

Original Research Article

Modulation of *Staphylococcus haemolyticus*-infected diabetic wounds using *Gongronema latifolium* extract

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Abstract

Purpose: To assess the antibacterial efficacy of *Gongronema latifolium* on resistant bacteria isolated from infected diabetic wound sites.

Methods: *Staphylococcus haemolyticus* was obtained from wound samples (n = 159) of hospitalised patients using standard cultural procedures and identified with 16S rDNA molecular typing. The following antibiotic resistance pattern (chloramphenicol, gentamicin, rifampicin, tetracycline, cefotaxime, ceftazidime, amikacin, ceftriaxone, ciprofloxacin and levofloxacin) was determined using the disc diffusion method. In vivo wound healing capacities of the leaf extract were carried out using four groups of rats (A - D), each containing four rats, induced intraperitoneally with freshly prepared alloxan monohydrate (150 mg/kg) injected after an 18-hour fast and compared with a standard antibiotic (ciprofloxacin, 500 mg/kg). Histopathology was done at the end of the study, haematology, and microbial counts were used as monitoring parameters for wound healing process of the *G. latifolium*.

Results: *Staphylococcus haemolyticus* showed resistance to 50 % of tested antibiotics. The extract exhibited a Minimum Inhibitory Concentration (MIC) of 62.5 mg/mL and a Minimum Bactericidal Concentration (MBC) of 500 mg/mL against *Staphylococcus haemolyticus*. There was histological evidence on phagocytosis and re-epithelization of traumatised tissue sites after periods of administering both medicinal plants. Inflammatory cells were seen to be evident in both untreated and treated groups of non-diabetic and diabetic rats, which portrays the role of innate immunity in modulating *S. haemolyticus* infection in the wound sites. Noticeable changes ($p < 0.05$) were observed in neutrophil and lymphocyte values, while monocyte values showed no significant variation. Eosinophils and Basophils were not expressed.

Conclusion: *Gongronema latifolium* is a potent, available and sustainable alternative to antibiotic use in the management of bacterial-infected wounds, especially with *Staphylococcus* infection.

Keywords: Antibiotic resistance, *Staphylococcus haemolyticus*, Diabetic wounds, *Gongronema latifolium*

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INTRODUCTION

Diabetic wounds/ulcers commonly occur in patients with either type 1 or type 2 diabetes [1].

This situation gets exacerbated when the ulcers get further infected, which most times may lead to amputation of affected body parts. The growing challenge of antibiotic resistance has

complicated infection management, driving the search for more sustainable alternatives. The use of medicinal plants as potent antibiotic alternatives has been proposed [2]. Furthermore, the use of phytotherapy for diabetes control has been investigated and documented by some researchers [3,4]. Phytotherapy involves the application of therapeutic plants with anti-hyperglycaemic activity for diabetes management. They have remained as dietary sources and have been reported to give a positive impact on fasting plasma glucose, arterial blood glucose and glycated haemoglobin (HbA1c) [5].

Among several uses, *Gongronema latifolium*, popularly called Utazi in Igbo, Arokeke in Yoruba, Utasi in Efik and Ibibio, is a medicinal plant as well as a food ingredient commonly used in different parts of West Africa, including Nigeria [6]. *Gongronema latifolium* is a member of the Apocynaceae family, though it was formerly classified under Asclepiadaceae. It is grown widely throughout the tropical and sub-tropical countries such as Nigeria, Guinea-Bissau, Western Cameroon, Ghana, Senegal, Côte d'Ivoire, and Sierra Leone, and is propagated easily using seed or stem cuttings. Leaves and stems of this plant have been reported to possess several health and nutritional benefits, such as antiparasitic [7], antihypertensive [8], anti-inflammatory [9], antibacterial [10] and also anti-diabetic activities [5]. These listed medicinal activities were achieved both through oral and topical applications of extracts of *G. latifolium*.

Staphylococcal infection of diabetic wounds or other body systems has been reported to occur from either the patient's personal environment or as a nosocomial infection [10]. Similarly, studies have reported microbial prevalence in diabetic patients, indicating high infection susceptibility in such conditions [11]. *S. haemolyticus* is a species of Staphylococcus that is coagulase-negative, mainly known for its involvement in nosocomial infections [12]. *Staphylococcus haemolyticus* strains isolated from clinical specimens have been said to be unaffected by several drugs [13] and possess antibiotic resistance against penicillin, oxacillin, ciprofloxacin, erythromycin, gentamicin and vancomycin [14]. It has been reported to possess genes that aid its antibiotic resistance and alternative measures to control infections caused by this microbe have been proposed. Among such opinions are improvement of disinfection and hygiene in households and hospitals, the use of probiotics, and medicinal plants [13].

This present study thus sought to control *S. haemolyticus* infection in diabetic wounds in a hospital-based system, using the leaves extract of *G. latifolium*.

EXPERIMENTAL

Sample size

The sample size for this study was determined using Cochran's formula (Eq 1)

$$n = \frac{Z^2 P q}{d^2} \dots\dots\dots (1)$$

Where n = sample size to be calculated; Z = standard normal deviation at 95% confidence interval (1.96); P = the proportion of prevalence from a previous study = 11.7% or 0.117; q = complementary probability of P (1 - P) = 1-0.117 = 0.883; d = error margin/precision 0.05² [15]. Therefore, n = 159.

Study location and population

The study was conducted in the Medical/Surgical wards at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Amaku, Awka, Nigeria. One hundred and fifty-nine (159) male and female patients with diabetic foot ulcers between the ages of 18 - 75 years were treated.

Animal study

Male and female albino rats with weights ranging between 180 and 350 g each were used for the animal study. They were obtained from the Faculty of Biological Sciences, University of Nigeria, Nsukka and acclimatised for fourteen days. The rats were housed in a well-conditioned animal house and were fed two times a day with standard pellets and had unrestricted access to clean drinking water.

Ethical approvals

Two ethical approvals were obtained for this study. The first ethical approval was obtained from the Research Ethical Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Amaku, Awka, Anambra State, Nigeria, with number (COOUTH/CMAC/ETH C/VOL.1/FN:04/262) for access to wounds from hospitalised patients, while the second was from the Faculty of Biological Sciences Ethical Board, University of Nigeria, Nsukka, UNN/FBS/EC/1079. All procedures on humans followed the conditions stated in the Helsinki declaration [16], while

animals were handled according to the guidelines on the care and use of laboratory animals [17].

Collection of wound samples (from hospitalised patients)

A total of 159 human wound samples were taken with sterile swab sticks and put in an ice bag, which was then transported to the Microbiology Research Laboratory, Owerri, for microbiological analysis. A total of eighty-one samples were obtained from male patients, while seventy-eight wound samples were obtained from female patients.

Characterisation of bacterial isolates

Staphylococcus species (*S. aureus*, *S. epidermidis* and *S. haemolyticus*) were isolated on Mannitol salt agar (Hi-Media Labs) and were subjected to Gram staining, coagulase testing, and other standard biochemical assessments. Thereafter, antibiogram of isolates was determined according to the previously reported method [18]. The most resistant isolates were subjected to 16s rDNA sequencing for molecular identification employing the primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3', at MacroGen, South Korea.

Antibiotic susceptibility/sensitivity test

Commercial antibiotics (Oxoid; Chloramphenicol (30 µg), Gentamicin (30 µg), Rifampicin (5 µg), Tetracycline (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Amikacin (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (30 µg) and Levofloxacin (30 µg)) of specified concentrations were evenly spaced on freshly prepared, surface-dried Mueller Hinton Agar (MHA), previously inoculated with standardised pure cultures of *Staphylococcus* species. The plates were incubated at 37 °C for 24 h, after which the diameter of the zone of inhibition (ZOI) was recorded. The resistance, sensitivity, and intermediate responses of the organisms were assessed according to guidelines of the Clinical and Laboratory Standards Institute [18]. The most resistant isolate was selected for *in vivo* rat study.

Collection and preparation of plant materials

Fresh leaves of *Gongronema latifolium* were collected within the premises of Owerri West Local Government Secretariat at about 7:45 am on the 12th of July 2023 and identified by Mr Iroka Finian, a botanist in the Department of

Botany, Nnamdi Azikiwe University, Awka. A voucher specimen (NAUH 034C) was archived in the university's herbarium. The leaves were separated from their stems and air-dried on a clean surface at room temperature for 21 days until a constant weight was achieved. Once dried, they were finely powdered using an electronic grinder (Black and Decker 3-in-1 Mixer Grinder), measured, and stored in clean, airtight containers for further use, following previously established procedures [19].

Aqueous extraction was conducted by immersing 50 g of powdered leaves in 400 mL of distilled water, followed by heating in a water bath. The mixture was stirred every five minutes and left to stand for 24 hours before filtration through Ashless No. 42 filter paper. The filtrate was concentrated in a hot water bath at 80°C for 5 hours and subsequently refrigerated at 4°C for further analysis [20].

Antibacterial activity of leaf extract on *Staphylococcus haemolyticus*

The tube dilution method was initially employed to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the plant extract. A double dilution of 500 mg of the aqueous extract was performed serially in sterile distilled water to obtain concentrations of 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, and 15.625 mg/mL. Following dilution, 1 mL of each extract concentration was added to test tubes containing 1 mL of phosphate-buffered saline (pH 6.5) and 0.1 mL of *Staphylococcus* culture, which had been incubated for 24 hours. The tubes were then incubated at room temperature for 24 hours, and turbidity was assessed. The agar well diffusion technique was used to measure the inhibition zones of the MIC, with ciprofloxacin (500 mg) serving as the reference standard. A 100 µL sample of each plant extract concentration was introduced into individual wells in sterile mannitol salt agar, previously inoculated with *S. haemolyticus*. The plates were incubated at 28 °C for 24 h and the diameters of the inhibition zones were measured using a meter ruler [21].

***In vivo* examination of plant extract in wound healing**

Induction of diabetes

A total of sixteen rats were used for this study and grouped into four groups (A - D), each containing four rats. Group A. Normal control, B. Diabetic wound, no treatment. C. Diabetic

wound, treatment using a standard drug. D. Diabetic wound treatment using *G. latifolium*. Rats were brought into the animal house and allowed to acclimatize for a period of fourteen days. They were housed in a well-conditioned animal house and were fed two times a day with standard pellets and had unrestricted access to clean drinking water. Diabetes was induced in rats chemically as described previously [22]. Alloxan monohydrate, freshly prepared, at a dosage of 150 mg for each kilogram of body weight, was intraperitoneally injected into the rats after an 18-hour fast. The method adopted to manage mortality rate and improve the efficiency of alloxan monohydrate was similar to previously outlined standards [23]. Rats were placed on 10 % sucrose water to avoid sudden hyperglycemia. Accu CHECK Glucometer was employed to measure fasting blood glucose level in each rat in the tail-vein blood before and after the induction of diabetes. Rats exhibiting fasting blood glucose concentrations exceeding 250 mg/dL were classified as diabetic.

Wound incision, infection, treatment and wound size measurement

Before wound incision, all surgical instruments (scissors, forceps, blades and needles) were sterilised using iodine. Prior to wound incision, the incision sites were properly disinfected with iodine before wound excision to prevent wound infection. Slight modification in the previously established method of wound incision by Ghanbari *et al* [24] was adhered to in creating wound on the experimental animals. Rats were numbed using ketamine at a dose of 75 mg/kg with xylazine at a dose of 10 mg/kg given via the intraperitoneal route prior to making the incision on the wound. Using a flexible, clear plastic template a complete-thickness circular wound was made on the dorsal surface of each diabetic rat, and the diameter of the wounds on the rats was measured and noted down. Rats were randomly selected and placed in different demarcations of the cage. Wound sites were infected with a 0.1 mL aliquot of 5.2×10^5 CFU/mL of *S. haemolyticus*. Indication of pus and fluid accumulation was noted at 72 hours, confirming the onset of infection. Wound areas received treatment through the topical application of the plant extract formulation reconstituted in sterile distilled water from which a desired concentration of 5 %w/v (50 mg/mL) and 10 %w/v (100 mg/mL) was achieved immediately before use on the surface of the wounds, starting on day 1. The treatments were administered twice daily (morning and evening) over a period of 21 consecutive days. The control group followed the same procedure, except that no

plant extract was applied to the wound surface. In order to optimize the effect of the plant extract debridement using a scalpel, scissors and forceps were used carefully by a trained physician in accordance with the method employed by the National Institute for Health and Care Excellence [25]. The method adopted to determine the size of wounds was similar to that of Tan *et al* [26]. To avoid bias, the diameters of the wound were recorded by observers neutral to the experiment on various days with the aid of a flexible meter rule, a twine and a digital vernier calliper. The relative wound areas (RWA) and the percentage wound closure (WC) were calculated using Eq 2 [27].

$$WC (\%) = \left\{ \frac{IWA - nth DWA}{IWA} \right\} 100 \dots\dots\dots (2)$$

Where, IWA = Initial wound area, nth DWA = wound area on nth day

The graph depicts the percentage of wound closure against the number of days since the wound was created, and a linear regression analysis was conducted. The value of X when Y is set at 50 % was identified as CT₅₀ (half-closure time, the duration required for the wound to close by 50 %). Staphylococcal levels in infected tissues were assessed by culturing 0.1 mL of serially diluted (one in ten-fold dilution) tissue homogenates on mannitol salt agar at 28 °C for 24 hours, employing the pour-plate method. Differential blood cell counts (neutrophils, monocytes and lymphocytes) were also observed in the experiment according to methods prescribed by Cheesbrough [28].

Histopathology

Rats were sacrificed at the end of the experiment by cervical dislocation in accordance with the procedure outlined by Aguwa *et al* [29]. Wound tissues were immediately removed and preserved in 10 % formalin. The immobilized sections were cut and stained using Hematoxylin and Eosin (H & E) for further histopathological analysis as described by Gurina and Simms [30].

Statistical analysis

Analyzed samples were obtained in triplicate and mean values were deemed significant at $P < 0.05$, which was determined using GraphPad Prism software version 8.

RESULTS

Antibiotic resistance patterns of wound isolates

Staphylococcus haemolyticus showed resistance to chloramphenicol, gentamicin, rifampicin, tetracycline, cefotaxime, and amikacin (Table 1).

In vitro antibacterial activity of extracts

Gongronema latifolium was examined for its antibacterial activity against *Staphylococcus haemolyticus*. Minimum Inhibitory Concentration (MIC) was recorded at 62.5 mg/mL, while Minimum Bactericidal Concentration (MBC) was 500 mg/mL (Table 2).

In vivo antibacterial activity of extracts in the wound healing modulation

Histopathological examination of excised wounded skin portions during and after complete treatment is shown in Figures 1 to 4. The role of immune cells during the wound-healing process was observed by examining differential blood counts; neutrophils, monocytes, and lymphocytes were found to be dominant, as shown in Tables 3 to 5.

Table 1: Antibiotic sensitivity profile of *Staphylococcus haemolyticus*

Antibiotics	Results	Diameter zone of inhibition (mm)
Chloramphenicol	-	0
Gentamicin	-	0
Rifampicin	-	0
Tetracycline	-	0
Cefotaxime	-	12.5
Ceftazidime	+	18
Amikacin	-	0
Ceftriaxone	+	15
Ciprofloxacin	+	21
Levofloxacin	+	18

+ = susceptible to antibiotic; - = resistant to antibiotic

Table 2: Bacterial inhibitor properties of *G. latifolium* extract against *Staphylococcus haemolyticus*

Concentration (mg/mL)	Outcomes	Diameter zones of inhibition (mm)
500	+	16.5
250	+	14
125	+	10.5
62.5	-	0
31.25	-	0
15.625	-	0
Ciprofloxacin	+	18

+ = zone of inhibition present; - = zone of inhibition absent

Notable ($p < 0.05$) variations were observed in neutrophil and lymphocyte counts, whereas monocyte counts showed no significant differences compared with the control group. The weights of the rats were monitored regularly through both phases of infection and treatment, and there were no notable variations in body weights among the different experimental groups, as presented in Table 6.

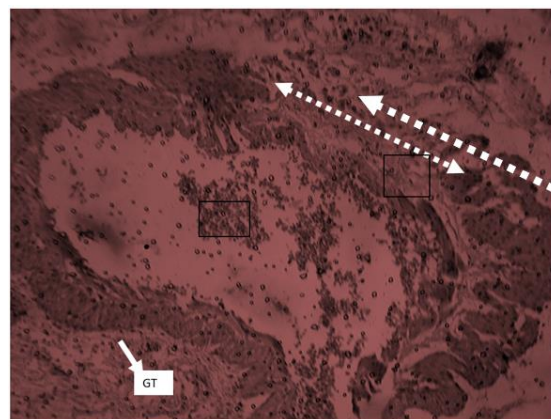


Figure 1: Group A-Histopathology of wound tissues from normal control H&E. mag.100X

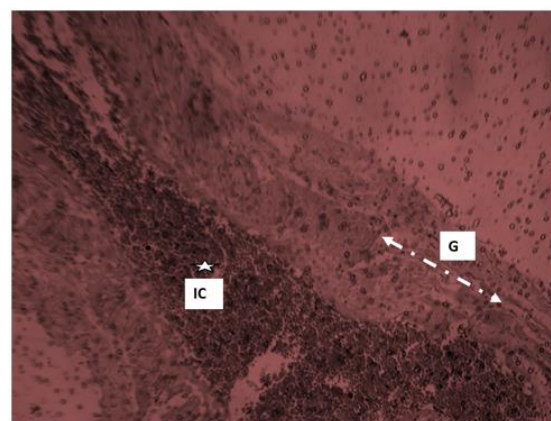


Figure 2: Group B- (Diabetic wound group; no treatment), H&E. mag.100X

Diagnostic features

The onset of wound healing is characterized by the emergence of granulation tissue (GT), identified by a white arrow. During this stage, inflammatory cells (IC), represented by the star, are widely dispersed throughout the wound site, increasing in number to combat pathogenic organisms. As a result, leukocytes are attracted to the wound site, intensifying the inflammatory response, which may lead to eliminating the infection without intervention. This process typically lasts a few days and represents an essential step in wound healing.

Pathological narratives

It was observed that the integrity of the capillaries appears to be compromised, resulting in frequent inflammatory reactions.

Diagnostic features

The newly formed epidermis (black arrow) is partly covered by a serocellular crust (SC; red arrow), which seems to have reduced in thickness in the epidermal region. Fibroblasts are present in the area, which is a good sign. Moreover, the granulation tissues (GT) seem to

be very adequate, indicating healthy tissue growth.

Diagnostic features

A limited number of proliferated Fibrinocytes (F; white arrow) in the granulation tissue suggests that the fibrin clot has disintegrated, resulting in an extracellular matrix and a reduced wound size. Wound healing commences with a positive contraction of the wound. Fibroblast (F; white arrow) is moderately present and is responsible for initiating epithelialization and collagen synthesis. A small number of inflammatory cells (red arrow) were noted.

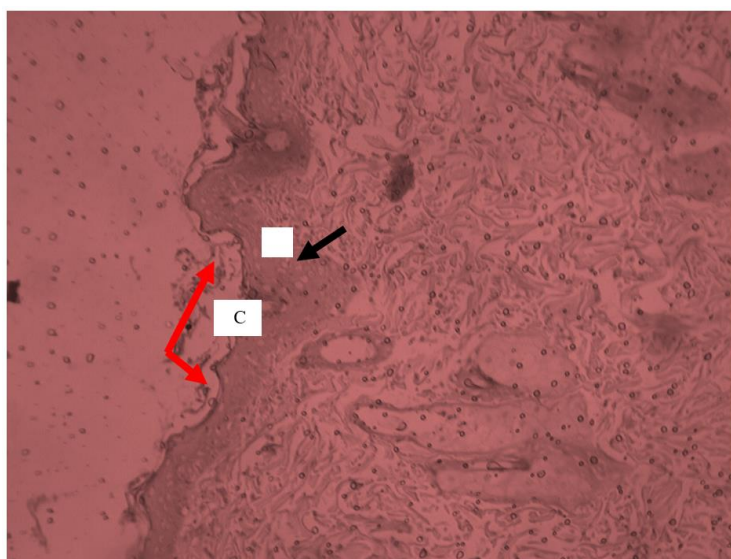


Figure 3: Group C- (Diabetic wound with standard drug (Ciprofloxacin) treatment, after complete days of experiment). H&E. mag.100X

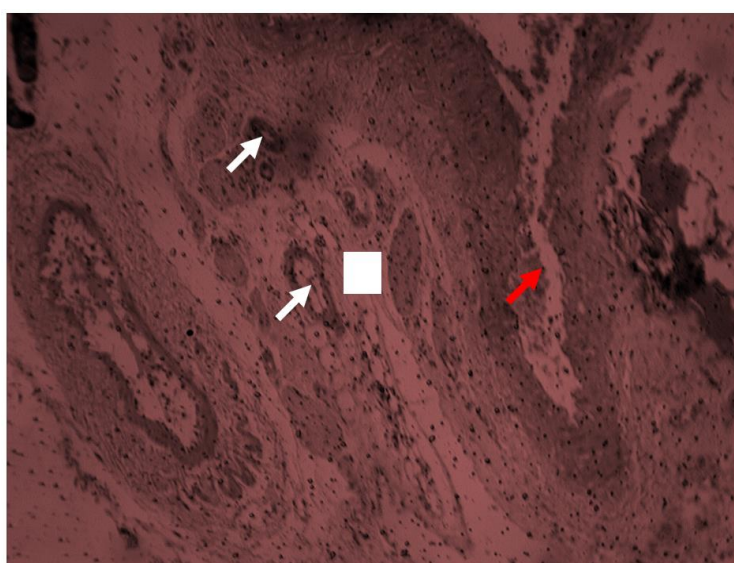


Figure 4: Group D-(Diabetic wound treatment with *G. latifolium* Extract 10%(w/v)) H&E. mag.100X

Table 3: Neutrophil counts (%) of test rat groups (n=16)

Schedule	Normal control	Diabetic wound with no treatment	Diabetic wound, treatment with standard drug	Diabetic wound treatment by <i>G. latifolium</i>
Baseline	48.00±2.00 ^a	48.67±12.65 ^a	47.33±2.52 ^a	51.33±7.02 ^a
During treatment	49.00±2.65 ^b	22.00±12.17 ^a	23.00±5.20 ^a	24.67±1.37 ^a
After treatment	49.00±2.65 ^{ab}	66.00±2.65 ^c	35.33±1.53 ^a	51.33±7.02 ^b

Mean values within the same row but with varying affixes are noticeably distinct ($p < 0.05$)

Table 4: Monocyte counts (%) of test rat groups (n=16)

Schedule	Normal control	Diabetic wound with no treatment	Diabetic wound, treatment with standard drug	Diabetic wound treatment by <i>G. latifolium</i>
Baseline	6.67±2.08 ^a	8.00±3.61 ^a	8.00±1.73 ^a	8.00±1.00 ^a
During treatment	6.67±2.08 ^a	2.00±0.00 ^a	5.67±3.51 ^a	8.00±1.00 ^a
After treatment	6.67±2.08 ^a	8.00±3.61 ^a	8.00±1.73 ^a	8.00±1.00 ^a

Mean values along similar line with varying affixes are considerably distinct ($p < 0.05$).

Table 5: Lymphocyte Counts (%) of Test Rat Groups, (n=16)

Schedule	Normal control	Diabetic wound with no treatment	Diabetic wound, treatment with standard drug	Diabetic wound treatment by <i>G. latifolium</i>
Baseline	43.67±1.16 ^a	42.67±10.07 ^a	43.67±0.58 ^a	39.33±7.02 ^a
During treatment	43.67±1.16 ^a	76.00±12.17 ^c	73.67±0.58 ^b	66.00±12.49 ^b
After treatment	43.67±1.16 ^c	25.67±4.04 ^a	55.6±2.89 ^d	39.33±7.02 ^b

Mean values within the same row but with varying affixes are noticeably distinct ($p < 0.05$).

Table 6: Body weight (kg) of test rat groups (n = 16)

Schedule	Normal control	Diabetic wound with no treatment	Diabetic wound, treatment with standard drug	Diabetic wound treatment by <i>G. latifolium</i>
Initial	243.2±10.99 ^a	271.2±33.59 ^a	259.4±31.76 ^a	249.2±43.30 ^a
Final	242.6±11.01 ^a	271.0±33.20 ^a	259.4±31.17 ^a	248.8±43.48 ^a

Mean values along similar line with varying affixes are considerably dissimilar ($p < 0.05$).

DISCUSSION

Antibiotic resistance is already known to be one of the top five public health concerns [31]. Antibiotic-resistant microorganisms make treatment and control of known diseases more difficult, leading to the use of heavier antibiotic generations, which in turn impacts the level of antibiotic resistance in the human ecosystem. There is a revolving cycle of high antibiotic float/persistence with high selection pressure, and consequent antimicrobial resistance spread. Studies centered on alternatives to antibiotic use for control of infections, both for humans and animals, have been encouraged in the past decade. A one health approach has also been adopted as one of the strategies to control antimicrobial resistance spread. Isolation of *Staphylococcus* species from wound samples in the hospital used for this study has been reported by Ukpuho *et al* [32]; however, *S. haemolyticus* was not reported as found in the present study. Antibiotic sensitivity showed a remarkable resistance to rifampicin, tetracycline,

amikacin, chloramphenicol, and gentamicin. This result is partly in alignment with a previous study, which reported varying percentages of resistance of wound bacterial organisms to tetracycline, amikacin and rifampicin [33]. These reported resistance patterns of wound infection bacteria reflect the potential problems to be faced by some hospitals in the management of wounds with regard to antibiotic resistance. This thus substantiates the objective of this study, which is to assess potential natural alternatives to wound management, especially for diabetic wounds.

Extract from *G. latifolium*, examined for its antibacterial activity against *Staphylococcus haemolyticus*, had a MIC of 62.5 mg/mL and a minimum bactericidal concentration (MBC) of 500 mg/mL against *S. haemolyticus*. This observation partly agrees with the earlier report for *G. latifolium* antibacterial activity against *Staphylococcus aureus*. They also reported the same outcome for *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [31,32].

In vivo experiment showed that aqueous extract of *G. latifolium* had wound healing activities on diabetic wounds elicited in Albino rats. There was histological evidence on phagocytosis and re-epithelization of traumatized tissue sites after periods of administering *G. latifolium* extract. Inflammatory cells were seen to be evident in both untreated and treated groups of normal control and diabetic rats, which portrays the role of innate immunity in modulating *S. haemolyticus* infection in the wound sites. There is a dearth of information on the histopathological findings of the wound healing capacity of *G. latifolium*, either in diabetic or non-diabetic superficial wounds, as in the present study. However, Okon *et al*, reported the protective effect of *G. latifolium* extract from myocardial injury triggered by the use of glucocorticoids for treating inflammatory disorders, in a rat study [9]. The experiment evaluated the role of innate immunity in wound healing by analyzing key blood parameters: lymphocytes, neutrophils, and monocytes. *G. latifolium* significantly enhanced neutrophil activity in diabetic rats ($p < 0.05$). Monocyte counts showed no significant variation across groups ($p > 0.05$), whereas ciprofloxacin markedly increased lymphocyte activity during wound modulation ($p < 0.05$). Eosinophils and basophils were undetectable and thus excluded from analysis. All albino rats maintained stable body weights throughout the study.

CONCLUSION

Antibacterial resistance poses a significant challenge in managing wound infections, particularly in cases complicated by comorbidities such as diabetes. *G. latifolium* has demonstrated potent antibacterial activity, offering a sustainable and accessible alternative to conventional antibiotics for treating bacterial wound infections, especially those caused by *Staphylococcus* species. Beyond its antimicrobial effects, *G. latifolium* appears to stimulate immune responses and exhibit anti-inflammatory properties, which promote wound re-epithelialization and healing while concurrently reducing bacterial load. Given these therapeutic benefits, there is a compelling need to integrate medicinal plants like *G. latifolium* into standard hospital treatment protocols for wound management.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Ethical considerations

The protocols were confirmed by two ethical committees and the authors of this manuscript observed ethical standards.

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