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Original Article

Prevalence of multidrug-resistant biofilm-forming pathogens in diabetic foot ulcers and antimicrobial activity of nanoparticles

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Abstract

Introduction: Diabetic foot ulcers (DFU) are the main devastating complications for diabetic patients. The involvement of multidrug-resistant microorganisms with the ability to produce biofilms in DFUs renders them difficult to treat. Nanotechnology has emerged as an innovative and promising technology in the therapy of diabetic foot lesions. Therefore, this study was designed to assess the prevalence of drug resistance and biofilm-forming pathogens in DFU and the antimicrobial activity of nanoparticles against these pathogens.

Methodology: A total of 111 adults with diabetic foot ulcers were randomly included. The clinical parameters and data of the classification and grading of the wound, along with microbiological factors, were analyzed.

Results: Nanoparticles were synthesized from *Withania coagulans* and *Fagonia cretica*. The results showed that the majority of patients were male (76%), with an average age of 54 years. The majority of ulcers were polymicrobial (56%), while *Staphylococcus aureus* (21.2%) was the predominant pathogen. A significant increase in methicillin-resistant *Staphylococcus aureus* (76.5%), extended-spectrum β-lactamase (ESBL) producers (55.8%), carbapenem-resistant *Pseudomonas aeruginosa* (46%), and vancomycin-resistant Enterococci (18.1%) was observed. Gram-negative isolates (31%), particularly *Pseudomonas aeruginosa*, exhibited strong biofilm formation activity compared to gram-positive (6%) and fungal isolates (24%).

Conclusions: The tested nanoparticles showed significant antimicrobial activity against strong biofilm forming bacterial and fungal isolates. Controlling certain extrinsic and metabolic parameters and comprehensively evaluating nanoparticle-based therapeutics can serve as powerful tools in curing chronic diabetic wounds.

Key words: Diabetes; ulcers; multidrug resistance; biofilm; nanoparticles.

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Introduction

Diabetes mellitus is a chronic metabolic disorder and the most prevalent non-communicable disease. The hallmark of diabetes mellitus is persistent hyperglycemia, which leads to insulin resistance, decreased insulin production, or both [1]. The disease is associated with multiple pathological complications such as high blood glucose levels causing immunosuppression, delayed wound healing, persistent chronic infections, and damage to kidneys, eyes, nerves, and blood vessels through the onset of nephropathy, retinopathy, and neuropathy, respectively. These complications further affect the quality of life of patients and their families [2,3]. Over the past three decades, there has been a steady rise in both the number of cases and incidence of diabetes worldwide. Sedentary lifestyles, aging populations, unhealthy eating habits, and rising obesity rates are some of the factors contributing to the rapid increase in the prevalence of diabetes. According to the World Health Organization, approximately 422 million individuals suffer from diabetes globally, with the majority residing in low- and middle-income countries. The disease is directly responsible for 1.5 million mortalities annually [4]. Pakistan is also predicted to rank among the four largest diabetic populations by 2030 owing to its steadily increasing diabetes prevalence rates [5].

Diabetic foot ulcers (DFU) are the most complex and devastating complications that are continuously increasing in diabetic patients owing to the complex interaction of factors, including ischemia and peripheral neuropathy [6]. DFUs are also considered the most expensive long-term complication of diabetes, with adverse consequences on the patient's life and imposing a substantial economic burden on the family of patients [7]. The exact cost of diabetic wound care at the global level is not known, but it is thought to be billions of

dollars [8,9]. In DFUs, the tendency of bacterial pathogens to produce biofilms is a major determinant of the chronicity and persistence of infections. A further challenge in managing biofilm-associated infections in DFU is multidrug resistance, as bacteria are present in close synergistic associations within the biofilm environment [10]. Consequently, in biofilms, non-pathogenic commensals or opportunistic bacteria can act symbiotically with pathogenic bacteria, leading to the development of chronic lesions that are extremely difficult to cure [11].

DFUs are mostly polymicrobial and are responsible for the emergence and dissemination of antibiotic resistance genes [12] Current therapeutic choices are severely limited by MDR bacteria, which are characterized as those that are resistant to three or more types of antibiotics. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, Proteus species, *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus*, and Enterococci are common MDR pathogens that are frequently isolated from DFUs [13]. Various resistance mechanisms, including β-lactamase production, efflux pumps, and modification of antibiotic target sites, are displayed by these superbugs. These mechanisms of resistance are further enhanced in biofilm conditions, which complicates therapy and calls for the adoption of different approaches to control these diseases [14,15]. Literature reports also emphasized that 49-85% of all diabetic foot-related problems, causing major socioeconomic collisions, are avertable if timely, suitable, and appropriate therapeutic strategies are adopted [16]. These measures can eventually result in decreased chances of lower-limb disability, reduced healthcare costs, and improved life expectancy and productivity. Unfortunately, conventional therapeutics are vanishing because of several underlying reasons, stressing the development of new therapeutics [16,17].

Nanotechnology has emerged as an innovative and promising technology in the therapy of diabetic foot lesions. Recently, many studies have emphasized the biological potential of nanomaterials in the treatment of diabetic ulcers. Some metal nanoparticles, such as silver nanoparticles, possess antibacterial and anti-inflammatory activities and help stimulate cellular mechanisms for curing chronic diabetic wounds [18]. Bioglass nanoparticles have significant angiogenic and antimicrobial effects. Some studies have shown progressive healing of chronic diabetic wounds using dressings containing metal nanoparticles [19,20].

Subsequently, this study comprehensively assessed recent trends in microbial resistance, the ability of pathogens to create biofilms in diabetic wound infections, and the antimicrobial action of iron and silver oxide nanoparticles. This study aimed to develop new strategies for treating diabetic foot complications and to offer a comprehensive dataset for empirically managing DFU. Ultimately, it protects patients from the traumatic experience of losing a foot or a leg.

Methodology

Study Design

This cross-sectional study was conducted at Dr. Ruth K. M. Pfau Civil Hospital Karachi from January 2021 and December 2021. Diabetic patients (≥ 18 years old) who visited the outpatient department with lesions/ulcers on either surface of their foot were included. A systematic questionnaire including general sociodemographic, behavioral, and clinical characteristics was used to conduct random interviews with the patients. Patients who satisfied the study selection criteria were enrolled in this study. Children, pregnant women, patients with significant ischemia, patients with osteomyelitis, and patients receiving systemic antibiotic treatment for chronic non-DFU infections, such as persistent urinary tract infections, within the previous two weeks were the main exclusion criteria.

Sample Collection

A standardized and systematic strategy was used to ensure reliable microbiological examination. The classification and grading of the wound or ulcer of the diabetic foot were performed according to the University of Texas diabetic wound classification [21]. The ulcer was washed with sterile normal saline and debrided aseptically. During the collection process, great care was taken to prevent unintentional contamination, and all specimens were quickly shifted into the proper transport media to maintain microbial viability. For traceability and contextual interpretation, extensive documentation was maintained, including the sample site, ulcer features, and patient data. All specimens were immediately transported to the Department of Microbiology, University of Karachi.

Institutional Ethics Approval

A summary of the study was provided to the patients before written consent was obtained. This study was approved by the Institutional Bioethical Committee of the University of Karachi (IBC KU-204/2021).

Microbial Profiling and Examination

All pus discharge and debrided tissue samples were

processed using standard protocols. Identification and antibiotic susceptibility of the isolated bacterial isolates were carried out using a BD Phoenix automated analyzer according to the recommendations of standards of the Clinical Laboratory Standard Institute [22].

Biofilm Formation Assay

The biofilm formation assay was performed following the protocol described previously by O'Toole, with slight modifications [23]. Muller Hinton broth (MHB) was used to perform biofilm assays in a flat-bottom 96-well microtiter plate. All wells were seeded with 1×10^5 CFU mL^{-1} of the test isolates. Control wells were also prepared, for example, positive (medium + known biofilm former) and negative (medium only). The biofilm microtiter plate was incubated for 24 hours at 37 °C. After incubation, the medium was discarded without disturbing the biofilm, and the 96-well microtiter plate was washed thrice with distilled water. Afterward, 125 µL of 0.1% crystal violet dye was added to each well of a 96-well microtiter plate, and the plate was left at room temperature for 15-20 minutes. After staining, the plate was washed three times by submerging it in a distilled water tub and left to dry in air for a few hours. Finally, the results were recorded by adding 125 µL of 30% glacial acetic acid (v/v) to each well and analyzed using an ELISA reader at a wavelength of 540 nm.

Synthesis of Iron oxide nanoparticles

Two samples of iron oxide nanoparticles were synthesized using the plant extract of *Withania coagulans*. $FeCl_3.6H_2O$ and $FeCl_2.4H_2O$ (1:2 molar ratios) were dissolved in 100 mL of double-distilled water in a 250 mL beaker for the synthesis of both iron oxide nanoparticles. One sample was heated for one minute, and the other was heated for 5 min at 80 ºC while being stirred with a magnetic stirrer. Following this, 20 mL of the *Withania coagulans* extract in an aqueous solution was added to each mixture. After 15 min, 20 ml of sodium hydroxide aqueous solution was added to both mixtures at a rate of 3 mL per minute, and a color change was noted [24].

Synthesis of silver oxide nanoparticles

Silver oxide nanoparticles were synthesized using the plant extract of *Fagonia cretica*. For the synthesis of silver oxide nanoparticles, 1 L of 0.1 mM silver nitrate solution was prepared. Adding 20 mL of $AgNO_3$ solution to 5 mL of *Fagonia cretica* extract in 50% ethanol, the reaction was carried out for 5 h at 50°C using a magnetic stirrer spinning at 150 rpm in a reagent bottle. A color change indicated the formation of nanoparticles. The pellet was separated by centrifugation for 20 min at 6000 rpm and stored at 4°C for further use. By adding 2 mL of water, the weight of the particles was determined as 2.7g/ 2 mL of water [24].

Antimicrobial Activity of Nanoparticles

The antimicrobial potential of the nanoparticles was screened using a disc diffusion assay, as described by Bauer *et al.* with slight modifications [25]. Mueller Hinton agar was used to determine the antibacterial activity of nanoparticles. A bacterial lawn was prepared by swabbing a bacterial count of 1×10^8 CFU mL^{-1} on an agar surface in three different directions. Presoaked sterile filter discs (nanoparticles impregnated at a concentration of 100 µg mL^{-1}) were placed on the inoculated plates and allowed to diffuse for 5 min. MHA plates were incubated for 18-24 hours at 37 °C. Gentamicin (10 µg/disc, Oxoid) was used as the positive control. The negative control was prepared by loading 10 µL of DMSO on a sterile filter disc.

Table 1. Microbiological factors of participants (n = 111).

Name of Variable	Occurrence	Percentage
Type of Infection		
Bacterial Infection	92/101	91
Fungal Infection	29/101	28.7
No Infection	10/111	9
Nature of Infection		
Polymicrobial Infection	56/101	56
Monomicrobial Infection	45/101	44
Gram Positive Pathogens		
Staphylococcus aureus	34/160	21.2
Methicillin resistant *Staphylococcus aureus*	26/34	76.5
Methicillin sensitive *Staphylococcus aureus*	8/34	23.5
Coagulase negative Staphylococci	14/160	8.8
Staphylococcus epidermidis	9/14	64.3
Staphylococcus luteus	3/14	21.4
Staphylococcus saprophyticus	2/14	14.3
Enterococcus faecalis	11/160	6.9
Beta haemolytic Streptococci	6/160	3.8
Gram Negative Pathogens		
Klebsiella pneumoniae	27/160	16.8
Proteus mirabilis	19/160	11.9
Proteus vulgaris	8/160	5
E. coli	24/160	15
Pseudomonas aeruginosa	13/160	8.1
Morganella morganii	3/160	1.9
Acinetobacter baumannii	1/160	0.6
Fungal Pathogens		
Candida species	18/29	62
Candida albicans	12/18	41.3
Candida tropicalis	4/18	13.7
Candida glabrata	2/18	6.9
Aspergillus niger	11/29	38

Statistical Analysis

Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) software version 22 (IBM SPSS Inc., Chicago, IL, USA). The study population was characterized using descriptive statistics with different variables. Bivariate correlation and chi-square tests were used to identify the qualitative factors associated with foot ulcers. The (significant) p was set at < 0.05, for predicting the association of different factors with DFUs.

Results

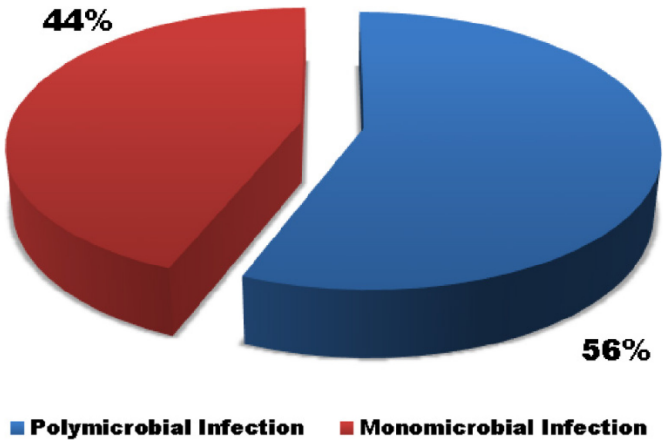
A total of 111 adults with diabetes were included in this study. Among the participants, 84 (76%) were male and 27 (24%) were female. Most of the patients' ages ranged from 25 to 85 years, with a mean age of 54 years ± 10. Greater infection rates were observed in the majority of patients with grade 2 ulcers, accounting for 61 cases (60.3%), followed by grade 1 ulcers with 23 cases (22.7%). Grade 3 and 4 ulcers were the least common, found in 10 patients (9.9%) and 7 patients (6.9%), respectively. Most of the patients (89%) with chronic DFU had uncontrolled HbA1c levels (> 7.0 %).

Microbiological Analysis

Among the 111 pus and tissue samples processed, 101 (91%) were positive for microbial growth, whereas 10 (9%) showed no microbial growth. Among the 101 positive samples, 92 (91%) showed bacterial growth, and 29 (28.7%) were positive for fungal infections. Most samples (56%) were polymicrobial (Figure 1). A total of 160 bacterial pathogens, including 65 (41%) Gram positive and 95 (59%) Gram negative pathogens, were isolated from the 111 samples.

Staphylococcus aureus (21.2%) was the predominant isolate, followed by *Klebsiella pneumoniae* (16.8%), *Escherichia coli* (15%), *Proteus mirabilis* (11.9%), coagulase-negative Staphylococci (8.8%), *Pseudomonas aeruginosa* (8.1%), *Enterococcus faecalis* (6.9%), *Proteus vulgaris* (5%), β-hemolytic Streptococci (3.8%), *Morganella morganii* (1.9%), and *Acinetobacter baumannii* (0.6%). Among the fungal isolates, *Candida* species (62%) were predominant, followed by *Aspergillus niger* (38%). *C. albicans* (41.3%) was the most common species isolated from the majority of samples, followed by *C. tropicalis* (13.7%), and *C. glabrata* (6.9%) (Table 1).

Figure 1. Polymicrobial vs monomicrobial infection among diabetic foot ulcer patients.



A significant increase in extended spectrum beta lactamase (ESBL) producers was observed among *E. coli*, *Klebsiella pneumoniae*, *Proteus* species, and *Morganella morganii*, with prevalence rates of 75%, 74%, 51.8%, and 33.3%, respectively. *Pseudomonas aeruginosa* exhibited greater resistance to carbapenem (46%), while notably increased resistance rates were observed among all members of Enterobacteriaceae against 3rd and 4th generation cephalosporins, carbapenems, and quinolone antibiotics. However, aminoglycosides and piperacillin/tazobactam showed

Table 2. Antibiotic resistant pattern of Gram-negative bacteria (Resistant %).

Antibiotics	*Klebsiella pneumoniae*	*E. coli*	*Proteus spp.*	*Pseudomonas aeruginosa*	*Morganella morganii*	*Acinetobacter baumannii*
Isolates (n)	27	24	27	13	3	1
ESBL	20 (74%)	18 (75%)	14 (51.8%)	-	1 (33.3%)	-
Ampicillin	-	23 (95.8%)	24 (88.8%)	-	-	-
Amoxicillin/ Clavulanic acid	25 (93%)	22 (91.6%)	21 (77.7%)	-	-	-
Ceftazidime	20 (74%)	18 (75%)	14 (51.8%)	7 (53.8%)	2 (66.6%)	1 (100%)
Ceftriaxone	20 (74%)	18 (75%)	14 (51.8%)	-	-	-
Cefepime	17 (63%)	14 (58.3%)	11 (40.7%)	3 (23%)	0 (0%)	1 (100%)
Imipenem	9 (33.3)	7 (29%)	6 (22%)	6 (46%)	0 (0%)	1 (100%)
Meropenem	9 (33.3%)	7 (29%)	6 (22%)	6 (46%)	0 (0%)	1 (100%)
Piperacillin/ Tazobactam	10 (37%)	11 (45.8%)	9 (33.3%)	7 (53.8%)	0 (0%)	1 (1000%)
Gentamicin	12 (44%)	10 (41.6%)	10 (37%)	6 (46%)	2 (66.6%)	1 (100%)
Amikacin	4 (15%)	0 (0%)	2 (7.4%)	3 (23%)	0 (0%)	1 (100%)
Ciprofloxacin	15 (56%)	19 (79%)	13 (48%)	5 (38.4%)	2 (66.6%)	1 (10%)
Trimethoprim/ Sulfamethoxazole	20 (74%)	17 (70.8%)	18 (66.6%)	-	3 (100%)	1 (100%)
Colistin	1 (3.7%)	2 (8.3%)	-	3 (23%)	-	0 (0%)

Table 3. Antibiotic resistant pattern of Gram-positive bacteria (Resistant %).

Antibiotics	*Staphylococcus aureus*	*Enterococcus faecalis*	BHS	Coagulase negative Staphylococci
Isolates (n)	34	11	6	14
Inducible Clindamycin Resistance	12 (35.2%)	-	-	3 (21.4%)
Ampicillin	-	4 (36.3%)	0 (0%)	-
Cefoxitin	26 (76.5%)	-	-	9 (64.2%)
Tetracycline	16 (47%)	5 (45.4%)	2 (33.3%)	8 (57.1%)
Erythromycin	21 (61.7%)	-	3 (50%)	10 (71.4%)
Clindamycin	15 (44%)	-	1 (16.6%)	6 (42.8%)
Ciprofloxacin	24 (70.5%)	7 (63.6%)	2 (33.3%)	9 (64.2%)
Trimethoprim/ Sulfamethoxazole	20 (58.8%)	5 (45.4%)	2 (33.3%)	11 (78.5%)
Gentamicin	15 (44%)	-	-	9 (64.2%)
Amikacin	3 (8.8%)	-	-	2 (14.2%)
Linezolid	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fusidic Acid	13 (38.2%)	-	-	8 (57.1%)
Teicoplanin	0 (0%)	3 (27.2%)	0 (0%)	0 (0%)
Vancomycin	0 (0%)	2 (18.1%)	0 (0%)	0 (0%)

comparatively lower resistance rates (Table 2).

Methicillin resistance in *Staphylococcus aureus* and Coagulase negative Staphylococci accounted for 76.5% and 64.2%, respectively. Twelve (35.2%) isolates of *Staphylococcus aureus* showed inducible clindamycin resistance. Vancomycin-resistant Enterococci accounted for 18.1%. None of the isolates showed resistance to linezolid (Table 3).

Biofilm Formation Assay

Among the 189 isolates from DFUs, 55% (104/160) of bacterial and 62% (18/29) of fungal isolates showed moderate to strong biofilm formation activity. Most of the strong biofilm formers (61.8%) possessed multidrug resistance. A total of 29 (31%) Gram-negative out of 95 isolates showed strong biofilm formation activity, followed by 4 (6%) out of 65 Gram-positive isolates, and 7 (24%) out of 29 fungal isolates. All Gram-negative isolates showed moderate to weak biofilm formation, except two isolates (Table 4). *Acinetobacter baumannii* (100%) exhibited the strongest biofilm forming activity, followed by *P. aeruginosa*. (54%), *K. pneumoniae*. (37%), and *E. coli* (29%) (Figure 2).

Most gram-positive isolates (40%) showed moderate biofilm-forming activity. Among the Gram-positive isolates, coagulase-negative staphylococci (14%) exhibited strong biofilm formation activity, followed by methicillin-resistant *S. aureus* (8%) (Table 5). Among the fungal isolates, *C. albicans* (31%)

Figure 2. Biofilm formation by Gram negative isolates.

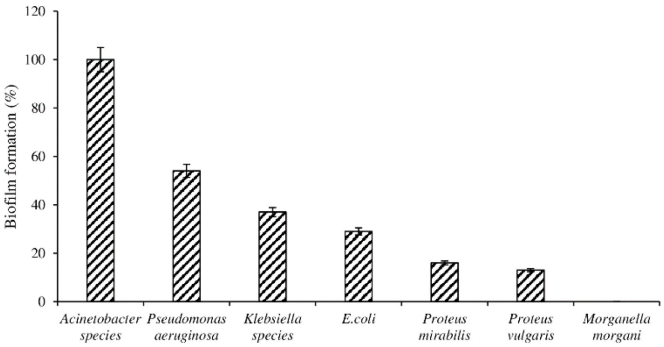


Figure 3. Inhibition of Gram negative, Gram positive and fungal isolates growth by nanoparticles.

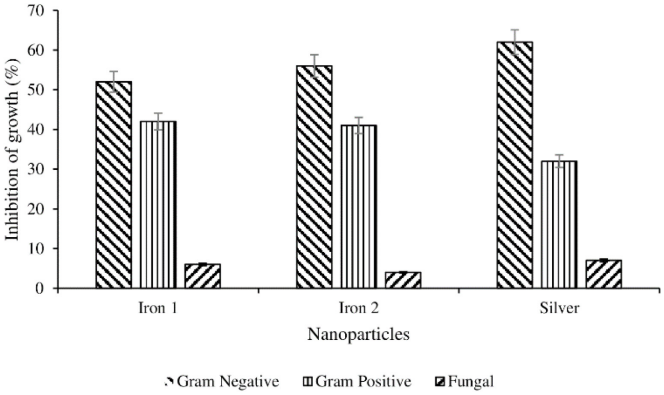


Table 4. Biofilm formation activity of Gram-Negative isolates (n = 95).

Pathogen	Isolates (n)	Strong	Moderate	Weak	Negative
Acinetobacter species	1	1	0	0	0
Escherichia coli	24	7	7	9	1
Klebsiella pneumoniae	27	10	13	4	0
Morganella morganii	3	0	3	0	0
Proteus mirabilis	19	3	11	4	1
Proteus vulgaris	8	1	5	2	0
Pseudomonas aeruginosa	13	7	6	0	0
Total isolates	95	29	45	19	2

Table 5. Biofilm formation activity of Gram-Positive isolates (n = 65).

Pathogen	Isolates (n)	Strong	Moderate	Weak	Negative
Beta Hemolytic *Streptococci*	6	0	0	3	3
Coagulase negative *Staphylococci*	14	2	4	4	4
Enterococcus faecalis	11	0	3	2	6
Staphylococcus aureus MRSA	26	2	15	2	7
Staphylococcus aureus MSSA	8	0	4	2	2
Total isolates	65	4	26	13	22

Table 6. Biofilm formation activity of Fungal isolates (n = 29).

Pathogen	Isolates (n)	Strong	Moderate	Weak	Negative
Aspergillus niger	11	3	4	1	3
Candida albicans	12	4	6	0	2
Candida tropicalis	4	0	1	0	3
Candida glabrata	2	0	0	1	1
Total isolates	29	7	11	2	9

showed the strongest biofilm-forming activity, followed by *A. niger* (27%) (Table 6).

Antimicrobial Activity of Nanoparticles

Iron oxide and silver oxide nanoparticles showed antimicrobial activity against 38-43% of the isolates (Figure 3). Gram-negative bacteria were more susceptible to these nanoparticles. Iron (I) oxide nanoparticles showed the highest antibacterial activity against *E. coli* (15%), followed by coagulase-negative Staphylococci (13.8%) and methicillin-resistant *S. aureus* (12.5%). Iron (II) oxide nanoparticles showed the highest activity against *E. coli* (17%), followed by *P. mirabilis* (13.5%), and methicillin-sensitive *S. aureus* (12.3%). Silver oxide nanoparticles also showed significant antibacterial activity against *E. coli* (20%), followed by *P. aeruginosa* (15.2%) and *K. pneumoniae*. (14%). Iron (I) oxide and silver oxide nanoparticles also exhibited moderate activity against *C. albicans* and *A. niger* (Figure 4a, 4b, and 4c).

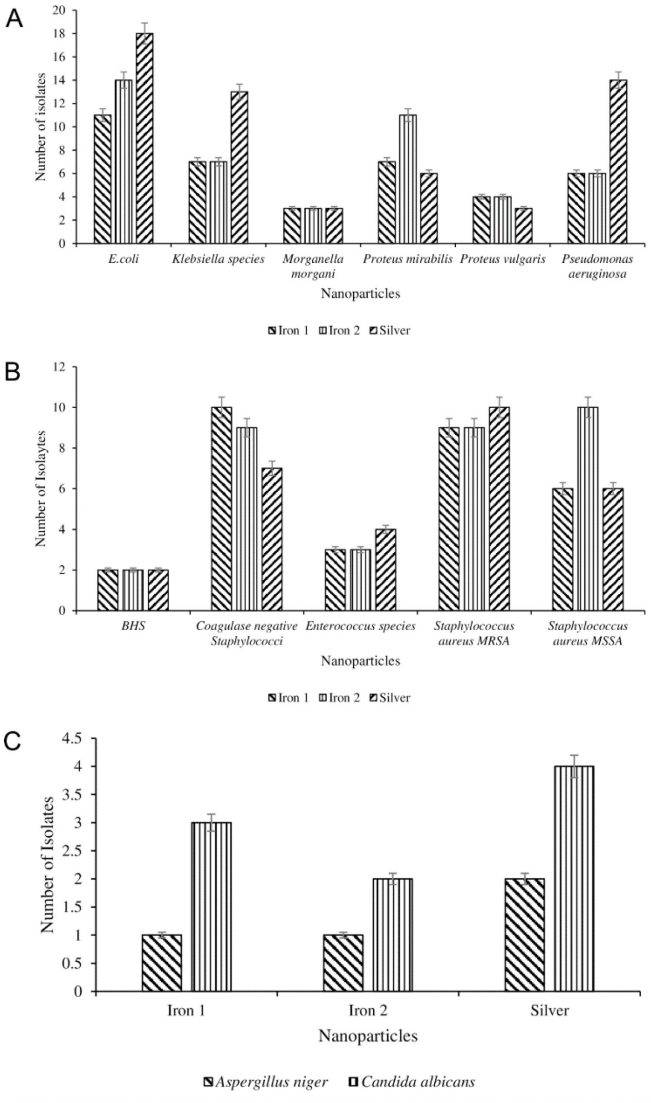
Discussion

Foot ulcers are a common complication that can cause permanent disability in patients with diabetes. The progression of foot ulcers or wounds to the amputation stage marks them as a serious public health issue. Understanding the underlying contributing risk factors can help control and manage this devastating complication among diabetic patients [26].

Microbiological Analysis

Infection burden is a common consequence and a major source of morbidity in DFUs, as evidenced by the high incidence of microbial growth (91%) in pus and tissue samples. Bacterial infections (91%) were predominant, with a notable presence of polymicrobial infections (56%). Polymicrobial infections often

Figure 4A. Number of Gram-negative isolates inhibited by nanoparticles; **B.** Number of Gram-positive isolates inhibited by nanoparticles; **C.** Number of fungal isolates inhibited by nanoparticles.



complicate treatment, necessitating the use of broad-spectrum or combination antibiotic therapies to effectively manage the diverse microbial community present [27].

S. aureus (21.2%), followed by Proteus species, and *K. pneumoniae* (16.8%) were the most frequent bacterial isolates. *S. aureus* is a well-documented, frequent pathogen in skin and soft tissue diseases while the prevalence of Gram-negative pathogens such as species of *Proteus* and *Klebsiella* indicates the diversity of the bacterial flora and the possibility of involvement of multiple drug-resistant strains. Significant fungal infections (28.7%) were also observed, with *Candida albicans* (41.3%) being the most common pathogen. Indeed, *Candida* species are frequent colonizers of diabetic foot ulcers. Fungal infections, especially *C. albicans*, can be difficult to treat since they require antifungal medication for a long duration and can result in mixed fungal-bacterial infections [28]. This emphasizes the stipulation of comprehensive microbial analysis and a multifaceted approach to infection control, integrating broad-spectrum antibiotics and antifungal agents where appropriate. Moreover, the isolation of biofilm-forming organisms, such as *Pseudomonas aeruginosa*, further complicates treatment because of their intrinsic resistance to conventional cures [29].

We observed methicillin-resistant *Staphylococcus aureus* (76.5%), extended spectrum beta lactamase (ESBL) producers (55.8%), carbapenem-resistant *Pseudomonas aeruginosa* (46%), vancomycin-resistant Enterococci (18.1%) with the potential of forming strong biofilms in our patients. This is in line with worldwide patterns, wherein *Pseudomonas* resistance to carbapenems has been documented more frequently, primarily as a result of the abuse of these drugs and the existence of carbapenemase producing strains. Carbapenem-resistant *Pseudomonas* can also develop biofilms, which makes eradication much more difficult and contributes to persistent and non-healing wounds. Limited treatment choices for this organism is a serious issue. One of several resistance mechanisms in these bacterial populations, including target-site modification, carbapenemase synthesis, and efflux pump overexpression can be involved [29-31]. However, aminoglycosides and piperacillin/tazobactam showed slightly lower resistance rates, suggesting that they could be used to treat these infections. However, these drugs should be used with caution because of the development of antibiotic resistance and other underlying physiological conditions in diabetic patients.

Table 7. Recent Clinical Trials on Nanoparticles in DFU.

Nanoparticle Type	Study Design	Findings	Reference
Colloidal silver nanoparticles	*In vitro* antimicrobial and antibiofilm activity on Gram positive organisms. *In-vivo* response of cAgNPs on chronic diabetic foot ulcers patients	Significant *in-vitro* bactericidal and bacteriostatic activity against MRSA, MSSA and VRE. Significant reduction in microbial load and in the number of moderate to strong biofilm producing organisms after treatment	34
Zinc oxide Nanoparticles	Calcium alginate dressings with ZnO nanoparticles	Better tissue regeneration, improves wound closure and reduce healing time	35
Cinnamon nanoparticles	Ointment containing cinnamon NPs loaded on chitosan- gelatin NPs to treat on burn wound healing in diabetic foot ulcers in rat	Accelerated repair of the wounds in DFU/CNP-CGNP group.	36
Zinc nanoparticles	Nanoparticles zinc paste bandage	Significant wound size reduction and faster healing times	37
Copper nanoparticles	2-layer antibacterial dressing embedded with copper oxide	Wound size reduction and bioburden reduction	38
Silver nanoparticles	Assess clinical efficacy of silver nanoparticles dressing vs standard moist wound dressing	Reduced bacterial burden and chronic interstitial wound fluid, increased vascularity and cytokine expression.	39
Toluidine blue conjugated chitosan coated gold–silver core–shell nanoparticles	*In vitro* efficacy of TBO–chit–Au–AgNPs mediated photodynamic therapy against polymicrobial biofilms. *In vivo* therapeutic potential	Promising antibacterial activity. Reduced Cytokines level, improved wound healing	40
Phenytoin-loaded chitosan-alginate nanoparticles	*In vivo* wound healing potential in rats using diabetic pressure ulcer model	Superior wound healing, granulation tissue formation, tissue maturation, and collagen content	41
Polylactic acid-based nanofibrous wound patch	Nanofibrous wound patch loaded with three drugs (phenytoin, sildenafil citrate and simvastatin) each in a separate layer in-vivo diabetic wound rat model	Proper wound healing, cell regeneration and arrangement without forming scars	42
Prussian blue nanoparticles	Thermosensitive poly (d,l-lactide)-poly(ethylene glycol)-poly(d,l-lactide) (PDLLA-PEG-PDLLA) hydrogel (PLEL)-based wound dressings	Improved diabetic wound healing, decreased ROS production, promoted angiogenesis, and reduced pro-inflammatory interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels within diabetic wounds	43
Tadalafil-loaded nanoparticles	In vivo wound healing potential in rat model	Improved wound closure in a diabetic rat model, reduced inflammation and enhanced angiogenesis	44

Biofilm Formation Assay

Biofilm production is a multi-step phenomenon that involves diverse groups of bacteria. The existence of biofilms is one of the primary causes of DFUs' resistance to healing and chronicity [32]. In this study, approximately 28.8% of DFU isolates exhibited strong biofilm formation in patients with uncontrolled diabetes, with HbA1c levels ranging from 10.2-16.9%. Approximately 68.8% of gram-negative isolates showed moderate to weak biofilm formation, and the majority were polymicrobial. Gram-negative bacteria were predominantly isolated from DFUs and demonstrated high antibiotic resistance and biofilm formation [33]. The adhesive matrix of biofilms is composed of extracellular DNA, proteins, and exopolysaccharides. In this study, *Acinetobacter* species, followed by *P. aeruginosa* and Enterobacteriaceae strains, demonstrated strong biofilms (Table 4). *P. aeruginosa* has great significance in causing chronic and recurrent diabetic foot infections due to its multidrug resistance and its potent ability to form biofilms. Quorum sensing is associated with biofilm formation by *P. aeruginosa*. Among gram-positive bacteria, the majority of methicillin-resistant *S. aureus* strains (23%) exhibited moderate biofilm formation, whereas *C. albicans* (34.4%) showed significantly strong to moderate biofilm formation among fungal isolates.

Antimicrobial Activity of Nanoparticles

In modern therapeutics, metal nanoparticles, including iron and silver nanoparticles, have received attention for the treatment of infectious diseases. Globally, researchers have investigated various types of nanoparticles to improve wound healing, antimicrobial properties, promotion of angiogenesis, antioxidant and anti-inflammatory effects, and controlled drug delivery. A summary of recent clinical trials employing nanoparticles over the past three years is presented in Table 7.

Certain topical medicines contain silver because of its exceptional catalytic properties, conductivity, stability, antibacterial, and anti-inflammatory characteristics. Silver nanoparticles have long been familiar for their strong antimicrobial properties and have been found effective against a wide range of bacteria, fungi, and even some viruses [45]. Silver nanoparticles cause oxidative stress by releasing silver ions, which kill microorganisms by intruding on their cell membranes and interfering with their normal functions. Another possible mechanism involves the generation of reactive oxygen species (ROS), such as superoxide ions and hydroxyl radicals, which can cause oxidative stress in microorganisms, ultimately killing them. There have been reports of some Ag nanoparticles interacting with microbial DNA, causing problems with transcription and replication that lead to cell death [46].

In advanced nanomedicine, iron nanoparticles are commercially used to treat iron deficiency and cancer. Magnetite (Fe_3O_4) and maghemite (Fe_2O_3) are iron oxide nanoparticles with antibacterial capabilities. A special characteristic of iron nanoparticles is their capacity to heat up in the presence of an alternating magnetic field, in addition to physically damaging microbial cell membranes and generating reactive oxygen species (ROS), contributing to the destruction of microorganisms. It is possible to use localized hyperthermia to kill certain bacteria in the vicinity of the lesion [47].

Silver and iron nanoparticles offer numerous therapeutic applications for the treatment of diabetic foot ulcers. They can be added to hydrogels, lotions, or wound dressings to help treat and prevent infections in foot ulcers. These nanoparticles may accelerate the healing process by improving the environment for wound healing by lowering the microbial burden and infection-related problems [48]. Iron nanoparticles can deliver localized hyperthermia therapy when combined with an external magnetic field. This method targets and destroys microorganisms in the wound region while preserving healthy tissues. The use of nanoparticles might lessen the need for conventional antibiotics, potentially preventing the emergence of multidrug-resistant bacterial strains [49,50]. In the present study, we found that both iron and silver nanoparticles had significant antibacterial activity against gram-negative bacteria. We observed slightly significant antibacterial activity of iron (II) nanoparticles against *E. coli* and *Proteus* species. Silver nanoparticles showed significant activity against *E. coli*, *P. aeruginosa*, and *Klebsiella* species, while moderate to weak activity was observed against gram-positive and fungal isolates. Most gram-negative isolates were multidrug-resistant and exhibited antibiotic resistance against ampicillin, cephalosporins, carbapenems, cephalosporins, co-trimoxazole, and chloramphenicol. Both iron oxide nanoparticles showed slightly higher antibacterial activity against gram-positive bacteria than silver nanoparticles (Figure 4b).

Conclusions

Our study concludes that multidrug-resistant biofilm-formers can increase the complexity and

chronicity of diabetic wounds. The findings emphasize the crucial need to create individualized interventions based on culture and sensitivity findings to achieve the best possible results and urge the implementation of targeted antibiotic stewardship programs to reduce the likelihood of developing multiple drug resistance superbugs. To prevent and treat DFU infections, it is also essential to provide education to patients with diabetes, appropriate wound care, and timely and correct diagnosis. Moreover, further comprehensive evaluation of nanoparticle-based therapeutics can serve as a powerful tool for curing chronic diabetic wounds in healthcare settings with resource limitations, where advanced therapeutic alternatives are not always available.

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Conflict of interests

No conflict of interests is declared.

References

1. Mendenhall E, Kohrt BA, Norris SA, Ndetei D, Prabhakaran D (2017) Non-communicable disease syndemics: poverty, depression, and diabetes among low-income populations. Lancet 389: 951–963. doi: 10.1016/S0140-6736(17)30402-6.
2. Edo A, Edo G, Ezeani I (2013) Risk factors, ulcer grade and management outcome of diabetic foot ulcers in a tropical tertiary care hospital. Nig Med J 54: 59–63. doi: 10.4103/0300-1652.108900.
3. Adeyemo AT, Kolawole B, Rotimi VO, Aboderin AO (2021) Multicentre study of the burden of multidrug-resistant bacteria in the aetiology of infected diabetic foot ulcers. Afr J Lab Med 10: 1261–1271. doi: 10.4102/ajlm.v10i1.1261.
4. Orfali R, Ghaffar S, AlAjlan L, Perveen S, Al-Turki E, Ameen F (2024) Diabetes-related lower limb wounds: antibiotic susceptibility pattern and biofilm formation. Saudi Pharm J 32: 102069. doi: 10.1016/j.jsps.2024.102069.
5. Adnan M, Aasim M (2020) Prevalence of type 2 diabetes mellitus in adult population of Pakistan: a meta-analysis of prospective cross-sectional surveys. Ann Glob Health 86: 1–8. doi: 10.5334/aogh.2679.
6. Jude EB, Tentolouris N, Appleton I, Anderson S, Boulton AJM (2001) Role of neuropathy and plasma nitric oxide in recurrent neuropathic and neuroischemic diabetic foot ulcers. Wound Repair Regen 9: 353–359. doi: 10.1046/j.1524-475x.2001.00353.x.
7. Sorber R, Abularrage CJ (2021) Diabetic foot ulcers: epidemiology and the role of multidisciplinary care teams. Semin Vasc Surg 34: 47–53. doi: 10.1053/j.semvascsurg.2021.02.006.
8. Los-Stegienta A, Katarzynska J, Borkowska A, Marcinek A, Cypryk K, Gebicki J (2021) Differentiation of diabetic foot ulcers based on stimulation of myogenic oscillations by transient ischemia. Vasc Health Risk Manag 17: 145–152. doi: 10.2147/VHRM.S307366.
9. Pemayun TGD, Naibaho RM, Novitasari D, Amin N, Minuljo TT (2015) Risk factors for lower extremity amputation in patients with diabetic foot ulcers: a hospital-based case-control study. Diabet Foot Ankle 6: 29629. doi: 10.3402/dfa.v6.29629.
10. Vatan A, Saltoglu N, Yemisen M, Balkan II, Surme S, Demiray T, Mete B, Tabak F (2018) Association between biofilm and multi/extensive drug resistance in diabetic foot infection. Int J Clin Pract 72: e13060. doi: 10.1111/ijcp.13060.
11. Afonso AC, Oliveira D, Saavedra MJ, Borges A, Simões M (2021) Biofilms in diabetic foot ulcers: impact, risk factors and control strategies. Int J Mol Sci 22: 8278. doi: 10.3390/ijms22158278.
12. Richard JL, Sotto A, Jourdan N, Combescure C, Vannereau D, Rodier M, Lavigne JP (2008) Risk factors and healing impact of multidrug-resistant bacteria in diabetic foot ulcers. Diabetes Metab 34: 363–369. doi: 10.1016/j.diabet.2008.02.005.
13. Iyamba J-ML, Bassom VMHN, Lukukula CM, Unya JW, Ngbandani BK, Vihembo GM, Ngoma NN, Wambale JM, Kantola PT, Takaisi-Kikuni NB, Iyamba J-ML, Bassom VMHN, Lukukula CM, Unya JW, Ngbandani BK, Vihembo GM, Ngoma NN, Wambale JM, Kantola PT, Takaisi-Kikuni NB (2021) Study of biofilm formation and antibiotic resistance pattern of bacteria isolated from diabetic foot ulcers in hôpital de référence Saint Joseph, Kinshasa, Democratic Republic of Congo. Adv Microbiol 11: 283–295. doi: 10.4236/aim.2021.115021.
14. Coşkun B, Ayhan M, Ulusoy S, Guner R (2024) Bacterial profile and antimicrobial resistance patterns of diabetic foot infections in a major research hospital of Turkey. Antibiotics 13: 599–607. doi: 10.3390/antibiotics13070599.
15. Akkus G, Sert M (2022) Diabetic foot ulcers: a devastating complication of diabetes mellitus continues non-stop in spite of new medical treatment modalities. World J Diabetes 13: 1106–1121. doi: 10.4239/wjd.v13.i12.1106.
16. Won SH, Chung CY, Park MS, Lee T, Sung KH, Lee SY, Kim TG, Lee KM (2014) Risk factors associated with amputation-free survival in patient with diabetic foot ulcers. Yonsei Med J 55: 1373–1378. doi: 10.3349/ymj.2014.55.5.1373.
17. Zubair M (2020) Prevalence and interrelationships of foot ulcer, risk-factors and antibiotic resistance in foot ulcers in diabetic populations: a systematic review and meta-analysis. World J Diabetes 11: 78–89. doi: 10.4239/wjd.v11.i3.78.
18. Choudhury H, Pandey M, Lim YQ, Low CY, Lee CT, Marilyn TCL, Loh HS, Lim YP, Lee CF, Bhattamishra SK, Kesharwani P, Gorain B (2020) Silver nanoparticles: advanced and promising technology in diabetic wound therapy. Mater Sci Eng C Mater Biol Appl 112: 1–16. doi: 10.1016/j.msec.2020.110925.

19. Fayyadh AA, Jaduaa Alzubaidy MH, Fayyadh AA, Jaduaa Alzubaidy MH (2021) Green-synthesis of Ag_2O nanoparticles for antimicrobial assays. J Mech Behav Mater 30: 228–236. doi: 10.1515/jmbm-2021-0024.
20. Ibraheem DR, Hussein NN, Sulaiman GM (2023) Antibacterial activity of silver nanoparticles against pathogenic bacterial isolates from diabetic foot patients. Iraqi J Sci 64: 2223–2239. doi: 10.24996/ijs.2023.64.5.11.
21. Oyibo SO, Jude EB, Tarawneh I, Nguyen HC, Harkless LB, Boulton AJM (2001) A comparison of two diabetic foot ulcer classification systems: the wagner and the University of Texas wound classification systems. Diabetes Care 24: 84–88. doi: 10.2337/diacare.24.1.84.
22. Humphries RM, Ambler J, Mitchell SL, Castanheira M, Dingle T, Hindler JA, Koeth L, Sei K (2018) CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J Clin Microbiol 56: e01934-17. doi: 10.1128/jcm.01934-17.
23. O'Toole GA (2011) Microtiter dish biofilm formation assay. J Vis Exp 30: 2347. doi: 10.3791/2437.
24. Jagathesan G, Rajiv P (2018) Biosynthesis and characterization of iron oxide nanoparticles using *Eichhornia crassipes* leaf extract and assessing their antibacterial activity. Biocatal Agric Biotechnol 13: 90–94. doi: 10.1016/j.bcab.2017.11.014.
25. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45: 493–496. doi: 10.1093/ajcp/45.4_ts.493.
26. Adam KM, Mahmoud SM, Mahadi SI, Widatalla AH, Shawer MAG, Ahmed ME (2011) Extended leg infection of diabetic foot ulcers: risk factors and outcome. J Wound Care 20: 440-444. doi: 10.12968/jowc.2011.20.9.440.
27. Lavery LA, Bhavan K, Wukich DK (2019) Biofilm and diabetic foot ulcer healing: all hat and no cattle. Ann Transl Med 7: 159–159. doi: 10.21037/atm.2019.03.33.
28. Malik A, Mohammad Z, Ahmad J (2013) The diabetic foot infections: biofilms and antimicrobial resistance. Diabetes Metab Syndr 7: 101–107. doi: 10.1016/j.dsx.2013.02.006.
29. Chatterjee D, Sivashanmugam K (2024) Insights on MDR Mechanism of *Pseudomonas aeruginosa* with emphasis on diabetic foot ulcer in the Indian subcontinent. J Pure Appl Microbiol 18: 837–852. doi: 10.22207/JPAM.18.2.08.
30. Chai W, Wang Y, Jiao F, Wu Y, Wang S (2020) A severe diabetic foot ulcer with intermediate cuneiform displacement and multidrug-resistant *Pseudomonas aeruginosa* infection: a rare case report. Front Med 7: 131. doi: 10.3389/fmed.2020.00131.
31. Guo H, Song Q, Mei S, Xue Z, Li J, Ning T (2023) Distribution of multidrug-resistant bacterial infections in diabetic foot ulcers and risk factors for drug resistance: a retrospective analysis. PeerJ 11: e16162. doi: 10.7717/peerj.16162.
32. Cavallo I, Sivori F, Mastrofrancesco A, Abril E, Pontone M, Di Domenico EG, Pimpinelli F (2024) Bacterial biofilm in chronic wounds and possible therapeutic approaches. Biology 13: 109. doi: 10.3390/biology13020109.
33. Pouget C, Dunyach-Remy C, Pantel A, Schuldiner S, Sotto A, Lavigne JP (2020) Biofilms in diabetic foot ulcers: significance and clinical relevance. Microorganisms 8: 1–15. doi: 10.3390/microorganisms8101580.
34. Singh A, Sharma S, Banerjee T, Pratap A, Shukla VK (2022) Significant in-vitro and in-vivo antimicrobial and antibiofilm activity of colloidal silver nanoparticles (cAgNPs) in chronic diabetic foot ulcers. Int J Low Extrem Wounds 24: 15347346221088690. doi: 10.1177/15347346221088690.
35. Loera-Valencia R, Neira RE, Urbina BP, Camacho A, Galindo RB (2022) Evaluation of the therapeutic efficacy of dressings with ZnO nanoparticles in the treatment of diabetic foot ulcers. Biomed Pharmacother 155: 113708. doi: 10.1016/j.biopha.2022.113708.
36. Hajati Ziabari A, Asadi Heris M, Mohammad Doodmani S, Jahandideh A, Koorehpaz K, Mohammadi R (2022) Cinnamon nanoparticles loaded on chitosan- gelatin nanoparticles enhanced burn wound healing in diabetic foot ulcers in rats. Int J Low Extrem Wounds 5: 15347346221101245. doi: 10.1177/15347346221101245.
37. Alwis R, Alwis R, Al-Raeei M (2023) Nanoparticles zinc paste bandages for the treatment of Syrian woman diabetic patient with ulcers in the foot: case images. Clin Case Rep 11: e7445. doi: 10.1002/ccr3.7445.
38. Longano D, Ditaranto N, Sabbatini L, Torsi L, Cioffi N (2011) Synthesis and antimicrobial activity of copper nanomaterials. Nano-Antimicrobials 26: 85–117. doi: 10.1007/978-3-642-24428-5_3.
39. Abdelaal A, Soliman M, Rafik H, Emam M, Mohamed Elsadek MM (2021) Blind comparative study between silver nanoparticles dressings and standard moist wound dressings (SMWD) in management of diabetic foot ulcers. QJM 114. doi: 10.1093/qjmed/hcab097.040.
40. Akhtar F, Khan AU, Qazi B, Kulanthaivel S, Mishra P, Akhtar K, Ali A (2021) A nano phototheranostic approach of toluidine blue conjugated gold silver core shells mediated photodynamic therapy to treat diabetic foot ulcer. Sci Rep 11: 24464. doi: 10.1038/s41598-021-04008-x.
41. Sheir MM, Nasra MMA, Abdallah OY (2022) Phenytoin-loaded bioactive nanoparticles for the treatment of diabetic pressure ulcers: formulation and in vitro/in vivo evaluation. Drug Deliv Transl Res 12: 2936–2949. doi: 10.1007/s13346-022-01156-z.
42. Ali IH, Khalil IA, El-Sherbiny IM (2023) Design, development, in-vitro and in-vivo evaluation of polylactic acid-based multifunctional nanofibrous patches for efficient healing of diabetic wounds. Sci Rep 13: 3215. doi: 10.1038/s41598-023-29032-x.
43. Xu Z, Liu Y, Ma R, Chen J, Qiu J, Du S, Li C, Wu Z, Yang X, Chen Z, Chen T (2022) Thermosensitive hydrogel incorporating Prussian blue nanoparticles promotes diabetic wound healing via ROS scavenging and mitochondrial function restoration. ACS Appl Mater Interfaces 14: 14059–14071. doi: 10.1021/acsami.1c24569.
44. Elsherbini AM, Sabra SA, Rashed SA, Abdelmonsif DA, Haroun M, Shalaby TI (2023) Electrospun polyvinyl alcohol/ *Withania somnifera* extract nanofibers incorporating tadalafil-loaded nanoparticles for diabetic ulcers. Nanomedicine 18: 1361–1382. doi: 10.2217/nnm-2023-0127.
45. Zhang J, Liu H, Che T, Zheng Y, Nan X, Wu Z (2023) Nanomaterials for diabetic wound healing: visualization and bibliometric analysis from 2011 to 2021. Front Endocrinol 14: 1124027. doi: 10.3389/fendo.2023.1124027.
46. Gudkov SV, Serov DA, Astashev ME, Semenova AA, Lisitsyn AB (2022) Ag_2O nanoparticles as a candidate for antimicrobial compounds of the new generation. Pharmaceuticals 15: 968. doi: 10.3390/ph15080968.
47. Sathiyaseelan A, Saravanakumar K, Mariadoss AVA, Wang MH (2021) Antimicrobial and wound healing properties of FeO fabricated chitosan/PVA nanocomposite sponge. Antibiotics 10: 524. doi: 10.3390/antibiotics10050524.

48. Essa MS, Ahmad KS, Zayed ME, Ibrahim SG (2023) Comparative study between silver nanoparticles dressing (SilvrSTAT Gel) and conventional dressing in diabetic foot ulcer healing: a prospective randomized study. Int J Low Extrem Wounds 22: 48–55. doi: 10.1177/1534734620988217.
49. Nor Azlan AYH, Katas H, Mh Busra MF, Salleh NAM, Smandri A (2021) Metal nanoparticles and biomaterials: the multipronged approach for potential diabetic wound therapy. Nanotechnol Rev 10: 653–670. doi: 10.1515/ntrev-2021-0046/html.
50. Gudkov SV, Burmistrov DE, Serov DA, Rebezov MB, Semenova AA, Lisitsyn AB (2021) Do iron oxide nanoparticles have significant antibacterial properties? Antibiotics 10: 884. doi: 10.3390/antibiotics10070884.