microorganisms surrounded by extracellular polymeric substance (EPS), playing a significant role in UKD by impeding the healing of wounds [8] . UKD biofilm infection is characterized by the presence of multiple types of bacteria that interact synergistically, forming the primary obstacle to the healing process [9] . In addition, *Staphylococcus aureus* (SA) is responsible for about 27.8% of UKD and contributes to the formation of biofilm. According to previous studies, biofilm protects bacteria and ensures resistance to antibiotics, a phenomenon known as MRSA, by preventing the antibiotics from reaching the bacterial cells [10]. The other bacteria responsible for causing UKD include

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Escherichia coli (10.15%), *Proteus mirabilis* (8.17%), *Proteus vulgaris* (6.19%), and *Pseudomonas aeruginosa* (6.19%). *Staphylococcus aureus* proved to be highly resistant to the antibiotics penicillin, azithromycin, clarithromycin, and erythromycin [11], fluoroquinolones, tetracyclines, with resistance rates ranging from 50% to 90% [12].

The potential of natural compounds from plant sources to serve as antibiofilm agents is explored, with essential oils demonstrating significant efficacy [13] . Combining the antimicrobial potential of lollipop leaf extract with a nanogel delivery system could provide a novel therapeutic approach to combat biofilm-associated infection in diabetic wounds [14].

To enhance the efficacy and durability of lollipop leaf extract, a suitable formulation is required. Nanogel is a highly promising drug delivery system due to its minute particle size, which enables superior penetration into biofilm and infected tissues. Nanogel can enhance the stability of active ingredients and facilitate the controlled release of drugs. Therefore, this study aims to evaluate the potential of lollipop leaf extract nanogels in accelerating wound healing in diabetic ulcers.

2. Experimental (Materials and Methods)

Samples of lolipop leaf (*Mitragyna speciosa*) were collected in the Forest of Kuranji District, Tanah Bumbu Regency, South Kalimantan. The process of identifying plants was conducted in the Basic Laboratory of the Faculty of Mathematics and Natural Sciences (FMIPA) at Universitas Lambung Mangkurat. Ethanol 95% (Merck, Germany), ethyl acetate (Merck, Germany), chloramphenicol (Sigma-Aldrich, Germany), phosphate-buffered saline (PBS; Sigma—Aldrich), McFarland (HiMedia, India), CMC-Na (Merck, Germany), Oxoid (Brain Heart infusion) (Merck, Germany), crystal violet (Merck, Germany), Carbopol (Merck, Germany), Trietanolamine (Merck, Germany), Gliserol (Merck, Germany), Propilenglikol (Merck, Germany) were the materials used in this study.

The instruments used were micropipette (Gilson, France), microtiter plate reader (OIS 2100, Spain), tube (Socorex, Swiss), LAF (Sakura, Japan), incubator 2B (Sakura, Japan), Microplate flat-bottom polystyrene 96 well (Iwaki, Japan), spektro genesys (Thermo Scientific Spectronic, USA), autoclave (Sakura, Japan), analytical balances (Ohaus, USA). Lollipop leaf samples were rinsed with flowing water and meticulously inspected for any contaminants. After washing, the plant samples were sliced and desiccated in an oven at 40°C for 3 hours. Sample standardization was conducted using precise criteria, and the Simplicia was extracted by fully immersing 300 g of powder in 96% ethanol. The mixture was agitated every 6 hours and replaced the solvent every 24 hours, resulting in a total of 5 repetitions or changes of solvent.

In this study, the liquid extract was subjected to evaporation in a water bath maintained at a temperature of 50°C until the weight reached a steady state. The levels of flavonoids, phenolics, alkaloids, saponins, quinones, steroids, and terpenoids were assessed using the tube technique. In addition, the antibacterial efficacy was assessed by using microdilution and well methods. The experiment was conducted on a 96-well flat-bottom polystyrene microtiter plate, applying different concentrations of test compounds (10%, 20%, 30% w/v) against *Staphylococcus aureus*. Chloramphenicol was used as the positive control. Concentrations of the bacterial suspension in BHI were adjusted to 0.5 McFarland units and then diluted to achieve a concentration of 5x106 colony-forming units per milliliter (CFU/mL). The microplate was filled with a solution of ethanol with a concentration of 96% and subjected to incubation for 15 minutes. Optical density measurements were conducted using ELISA Reader at a wavelength of 570 nm.

Evaluation of Biofilm Formation during the Intermediate Phase (24 hours) and the Maturation Phase (48 hours) was conducted using the Microbroth Dilution Technique. The microtiter plates were washed with distilled water to eliminate cells that were not adhered to and then left to dry in the ambient air at room temperature for 5 minutes. This was performed to assess the efficacy of the tested extract in preventing the development of biofilm. Subsequently, a 125 µL aliquot of a 1% crystal violet solution was added to each well to stain the viable and non-viable cells, as well as any other constituents of biofilm. After incubating the plate for 15 minutes at room temperature, it was rinsed with running water to remove the purple coloration. Each well was supplemented with 200 µL of 96% ethanol, and the optical density (OD) was quantified using a microplate reader at a wavelength of 595 nm [15].

Nanogel lolipop leaf extract, with the bacterial test suspension, was placed onto a microtiter plate, including coverslips. The plate was then placed in an incubator set at a temperature of 37°C for 24 to 72 hours to facilitate biofilm formation process. In vivo antibiofilm studies were tested using adult rats (*Rattus norvegicus*) weighing between 150 to 200 g. The animals were housed in a regulated setting and given unrestricted access to water and food.

A combination of streptozocin and nicotinamide was administered to induce type II diabetes. The animals received an intraperitoneal injection of nicotinamide at a dosage of 240 mg/kg after a 12-hour fasting period. I.P. administration of streptozocin at a dosage of 100 mg/kg occurred after 15 minutes. After the streptozocin treatment, the animals were fasted for 12 hours, and measured their fasting blood glucose levels 72 hours later. This study classified rats as diabetic only when their fasting blood glucose level exceeded 150 mg/dL [16]. Rats were divided into 3 groups, namely positive, negative, and the highest concentration of gel, and were sedated by inhaling 10% ether before the injury. The fur surrounding the back was cleansed with 70% alcohol and shaved to a 3 cm diameter. In addition, every sample animal received the same treatment. Rats' back was cut with a sterile slingshot, creating an incision that was 1 cm long and 2 mm deep, and administered bacteria to prevent infection, positive control using chloramphenicol, and applied once a day to the wound until the wound was closed.

After incubating the plate for 15 minutes at room temperature, we rinsed it with running water to remove the purple coloration. Each well was supplemented with 200 µL of 96% ethanol, and the optical density (OD) was quantified using a microplate reader at a wavelength of 595 nm [15].

The nanogel lolipop leave extract, together with the bacterial test suspension, was placed onto a microtiter plate including coverslips. The plate was thereafter placed in an incubator set at a temperature of 37°C for a duration of 24-72 hours to facilitate the biofilm formation process.

In vivo antibiofilm studies were tested using adult mice (Rattus norvegicus) weighing between 150-200 g. The animals were housed in a regulated setting and given unrestricted access to water and food. A combination of streptozocin and nicotinamide was administered to induce type II diabetes. The animals received an intraperitoneal injection of nicotinamide at a dosage of 240 mg/kg after a 12-hour fasting period. 15 minutes later, I.P. administration of streptozocin at a dosage of 100 mg/kg occurred. After the streptozocin treatment, we fasted the animals for 12 hours and measured their fasting blood glucose levels seventy-two hours later. The research classified animals as diabetic only if their fasting blood glucose level exceeded 150 mg/dL [16] . Mice were divided into 3 groups, namely positive, negative, and the highest concentration of gel. The researchers made an open incision on the rat, measuring 1 cm in diameter, and administered bacteria to prevent infection. positive control using chloramphenicol and applied once a day to the wound until the wound is closed.

3. Results and discussion

In this study, the formulation and evaluation of nanogel lollipop leaf extract as a promising antibiofilm agent for the treatment of diabetic ulcer infection was reported. The extract was first characterized for its phytochemical composition and antimicrobial activities, followed by the development and optimization of a nanogel formulation to improve the stability and bioavailability of the active compounds. In vitro, antibiofilm assays were conducted to assess the efficacy of nanogel formulation against clinically relevant biofilm-forming bacteria isolated from diabetic ulcers. The potential of this novel nanogel system to improve wound healing and reduce the burden of biofilm-associated infection in diabetic ulcers was discussed. The presence of biofilm created by pathogenic bacteria exacerbated the infection in diabetic ulcers [17].

Fig 1. Percentage of Inhibitory Activity of Lolipop Leaf Extract Against Mid-Phase (24 hours) *Staphylococcus aureus* Biofilm.

The presence of this biofilm conferred protection to microorganisms against the immune response of the body and conventional antibiotic therapy, thereby posing significant challenges in the treatment of infection [18] . Flavonoids and quinones in the extract had gained significant attention in antimicrobial studies due to their potent

antibacterial and antibiofilm activities [19]. Flavonoids were effective at killing bacteria because of their damage to the membranes, stopping the imitation of DNA, lowering the function of their efflux pumps, and stopping the creation of adenosine triphosphate [20].

During the mid-phase biofilm, lolipop-derived nanogel extract (LDNE) at a concentration of 30% w/v indicated strong antibiofilm activity against *Staphylococcus aureus*, with an inhibition percentage of 86.94% ± 0.006. This inhibition was not significantly different ($p > 0.05$) from the control drug at a concentration of 1% w/v, which showed an inhibition percentage of 83.58% ± 0.0042 (Figure 1). The result in this study suggested that inhibiting bacteria in biofilm formation was more challenging, as the inhibition percentage decreased when compared to bacteria in planktonic form [21].

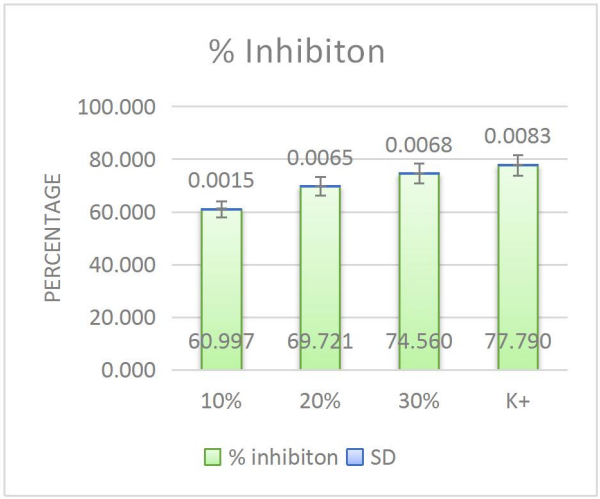


Fig 2. Percentage of Inhibitory Activity of LDNE Against Maturation Phase (48 hours) *Staphylococcus aureus* Biofilm

Presenting LDNE at a concentration of 30% w/v resulted in an inhibitory activity of 74.56% ± 0,0068 during biofilm development phase. This result did not exhibit a significant difference ($p>0.05$) compared to the inhibitory efficacy of the pharmacological control, which revealed a higher inhibitory efficacy of 77.79% ± 0.083. The available data provided evidence that an increase in the length of biofilm formation was associated with an increase in the organization of the matrix, resulting in a more robust and intricate biofilm structure [22].

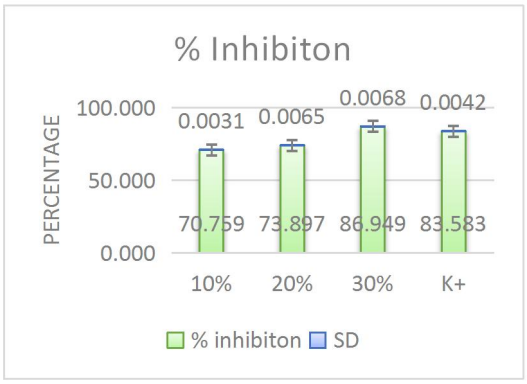
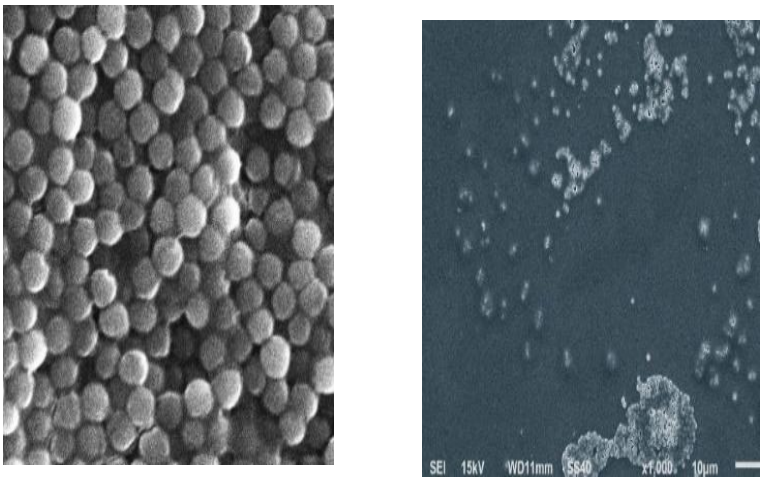


Fig 3. Percentage of *Staphylococcus aureus* Biofilm eradication activity from LDNE

During the eradication, LDNE at a concentration of 30% w/v indicated strong antibiofilm activity against *Staphylococcus aureus*. This inhibition was not significantly different ($p > 0.05$) from the control drug with 1% concentration. The primary mode of action when plant-based solutions combated mature or pre-formed biofilm appeared to be specifically targeting EPS [23]. Plant extracts, derivatives, or chemicals mostly exerted their effects on different structural elements of EPS [24]. This suggested that lolipop leaf extract contained compounds that could disrupt the formation or integrity of bacterial biofilm, potentially enhancing the efficacy of existing

antibiotics or serving as an alternative treatment option. In addition, this also indicated that lollipop leaf extract had chemicals in it that could stop bacterial biofilm from forming or staying together. This could make antibiotics perform better or suggest another way to treat the infection [25].



(a) (b)

Fig 4. Figure 4a Result of Scanning Electron Microscopy Biofilm with No Treatment, Figure 4b Result of Scanning Electron Microscopy Biofilm with administration of kratom extract 30% b/v.

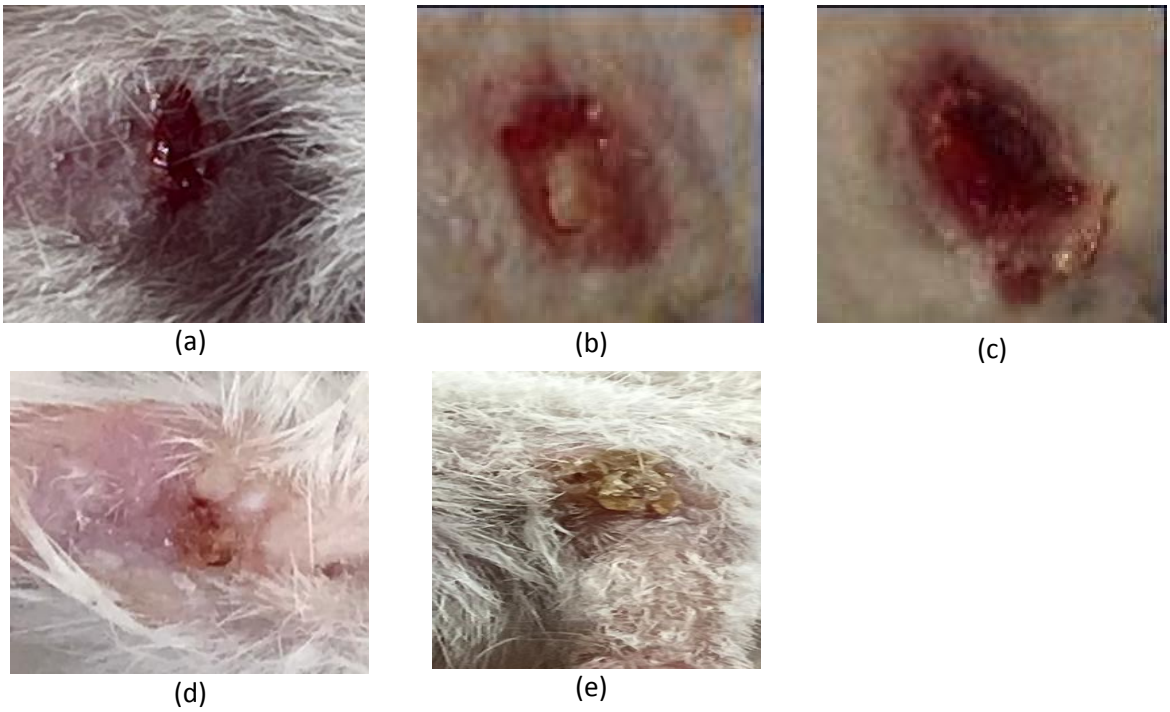


Fig 5. 5a. untreated group; 5b. erythema and swelling after administration of *Staphylococcus aureus*; 5c. Occurrence of Ulcers; 5d. the ulcer begins to dry up; 5e. The wound begins to close.

Pathogenic bacteria could colonize the ulcer, and immunological deficiencies associated with diabetes made infection more likely [26]. The pathogens involved in this infection ranged from aerobic to anaerobic species, with *Staphylococcus aureus* serving as the causative agent. When bacteria formed biofilm, their cells were enclosed in a self-produced polymer matrix, which shielded the cells from the immune system and antibiotics [25]. Consequently, the presence of biofilm on an ulcer was responsible for delayed healing and the chronicity of the subsequent infection.

Table 1. Results of the Effectiveness Test of Nanogel Preparations on Infected Mice

Sample	Wound Healing (Day)											
	1	2	3	4	5	6	7	8	9	10	11	12
K-	•• **	•• **	•• **	•• **	•• **	•• **	•• **	•• **	•• * +	• * +	• +	• +
K+	•• **	•• **	•• **	• • * +	• • * +	•* +	•* +	• * +	•+	+	√	
Gel 30%	•• **	•• **	•• **	•• **	•• *+	•• *+	•* +	•* +	•* +	• +	+	√

Description: K-: group without treatment; K+: Group given chloramphenicol; Gel 30%: Group given K gel; •: Erythema and Infection; *: Swelling; +: Wound begins to close; √: Wound closes

Table 2. Wound Diameter

Sample	Wound Healing Day (cm)											
	1	2	3	4	5	6	7	8	9	10	11	12
K-	1,00	0,98	0,97	0,94	0,90	0,88	0,85	0,79	0,76	0,73	0,71	0,69
K+	1,00	0,96	0,92	0,87	0,83	0,76	0,71	0,66	0,59	0,52	0,47	0,41
Gel 30%	1,00	0,95	0,92	0,89	0,87	0,83	0,78	0,76	0,72	0,67	0,56	0,49

Nanogel administration yielded results that were not significantly different based on healing time or wound area closure (Tables 1 and 2). The results indicated that LDNE exhibited a concentration-dependent inhibitory effect on the growth of *Staphylococcus aureus* biofilm. Moreover, the significant inhibitory activity observed at higher concentrations suggested that LDNE could potentially serve as a natural antibacterial agent, which was consistent with previous studies that documented the effectiveness of plant-derived substances against several bacterial strains, including *Staphylococcus aureus* [27]. The identification of these active compounds could lead to the discovery of novel therapeutic agents that not only inhibited bacterial growth but also modified resistance mechanisms in *Staphylococcus aureus*, thereby enhancing the overall effectiveness of antibacterial treatments derived from natural sources [28] . In addition, the synergistic effects of combining lolipop leaf extract with conventional antibiotics could further improve the therapeutic strategies against *Staphylococcus aureus*, as evidenced by studies showing that natural products could enhance the efficacy of existing antibacterial treatments when used together [29].

4. Conclusions

In conclusion, the results suggested that LDNE had a concentration-dependent suppressive impact on the proliferation of *Staphylococcus aureus* biofilm. The effectiveness of plant-derived substances against various bacterial strains, including *Staphylococcus aureus*, potentially served as a natural antibacterial agent.

5. Conflicts of interest

The authors declare no conflict of interests.

6. Formatting of funding sources

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