**Republic of Iraq**
**Ministry of Higher Education and Scientific Research**
**University of Misan**
**College of Science**
**Department of Biology**

# Isolation and identification of bacteria and fungi and study of their sensitivity to BiONPs, with a focus on immunological aspects in diabetic foot ulcer patients

**A search submitted to the college of Science (University of Misan ) in partial Fulfillment of the Requirements for the Degree of Baccalaureate in Biology**

**By**
**Fatima E. Faleh & Kawthar S. Zwaid**

**Supervised by**
**Dr . Raed S. Madhi**

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بسم الله الرحمن الرحيم

(وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ ٱللَّهِ عَلَيْكَ عَظِيمًا)

صدق الله العلي العظيم

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# Certification

I certify that this project was prepared under my supervision at College of Science - University of Misan as a partial requirement for the degree of Bachelor of Science in Biology.

**Instructor**
**Dr. Raed Saddam Madhi**
**Supervisor**

In view of the available recommendations, I forward this project for debate by the examining committee.

**Prof. Dr. Saleh Hassan Jazza**

Head of biology Department

College of Science -University of Misan

# Dedication

All praise and thanks are due to Allah, the Most Gracious, the Most Merciful. With His guidance and grace, this research has been completed in the best and most fulfilling way.

We dedicate this work to our beloved families, whose unwavering support, prayers, and love have been the light that guided us through every challenge.

To our loyal friends, who stood by our side with encouragement, patience, and heartfelt words that lifted our spirits time and time again.

To everyone who believed in us, who saw potential in our efforts, and who inspired us—whether through words, actions, or silent prayers.

To all of you who were a part of this journey,
we say: Thank you. This achievement is as much yours as it is ours.

And with your support, we step into the future with steady hearts and deep gratitude.

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# Summary

# Summary

Diabetic foot ulcer (DFU) is one of the most prevalent and severe chronic complications of diabetes mellitus. It is mainly caused by peripheral neuropathy and impaired blood circulation, which delay wound healing and increase the risk of infection. DFUs are among the leading causes of non-traumatic lower-limb amputations and represent a significant physical, psychological, and economic burden on patients.

This study specifically focused on grade 2 diabetic foot ulcers, which involve subcutaneous tissue and often reach the tendons without bone involvement. This stage reflects disease progression and presents challenges for conservative treatment, especially due to the presence of polymicrobial infections that hinder healing.

A total of 50 clinical samples were collected from patients with diabetic foot ulcers at the Maysan Specialized Center for Diabetes and Endocrinology between December 2024 and February 2025. Fasting blood sugar (FBS) and HbA1c were measured. Sterile cotton swabs were used for sample collection. The isolates were distributed as follows; 30 bacterial isolates and 20 fungal isolates.

Demographic analysis showed that Females were more affected than males, representing 57% of cases compared to 43%. The ≥50 age group had the highest incidence (43%), followed by the 40–49 (34%) and 30–39 (23%) age groups. The results also showed that FBS and levels were significantly increased ($p \leq 0.05$) in patients with DFU.

Bacterial samples were cultured on Blood agar and MacConkey agar. Fungal samples were cultured on Sabouraud Dextrose Agar. Microbial identification was performed using the VITEK-2 Compact System, which revealed the presence of :

• Gram-positive bacteria:

– *Staphylococcus aureus*

– *Staphylococcus pseudintermedius*

• Gram-negative bacteria:

– *Proteus mirabilis*

– *Pseudomonas oryzihabitans*

For fungal identification, Germ Tube Test and CHROMagar Candida medium were used. The isolates exhibited green colonies and formed germ tubes, suggesting they may be either *Candida albicans* or *Candida dubliniensis*. Due to their phenotypic similarity, definitive species identification was not possible without molecular testing.

Antimicrobial susceptibility testing was performed using Mueller-Hinton agar and standard antibiotic discs. The results showed that gram-positive strains showed high sensitivity to Levofloxacin. Gram-negative strains were mostly sensitive to Amikacin and Ceftazidime, except *Proteus mirabilis*, which exhibited partial resistance to Ceftazidime. *Candida* sp isolates were sensitive to Caspofungin and resistant to Voriconazole.

Additionally, bismuth oxide nanoparticles (BiONPs) were synthesized using green methods from three plant extracts:

• *Rosmarinus officinalis* (rosemary)

• *Camellia sinensis* (green tea)

• *Silybum marianum* (milk thistle)

• Rosemary-based BiONPs had strong antibacterial and antifungal activity even at 25% concentration.

• Green tea BiONPs were effective against *Staphylococcus pseudintermedius* and *Pseudomonas oryzihabitans* at all tested concentrations but showed limited activity against *Proteus mirabilis.*

• Milk thistle BiONPs exhibited minimal antimicrobial effect, with activity limited to *Staphylococcus aureus*.

These findings support the promising antimicrobial potential of plant-based BiONPs, especially in the context of increasing microbial resistance to conventional antibiotics.

Furthermore, the study highlighted the immunological aspect of diabetic foot ulcers, particularly the dysfunction of innate immune cells. The results found a strong association between neutrophils and monocytes with bacterial infections (gram positive and gram negative bacteria) in patients with

diabetic foot ulcers and as follows: ($y = -10.573x + 87.118$; $R^2 = 0.6417$), ($y = -11.084x + 88.133$; $R^2 = 0.615$), ($y = 3.9429x + 7.9571$; $R^2 = 0.5807$), and ($y = 3.9279x + 8.125$; $R^2 = 0.622$). Thus, study the exact mechanism of neutrophils and monocytes in diabetic foot ulcer could greatly participate in healing of diabetic foot ulcer.

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## List of Abbreviations

| ANOVA | Analysis of variance |
|---|---|
| **R0129** | Diamino-6,7-Diisopropylpteridine Resistance-2,4 |
| **AGAL** | Alpha-Galactosidase |
| **AGLU** | Alpha-Glucosidase |
| **APPA** | Ala-Phe-Pro-Arylamidase |
| **AST-P580 card** | Antimicrobial Susceptibility Testing P580 Card |
| **BGAL** | Beta-Galactosidase |
| **BGUR** | Beta-Glucuronidase |
| **BiONPs** | Bismuth Oxide Nanoparticles |

| | |
|---|---|
| **CLSI** | Clinical and Laboratory Standards Institute |
| **CRP** | C-Reactive Protein |
| **CS** | *Camellia sinensis* |
| **DFIS** | Diabetic Foot Infections |
| **DFUS** | Diabetic Foot Ulcers |
| **dMAL** | D-Maltose |
| **dMAN** | D-Manitol |
| **DMSO** | Dimethyl Sulfoxide |
| **dSOR** | D-Sorbitol |
| **dTRE** | D-Trehalose |
| **ESR** | Erythrocyte Sedimentation Rate |
| **FBS** | Fasting Blood Sugar |
| **FE-SEM** | Field Emission Scanning Electron Microscopy |
| **GN card** | Gram-Negative identification card |
| **GP card** | Gram-Positive identification card |
| **GTT** | Germ Tube Test |
| **HbA1c** | Hemoglobin A1c |
| **HLA types** | Human Leukocyte Antigen types |
| **IGF-1** | Insulin-like Growth Factor 1 |
| **ILATk** | L-Lactate Alkalinisation |
| **LPCB** | Lactophenol Cotton Blue |
| **MALDI-TOF** | Matrix-Assisted Laser Desorption/Ionization - Time of Flight |
| ***MDR-TB*** | Multi-Drug Resistant Tuberculosis |
| **MRI** | Magnetic Resonance Imaging |
| **MRSA** | Methicillin-Resistant *Staphylococcus aureus* |
| **NETS** | Neutrophil Extracellular Traps |

| | |
|---|---|
| **NLRP3** | Nucleotide-Binding Domain, Leucine-Rich–Containing Family, Pyrin Domain–Containing-3 |
| **NPs** | Nanoparticles |
| **PCR** | Polymerase Chain Reaction |
| **PHOS** | Hosphatase |
| **ProA** | L-Proline Arylamidase |
| **PVD** | Peripheral Vascular Disease |
| **PyrA** | L-Pyrrolydonyl-Arylamidase |
| **RO** | *Rosmarinus officinalis* |
| **ROS** | Reactive Oxygen Species |
| **SAC** | Saccharose/Sucrose |
| **SM** | *Silybum marianum* |
| **TIMPS** | Tissue Inhibitors of Metalloproteinases |
| **TyrA** | Tyrosine Arylamidase |
| **UV-visible** | Ultraviolet-Visible Spectroscopy |
| **XRD** | X-Ray Diffraction |

# Chapter One
# Introduction

# Introduction

## 1.1 Introduction

Diabetic foot ulcer (DFU) represents one of the most severe and persistent complications associated with uncontrolled diabetes mellitus, frequently leading to infection, amputation, or even mortality. With the global rise in diabetes prevalence, the burden of DFUs has reached alarming levels, especially in low- and middle-income countries, where limited access to advanced wound care exacerbates patient outcomes (Armstrong *et al*., 2017). Beyond the metabolic dysfunction, the chronic nature of DFUs is largely sustained by polymicrobial infections—bacterial and fungal—that thrive in the necrotic tissue and immunocompromised microenvironment of the diabetic foot.

Traditional treatments, including antibiotics and debridement, have shown limited efficacy due to rising antimicrobial resistance and biofilm formation (Lipsky *et al*., 2020). In this context, there is growing interest in harnessing the potential of nanotechnology to revolutionize infection control and tissue regeneration. Among the most promising agents are metal-based nanoparticles, which offer dual roles in antimicrobial action and wound healing modulation (Ranjbar-Mohammadi & Bahrami, 2018).

Bismuth nitrate [$Bi(NO_3)_3$] is commonly used as a precursor in the synthesis of bismuth oxide nanoparticles ($Bi_2O_3$) due to its availability and ease of thermal decomposition (Arora *et al*., 2018). Bismuth oxide nanoparticles have gained attention for their unique physicochemical properties, including antimicrobial activity, photocatalytic efficiency, and low toxicity, which make them suitable for environmental and biomedical applications (Arora *et al*., 2018).

Recently, green synthesis approaches using plant extracts have become popular due to their sustainability and environmental friendliness (Khan *et al*., 2020). These extracts contain phytochemicals such as phenolics, flavonoids, and antioxidants that act as natural reducing and stabilizing agents in the nanoparticle formation process (Sharma *et al*., 2015). This method not only reduces the reliance on hazardous chemicals but also aligns with the principles of green chemistry (Khan *et al*., 2020).

Furthermore, the interaction between these nanoparticles and host immune components, especially white blood cells (WBCs), opens an underexplored avenue for understanding immune modulation in diabetic wounds. WBCs play a critical role in inflammation and infection resolution, yet their dysfunction in diabetic individuals contributes to prolonged healing (Wetzler *et al*., 2020). Evaluating how bismuth-based nanoparticles influence WBC behavior may unveil novel therapeutic mechanisms for immunocompromised patients.

**Aims of the study**

- To isolate and characterize microbial pathogens—both bacterial and fungal—from diabetic foot ulcers (DFUs).

- To evaluates the antimicrobial efficacy of green-synthesized bismuth nanoparticles against the isolated pathogens.

- It highlights a multifaceted approach to DFU management that merges microbiology, nanotechnology, and immunology.

- The study investigates the potential correlation of white blood cells (WBCs) to the diabetic foot infection.

# Chapter Two
# Review of Literatures

# Literature review

## 2.1 Diabetes

Diabetes stands as a chronic condition which disrupts normal food energy processing in your body. Your body transforms most of the food you consume into sugar, specifically glucose that enters your bloodstream. The pancreas releases insulin when blood sugar levels rise. The hormone insulin acts as a key that permits blood sugar to enter the body's cells where it becomes an energy source(Karpińska & Czauderna, 2022).

Diabetes causes an inability to generate sufficient insulin or proper utilization of it by the body. When there isn't enough insulin or cells stop responding to insulin blood sugar levels rise above normal within your bloodstream. Over time this situation can provoke serious health problems including kidney disease as well as heart disease and vision deterioration (Galicia-Garcia *et al*., 2020).

### 2.1.1 Signs and symptoms

Excessive thirst, weight loss, and excessive urination are some of the common symptoms of unidentified diabetes. Other undefined symptoms are fatigue, blurred vision, and genital itching caused by a Candida infection (Stefaniak *et al*., 2021). Symptoms may not show in half of those who have diabetes. Type 2 diabetes starts gradually; patients can be symptom-free for years. Type 1 arises suddenly after a pre-clinical phase (L. Huang *et al*., 2022).

A diabetic ketoacidosis, a medical emergency, typically occurs in type 1 diabetes. However, it can also occur in type 2 diabetes if the condition is of very long duration or if the patient's β-cells are not functioning effectively (Elendu *et al*., 2023).

Symptoms that excess ketone bodies are being produced include vomiting, nausea, abdominal pain, fruity smell on the breath, deep breathing (also known as Kussmaul breathing), and in severe cases, an altered level of consciousness. Hyperosmolar hyperglycemic state is another emergency that happens when there is very high blood sugar that results in dehydration, which can cause high sodium levels in the blood and even coma (Pasquel & Umpierrez, 2014)

One of the side effects of using insulin in diabetes is low blood sugar, or hypoglycemia. Symptoms can range from intense sensations like sweating, trembling, and fast heartbeat to more serious problems like confusion, disorientation, seizures, coma, and in severe cases, even death. Mild symptoms may not manifest prior to the onset of thinking problems since having low blood sugar regularly can lower the threshold at which symptoms are felt (Agrawal *et al*., 2022).

### 2.1.2 Types of diabetes

There are two major types of diabetes, which are type 1 and type 2, but there are others. Those with type 1 usually need injections or insulin therapy to control blood glucose. In the case of type 2, metformin or semaglutide, which are anti-diabetic medications, and lifestyle changes are normally prescribed by physicians. There are some women who develop gestational diabetes during pregnancy, but it usually disappears a few weeks after delivery (Marín-Peñalver *et al*., 2016).

#### Type 1

Type 1 is the most common type of diabetes in children under the age of 20 years and represents 5 to 10% of all cases (Mobasseri *et al*., 2020). "Juvenile-onset diabetes" is no longer a preferred term because the condition is mostly seen in adults (American Diabetes Association, 2009).

The condition has two categories: immune-mediated or idiopathic (where there is no known cause). It is marked by the devastation of insulin-producing beta cells within pancreatic islets, leading to an extreme lack of insulin (American Diabetes Association, 2009). Most are immune-mediated; a T cell-mediated assault annihilates beta cells, and this leads to a lack of insulin production (Toren *et al*., 2021). Because of low insulin and impaired reaction to low blood glucose, patients have irregular and unpredictable blood glucose levels (Christou *et al*., 2023). Type 1 diabetes is affected by several genes, some of which are HLA types, and is partially inherited. One or more of the environmental factors, like a viral infection or diet, trigger diabetes in people who are genetically susceptible to develop the disease (Knip & Simell, 2012). Several viruses have been implicated in this hypothesis, but there is still no strong evidence to support it in humans.

**Type 2**

Insulin resistance, which can be accompanied by impaired insulin secretion, is a key feature of type 2 diabetes (Czech, 2017). The impaired responsiveness of tissues in the body to insulin is believed to be mediated through the insulin receptor (Zhao *et al*., 2023). The exact defects are not defined. Diabetic mellitus due to a known defect is classified differently. Type 2 diabetes is responsible for the highest proportion of diabetes at 95% (American Diabetes Association 2009). Most individuals with type 2 diabetes have prediabetes, which signals impaired glucose tolerance and/or impaired fasting glucose, before reaching the diagnostic criterion (American Diabetes Association 2009). You can reverse or delay prediabetes from developing into type 2 diabetes by medication or lifestyle change. They make your body utilize insulin more effectively or reduce the sugar your liver makes (Portero McLellan *et al*., 2014).

Lifestyle and genetics play a major role in most cases of type 2 diabetes (Murea *et al*., 2012). Numerous lifestyle elements, such as obesity (i.e., a body mass index of more than 30), stress, poor diet, lack of exercise, and urban residence, are well-documented to significantly play a role in the onset of type 2 diabetes (Wu *et al*., 2014). 30% of Chinese and Japanese, 60–80% of European and African, and 100% of Pima Indian and Pacific Island populations' cases are caused by excess body fat (Ma & Chan 2013).

Beverages containing added sugar are associated with an increased risk (Rippe & Angelopoulos, 2016).

The second factor to note is the type of fats consumed; polyunsaturated and monounsaturated decrease the risk, while saturated and trans increase the risk (DiNicolantonio & O'Keefe, 2022). Eat too much white rice and one increases the risk of developing diabetes, especially among Chinese and Japanese populations (Hu *et al*., 2012). Physical inactivity may increase the risk of developing diabetes among some people (Qin *et al*., 2010). Neglect is the most significant factor since it increases the risk of type 2 diabetes by 32% in later life (Rich-Edwards *et al*., 2010). Additional adverse childhood events include abuse, neglect, and home problems. Potential risk factors include unhealthy lifestyle (such as poor diet and physical inactivity) and side effects of antipsychotic medications (such as dyslipidemia, weight gain, and metabolic disturbances) (Monnat & Chandler, 2015).

### 2.1.3 Pathophysiology of diabetes

Insulin is the main hormone that controls how much sugar, called glucose, is taken in by the body's cells. This mainly happens in the liver, fat tissue, and muscle, but not in smooth muscle because insulin works through a different receptor called IGF-1. Because of this, the main reason for all types of diabetes mellitus is either not having enough insulin or the body's

receptors not responding to it (Leroith *et al*., 2011). Beta cells, or β-cells, are found in the islets of Langerhans in the pancreas. They release insulin into the bloodstream when the blood glucose level rises after eating. Two thirds of the body's cells use insulin to take up glucose from the blood. They either use it for energy, convert it into other molecules that they need, or store it. Low blood glucose triggers the breakdown of glycogen to glucose and the reduced release of insulin from the beta cells. Insulin works in the opposite direction of glucagon, which is the hormone mainly accountable for regulating this process (Rorsman & Ashcroft, 2018). Body cells need insulin in order to take up glucose in a manner that is appropriate. Muscles and the liver are not ideal places to store glucose in case of insufficient insulin, insulin-resistant cells, or if insulin is not working effectively. Ultimately, this leads to very elevated blood sugar levels, low protein synthesis, and other metabolic complications, including metabolic acidosis when there is a lack of insulin (Wilcox, 2005).

### 2.1.4 Diabetic foot ulceration

Diabetic foot disease is used to describe any condition that most directly affects the feet of diabetics due to sensory neuropathy or PAD. Diabetes potentially causes both chronic and acute complications, including diabetic foot disorders (Altoijry *et al*., 2021). The diabetic foot syndrome term captures the combination of most frequent diabetic foot disorders, some of which include neuropathic osteoarthropathy, diabetic foot ulcer, and complicating infections. Charcot foot is the bony deformity that occurs as a consequence of this. Diabetic individuals possess dry skin and have a diminished capacity for nociception, which is the sensation of pain, due to ongoing peripheral nerve deterioration, or as more commonly referred to, diabetic neuropathy. Consequently, minor trauma can be overlooked and ultimately progress to a full-thickness diabetic foot ulcer. Furthermore, foot

surgery procedures can be carried out effectively without the use of anesthetics. With 512 mN quantitative pinprick stimulation, it is easy to assess the in sensivity of the foot to pain (Konarzewska *et al*., 2018). Diabetic angiopathy, or defective blood flow to the extremities, may be caused by the coupling of peripheral nerve impairment and peripheral artery disease (PAD) in patients with diabetes (Soyoye *et al*., 2021). Concomitant peripheral artery disease (PAD) occurs in nearly half of the patients with diabetic foot ulcers (Altoijry *et al*., 2021). Recent studies have associated a deficiency of vitamin D with diabetic foot infection, in addition to a heightened risk of amputation and mortality (Danny Darlington *et al*., 2019). Literature has demonstrated that the lifetime prevalence of foot ulcers among individuals with diabetes has been approximated to be 15% and could even be as high as 25% (McDermott *et al*., 2023). In situations where wounds have compromised healing, there is also a possibility of infection that may progress to bones and joints and, as a result, necessitate the amputation of a lower extremity. The most prevalent reason for non-traumatic amputation among diabetics is foot infection (Armstrong & Lipsky, 2004).

#### 2.1.4.1 Diabetic Foot Problems' Warning Signs

1. Change in color of the skin.
2. Variations in the temperature of the skin.
3. Swelling of the ankle or foot region.
4. legs hurting.
5. Open lesions on the foot that show drainage or take a long time for healing.
6. Ingrown toenails or fungal infections.
7. dry skin fissures, particularly at the heel.
8. An unusual or persistent odor emanating from the feet.

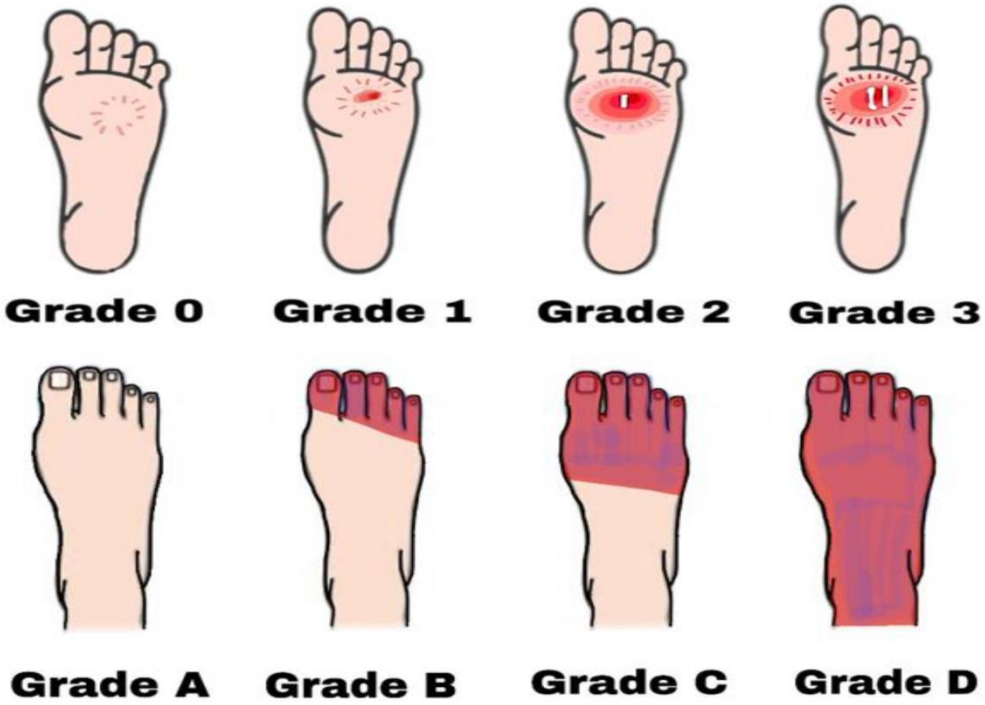



**Figure 2-1.** Diabetic foot ulcers according to Brodsky's pattern. Although there are no signs of ulcers in Grade 0, there is ongoing pain. A superficial ulcer is grade 1. A profound ulceration near the bones is indicated by a grade of 2. Grade 3 denotes the presence of osteomyelitis, a serious infection. Grade A is ischemia-free. Ischemia but not gangrene is indicated by a grade B. Partial and total gangrene infections with ischemia are classified as grades C and D, respectively (Ansari *et al*. 2022).

### 2.1.4.2 Diagnosis of diabetic foot infection

A comprehensive assessment of the lower extremities is imperative for the early identification of pathologies. Individuals predisposed to the development of foot ulcers may derive significant advantages from evaluations aimed at detecting peripheral neuropathy and peripheral artery disease. Suboptimal glycaemic control, along with a documented history of ulcerations or amputations, significantly elevates the risk (Alexiadou & Doupis, 2012).

Conduct a thorough evaluation of the patient's general health status for signs indicative of toxicity or sepsis, such as the presence or absence of

fever, malaise, atypical behaviors, compromised circulation, or respiratory difficulties. During each follow-up consultation, it is crucial to assess the feet for manifestations of active disease, including gangrenous tissue or ulceration. Scrutinize lesions that may predispose to ulceration, encompassing fungal infections, skin fissures, cracks, malformed nails, macerated interdigital areas, calluses, and deformities such as pes cavus, hammer toes, and claw toes (Tol *et al*., 2014). Employ the dorsum of your hand to gauge the thermal status of the feet. A cold foot may signify ischaemia, whereas a warmer foot accompanied by erythema and edema may suggest an inflammatory pathology, such as cellulitis or acute Charcot foot.

### 2.1.4.3 Risk factors

1- Neuropathy.

2- Peripheral Vascular Disease.

3- Presence of Pre-ulcerative Lesions.

4- Hypertension.

5- Sex and Age.

6- Smoking.

7- Charcot Joint.

## 2.2 Bacterial infection in diabetic foot ulceration

Bacterial infection significantly complicates the treatment of DFU, primarily due to biofilm formation, which is the cause of chronicity and antibiotic resistance (Pouget *et al*., 2020). An estimated 50% to 60% of DFUs are likely to become infected, with moderate to severe infection carrying a 20% risk of amputation (Armstrong *et al*., 2023).

### 2.2.1 Causes and Predisposing Factors for Bacterial Infection in Diabetic Foot Ulcers

Bacterial infections in DFUs are influenced by a combination of host-related factors, including weakened immune systems, poor blood flow, diabetic neuropathy, and the unique environment of the ulcer itself.

1. Weakened Immune System

Diabetic patients often exhibit impaired immune responses, which increase their susceptibility to infections. Hyperglycemia, a hallmark of diabetes, disrupts various components of the immune system, including neutrophil function, humoral immunity, and the antioxidant defense system (Casqueiro *et al*., 2012 ; Bessman & Sapico, 1992).

2. Poor Blood Flow

Peripheral arterial disease (PAD) and microvascular dysfunction are common in diabetic patients and significantly contribute to the development and progression of DFUs. Reduced blood flow impairs oxygen delivery and nutrient supply to the affected tissues, delaying wound healing and increasing the risk of infection (Kim, 2024 ; Uçkay *et al*., 2014). Hypoxia in the wound tissue not only hinders the immune response but also creates an anaerobic environment that favors the growth of anaerobic bacteria, such as *Bacteroides* and *Fusobacterium species* (Villa *et al*., 2024 ; Pitocco *et al*., 2019).

3. Diabetic Neuropathy

Diabetic neuropathy is a key predisposing factor for DFUs. Sensory neuropathy reduces pain perception, leading to unnoticed injuries and trauma to the foot. Motor neuropathy can cause muscle imbalances and gait abnormalities, increasing the risk of repetitive stress and ulcer formation

(Kim, 2024 ; Uçkay *et al*., 2014). Autonomic neuropathy further exacerbates the problem by altering sweat and oil gland function, leading to dry, cracked skin that is more susceptible to bacterial invasion (Kim, 2024 ; Yousif *et al*., 2024).

4. The Ulcer Environment

The environment of diabetic foot ulcers provides an ideal breeding ground for bacterial growth. The warm, moist, and nutrient-rich conditions of the ulcer attract a wide range of microorganisms, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and anaerobic bacteria (Villa *et al*., 2024 ; Bansal *et al*., 2008).

5. Antimicrobial Resistance

The rise of antimicrobial resistance among bacterial pathogens in DFUs poses a significant challenge to treatment. Gram-negative bacteria, in particular, exhibit high levels of resistance to commonly used antibiotics, necessitating the use of broad-spectrum therapies (Maity *et al*., 2024 ; Zubair *et al*., 2010).

### 2.2.2 Types of Bacteria Associated with Diabetic foot ulcers

Diabetic foot infections (DFIs) are commonly associated with a variety of bacterial pathogens, including Gram-positive, Gram-negative, and anaerobic bacteria. These infections are often polymicrobial, involving multiple bacterial species, and their microbiological profiles can vary based on geographic location, patient demographics, and the severity of the infection .

### 2.2.2.1 Gram-Positive Bacteria

1. *Staphylococcus aureus*

*Staphylococcus aureus* is one of the most common Gram-positive bacteria isolated from diabetic foot infections. It is known for its ability to form biofilms and develop resistance to antibiotics, including methicillin-resistant *Staphylococcus aureus* (MRSA) strains. MRSA is particularly problematic due to its resistance to beta-lactam antibiotics, which are commonly used in clinical settings. Studies have shown that *S. aureus* is frequently isolated in both monomicrobial and polymicrobial infections, and it is often associated with severe infections and poor outcomes (Meloni *et al*., 2023 ; Goh *et al*., 2020 ; Evran *et al*., 2018).

2. *Staphylococcus species*

*Staphylococcus species* are the most common organisms isolated in cases of a DFU with *Staphylococcus pseudintermedius* being the most common. They are well known for their biofilm formation ability, which is a protective shield of bacteria and the host protective system working and the established environment resulted in the molecular effects of the bacterial genes expression controlling("Screening and Characterization of Biofilm Formation by *Staphylococcus Spp* and *Pseudomonas Spp* Isolates from Diabetic Foot Ulcer", 2022) (Mamdoh *et al*., 2023).

### 2.2.2.2 Gram-Negative Bacteria

1. *Pseudomonas oryzihabitans*

*Pseudomonas spp*, such as *Pseudomonas oryzihabitans*, are also prevalent pathogens of DFUs. These bacteria are aerobic pathogens which pass the polymicrobial character of those infections (Suryaletha *et al*., 2018).

*Pseudomonas spp* is known to form films, making the infection more difficult to treat because of their multidrug resistant properties(Mansour & Abdulwaha, 2024).

2. *Proteus spp*

*Proteus species*, such as *Proteus mirabilis*, are also commonly isolated from diabetic foot infections. These bacteria are known for their ability to produce urease, which can lead to the formation of kidney stones and other complications. Proteus infections are often associated with polymicrobial infections and are more common in patients with severe ulcers or osteomyelitis (Álvaro-Afonso *et al*., 2023 ; Liu *et al*., 2023).

### 2.2.3 Classification of Bacterial Infections in Diabetic Foot Ulcers

DFIs can be categorized into three main types :

#### 2.2.3.1 Superficial Infection

Superficial infections are the mildest form of DFIs and are typically limited to the skin and soft tissue. These infections are often caused by Gram-positive cocci, such as *Staphylococcus aureus* and *Streptococcus species*. Superficial infections are usually associated with minimal inflammation and do not involve deeper tissues (Matheson *et al*., 2021 ; Noor *et al*., 2017 ; Lipsky *et al*., 2012).

Erythema, induration, tenderness, warmth, and drainage are common signs of superficial infection (Matheson *et al*., 2021 ; Noor *et al*., 2017).

Superficial infections can often be treated with oral antibiotics targeting Gram-positive cocci (Rao & Lipsky, 2007 ; Lipsky, 1999).

Wound care, including debridement and off-loading, is essential for healing (Lipsky *et al*., 2012 ; Gemechu *et al*., 2013).

#### 2.2.3.2 Deep Tissue Infection

Deep tissue infections involve the fascia, muscle, or tendons and are more severe than superficial infections. These infections are often polymicrobial, involving both Gram-positive and Gram-negative bacteria, as well as anaerobes in some cases (Noor *et al*., 2017 ; Lipsky *et al*., 2012 ; Gadepalli *et al*., 2006).

Deep tissue infections are associated with more severe inflammation, such as increased erythema, swelling, and purulence (Noor *et al*., 2017 ; Lipsky *et al*., 2012). Systemic signs, such as fever and leukocytosis, may also be present in severe cases (Lipsky *et al*., 2012).

Deep tissue infections often require intravenous antibiotics and surgical intervention, such as debridement or drainage (Lipsky *et al*., 2012 ; Gemechu *et al*., 2013).

#### 2.2.3.3 Osteomyelitis

Osteomyelitis is a serious complication of DFIs and involves infection of the bone. It is often a consequence of untreated or inadequately treated deep tissue infections. Osteomyelitis is associated with a high risk of amputation and mortality if not managed promptly (Lavery *et al*., 2024 ; Lavery *et al*., 2020 ; Lipsky *et al*., 2012).

Imaging, such as plain radiographs, MRI, or bone biopsy, is essential for confirming the diagnosis (Cook *et al*., 1996 ; Woo *et al*., 2023 ; Hockney *et al*., 2022).

Osteomyelitis requires prolonged antibiotic therapy, often for more than 6 weeks, and may necessitate surgical debridement or resection of infected bone (Lipsky *et al*., 2012 ; Lipsky, 1999 ; Hockney *et al*., 2022).

### 2.2.4 Mechanisms of Infection's Effect on Wound Healing

Wound healing is a complex process that can be significantly disrupted by infections, particularly those involving bacterial biofilms. The presence of bacterial infections in wounds can lead to the production of toxins, the stimulation of a chronic inflammatory response, and the formation of biofilms, all of which can impede the healing process .

The mechanisms of bacterial toxin production, chronic inflammation, and biofilm formation are closely interconnected. For instance, the production of toxins can stimulate an inflammatory response, which in turn can create an environment that is conducive to biofilm formation (Vestweber *et al*., 2024 ; Zhang, 2023). Similarly, the chronic inflammatory response can lead to the release of ROS and other inflammatory mediators, which can damage the tissue and create a nutrient-rich environment that supports bacterial growth and toxin production (Gajula *et al*., 2020 ; Trøstrup *et al*., 2020).

Moreover, the formation of biofilms can perpetuate the cycle of toxin production and inflammation. Biofilms can act as a reservoir for bacteria, allowing them to persist in the wound and continuously produce toxins and other virulence factors (Bose & Das, 2024 ; Vestweber *et al*., 2024). This persistence of bacteria in the wound can lead to a prolonged inflammatory response, which can further delay healing .

Strategies to Combat Infection-Induced Impediments to Wound Healing Given the complex interplay between bacterial toxin production, chronic inflammation, and biofilm formation, effective strategies for promoting wound healing must address all three mechanisms. Several approaches have been explored, including the use of antimicrobial peptides,

immunomodulatory agents, and biofilm-disrupting therapies (Bose & Das, 2024 ; Jeon *et al*., 2023 ; Sankar & Muthukaliannan, 2024).

For example, nanodecoys and nano-on-nanodroplets have been developed to target bacterial biofilms and neutralize toxins (Bose & Das, 2024 ; Peng *et al*., 2024). These nanotechnology-based approaches can disrupt the biofilm matrix, making bacteria more susceptible to antibiotics and host immune defenses. Additionally, immunomodulatory agents, such as those that polarize macrophages towards the M1 phenotype, can enhance the host's immune response and promote the clearance of bacteria and biofilms (Bose & Das, 2024 ; Jeon *et al*., 2023).

### 2.2.5 Diagnosis and Treatment of Bacterial Infection of Diabetic Foot Ulcers

The diagnosis and treatment of bacterial infections in diabetic foot ulcers (DFUs) are critical due to the high risk of complications, including amputations and increased mortality.

#### 2.2.5.1 Diagnosis

1. Clinical Examination and Signs of Infection: Diagnosis begins with a clinical assessment, looking for signs such as purulent discharge, erythema, and edema. The severity of the infection is classified using systems like the IDSA/IWGDF classification, which considers the depth and systemic signs of infection(Kadanali *et al*., 2024).

2. Laboratory Tests: Bacterial cultures and antibiotic sensitivity testing are crucial for identifying pathogens and guiding antibiotic therapy. Inflammatory markers like CRP and ESR can help differentiate infection from colonization(Kadanali *et al*., 2024).

3. Advanced Techniques: Techniques such as PCR and MALDI-TOF are employed to accurately identify bacterial species, which is particularly important in polymicrobial infections(Soula, 2023 ; Grigoropoulou *et al*., 2017).

### 2.2.5.2 Treatment

1. Wound Cleaning and Debridement: Aggressive debridement is necessary to remove necrotic tissue and reduce bacterial load, promoting healing and preventing further infection(Kadanali *et al*., 2024).

2. Antibiotic Therapy: Treatment typically starts with empiric antibiotics, adjusted based on culture results. The duration varies from 1-2 weeks for mild infections to longer for severe cases(Grigoropoulou *et al*., 2017).

3. Alternative Treatments: The use of nanoparticles and plant extracts is being explored as adjunctive therapies to enhance healing and reduce reliance on antibiotics(Soula, 2023).

4. Preventing Antibiotic Resistance: Strategies include using targeted antibiotics based on culture results and employing non-antibiotic therapies to minimize resistance development(LaSalvia & Karchmer, 2024).

While the primary focus is on effective diagnosis and treatment, it is also important to consider preventive measures. Regular patient education, proper foot care, and interdisciplinary collaboration are vital in preventing the development and recurrence of DFUs. Additionally, the use of orthoses and hyperbaric oxygen therapy can support healing and reduce complications(Kadanali *et al*., 2024).

## 2.3 Fungal infection in diabetic foot ulceration

Fungal infections in diabetic foot ulcers (DFUs) are increasingly recognized as a significant factor in the nonhealing nature of these wounds,

often complicating the clinical picture and leading to higher rates of amputation. Differentiating between bacterial and fungal infections in DFUs is crucial for effective treatment and management. Fungal infections, particularly those caused by *Candida species*, are prevalent in DFUs and often coexist with bacterial infections, complicating the healing process and increasing the risk of severe outcomes such as osteomyelitis and amputation.

In a study of patients with osteomyelitis, 22% had mixed fungal and bacterial growth, highlighting the role of fungi in complicating DFUs(Khalifa *et al*., 2022).

### 2.3.1 Differentiating Fungal from Bacterial Infection

Fungal infections are often diagnosed through deep tissue biopsies and specific fungal cultures, which are essential for accurate identification and treatment(Sk *et al*., 2023 ; Sanniyasi *et al*., 2015).

The presence of fungal infections is often linked to prolonged antibiotic use, which can disrupt normal flora and promote fungal overgrowth(Sanniyasi *et al*., 2015).

### 2.3.2 Common fungi in diabetic foot ulcers

Fungal infections in diabetic foot ulcers (DFUs) are a significant concern due to their potential to complicate wound healing and increase the risk of severe outcomes, such as amputations. The most common fungi isolated from DFUs include *Candida species*, *Aspergillus species*, and *Zygomycetes* like *Rhizopus species*. These fungi can exacerbate the condition of DFUs by contributing to chronic infection and inflammation, which impedes healing.

1.*Candida Species*

*Candida species* are the most frequently isolated fungi in DFUs, with *Candida tropicalis* and *Candida albicans* being the predominant species(Sk *et al*., 2023 ; Kalshetti *et al*., 2021 ; Al-Chalabi *et al*., 2024).

These yeasts are opportunistic pathogens that thrive in moist environments, such as those found in chronic wounds. They can form biofilms, which protect them from the host immune response and antifungal treatments(Aring *et al*., 2021).

2. *Aspergillus Species*

Although less common than Candida, Aspergillus species, such as *Aspergillus fumigatus*, have been isolated from DFUs(Kalshetti *et al*., 2021).

*Aspergillus* is a mold that can produce spores, which are highly resistant to environmental stresses and can colonize wounds.

*Aspergillus* can cause invasive infections, particularly in immunocompromised individuals, leading to severe complications in DFUs(Kalshetti *et al*., 2021).

While *Candida species* are the most common fungi associated with DFUs, the presence of other fungi like *Aspergillus* highlights the complexity of fungal infections in these wounds.

### 2.3.3 Risk Factors for Fungal Infections in Diabetic Foot Ulcers

Numerous risk factors have been delineated that render diabetic individuals susceptible to fungal infections in their foot ulcers.

1. Poor Glycemic Contro

A prominent risk factor for the incidence of fungal infections in DFUs is suboptimal glycemic control. Elevated blood glucose levels undermine the efficacy of the immune system, diminishing the organism's capacity to combat infections. Empirical studies have demonstrated that individuals exhibiting heightened HbA1c levels exhibit an increased vulnerability to fungal infections, as hyperglycemia fosters an environment favorable to fungal proliferation (Akkus *et al*., 1969 ; Assadamongkol *et al*., 2016 ; Lee *et al*., 2003).

2. Duration of Ulcer

The temporal duration of the diabetic foot ulcer serves as another significant risk factor. Fungal infections are more prevalently documented in chronic, non-healing ulcers. Research indicates that ulcers persisting for a duration of 4-5 months or longer are more susceptible to fungal infections, potentially due to extended exposure to pathogens and hindered healing processes (kala *et al*., 2016 ; Sk *et al*., 2023 ; Khalifa *et al*., 2022).

3. Prior Antibiotic Therapy

The prolonged or inappropriate administration of antibiotic therapy has been correlated with an augmented risk of fungal infections in DFUs. Antibiotics may disrupt the equilibrium of the natural flora present on the skin and mucous membranes, engendering a milieu conducive to fungal proliferation. Patients undergoing long-term antibiotic treatment are at an increased likelihood of developing fungal infections, particularly those caused by *Candida species* (kala *et al*., 2016 ; Sanniyasi *et al*., 2015 ; Devasia *et al*., 2023).

4. Advanced Age

Elderly diabetic individuals are predisposed to a heightened risk of developing fungal infections in their foot ulcers. The immunological capacity of aging individuals is less proficient at counteracting infections, and older patients frequently present with additional comorbid conditions, such as peripheral vascular disease, which can complicate the wound healing process (Assadamongkol *et al*., 2016 ; "Study of Co-existence of Fungal infection in Diabetic Foot ulcers in Delhi Population", 2023).

5. Male Gender

Numerous investigations have identified male gender as a pertinent risk factor for fungal infections in DFUs. This predisposition may be ascribed to variances in lifestyle, hygiene practices, or hormonal influences. For example, one investigation discovered that males exhibited a greater propensity for fungal infections occurring between the toes in comparison to their female counterparts (Akkus *et al*., 1969 ; Assadamongkol *et al*., 2016).

6. Presence of Onychomycosis or Tinea Pedis

Fungal infections, specifically onychomycosis (a fungal infection affecting the toenails) and tinea pedis (commonly referred to as athlete's foot), are prevalent among individuals with diabetes and possess the capacity to propagate to foot ulcers, thereby elevating the risk of fungal contamination. Empirical research has indicated that patients afflicted with these conditions exhibit a heightened propensity for developing fungal infections within their diabetic foot ulcers (DFUs) (Akkus *et al*., 1969 ; Lee *et al*., 2003).

7. Peripheral Vascular Disease

Peripheral vascular disease (PVD) is frequently observed as a comorbid condition in diabetic patients and can significantly detract from blood circulation to the feet, thereby prolonging the wound healing process. Diminished circulation fosters an environment conducive to fungal colonization and subsequent infection of the ulcer, rendering PVD a critical risk factor for fungal infections in DFUs (Assadamongkol *et al*., 2016 ; Lee *et al*., 2003).

8. Wound Grade and Depth

The degree of severity of diabetic foot ulcers, delineated by their grade and depth, constitutes a pertinent risk factor for fungal infections. Ulcers classified as higher-grade (e.g., grade III or IV) with extensive tissue involvement are predisposed to harbor fungal pathogens due to the intricate nature of the wound environment (Sk *et al*., 2023 ; "Study of Co-existence of Fungal infection in Diabetic Foot ulcers in Delhi Population", 2023).

9. Immunosuppression

Individuals with diabetes frequently encounter immune dysfunction attributable to chronic hyperglycemia, which impairs the operational capacity of polymorphonuclear leukocytes and diminishes the host's ability to combat infections. This state of immunosuppression renders diabetic patients increasingly vulnerable to fungal infections within their foot ulcers (Akkus *et al*., 1969 ; Lee *et al*., 2003).

### 2.3.4 Diagnosis of Fungal Infections in DFU

The diagnosis of fungal infections in diabetic foot ulcers (DFU) involves both traditional and modern methods, each with distinct advantages and limitations. Traditional methods, such as direct microscopy and fungal

culture, remain foundational in clinical mycology, providing essential preliminary diagnoses. However, modern techniques like PCR technology.

### 2.3.4.1 Traditional Methods

1. Direct Microscopy: Utilizes KOH and Calcofluor white staining to provide rapid presumptive diagnoses(Shahid *et al*., 2010).

2. Fungal Culture: Involves growing fungi on selective media, which can take up to 30 days for results, making it time-consuming(El-Aal *et al*., 2017 ; Shahid *et al*., 2010).

### 2.3.4.2 Modern Methods

1. PCR Technology: Allows for the detection of fungal DNA, offering rapid and specific identification of pathogens, particularly useful in immunocompromised patients(Kuba, 2008).

2. Immunological Tests: Detect fungal antigens in biological fluids, although they are limited to specific genera(Kuba, 2008).

While traditional methods are still considered the gold standard, the integration of modern techniques is crucial for timely and accurate diagnosis, especially in high-risk populations. However, reliance on advanced methods may overlook the importance of understanding fungal biology and taxonomy, which remains essential for effective diagnosis(Willinger *et al*., 2014).

### 2.3.5 Treatment Strategies

The treatment of fungal infections in diabetic foot ulcers (DFUs) involves a combination of antifungal medications and surgical interventions. Diabetic patients are particularly susceptible to fungal infections due to

compromised immune responses and high blood sugar levels, which can exacerbate the severity of foot ulcers.

### 2.3.5.1 Antifungal Treatments

1. Echinocandins: Caspofungin is another option, particularly for infections resistant to azoles. It works by inhibiting the synthesis of fungal cell walls, making it effective against certain resistant strains(Kandregula *et al*., 2022).

2. Amphotericin B: This is reserved for severe cases due to its potent antifungal properties and potential side effects. It is often used when other treatments fail(Raiesi *et al*., 2018).

### 2.3.5.2 Role of Debridement

Debridement is a critical component of DFU management. It involves the removal of necrotic tissue, which helps reduce the microbial load and promotes healing. This procedure is essential in controlling infections and is often performed alongside antifungal treatments(Jude & Unsworth, 2004).

### 2.3.5.3 Challenges in Treatment

1. Polymicrobial Nature: DFUs often involve mixed infections, including bacteria and fungi, complicating treatment strategies. Fungal infections can be overlooked, leading to inadequate treatment(Kandregula *et al*., 2022).

2. Drug Resistance: There is a growing concern about antifungal resistance, which necessitates careful selection of antifungal agents and may require combination therapies(Raiesi *et al*., 2018).

3. Delayed Healing: Fungal infections can prolong the healing process, increasing the risk of complications such as amputations(Chang & Nguyen, 2021).

## 2.4 Nanoparticles and microbial sensitivity

Nanoparticles, characterized as particles with dimensions ranging from 1 to 100 nanometers, have emerged as pivotal instruments within the medical and biological domains owing to their distinctive properties, including an elevated surface area to volume ratio and the capacity for functionalization with a diverse array of molecules. These attributes empower nanoparticles to act as formidable antibacterial agents, presenting innovative paradigms to address microbial resistance to antibiotics. The proliferation of antibiotic-resistant strains, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant tuberculosis (MDR-TB), has prompted the investigation of alternative therapeutic modalities, with nanoparticles offering a promising pathway due to their potential to disrupt bacterial membranes, induce the generation of reactive oxygen species, and release metal ions that interfere with bacterial metabolic processes (Ahmad, 2022 ; Vikal *et al*., 2024).

### 2.4.1 Significance of Nanoparticles in Medicine

1. Antibacterial Applications: Nanoparticles exhibit superior capabilities in traversing biofilms and penetrating bacterial cell membranes compared to traditional antibiotics, thereby augmenting their antibacterial effectiveness (Vikal *et al*., 2024).
2. Medical Device Coatings: Nanoparticles are employed in the coatings of medical devices to mitigate the formation of biofilms and prevent infections, particularly within urinary stents and implants (Grasse, 2022).

3. Targeted Drug Delivery: Nanoparticles can be engineered to facilitate the targeted delivery of pharmacological agents directly to sites of infection, thereby enhancing therapeutic efficacy while minimizing adverse effects (Ahmad, 2022).

### 2.4.2 Role in Combating Antibiotic Resistance

1. Mechanisms of Action: Nanoparticles operate through a variety of mechanisms, including physical disruption and the induction of oxidative stress, thereby complicating the ability of bacteria to acquire resistance via singular genetic mutations (Vikal *et al*., 2024).
2. Enhanced Antibacterial Activity: Empirical research has demonstrated that nanoparticles manifest superior antibacterial properties against both Gram-positive and Gram-negative bacteria in comparison to conventional antibiotics (Modi *et al*., 2022).
3. Combination Therapies: The incorporation of nanoparticles alongside established antibiotics may significantly augment their efficacy in combating resistant bacterial strains (Thambirajoo *et al*., 2021).

While the utilization of nanoparticles presents promising avenues for intervention, it is imperative to address potential challenges, including toxicity and environmental ramifications.

### 2.4.3 Mechanism of Nanoparticle Effect on Microbes

These mechanisms include disruption of cell membranes, production of reactive oxygen species (ROS), and inhibition of essential cellular functions .

1. **Disruption of Cell Membrane**

Nanoparticles can physically damage microbial cell membranes, leading to cell lysis and death. This is particularly evident with metal nanoparticles like silver and copper, which create gaps in the cell wall structure(Kodaparthi *et al*., 2023 ; Phakatkar *et al*., 2022).

The size and surface properties of NPs influence their ability to penetrate and disrupt membranes, with smaller particles often exhibiting greater efficacy(Eduok *et al*., 2017).

2. **Production of Reactive Oxygen Species (ROS)**

Many metal oxide nanoparticles, such as zinc oxide and magnesium oxide, generate ROS upon interaction with microbial cells. These reactive species cause oxidative stress, leading to lipid peroxidation and subsequent cell damage(Kodaparthi *et al*., 2023 ; Maksimova & Зорина, 2024).

ROS production is a significant contributor to the bactericidal effects of NPs, as it disrupts cellular homeostasis and damages vital biomolecules(Pal *et al*., 2021).

3. **Inhibition of Vital Enzymes and Cellular Functions**

NPs can inhibit key metabolic processes by interfering with enzyme activity and protein synthesis. This disruption can lead to impaired growth and reproduction of microbes(Pal *et al*., 2021 ; Phakatkar *et al*., 2022).

The interaction of NPs with microbial physiology can also affect quorum sensing and biofilm formation, further complicating microbial survival strategies(Maksimova & Зорина, 2024).

While the mechanisms of nanoparticle toxicity are well-documented, it is essential to recognize that the effects can vary significantly based on the type of nanoparticle, concentration, and environmental conditions.

### 2.4.4 Bio-Nanoparticles

Bio-nanoparticles are synthesized using plant extracts, which provide a green and eco-friendly method for nanoparticle production .

Plant-based nanoparticles, such as those derived from spinach and orange extracts, loaded with Cu and Zn salts, have shown potent antimicrobial activity against multidrug-resistant bacteria(Sahu *et al*., 2024).

The phytochemical composition of plant extracts contributes to the antimicrobial properties of these nanoparticles, offering a novel approach to combatting resistant pathogens(Sahu *et al*., 2024 ; "Antimicrobial nanoparticles: Synthesis, mechanism of actions", 2023).

### 2.4.5 Challenges and Future Prospects

Nanoparticles present both significant challenges and promising future prospects, particularly in the fields of medicine and pharmaceuticals. The primary concern revolves around their potential toxicity to human cells, which necessitates further research to ensure safe application. Despite these challenges, nanoparticles hold immense potential for advancing medical treatments and drug delivery systems. This dual nature of nanoparticles underscores the need for a balanced approach in their development and application .

#### 2.4.5.1 Toxic effects

1. Allergy

2. Organ failure

3. Immune functions as Liver, kidney, spleen, lung,

membrane integrity and oxidative stress.

4. Tissue damage

5. DNA damage

6. Increase inexpression of genes

7. Decreases the rate of aerobic respiration

Comprehensive studies are required to evaluate the stability and potential human exposure to nanoparticles, especially as their production is expected to increase(Alam, 2023 ; "Toxicology Related to Nanoparticles – Challenges and Future Prospects", 2023).

Research into the mechanisms of nanoparticle uptake, dosage, and toxicity levels is crucial to developing safer nanomaterials(Ramanathan, 2019).

Rigorous studies and stringent guidelines are necessary to ensure the effective and safe development of nanomedicine-based formulations(Thapa & Kim, 2022).

### 2.4.5.2 Future Applications in Medicine and Pharmaceuticals

Nanoparticles offer promising applications in nanomedicine, including enhanced pharmaceutical effectiveness and novel therapies for complex diseases like cancer(Bhuiyan *et al*., 2024 ; Thapa & Kim, 2022).

The development of nanomedicine-based formulations has led to several approved and marketed products, highlighting their potential in targeted drug delivery and reduced toxicity(Thapa & Kim, 2022).

## 2.5 Bismuth nitrate

Bismuth nitrate, with the chemical formula $Bi(NO_3)_3$, is a compound of bismuth that is commonly used in various chemical reactions due to its ability to provide bismuth ions. It is often utilized in organic synthesis and medicinal chemistry because of its relatively low toxicity and effectiveness as a catalyst.

### 2.5.1 Role in Medicinal Chemistry

Bismuth compounds, including bismuth nitrate, are valued in medicinal chemistry for their low toxicity and potential in synthesizing active pharmaceutical ingredients. They have historical and ongoing applications in treating bacterial infections and gastrointestinal disorders(Salvador *et al*., 2012).

While bismuth nitrate is a valuable reagent in chemical reactions, it is important to consider its handling and storage requirements due to its hygroscopic nature and potential decomposition in water. This highlights the need for careful management to maintain its effectiveness and safety in laboratory settings(Ollevier *et al*., 2016).

### 2.5.2 Physical and Chemical Properties of Bismuth Nitrate

#### 2.5.2.1 Solubility in Water and Other Solvents

Bismuth nitrate exhibits varying solubility depending on the solvent and conditions. It is soluble in water containing nitric acid but decomposes in pure water, forming oxynitrate ($BiONO_3$) (Ollevier *et al*., 2016). In other solvents, it is soluble in glycerol, diluted acids like acetic acid, and acetone, while its solubility is low in alcohol and ethyl acetate (Ollevier *et al*., 2016).

### 2.5.2.2 Stability and Sensitivity to Temperature and pH

Bismuth nitrate is hygroscopic and decomposes upon exposure to water, forming $BiONO_3$ (Ollevier *et al*., 2016). Its stability is also affected by temperature and pH. For instance, basic bismuth nitrate precursors have been shown to transform into α-$Bi_2O_3$ microparticles upon annealing at 600°C, indicating thermal stability changes (Karen *et al*., 2021). The pH sensitivity is evident in its catalytic applications, where the maximum sorption performance for dye removal occurs at pH 2.0 (Najdanović *et al*., 2019).

### 2.5.3 Conventional Methods for Preparing Bismuth Oxide from Bismuth Nitrate

Bismuth nitrate ($Bi(NO_3)_3 \cdot 5H_2O$) is a common precursor for the synthesis of $Bi_2O_3$ NPs. Several conventional methods have been employed to prepare $Bi_2O_3$ NPs, including :

1. Liquid Phase Precipitation Method

This method involves the use of bismuth nitrate and sodium hydroxide (NaOH) as raw materials, with lignosulfonate as a surfactant to control particle size and morphology. The addition of surfactants reduces surface tension and prevents particle aggregation, resulting in stable and uniform nanoparticles (Chen *et al*., 2015).

2. Sol-Gel Method

The sol-gel method involves the hydrolysis of bismuth nitrate in the presence of a stabilizing agent, followed by calcination to obtain crystalline $Bi_2O_3$ NPs. This method allows precise control over particle size and crystallinity (Singh *et al*., 2023).

3. Flame Spray Pyrolysis

In this method, bismuth nitrate is dissolved in a solvent (e.g., ethanol or acetic acid) and subjected to flame spray pyrolysis. The resulting nanoparticles are hollow, shell-like, or solid, depending on the precursor solution composition (Mädler & Pratsinis, 2004).

### 2.5.4 Role of Plant Extracts as Reducing and Stabilizing Agents

Plant extracts have emerged as eco-friendly reducing and stabilizing agents in the green synthesis of $Bi_2O_3$ NPs. These extracts contain phytochemicals, such as phenols, flavonoids, and terpenoids, which facilitate the reduction of bismuth ions and stabilize the nanoparticles, preventing agglomeration (Villagrán *et al*., 2024 ; Singh *et al*., 2023).

#### 2.5.4.1 Advantages of Plant Extracts

1. Eco-Friendly and Cost-Effective: Plant extracts are abundant, biodegradable, and non-toxic, making them a sustainable alternative to conventional chemical methods (Villagrán *et al*., 2024 ; Phougat *et al*., 2017).
2. Dual Role as Reducing and Capping Agents: Phytochemicals in plant extracts not only reduce bismuth ions to form nanoparticles but also stabilize the particles, ensuring uniform size distribution and high colloidal stability (Singh *et al*., 2023 ; Abuzeid *et al*., 2023).
3. Enhanced Biological Activity: $Bi_2O_3$ NPs synthesized using plant extracts exhibit improved biological properties, such as antioxidant, antibacterial, and anticancer activities, due to the synergistic effects of phytochemicals and nanoparticles (Dandge *et al*., 2024 ; Li *et al*., 2022).

### 2.5.4.2 Examples of Plant Extracts

1.Green tea extract of Camellia sinensis is rich in powerful antioxidant compounds, primarily due to flavonoids such as catechins("Herbal flavonoids in healthcare", 2022). It can selectively cause cell death in triple-negative breast cancer cells and enhance the effectiveness of chemotherapeutic drugs such as cisplatin and paclitaxel(Raad *et al*., 2024). Green tea extract is used in the food industry to prolong the storage shelf of foods containing lipids by preventing oxidation(Senanayake, 2013).

2. Salvia rosmarinus rosemary extract is a strong antioxidant with great potential as a natural alternative to synthetic antioxidants in food stabilization(Senanayake, 2013). It also has anticancer activity, particularly in combination with green tea extract, by sensitizing cancer cells to chemotherapeutic agents(Raad *et al*., 2024). The extract is used extensively as a color and flavoring agent in foods(Mir *et al*., 2022).

3. Milk thistle constituent silymarin is used extensively because of its hepatoprotective activity and therapeutic efficacy for the treatment of liver diseases ("Herbal flavonoids in healthcare", 2022). It is flavonoids that are responsible for its anti-inflammatory and antioxidant activity, therefore a component of herbal medicines ("Herbal flavonoids in healthcare", 2022).

### 2.5.5 The Importance of Using Plant Extracts in Nanopharmaceutical Preparation

The utilization of plant extracts for the green synthesis of nanoparticles is increasingly preferred over conventional chemical methodologies owing to its environmental sustainability and economic advantages. Conventional

approaches frequently entail the use of hazardous chemicals and substantial energy expenditure, whereas synthesis through plant-derived materials is more sustainable and environmentally benign. Plant extracts encompass intrinsic biomolecules that function as reducing and capping agents, thereby promoting the formation of nanoparticles devoid of deleterious byproducts. This methodology not only mitigates ecological repercussions but also augments the biocompatibility and functional characteristics of the nanoparticles, rendering them appropriate for diverse applications in the fields of medicine, agriculture, and industry (Azad *et al*., 2023 ; Antonio-Pérez *et al*., 2023 ; Phougat *et al*., 2017).

#### 2.5.5.1 Advantages of Green Methods

1. Environmental Safety: The green synthesis process circumvents the use of toxic chemicals, thereby diminishing environmental pollution and associated health hazards (Azad *et al*., 2023 ; Kameswaran *et al*., 2025).

2. Cost-Effectiveness: The employment of plant extracts is economically advantageous as it lessens the reliance on costly chemicals and energy-demanding procedures (Phougat *et al*., 2017).

3. Biocompatibility: The nanoparticles generated exhibit enhanced compatibility for therapeutic applications due to the absence of toxic residues (Phougat *et al*., 2017).

#### 2.5.5.2 Influence of Reaction Conditions

Extract Concentration: Increased concentrations of extracts can yield smaller nanoparticles due to the heightened availability of reducing agents (Azad *et al*., 2023).

Temperature: Elevated reaction temperatures typically expedite the synthesis process, consequently impacting the size and morphology of the resulting nanoparticles (Antonio-Pérez *et al*., 2023).

Although green synthesis presents numerous benefits, it is imperative to acknowledge the variability inherent in the composition of plant extracts, which may influence the consistency and reproducibility of nanoparticle production. Additional research is warranted to refine these methodologies and to comprehensively elucidate the mechanisms at play (Azad *et al*., 2023 ; Antonio-Pérez *et al*., 2023).

## 2.6 Leukocytes and infections

Immune system cells, referred to as immunocytes, play a critical role in the body's defense against exogenous pathogens and infectious diseases. There exist three principal subtypes of leukocytes: monocytes, lymphocytes, and granulocytes (Monga *et al*., 2022). The eventual destiny of "aged" neutrophils remained elusive until the advent of the novel paradigm of apoptosis, which elucidated the process of regulated cellular demise as a concluding phase in cellular differentiation, distinguishing it from necrosis induced by trauma, toxins, or immune responses (Kobayashi & DeLeo, 2009). It has been acknowledged that neutrophils possess a relatively brief lifespan following their release from the bone marrow. The rapid innate immune response against the majority of bacterial and fungal pathogens, which occurs prior to the activation of the complex humoral and cellular mechanisms of acquired immunity to address an infection, is predominantly facilitated by neutrophils. The autoimmune mechanisms involving antibodies targeting neutrophils or their constituents can precipitate lysis or phagocytosis of neutrophils before the normal apoptotic mechanisms can transpire; alternatively, other factors leading to cytolysis may result in the premature replacement of neutrophil apoptosis with

necrosis, along with the release of potent proteolytic enzymes contained within neutrophils, induced by microorganisms that secrete toxins capable of lysing the neutrophils (Lionakis *et al*., 2023). Neutrophil extracellular traps (NETs), which are composed of cationic granule proteins and histones, represent filamentous DNA structures formed when the nucleus is extruded extracellularly as a consequence of such cytolytic events (Rada, 2019). These fascinating structures may contribute to host defense by ensnaring bacteria and fungi and, according to certain studies, even exhibiting the capacity to eliminate microorganisms.

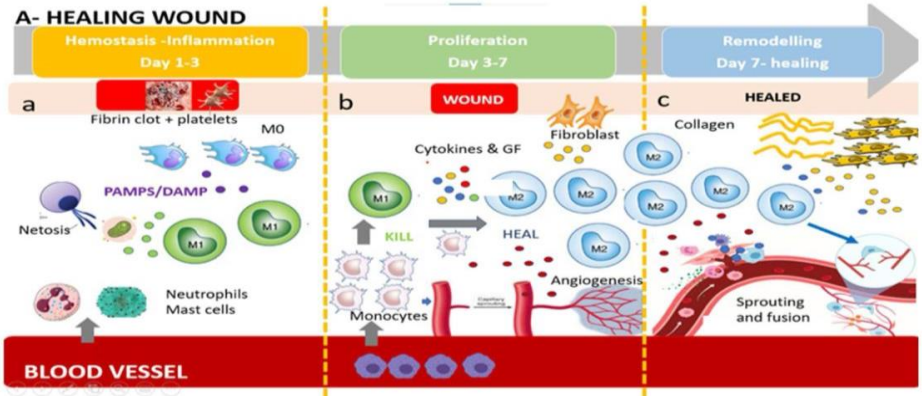



**Figure 2-2**: The healing wound: (a) Platelets create a fibrin clot; pro-inflammatory cytokines are generated as a result of the release of chemo-attractants, which draw in inflammatory cells such mast cells and neutrophils. Neutrophil Extracellular Trap, or NETosis, aids in the capture and elimination of pathogens. Tissue-resident macrophages respond to molecular patterns (PAMPs and DAMPs) linked to pathogens and damage. M1 monocytes differentiate in the first wave (phagocytoses stage). (c) The proliferative phase begins after the inflammation resolves.

Through vessel sprouting, angiogenesis develops. Monocytes that infiltrate develop into M1 and M2 macrophage subsets. M1 macrophages consume dead bacteria and neutrophils while also producing inflammatory cytokines and reactive oxygen species (ROS). Following M1 polarization, M2 pro-regenerative phenotypes release growth factors, proteases, and anti-inflammatory cytokines that cause collagen to replace the temporary extracellular matrix, promote fibroblast proliferation, and cause the development of new blood vessels. Granular tissue and keratinocyte coverage are the

end products of this process. (c) Remodeling is aided by myofibroblasts, fibroblasts, and macrophages that reorganize the temporary extracellular matrix (ECM) into definitively healed tissue. This process is mostly accomplished by matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), producing tissue with robust functioning and tensile strength. When angiogenesis is nearly finished, macrophages create molecular bypass, or fusion, between newly created vessels, resulting in the formation of a useful network.

## 2.7 Leukocytes and diabetic foot

Leukocytes have been demonstrated to play a pivotal role in the pathogenesis of diabetic foot ulceration. A defining characteristic of the inflammatory phase is the recruitment of inflammatory cells, which is accompanied by the secretion of various "danger signals" at the injury site. Neutrophils are among the first responders to these signals. In the context of acute wounds, circulating neutrophils are primarily responsible for the recruitment to the wound site (F. Huang *et al*., 2023). Neutrophils combat pathogens through phagocytosis, the generation of reactive oxygen species (ROS), the secretion of cytokines that amplify inflammatory signals, and the production of matrix metalloproteinases to clear necrotic debris. Moreover, neutrophils facilitate the release of vascular endothelial growth factor (VEGF) in preparation for the proliferative phase. Ultimately, the resolution of inflammation occurs when neutrophils are removed from the site via reintegration into the circulatory system or through phagocytosis by macrophages (Selders *et al*., 2017). The recruitment and infiltration of neutrophils into diabetic ulcers may hinder the healing trajectory (Su & Richmond, 2015). Neutrophils eliminate extracellular traps (NETs), secrete cytotoxic enzymes, and phagocytize pathogens (Domínguez-Díaz *et al*., 2021). Recent studies have elucidated that NETs play a critical role in the delayed healing of wounds. Elevated blood glucose levels stimulate neutrophils, which are markedly expressed at the wound sites of diabetic

ulcer patients, to generate extracellular traps (NETs). In order to perpetuate the inflammatory response, NETs activate the NLRP3 inflammasome, prolong the repair of diabetic ulcers, and stimulate macrophages (Sabbatini *et al*., 2021).

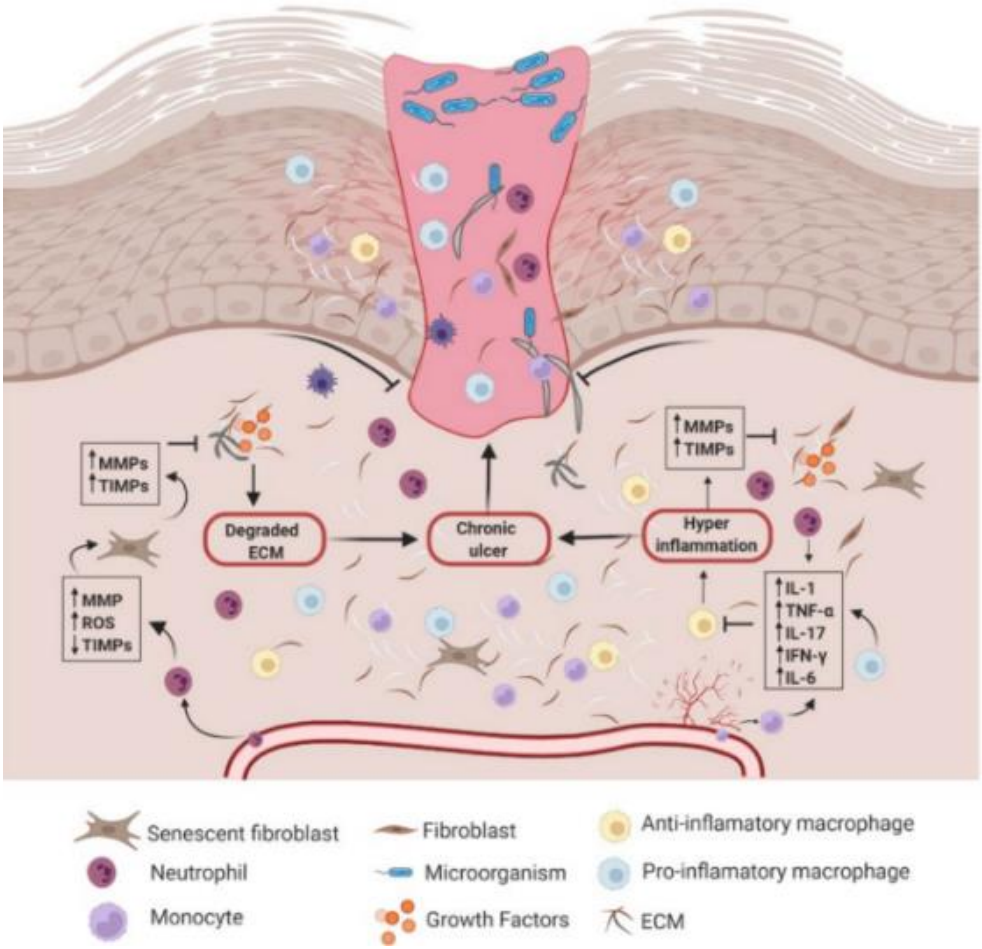



**Figure 2-3:** The healing phase of a chronic wound. Necrosis, chronic infections, and ongoing inflammation are the hallmarks of diabetic foot ulcers. The extracellular matrix (ECM) cannot be properly remodelled in these wounds due to an imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Additionally, inflammation is long-lasting and has a significant immune cell infiltration. This is due to an increase in pro-inflammatory cytokines including interleukins (IL) and neutrophils and type I monocytes, which are pro-inflammatory cells that react to infectious pathogens. In a similar vein, there is an increase in reactive oxygen species (ROS), which exacerbates the tissue component deterioration. Together, these processes restrict angiogenesis, cell migration, and extracellular matrix remodeling, which causes persistent wounds (Güiza-Argüello *et al*., 2022).

# Chapter Three
# Patients
# Materials and Methods

# Materials and Methods

## 3.1 Materials

### 3.1.1 Equipment

**The equipment utilized in this study are shown in Table (3-1):**

**Table (3-1) : Equipment Used in the Study**

| No | Equipment | Supplier | Origin |
|---|---|---|---|
| 1 | Laminar Flow Cabinat | Daihan Labtech | Korea |
| 2 | Auto Clave | Hirayama | Japan |
| 3 | Electric Oven | Memmert | Germany |
| 4 | Sensitive Balance | Sartorius | Korea |
| 5 | Incubater | Human Lab | Korea |
| 6 | Light Microscope | Olympus | Japan |
| 7 | Refrigerator | Vitals | Poland |
| 8 | Centerfuge | Hettich | Germany |
| 9 | Vortex mixture | Medilab | Korea |
| 10 | Magnatic Stirror | VISION | Korea |
| 11 | Vitek-2 Compact System | BioMerieux | France |

### 3.1.2 Instruments and Supplies

**The instrument listed in Table (3-2) were used in this study:**

**Table (3-2) : Instruments and Supplies Used in the Study**

| No | Equipment | Supplier | Origin |
|---|---|---|---|
| 1 | Petri Dishes | Sterilin | England |
| 2 | Slides and Cover Slides | Mehecal | China |
| 3 | Loop | Himedia | India |
| 4 | Benzene Burner | Shandon | England |
| 5 | Cloves | Tanedial | China |
| 6 | Sterile Cotton Swabs | AFCO | Jordon |
| 7 | Graduated Glass Cylinders | Supcorior | Germany |
| 8 | Plastic Tubes | AFMA-Dispo | China |
| 9 | Filter Papers | Meheco | China |
| 10 | Flasks Glass | Meheco | China |
| 11 | Parafilm | Pechiney | USA |
| 12 | Forceps | Behring | Germany |
| 13 | Micropipettes 100-1000 µL | Dragon | China |
| 14 | Funnal Glass | Pyrex | USA |
| 15 | Beaker | ISOLAB | Germany |
| 16 | Syringes | Meheco | China |

## 3.1.3 Solutions

**The solutions utilized in this study are documented in Table (3-3) :**

**Table (3-3): Solutions Used in the Study**

| No | Solution | Supplier | Origin |
|---|---|---|---|
| 1 | Gram stain solutions | Himedia | India |
| 2 | Lactophenol-cotton blue stain | Bio neer | Korea |
| 3 | (DMSO)Dimethyl Sulfoxide | Alpha Chemica | India |
| 4 | Sodium Hydroxide Solution | Merck | Germany |

## 3.1.4 Chemical and Biological Material

**The chemical and biological materials used in this study are listed in Table (3-4) :**

**Table (3-4): Chemical and Biological Material Used in the Study**

| No | Material | Supplier | Origin |
|---|---|---|---|
| 1 | Agar | Biobasic | Canada |
| 2 | Ethanol 70% | Ajax | Australia |
| 3 | Humen Blood | - | - |
| 4 | Bismuth Nitrate | Sigma-Aldrich | Mexico |
| 5 | Seder Oil | BDH | England |
| 6 | Tetracycline | Anhui Medipharm | China |
| 7 | *Silybum marianum* (milk thistle) | - | - |
| 8 | *Camellia sinensis* (green tea) | Altunkaya | Turkey |
| 9 | *Rosmarinus officinalis* (rosemary) | - | - |

## 3.1.5 Cultural Media

**The culture media shown in Table (3-5) were used :**

**Table (3-5): Culture Media used in the Study**

| NO | Media | Supplier | Origin |
|---|---|---|---|
| 1 | Nutrient Agar | Himedia | India |
| 2 | Sabouraud Dextrose Agar | Himedia | India |
| 3 | HiCrome™ Candida Differential | Himedia | India |

| | | | |
|---|---|---|---|
| | Agar | | |
| 4 | MacConkey Agar | Himedia | India |
| 5 | Mueller Hinton Agar | Oxoid | England |
| 6 | Blood Agar Base | Himedia | India |

### 3.1.6 Antimicrobials Disks

**The Antimicrobials used are shown in the table (3-6) :**

**Table(3-6): Antimicrobials Disks Used in Study**

| Antibacterial | | | |
|---|---|---|---|
| Antibacterial | | Symbol | Concentration( µg/disk) |
| 1 | Amikacin | AK | 30 µg |
| 2 | Ceftazidime | CAZ | 30 µg |
| 3 | Levofloxacin | LE | 5 µg |
| Antifungal | | | |
| Antifungal | | Symbol | Concentration (µg/disk) |
| 1 | Caspofungin | CAS | 5 µg |
| 2 | Voriconazole | VO | 1 µg |

## 3.2 Working Methods

### 3.2.1 Sterilization

- Moist sterilization

  An autoclave was utilized to verify the sterilization of all culture media that had been utilized during the experiment. The autoclave was properly set to operate at a temperature of 121°C, with a pressure of 15 psi, for 15 minutes (Greenwood *et al*., 2012).

- Dry sterilization

Glassware and instruments were sterilized in an electric oven at 180°C for 2 hours (Greenwood *et al*., 2012).

### 3.2.2 Preparation of Culture Media

- Isolation and Growth Media

Culture media utilized in this study comprised various specific formulations for the isolation and identification of different kinds of bacteria and fungi isolated from diabetic foot ulcer patients.

#### 3.2.2.1 Culture Media of Bacteria

1. Nutrient Agar

It was prepared according to the instructions of the company equipped, sterilized by the autoclave, and left it to cool to a temperature of 45-50°C, and then it was poured into sterile dishes so that it was used as a medium general for the transplantation of bacterial insulation.

2. Blood Agar Medium

Blood agar was prepared according to the company instructions, autoclaved to sterilize, and cooled to 45-50°C. 5-10% human blood was added and thoroughly mixed with the medium. It was poured into sterile Petri plates and incubated for solidification. This enrichment medium was utilized for the identification and isolation of bacteria, showing their ability for the lysis of blood cells through the action of the enzyme hemolysin (Forbes *et al*., 2007).

3. MacConkey agar

Following the detailed instructions of the manufacturer, the medium was autoclaved to sterilize it and left to cool to a temperature of 45-50°C. After it had cooled to this specific temperature, the prepared medium was carefully poured into sterile dishes prepared for this purpose. This medium is both a

selective and differential medium and is used particularly for the isolation of Gram-negative bacteria and can differentiate between those bacteria that can ferment lactose and those that cannot (Alexander *et al*., 2004).

### 3.2.2.2 Culture Media of Fungi

1. Sabouraud Dextrose Agar

This medium was prepared according to the recommendations of the supplier company (Himedia) by dissolving 65 g of powder in 1000 ml of distilled water and sterilizing it in an autoclave at 121°C and 15 psi for 30 minutes. After sterilization, it was left to cool and before solidification, the antibacterial Tetracycline was added to the medium at a rate of 250 mg/L. It was then poured into 9 mm diameter plastic dishes. This medium was used to isolate dermatophytes (Emmons *et al*., 1974).

2. HiCrome™ Candida Differential Agar

This medium was prepared according to the recommendations of the supplier company (Himedia) and it is used to distinguish *Candida sp*.

A few colonies of pure culture of Sabouraud agar were taken with a sterile loop and streaked upon Candida agar medium. They were incubated aerobically at 37°C for 48 hours (Beighton *et al*., 1995).

### 3.2.2.3 Sensitivity test medium

Mueller Hinton Agar

Prepared according to the manufacturer's instructions, it was autoclaved and allowed to cool to 45-50°C. It was then poured into sterile Petri dishes. This medium was used for antibiotic susceptibility testing (Murray *et al*., 1995).

### 3.2.3 Isolates

- **Collection of Specimens**

  A total of 50 samples were collected from diabetic patients with diabetic foot ulcers of both genders. 30 of them were bacterial isolates and 20 of them were fungal isolates. The samples were obtained from Maysan

Specialized Center for Diabetes and Endocrinology during the period from December 2024 to the end of February 2025. Sterile cotton swabs were used for sample collection, and the collected samples were then transported to the laboratory for further analysis.

- **Isolation**

1. Bacterial isolation

The swabs collected from patients with diabetic foot ulcers were inoculated onto Blood Agar, MacConkey Agar and Nutrient Agar using the streaking method. The plates were then incubated at 37°C for 24 hours. A subculture was performed for the growing colonies to obtain pure isolates, followed by incubation under the same conditions (37°C for 24 hours).

2. Fungal isolation

For fungal isolation, the swabs were inoculated onto Sabouraud Dextrose Agar using the streaking method and incubated at 25°C for 5 to 7 days. A subculture was then performed for the growing fungal colonies under the same conditions.

- **Identification of Isolates**

The isolates were identified based on their cultural characteristics and microscopic examination.

- **Cultural Characteristics**

1. Bacterial

Selective and rich culture media (blood agar and MacConkey's agar) have been used for the phenotypic characterization of bacteria with respect to colony shape, color, size, and odor, as well as their hemolytic potential (if blood is present) (Baron *et al*., 2007 ; Rajamanikandan *et al*., 2022).

2. Fungal

Selective culture media (Sabouraud Dextrose Agar and HiCrome™ Candida Differential Agar) were used to study the morphological characteristics of

dermatophytes, and several characteristics that must be taken into consideration include the growth rate or speed of growth, the surface of the fungal colony (flat or containing regular or irregular folds), the texture of the colony (yeasty, smooth or powdery, granular, velvety or cottony), the morphology of the colony and its texture (powdery, cottony, fuzzy), its color, and the color of the back of the colony (Ajello *et al*., 1977).

- **Microscopic Examination**

1. Bacterial

Gram stain was used to detect bacteria isolated from culture media. A drop of distilled water was placed on a glass slide using a cooled, sterile bacterial carrier, then mixed with an isolated bacterial colony and left to dry at room temperature. The bacteria placed on the slide were then fixed by passing it three times through a Benzene burner. The dried bacterial smear was stained with Gram stain and examined using a 100X oil-based objective lens (Baron *et al*., 2007).

2. Fungal

The microscopic examination was conducted to note the various innate structures, such as the fungal threads, shapes and branches microconidia and its shapes, shapes, sizes, number and thickens and thickens its wall, chlamydospores and arthrophy (arthospores) and by taking part of the developing fungal colony by using a sterile Needle and placing it in a drop of dye LPCB theme on a glass slice and then checked with light microscope(Light Microscope) the power of the 10 and 40 lens enlargement and depending on the following category sources (Dismukes and others, 2003 ; Ellis, and others 2007 ; JorgenSen *et al*. 2015).

### 3.2.4 Germ Tube Test (GTT)

This test is a rapid method for differentiation between *Candida dubliniensis* and *Candida albicans* by its efficiency to produce short tube delicate like structure known as germ tubes when it is incubated in serum of human blood at 37 °C for two hours. Germ tubes differs from pseudo-hyphae because it is

elongations of daughter cells from the mother cell without shrinkages at the origins (Deorukhkar and Saini, 2014).
The germ tube test procedure encompasses the subsequent stages:

1. Preparation of the Fungal Isolate
A freshly isolated specimen of Candida species is procured from a clinical specimen or culture. (Garcia, 2010 ; Hidayati *et al*., 2023).

2. Inoculum Preparation
A suspension of the fungal isolate is formulated in sterile saline to attain a 0.5 McFarland standard (approximately 1 x 10^6 cells/mL) (Ruby *et al*., 2023 ; Hilmioglu *et al*., 2007).

3. Inoculation of the Medium
One milliliter of the prepared inoculum is introduced into one milliliter of the chosen medium (e.g., human serum ) within a test tube (Minh *et al*., 2023 ; Deorukhkar *et al*., 2012).

4. Incubation
The test tube undergoes incubation at a temperature of 37°C for a duration of 2 to 3 hours. The incubation periods may fluctuate based on the medium employed, with certain media necessitating extended incubation intervals (Minh *et al*., 2023 ; Kotgire & Hatkar, 2024 ; Hilmioglu *et al*., 2007).

5. Observation Under the Microscope
Following the incubation phase, a droplet of the medium-inoculum amalgamation is positioned on a pristine glass slide and scrutinized under a light microscope.

Germ tubes are characterized as elongated formations emanating from the yeast cells, typically measuring at least three times the length of the parental cell and exhibiting no constriction at the base (Garcia, 2010) (Hidayati *et al*., 2023). A positive outcome is denoted by the presence of germ tubes. *C. albicans* generally produces germ tubes within 2 to 3 hours of incubation in human serum or other appropriate media (Minh *et al*., 2023 ; Kotgire &

Hatkar, 2024). A negative outcome is indicated by the absence of germ tubes, suggesting that the isolate is not *C. albicans* or one of the other species capable of germ tube production (Garcia, 2010 ; Hidayati *et al*., 2023).

### 3.2.5 VITEK-2 compact system

is an automated tool that determines the kind of pathogen by determining whether it is yeast, Gram-positive bacteria, or Gram-negative bacteria. Two kits that were connected to the device were used for diagnosis: one for susceptibility testing (AST-P580 card) and the other for diagnosis (GN card and GP card). 64 wells with a dried medium and a color indicator for biochemical testing are included in the diagnostic kit. Color changes brought on by bacterial growth in the table are recorded by the device. 18–20 antibiotics in 64 wells, each with varying concentrations, make up the antibiotic susceptibility testing kit. After bacterial growth, the device logs variations in turbidity. According to (Maina & Kagotho, 2014 ; Karagoz *et al*., 2015 ; Khalaf & Al-Kaabi, 2023), the device functions as follows:

1. For 24 hours, bacteria were cultivated and incubated aerobically at 37°C.

2. An 18–24 hour-old colony from a pure culture was transferred to a sterile tube with 3 ml of regular saline to create a bacterial suspension.

3. A Densi-Chek device was used to measure the suspension's density, which ranged from 0.5 to 6.3.

4. After inserting the card into the tube and fixing it in the apparatus, the outcome was displayed.

### 2.2.6 Susceptibility Test

**A. Antibacterial Susceptibility Test (AST)**

The susceptibility test of the bacterial isolates under study was performed using the Kirby-Bauer method, according to Bauer *et al*., 1966, as follows:

1. 3-5 colonies of the bacterial isolate, which had been cultured on solid MacConkey agar and blood agar for 24 to 48 hours, were moved to a test tube with three milliliters of saline. A standard Macfarland turbidity-

fixed solution of 1% concentrated sulfuric acid (H2S) and 1% anhydrous barium chloride (BaCl2), which produces a count of between $10^8$ ×1 and $10^8$ ×2 cells per milliliter, was used to compare the density. The standard tube and the inoculum tube were held side by side in adequate light, no more than an inch from the Wickerham card's face, in order to conduct the comparison. The lines' looks in the two suspensions were contrasted.

2. A sterile cotton swab was inserted into the tube containing the bacterial suspension, rotated, and pressed against the inner wall of the tube to remove excess inoculum. The swab was then passed over the solid Müller-Hinton medium by wiping the swab back and forth, moving very close together. The plates were left to dry at room temperature for at least 3 to 5 minutes.

3. Antibacterial discs were placed onto the surface of the inoculated Müller-Hinton medium using sterile forceps and gently pressed they were then incubated at 37°C for 24 hours.

4. After incubation, the results were recorded by measuring the diameter of the inhibition zone in mm around each disc, and then compared to the standard tables provided in (2025 CLSI), this table(3-7)shows the inhibition zone diameters for the bacterial species identified using the VITEK system :

**Table (3-7) Inhibition Zones *Staphylococcus aureus and Staphylococcus pseudintermedius* with Levofloxacin , *Pseudomonas oryzihabitans* and *Proteus mirabilis* with Amikacin and Ceftazidime (CLSI, 2025).**

| Inhibition zones in millimeters (mm) | | | | | |
|---|---|---|---|---|---|
| *Staphylococcus aureus* | | | *Staphylococcus pseudintermedius* | | |
| Levofloxacin | | | | | |
| Sensitive | Medium | Resistance | Sensitive | Medium | Resistance |
| ≥ 19 | 16–18 | ≤ 15 | ≥ 19 | 16–18 | ≤ 15 |
| *Proteus mirabilis* | | | *Pseudomonas oryzihabitans* | | |
| Amikacin | | | | | |
| Sensitive | Medium | Resistance | Sensitive | Medium | Resistance |
| ≥ 20 | 17–19 | ≤ 16 | ≥ 17 | 15–16 | ≤ 14 |
| Ceftazidime | | | | | |
| Sensitive | Medium | Resistance | Sensitive | Medium | Resistance |
| ≥ 21 | 18–20 | ≤ 17 | ≥ 18 | 15–17 | ≤ 14 |

## B. Antifungal Susceptibility Test (AST)

The procedure was performed as mentioned above in the paragraph(A) and in comparison with the standard tables in CLSI 2022, this table (3-8) shows the diameters of the inhibition zone for *Candida albicans* :

**Table (3-8) Inhibition Zones *Candida albicans* with Caspofungin and Voriconazole(CLSI,2022)**

| Inhibition zones in millimeters (mm) | | |
|---|---|---|
| *Candida albicans* | | |
| Caspofungin | | |
| Sensitive | Medium | Resistance |
| ≥ 17 | 15–16 | ≤ 14 |
| Voriconazole | | |

| Sensitive | Medium | Resistance |
|---|---|---|
| ≥ 17 | 15-16 | ≤ 14 |

### 3.2.7 Steps for the preparation of nanoparticles

#### 3.2.7.1 Preparation of Plant Leaf Extracts :

Three different plant leaf extracts were used in this study: *Silybum marianum* (milk thistle), *Camellia sinensis* (green tea), and *Rosmarinus officinalis* (rosemary). Each extract was individually prepared according to the following described procedure (Moorthy & Kavitha, 2023) :

1. Fresh leaves were collected and thoroughly washed several times with distilled water to remove dust and surface impurities.

2. The leaves were then air-dried in the shade for 4–5 days to eliminate any residual moisture.

3. Once completely dried, the leaves were ground into a fine powder using an electric grinder.

4. Five grams (5 g) of the powdered leaves were weighed and added to 100 ml of distilled water in a 250 ml beaker.

5. The mixture was heated to 60°C on a hot plate.

6. The solution was stirred at 600 rpm using a magnetic stirrer until it turned dark brown in color.

7. The aqueous extract was filtered using filter paper to remove solid residues.

8. The filtrate was stored at 4°C for further use.

#### 3.2.7.2 Synthesis of Green BiONPs :

According to (Moorthy & Kavitha, 2023):

1. A volume of 30 mL of leaf extract was mixed with 1 mM of bismuth nitrate ($BiNO_3$ ) and 120 mL of distilled water in a 250 mL beaker.

2. Two drops of sodium hydroxide (NaOH) solution were added to adjust the pH to an alkaline level.

3. The mixture was kept at room temperature for 12 hours to allow the reaction to proceed.

4. A color change from yellowish brown to dark brown indicated the formation of bismuth oxide nanoparticles (BiONPs).

5. The resulting solution was centrifuged continuously for 2 minutes at 5,000 rpm to separate the nanoparticles.

6. The obtained BiONPs were then dried at 100°C to yield a powder, as shown in( Figure 3-1):

**Figure 3-1** Final product of the green synthesis – bismuth oxide nanopowder.

### 3.2.7.3 Preparation of Serial Dilutions of BiNPs Using DMSO :

A stock solution of the synthesized bismuth nanoparticles (BiNPs) was prepared by dissolving 1 gram of the nanoparticle powder in 10 mL of dimethyl sulfoxide (DMSO), yielding a 100% (w/v) concentration.

Subsequently, a two-fold dilution was performed as follows:

1. 50% Concentration:

5 mL of the 100% stock solution was mixed with 5 mL of pure DMSO to obtain a final concentration of 50%.

2. 25% Concentration:

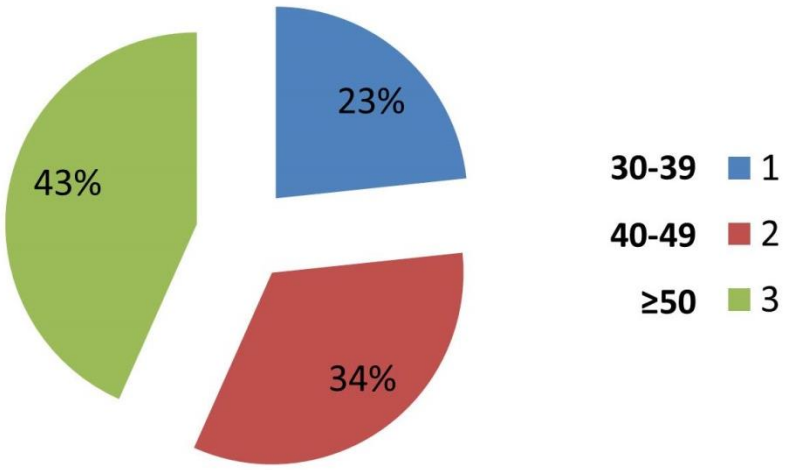
5 mL of the 50% solution was further diluted with 5 mL of DMSO to achieve a 25% concentration.


**Figure 3-2** The three concentrations of bismuth oxide nanoparticles.

# Chapter Four
# Results

## 4.1 Age distribution

Age factor has been shown to be one of the critical factors that involved in the incidence of different diseases. Our study did survey for age distribution for patients with diabetic foot ulcer. Our results found that the highest percentage of patients was in age group (≥50) years corresponding to percentage (43%), and followed by age group (40-49) years (34%) and then (23%) in age group (30-39) (Figure 4-1).




## 4.2 Sex distribution

We also did survey for sex distribution for patients with diabetic foot ulcer. Our results found that the highest percentage of diabetic foot ulcer was in female ( 57%) and followed by male (43%) (**Figure** 4-2).

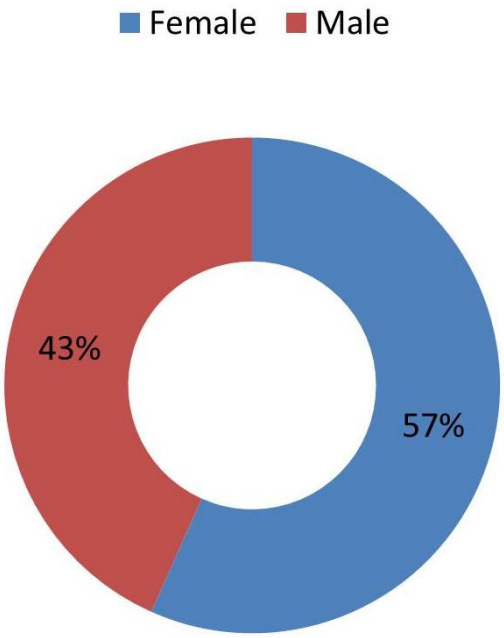



**Figure 4-2:** Male and female sex distribution in diabetic foot ulcers

## 4.3 Grades of diabetic foot ulcer

The present study was included grade 2 Patients who severed from diabetic foot ulcer. Grade 2 showed a great penetration in the foot tendon, as shown in figure 4-3.

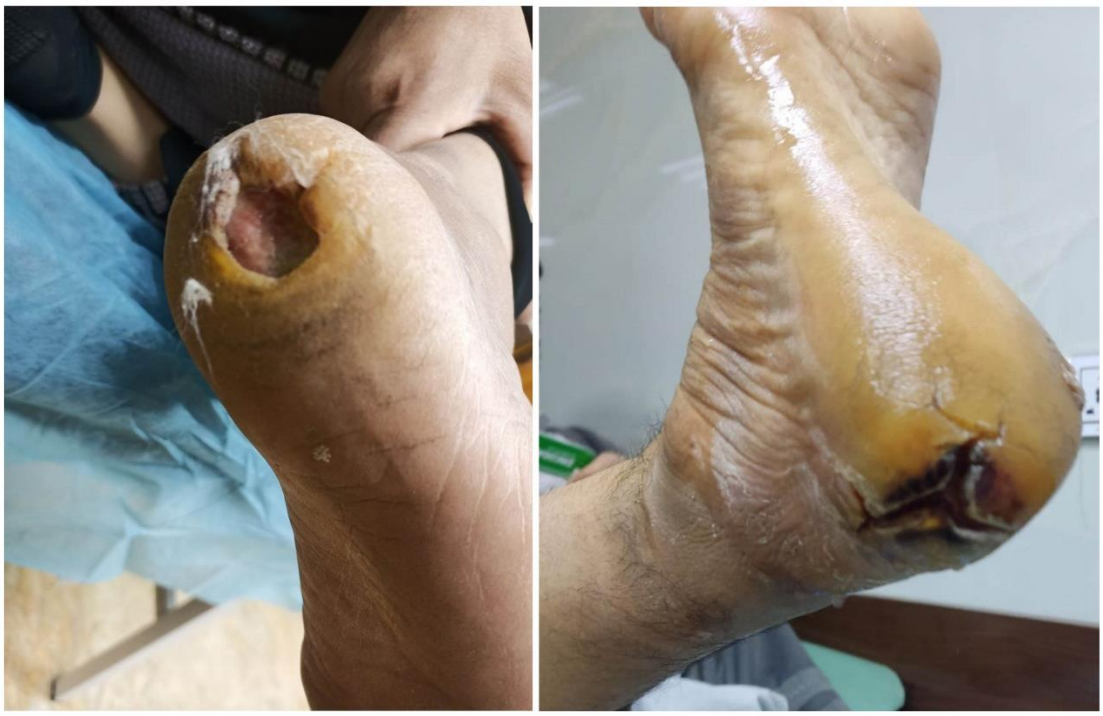


**Figure 4-3: Grade 2 diabetic foot ulceration.**

## 4.4 FBS levels in diabetic foot ulcer patients and control groups

It is commonly known that FBS levels are utilized as a predictor of diabetes mellitus. FBS levels were assessed in the current investigation for both the patient and control groups. (**Figure 4-4A**). The statistical analysis found that levels of FBS were significantly increased ($p \leq 0.05$) in patients with diabetic foot ulcer as compared to control group (**Figure 4-4A**). Additionally, HbA1c levels were also examined in both control groups and diabetic foot ulcer patients (**Figure 4-4B**). The present results found that the levels of HbAc1 were significantly increased by 2.2 fold in patients with diabetic foot ulcer as compared to control group (**Figure 4-4B**).

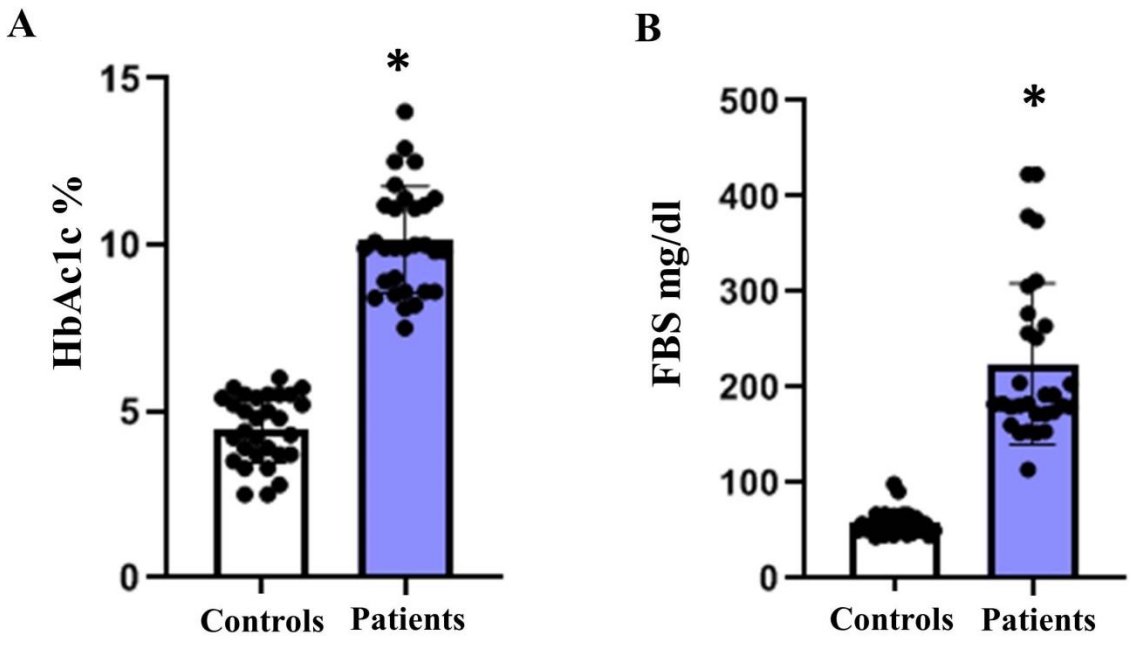



**Figure 4-4:** Levels of HbAc1 and FBS in patients and control group.

## 4.5 Cultural characteristics of bacteria

Isolated samples from ulceration of patients with diabetic foot were cultured on blood agar. Growth on blood agar was abundant in 18 to 24 hours. Colonies that are round, elevated, opaque, and yellow to golden yellow (Figure 4-5A).

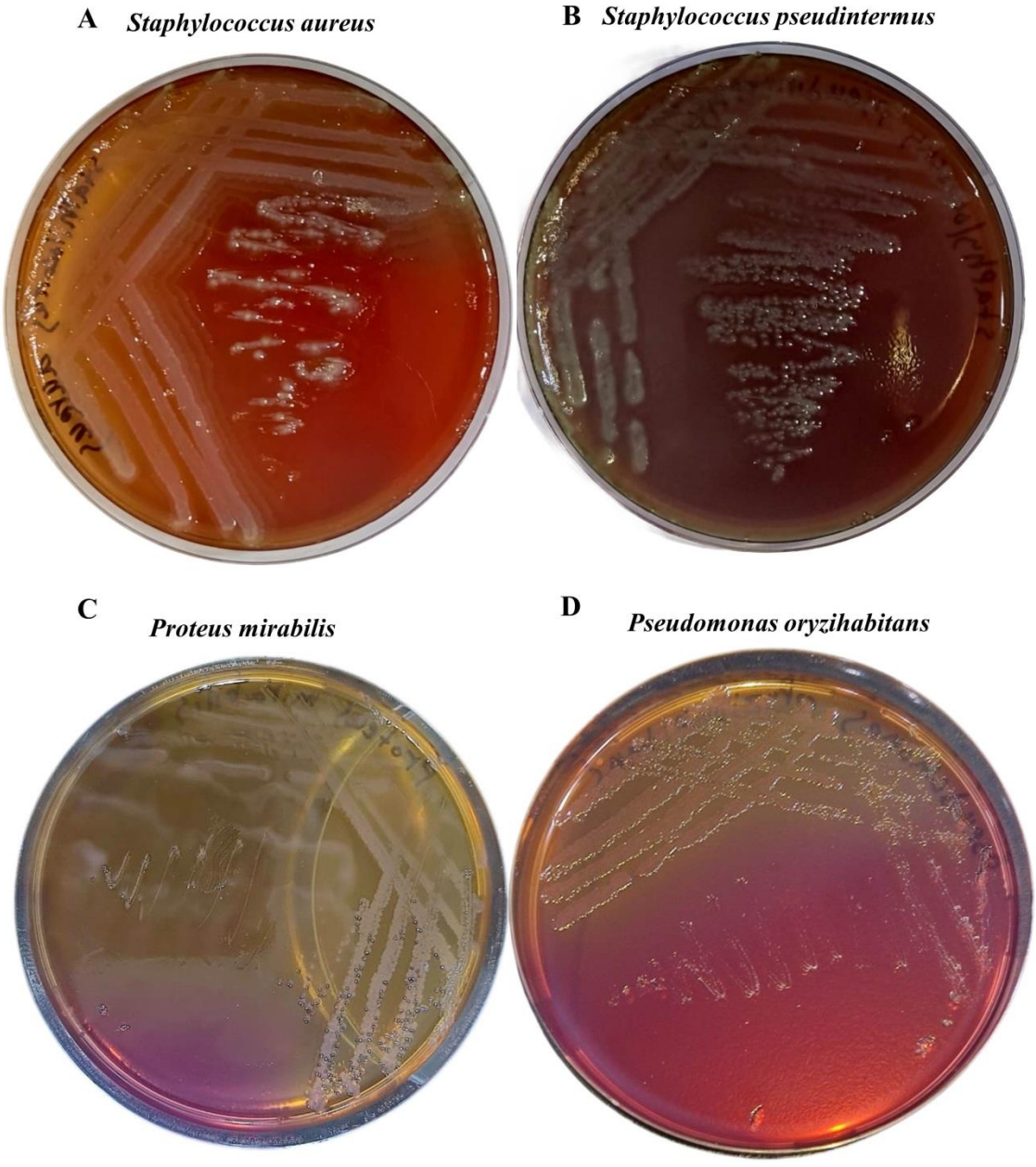



**Figure 4-5:** Culture characteristics of gram positive and negative bacteria isolated from diabetic foot ulceration patients.

In addition, *Staphylococcus pseudintermedius* colonies showed small round, greyish white, opaque colonies (Figure 4-5C). On other hand, gram negative bacteria, *Proteus mirabilis*, growth on MacConkey agar (MAC) showed smooth, pale or colorless colonies and it doesn't swarm. Moreover, *pseudomonas oryzihabitans* had yellow pigmented, often wrinkled colonies on MAC (Figure 4-5D).

## 4.6 Results of microscopic examinations

Diabetic foot ulcer-isolated bacteria were examined under light microscope. The microscopic examinations showed a spherically shaped gram positive bacteria , *Staphylococcus aureus* and *pseudintermedius* (**Figure 4-6A and B** ). Moreover, the present results also showed that gram negative bacteria (*proteus mirabilis and pseudomonas oryzihabitans*) have rod-shape under microscope (**Figure 4-6 C and D**).

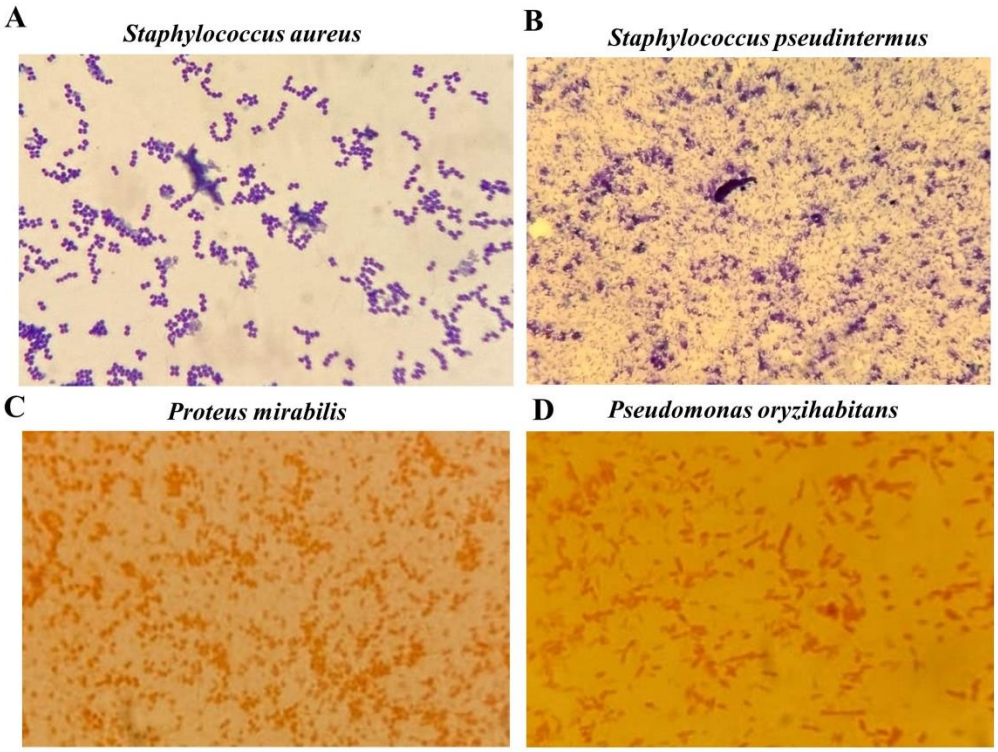



**Figure 4-6:** Microscopic examinations of gram positive and negative bacteria isolated from diabetic foot ulceration pateints.

## 4.7 Identification and sensitivity of diabetic foot-isolated bacteria

It was important to identify the isolated bacteria from diabetic foot ulceration. Biochemical tests were performed using VITEK2 System to identify the isolated bacteria. According to the biochemical tests, the results found that diabetic foot-isolated bacteria were as followed: *Staphylococcus aureus, Staphylococcus pseudintermedius, Proteus mirabilis* and *Pseudomonas oryzihabitans*, as summrized in table 4-1

Moreover, antibiotic sensitivity tests were also performed using VITEK2 System. The results of VITEK2 System found that *Staphylococcus aureus* and *Staphylococcus pseudintermedius* were sensitive to Levofloxacin (Figure 2C and 3C and table 4-2 and 3). In addition, result analysis of VITEK2 System showed that *Proteus mirabilis* and *Pseudomonas oryzihabitans* were sensitive to Amikacin and Ceftazidime (Figure 3C and 4C and table 4-4 and 5). However, using Muller Hinton Agar, the results found that *Proteus mirabilis* showed resistance against Ceftazidime.

Table 4-1: Biochemical test of positive and negative bacteria using VITEK2.

| Tests | *Staphylococcus aureus* | *Staphylococcus pseudintermedius* | *Proteus mirabilis* | *Pseudomonas oryzihabitans* |
|---|---|---|---|---|
| URE | - | + | + | - |
| APPA | - | - | - | - |
| PHOS | + | + | + | - |
| dMAN | + | - | - | + |
| AGAL | - | - | - | - |
| SAC | + | - | - | - |
| BGAL | - | - | - | - |
| dSOR | - | - | - | - |
| TyrA | - | - | + | + |
| PyrA | + | - | - | + |
| dTRE | + | + | + | + |
| BGUR | - | - | - | - |
| ProA | - | - | - | + |
| AGLU | - | - | - | - |
| dMAL | + | - | - | - |
| ILATk | - | + | + | + |
| 0129R | + | + | + | - |

**Table 4-2 : Antibiotic activity against *Staphylococcus aureus***

| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
|---|---|---|---|---|---|
| Benzylpenicillin | >=0.5 | R | Teicoplanin | >=32 | R |
| Oxacillin | >=4 | R | Vancomycin | >=32 | R |
| Gentamicin | 8 | •R | Tetracycline | >=16 | R |
| Tobramycin | >=16 | R | Tigecycline | <=0.12 | S |
| Levofloxacin | 0.25 | S | Nitrofurantoin | <=16 | S |
| Moxifloxacin | 0.5 | S | Fusidic Acid | 8 | R |
| Erythromycin | <=0.25 | S | Rifampicin | <=0.5 | S |
| Clindamycin | 2 | •R | Trimethoprim/ Sulfamethoxazole | <=10 | S |
| Linezolid | 2 | S | | | |

**Table 4-3 : Antibiotic activity against *Staphylococcus pseudintermedius***

| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
|---|---|---|---|---|---|
| Benzylpenicillin | >= 0.5 | R | Clindamycin | >= 8 | R |
| Oxacillin | >= 4 | R | Teicoplanin | >= 32 | R |
| Gentamicin | >= 16 | R | Vancomycin | >= | R |

| | | | | | |
|---|---|---|---|---|---|
| | | | | 32 | |
| Tobramycin | 2 | S | Tetracycline | >= 16 | R |
| Levofloxacin | <= 0.12 | S | Fusidic Acid | >= 32 | R |
| Moxifloxacin | <= 0.25 | S | Rifampicin | >= 32 | R |
| Erythromycin | >= 8 | R | | | |

**Table 4-4 : Antibiotic activity against *Proteus mirabilis***

| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
|---|---|---|---|---|---|
| Piperacillin/Tazobactam | 8 | S | Meropenem | <= 0.25 | S |
| Ceftazidime | 0.5 | S | Amikacin | 4 | S |
| Ceftazidime/ Avibactam | <= 0.12 | S | Gentamicin | >= 16 | R |
| Ceftolozane/Tazobactam | <= 0.25 | S | Ciprofloxacin | <= 0.06 | S |
| Cefepime | 16 | R | Colistin | >= 16 | R |
| Imipenem | 4 | R | | | |

**Table 4-5 : Antibiotic activity against *Pseudomonas oryzihabitans***

| Antimicrobial | MIC | Interpretation | Antimicrobial | MIC | Interpretation |
|---|---|---|---|---|---|
| Ceftazidime | 1 | S | Amikacin | <=1 | S |

| | | | | | |
|---|---|---|---|---|---|
| Cefepime | 0.25 | S | Gentamicin | <=1 | S |
| Imipenem | <=0.5 | S | Ciprofloxacin | <=0.06 | S |
| Meropenem | <=0.25 | S | Trimethoprim/ Sulfamethoxazole | <=20 | S |

## 4.8 Cultural characteristics and microscopic examinations of *Candida sp*

Isolated samples from ulceration of patients with diabetic foot were cultured on Sabouraud Dextrose Agar (SDA) which has been used as a selective media for dermatophytes. Candida colonies showed white colored, smooth, and yeast-like appearance and these characters were more close to *C. albicans* phenotypically (Figure 4-7A). Moreover , the microscopic examinations showed a spherically shaped of *Candida sp* using Lactophenol-cotton blue stain (Figure 4-7B).

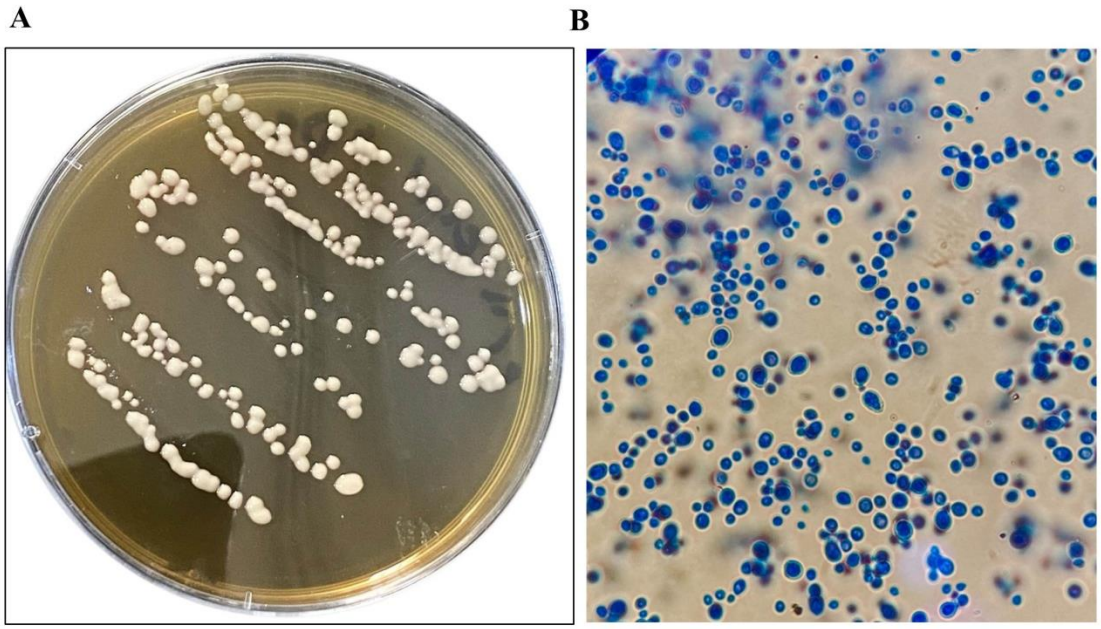



**Figure 4-7:** Culture characteristics and microscopic examinations of *Candida* sp isolated from diabetic foot ulceration patients.

## 4.9 Diagnosis and sensitivity of diabetic foot-isolated yeast

Next, the diabetic foot ulceration was examined whether it has fungal infection. The fungal culturing of diabetic foot samples on HiCrome™ Candida differential agar showed a growth of *Candida sp* (Figure 4-9). The yeast demonstrated the ability to form germ tubes, and its colonies appeared green on CHROMagar Candida medium. These results suggest that the isolate may be either *Candida albicans* or *Candida dubliniensis*, as both species share these phenotypic traits. Moreover, *Candidia sp* was sensitive to antifungal drug , Caspofungin, that used as a control treatment (Figure 4-24)

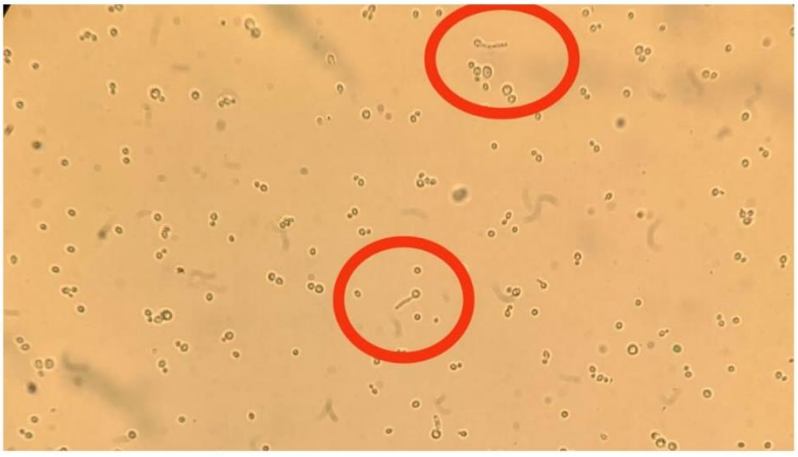


**Figure 4-8:** Microscopic examinations of yeast isolated from diabetic foot. Red circles show the germ tube of *Candida sp*.

**Figure 4-9:** Cultural characteristics of *Candida sp*. on HiCrome™ Candida Differential Agar.

## 4.10 Characterization Techniques of BiONPs

### 4.10.1 XRD studies

XRD analysis is performed to determine the nature of the crystalline phase and the interplanar distance. the XRD patterns of three distinct BiONPs, which display two value peaks at 27.7, 29.2, 31.07, 32.7, 45.76, 45.9, 53.2, 55.47, 57.4, and 73.7°, corresponding to the crystalline planes (201), (101), (002), (220), (004), (400), (131), (421), (402), and (113), respectively, which well matches with the standard JCPDS card no. 27.0050. The appearance of a sharp diffraction peak confirms the good crystalline nature associated with BiONPs. The average crystalline sizes of the BiONPs calculated using the Debye−Scherrer equation $D = k\lambda/\beta \cos \theta$ were found to be 19.55, 19.78, and 20 nm, respectively, for M-BiONPs, G-BiONPs, and R-BiONPs. The obtained XRD peak confirms the tetragonal phase for the synthesized BiONPs. From Figure (4-10), it is confirmed that the synthesized BiONPs do not contain any impurities.

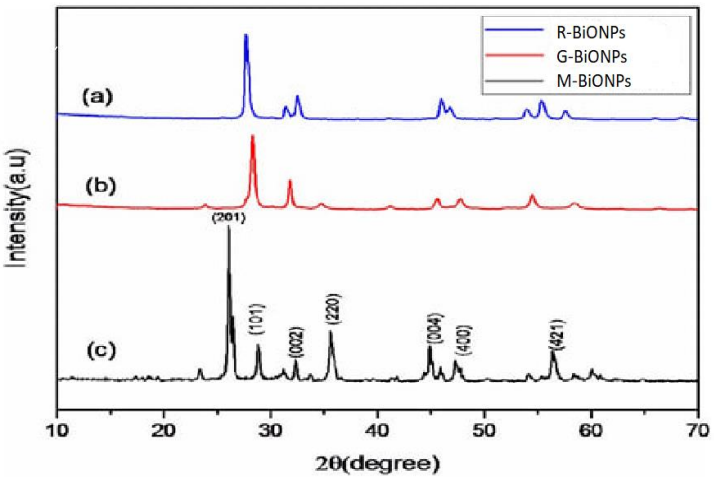



**Figure (4-10):** XRD patterns of BiONPs .

## 4.10.2 FE-SEM studies

FE-SEM image for investigate a morphological characteristics of BiO NPs .The figure (4-11) show two images for all three samples, once 1µm and other with scale 500 (nm). The FESEM images clearly show that the synthesized BiONPs are irregularly distributed with morphology comprising a number of microscopic holes scattered over the surface. As notable from the images, R-BiONPs, G-BiONPs, and M-BiONPs exhibit rod-shaped, and rock-shaped nanostructures, respectively. Therefore, we presume that the surface morphology, particle shape, and size are highly dependent upon the nature of the leaf extract used as a reducing agent, as evident from the results. We presume that the presence of a fibrous layer formed around the BiONPs is due to the presence of phytochemicals surrounding them. The presence of agglomerated bismuth nanoparticles, as evident from FESEM images, is inevitable during green synthesis due to the lack of control in the phytochemical composition of the bioextract. neous porous nature associated with BiONPs is attributable to differences in nucleation time and nonuniform distribution of heat zones within the reaction time. This is likely because the bioextract contains surfactants, capping, structure-directing, and protective agents. The BiONPs also

display agglomeration, although the borders between single crystallites are visible.

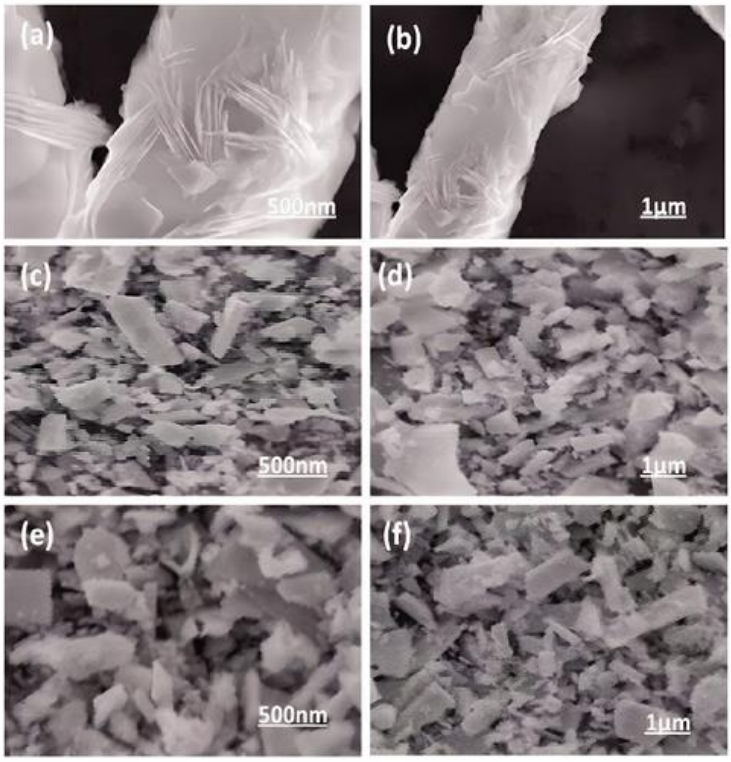



**Figure (4-11) :** FE-SEM micrograph of the synthesized (a, b) R-BiONPs, (c, d) G-BiONPs, and (e, f) M-BiONPs**.**

## 4.10.3 UV−visible study

The results of UV spectra disclose the optical properties of aqueous leaf extracts as well as synthesized BiONPs, as depicted in Figure 3, which are evaluated by a UV−visible spectrophotometer in the range of 250−500 nm. The synthesized BiONthe Ps showed maximum absorbance at 444 nm (M−L), 418 nm (G−L), and 404 nm (R−L), which confirms the formation of BiONPs. Figure 12 also shows the absorbance spectrum of (R−L, G−L, M−L) leaf extracts. The absorption edge at 292 nm (M−L), 274 nm (G−L), and 274 nm (R−L) for the aqueous leaf extract revealed the presence of active phytochemicals in the extract and confirmed that the aqueous leaf extract possesses phytochemicals like alkaloids, polyphenols, terpenoids, etc.

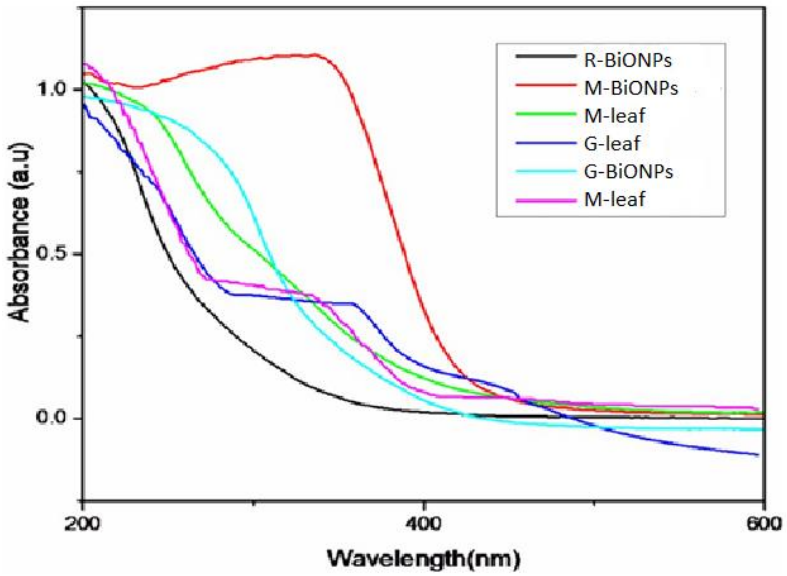



**Figure (4-12) :** UV−visible spectra of aqueous plant extract (R−L, G−L, M−L) and synthesized BiONPs.

## 4.11 Antibacterial activity of BiONPs

Nanoparticles Greenly synthesized have been shown to have a great positive effect in different disease. In this study, we did a comparative studies of BiONPs that synthesized from different plants to explore its antibacterial activity against gram positive and gram negative bacteria isolated form ulceration of patients with diabetic foot. Firstly, the antibacterial activity of BiONPs, that synthesized from *Rosmarinus officinalis*, was examined against gram positive bacteria (*Staphylococcus aureus*, *Staphylococcus pseudintermedius*) and gram negative bacteria (*Proteus mirabilis* and *Pseudomonas oryzihabitans*) isolated form ulceration of diabetic foot (Figure 4-13,14,15 and 16). The results found that RO-BiONPs (100%, 50% and 25%) showed a significant antibacterial activity ($P \leq 0.05$) against *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Proteus mirabilis*, and *Pseudomonas oryzihabitans* (Figure 4-13,14,15 and 16).

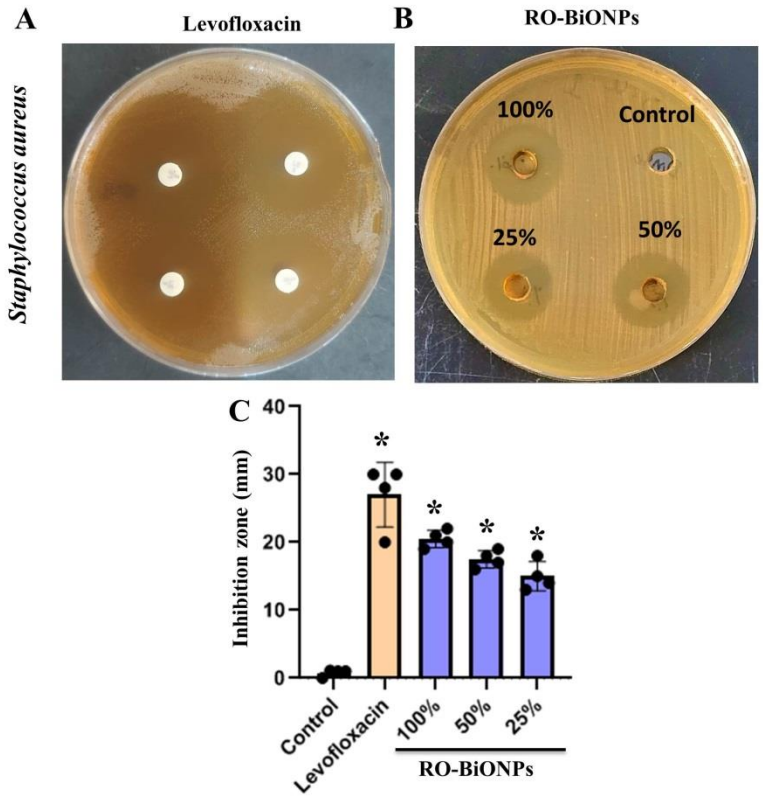



**Figure 4-13:** Antibacterial activity of RO-BiONPs against *Staphylococcus aureus*.

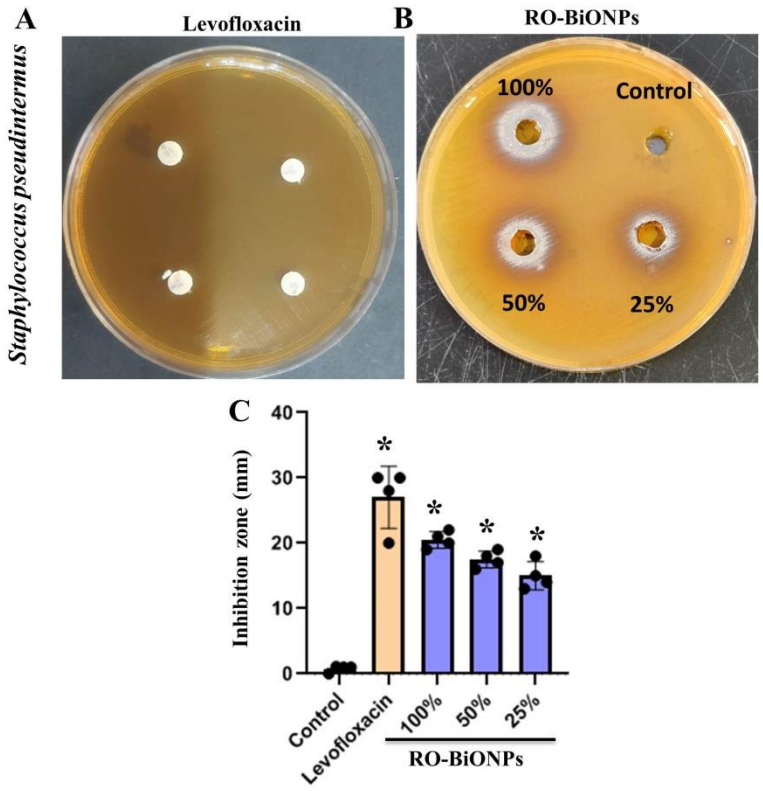



**Figure 4-14:** Antibacterial activity of RO-BiONPs against *Staphylococcus Pseudintermus.*

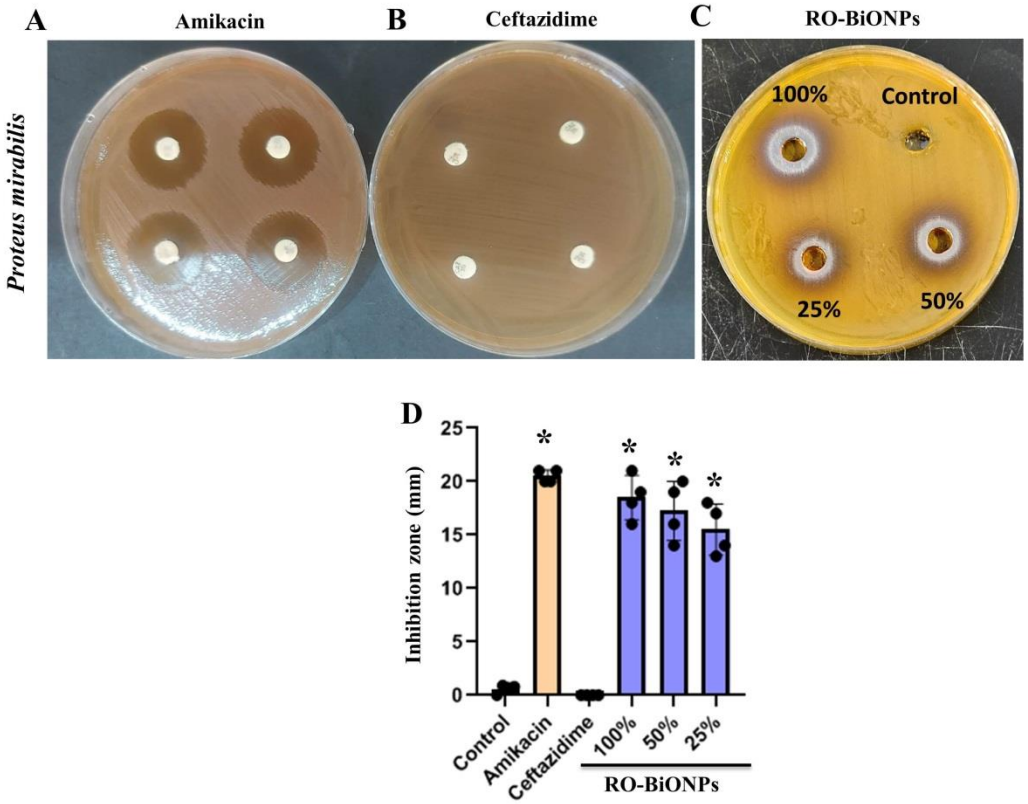


**Figure 4-15:** Antibacterial activity of RO-BiONPs against *Proteus mirabilis.*

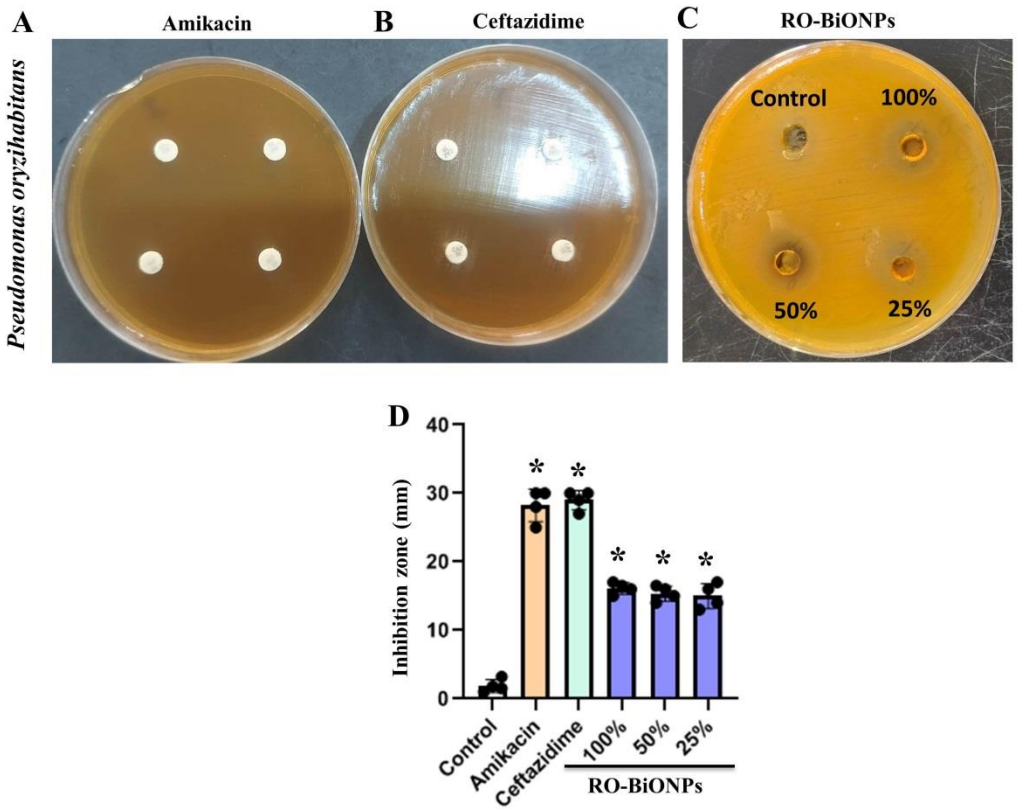



**Figure 4-16:** Antibacterial activity of RO-BiONPs against *Pseudomonas oryzihabitans.*

Secondly, the antibacterial activity of BiONPs, that synthesized from *Camellia sinensis*, was also explored against *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Proteus mirabilis*, and *Pseudomonas oryzihabitans* isolated from diabetic foot ulceration (Figure 4-17, 18, 19, and 20). CS-BiONPs showed a great antibacterial activity against against *Staphylococcus pseudintermedius* and *Pseudomonas oryzihabitans* at concentrations 100%, 50%, and 25% , respectively (Figure 4-17 and 18).

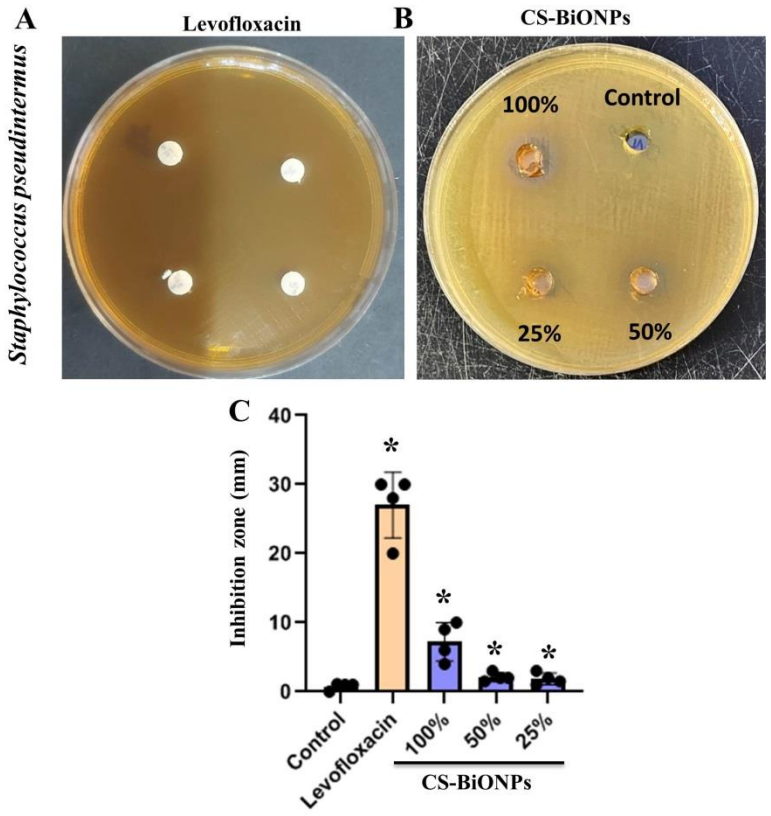



**Figure 4-17:** Antibacterial activity of CS-BiONPs against *Staphylococcus Pseudintermus.*

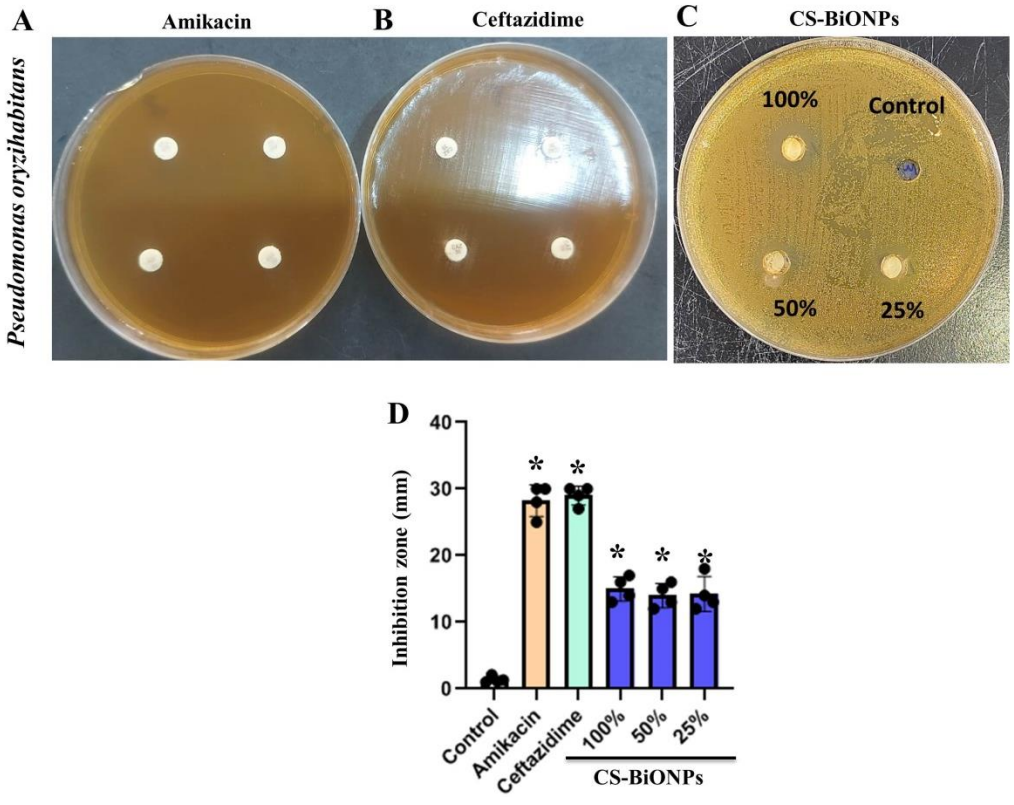


**Figure 4-18:** Antibacterial activity of CS-BiONPs against *Pseudomonas oryzihabitans.*

However, CS-BiONPs showed antibacterial effect against *Staphylococcus aureus* at concentrations 100% and 50%, respectively, and against *Proteus mirabilis* only at concentration 100%. CS-BiONPs did not showed any antibacterial activity at concentrations 25% against *Staphylococcus aureus and* at concentrations 50% and 25% against *Proteus mirabilis* (Figure 4-19 and 20).

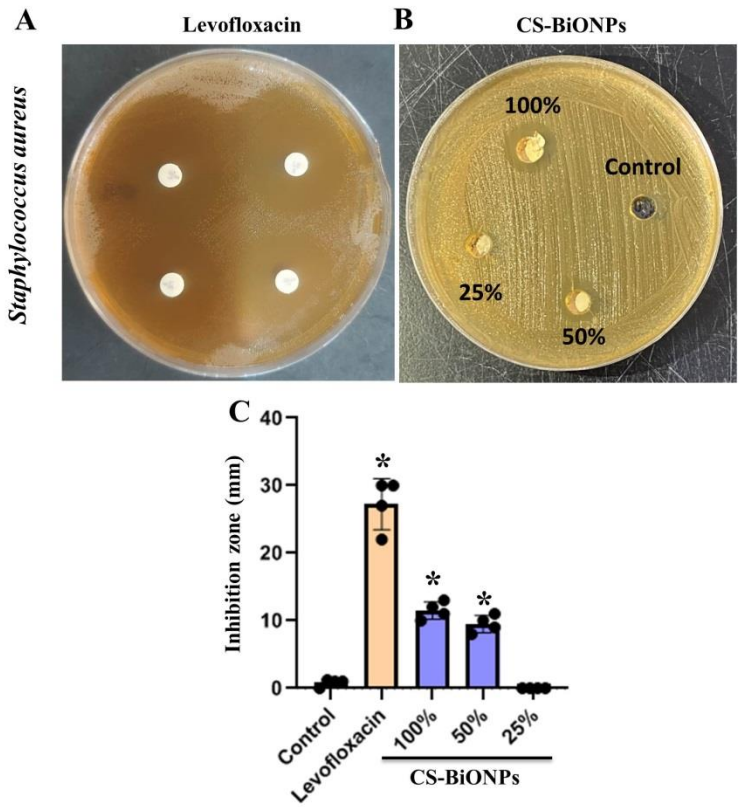



**Figure 4-19:** Antibacterial activity of CS-BiONPs against *Staphylococcus aureus.*

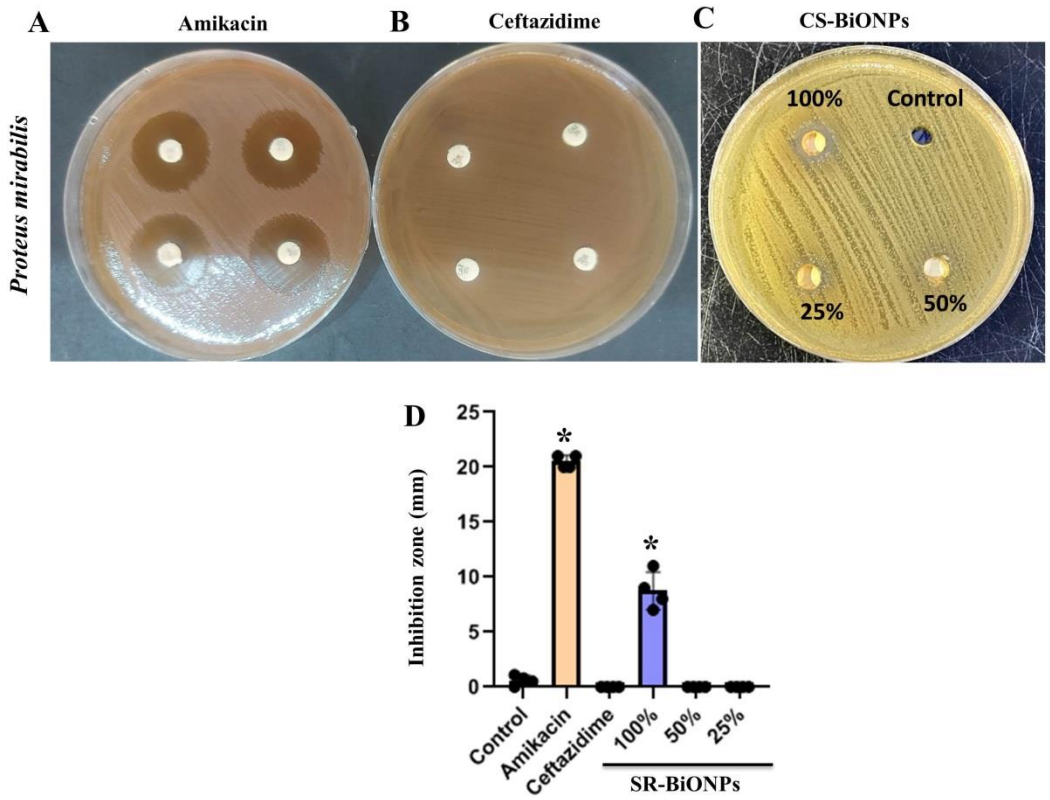



**Figure 4-20:** Antibacterial activity of CS-BiONPs against *Proteus mirabilis.*

Finally, the antibacterial activity of BiONPs, that synthesized from *Silybum marianum*, was also explored against *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Proteus mirabilis*, and *Pseudomonas oryzihabitans* isolated from diabetic foot ulceration (Figure 4-21, 22, 23, and 24). SM-BiONPs showed a great antibacterial activity only against *Staphylococcus aureus* (Figure 4-21).

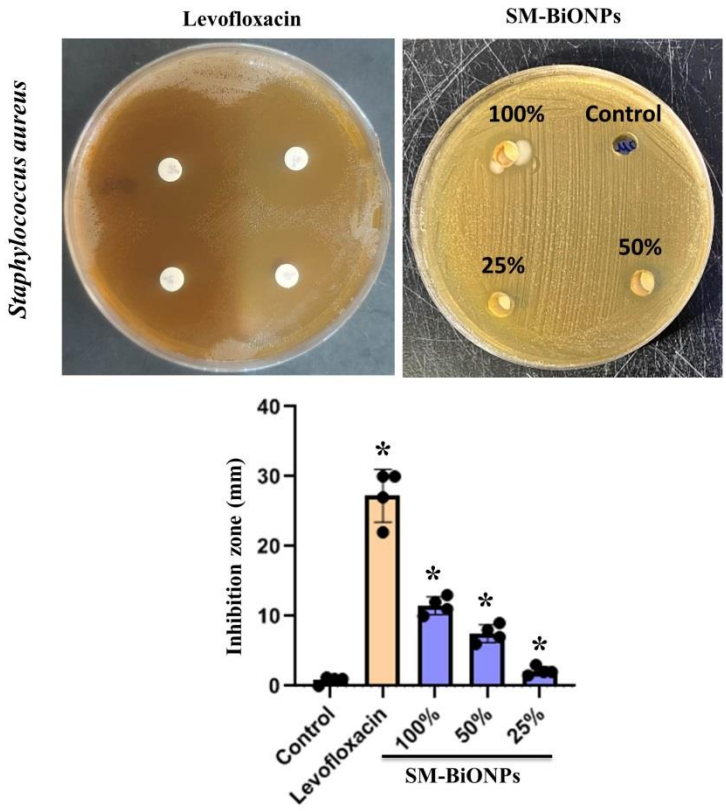



**Figure 4-21:** Antibacterial activity of SM-BiONPs against *Staphylococcus aureus.*

However, SM-BiONPs did not show any bacterial effect against *Staphylococcus pseudintermedius*, *Proteus mirabilis*, and *Pseudomonas oryzihabitans* (Figure 4-22, 23 and 24).

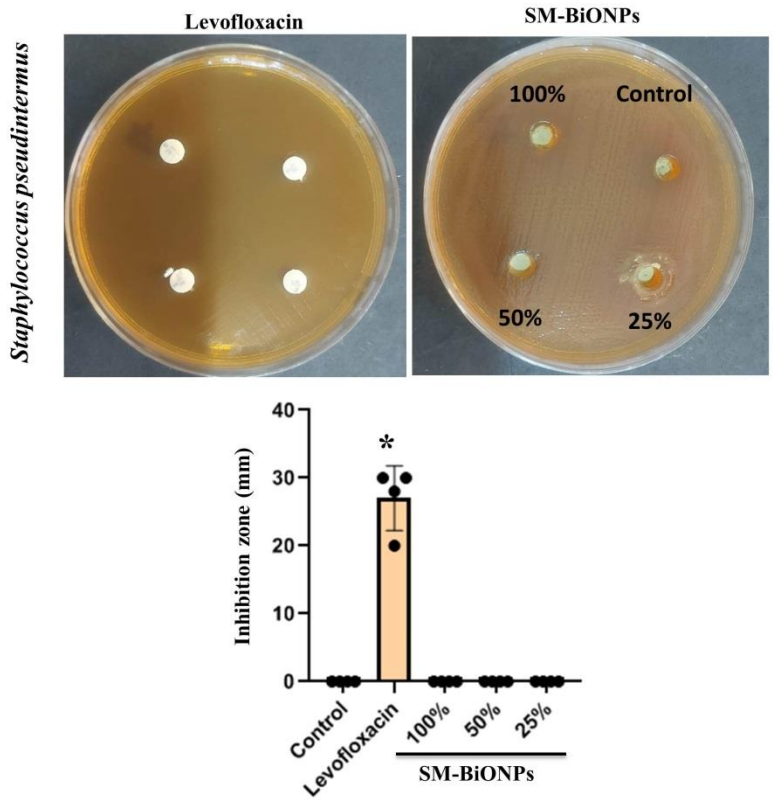



**Figure 4-22:** Antibacterial activity of SM-BiONPs against *Staphylococcus Pseudintermus.*

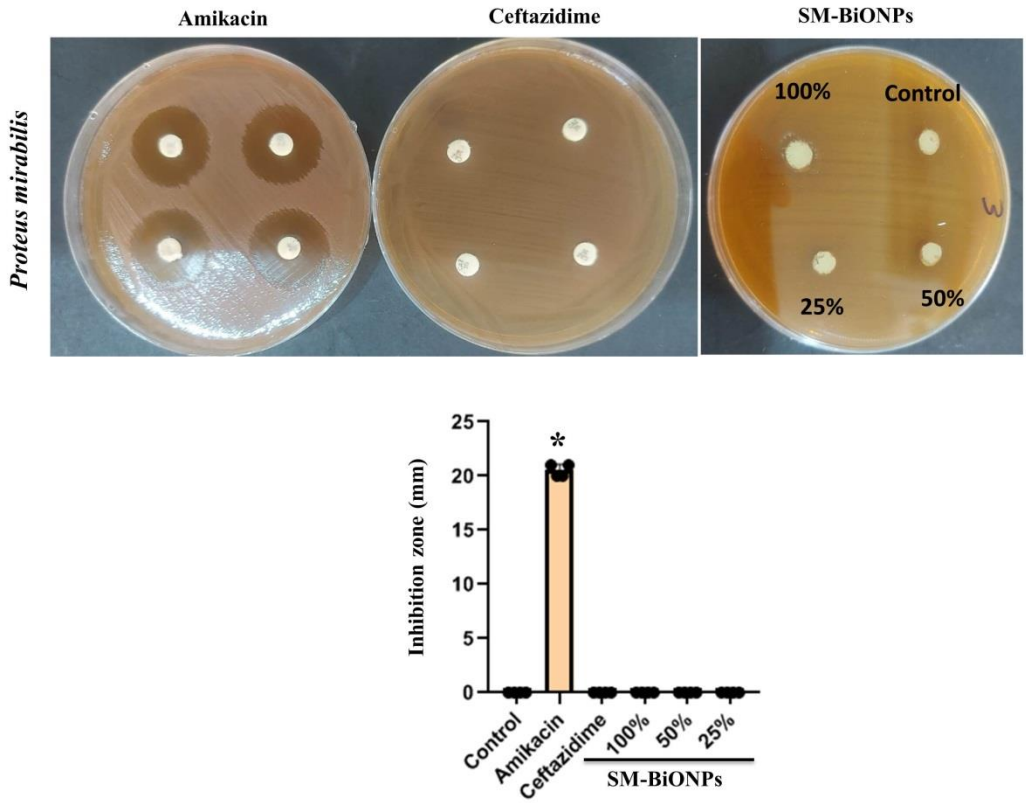



**Figure 4-23:** Antibacterial activity of CS-BiONPs against *Proteus mirabilis.*

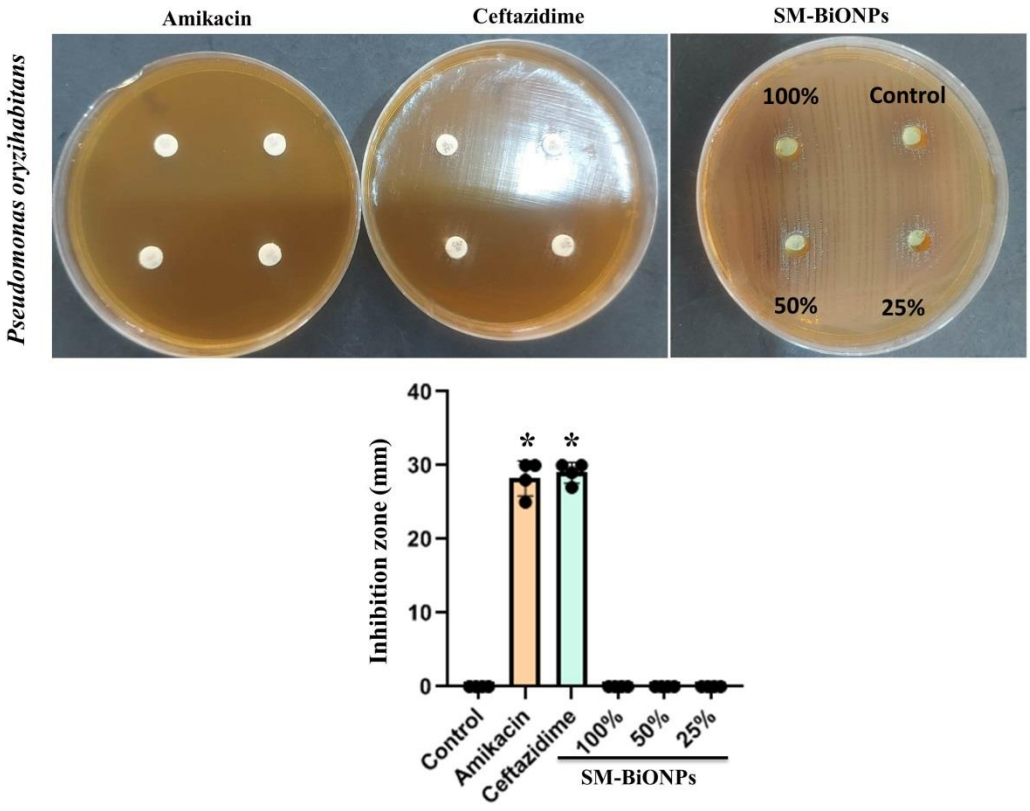



**Figure 4-24 :** Antibacterial activity of SM-BiONPs against *Pseudomonas oryzihabitans.*

## 4.12 Antifungal activity of BiONPs

Next, antifungal activity of BiONPs was investigated against the yeast isolated form ulceration of patients with diabetic foot (Figure 4- 25, 26, and 27). RO -BiONPs, that synthesized from *Rosmarinus officinalis* and *Camellia sinensis*, was examined against yeast that isolated form ulceration of diabetic foot patients. It was noticed that RO -BiONPs and CS- BiONPs show a significant antifungal effect against *Candidia* sp as compared to control group (Figure 4-25 and 26).

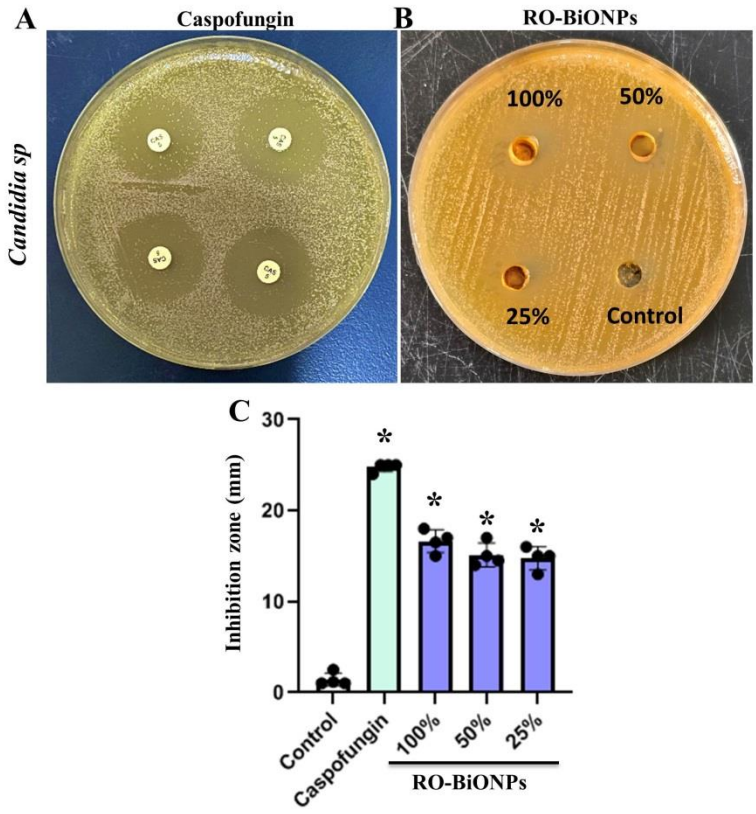



**Figure 4-25 :** Antifungal activity of RO-BiONPs against *Candida sp.*

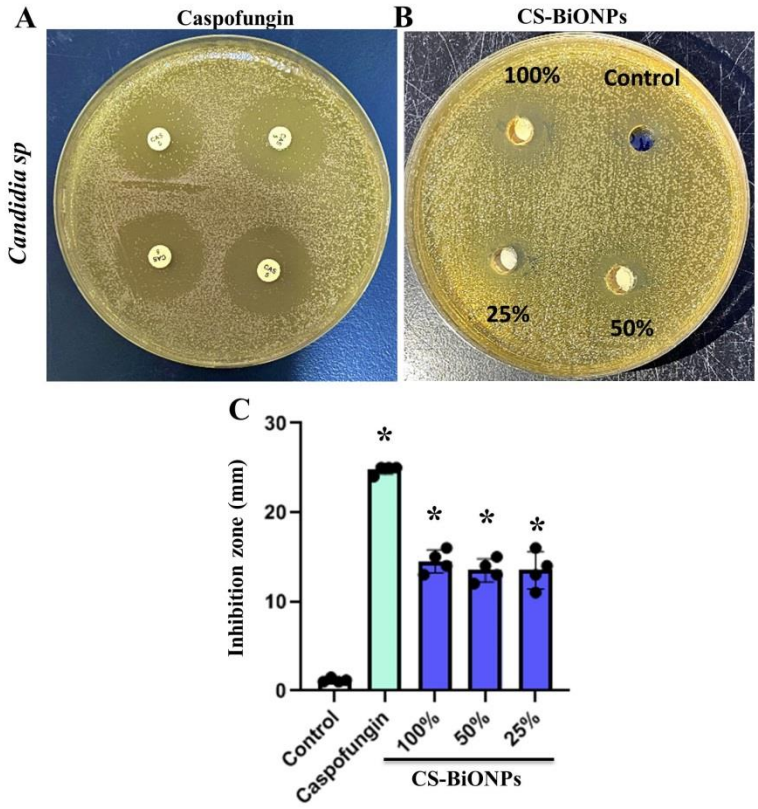



**Figure 4-26 :** Antifungal activity of CS-BiONPs against *Candida sp.*

However, BiONPs that synthesized from *Silybum marianum* did not any effect against *Candidia* sp (Figure 4-27)**.**

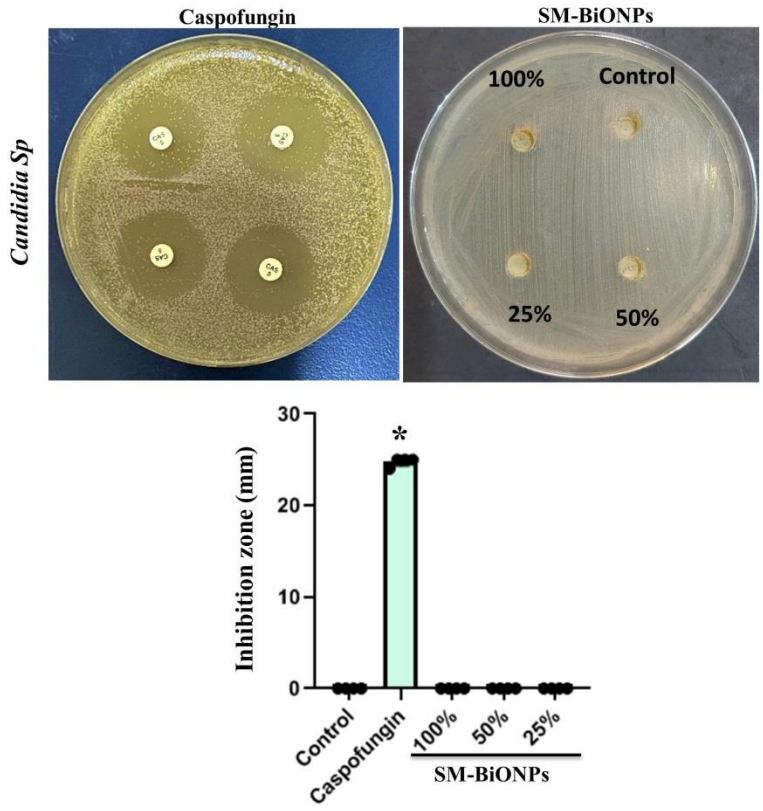



**Figure 4-27:** Antifungal activity of SM-BiONPs against *Candida sp.*

## 4.13 Association of neutrophils and monocytes with diabetic foot infections

It is commonly known that leukocytes are crucial in diabetic foot ulcers. The relationship between leukocytes and gram-positive and gram-negative bacteria was the first thing we looked at in this study. According to the statistical study, neutrophils and both gram positive and gram negative bacteria are highly correlated (Figure 4-28 A and B).

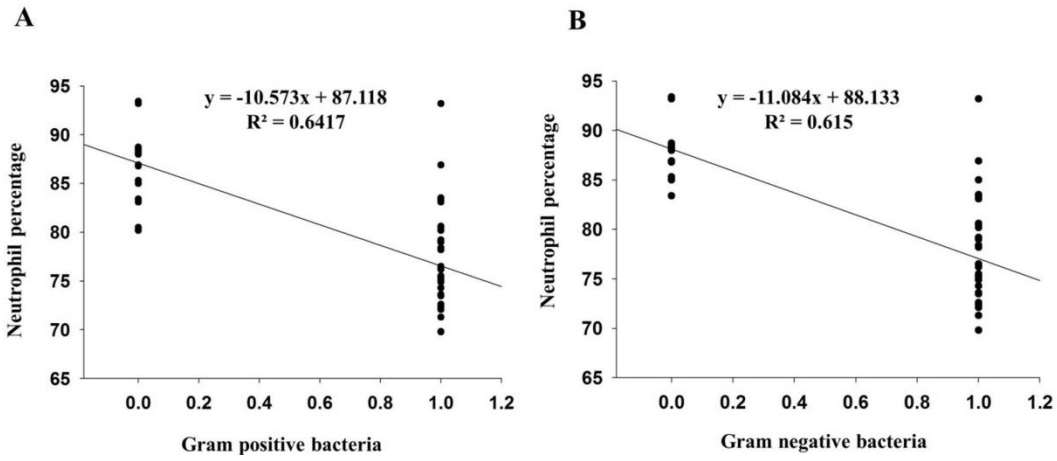



**Figure 4-28:** Association between neutrophils and gram positive and negative bacteria in patients with diabetic foot ulcers. A) correlation between neutrophil percentage and gram positive bacteria (y = -10.573x + 87.118; $R^2$ = 0.6417). B) correlation between neutrophil percentage and gram negative bacteria (y = -11.084x + 88.133; $R^2$ = 0.615).

Furthermore, a comparison of monocytes containing gram-positive and gram-negative bacteria was conducted (Figure 4-29A and B). Monocyte percentage and gram positive and negative bacteria were found to be significantly correlated (Figure 4-29).

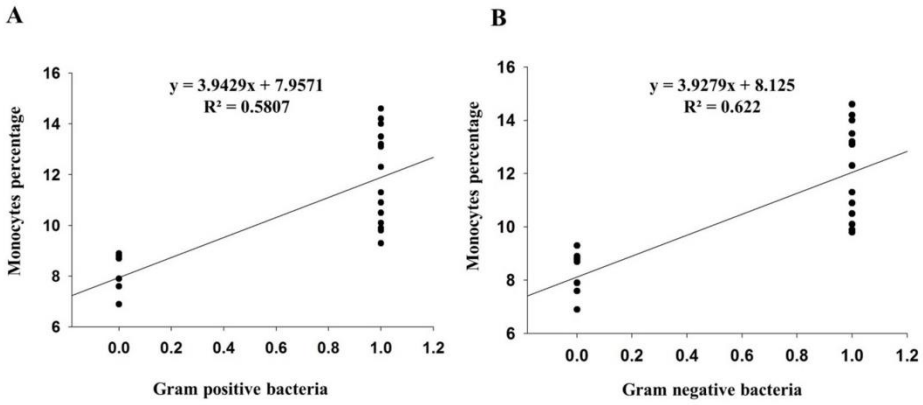



**Figure 4-29:** Association between monocytes and gram positive and negative bacteria in patients with diabetic foot ulcers. A) correlation between neutrophil percentage and gram positive bacteria (y = -10.573x + 87.118; $R^2$ = 0.6417). B) correlation between neutrophil percentage and gram negative bacteria (y = -11.084x + 88.133; $R^2$ = 0.615).

# Chapter Five
# Discussion

# Discussion

The diabetic foot ulcer (DFU) is one of the most frequent and severe complications of diabetes mellitus. The objective of this research was to investigate the demographic, microbiological, and biochemical features of individuals, and to assess the antimicrobial potential of plant-mediated prepared nanocompounds.

Age distribution results showed that majority of the patients were in the ≥50 (43%), 40–49 (34%) and 30–39 (23%) age groups respectively. These results support earlier findings that aging enhances the risk of diabetic foot complications with advancing age due to the gradual decline in blood circulation and peripheral neuropathy. Older people tend to have had diabetes for longer, one of the most important risk factors for foot ulcers given the accumulation of damage to nerves and blood vessels (Tang *et al*., 2023). In addition, with aging, the comorbidities such as neuropathy and nephropathy are much more common, which are both key risk factors for DFU (Tang *et al*., 2023). Ulcer of foot is also a problem in the aged and symptoms may start later in life and leads to greater severity of complications (Nwabudike *et al*., 2008).

The distribution of sex was also worth mentioning, women were more affected (57%) than men (43%). It may be caused by hormonal levels, patterns of health behaviours or social factors impacting upon access to medical care. In contrast, some research indicates that when confounders such as neuropathy are considered, females also have a similar risk to suffer from FU (Hussein *et al*., 2022; Dinh & Vives, 2008). Although men compared with women have more neuropathy and plantar pressure and women tend to have more joint mobility, favoring an initial lowering of the risk of ulceration. The only difference reported is the earlier onset of ulcers in men (Dinh & Vives 2008); however, once

they develop neuropathy women become susceptible to ulcers as well. Hormonal differences and differences in the structure of skin and subcutaneous tissue may also be involved although this is less described (Dinh & Vives 2008).

Regarding ulcer stage, this investigation centred on subjects with stage 2 ulcers, that is, involving tendons in foot. This degree of represents advancing disease and is too severe to be treated conservatively, representing delayed identification or poor glycemia. Grade 2 ulcers also produce a polymicrobial environment that obfuscates the healing process. Grades 2 ulcers were reported as one of the most common stages in diabetic foot occurrences (deep ulcer penetrating to tendon or capsule joint without bone involvement) (Diabetes Research Journal, 2016). This stage usually results from late diagnosis and poor control of blood glycemia leading to grossly overlooked ulcer before intervention. The bulk of the diabetic foot ulcers occur secondary to peripheral neuropathy, peripheral arterial disease, and mechanical pressure, particularly from inappropriate footwear. Neuropathy is responsible for the loss of protective sensation and vascular disease is associated with decreased blood supply, affecting tissue healing and increasing ulceration risk (Bolton *et al*., 2004).

Biochemical parameters showed that FBS levels in patients were significantly higher than controls ($p \leq 0.05$); HbA1c levels were 2.2 times higher, indicating a chronic hyperglycemic state. This elevation is a primary cause of delayed wound healing and heightened inflammation of diabetic foot ulcers. High fasting blood glucose levels and HbA1c in patients with DFU are commonly the manifestation of long-term inadequate glycaemic control, compounded by continuing inflammation and infection. These conditions contribute to insulin resistance and the

release of stress hormones (e.g. cortisol), which hike blood glucose levels still higher (Diabetes Care, 2006).

Regarding microbiology, several bacterial species were present, such as the Gram-positives *Staphylococcus aureus* and *Staphylococcus pseudintermedius* and the Gram-negatives *Proteus mirabilis* and *Pseudomonas oryzihabitans*. Also the fungus *Candida albicans* was recovered. In this study, the germ tube test and CHROMagar Candida medium were employed to identify the isolated *Candida species*. The yeast demonstrated the ability to form germ tubes, and its colonies appeared green on CHROMagar Candida medium. These results suggest that the isolate may be either *Candida albicans* or *Candida dubliniensis*, as both species share these phenotypic traits, necessitating further confirmatory tests (Pinjon *et al*., 1998).The green coloration observed on CHROMagar Candida medium is attributed to the activity of the enzyme β-N-acetylgalactosaminidase, produced by both *Candida albicans* and *Candida dubliniensis*, which reacts with chromogenic substrates present in the medium, resulting in a characteristic green pigment. Since both species exhibit this enzymatic activity, they produce similarly colored green colonies, making it difficult to distinguish between them based on colony color alone (Odds & Bernaerts, 1994). These results are in an agreement with previous studies that have shown that diabetic foot ulcers are generally polymicrobial, with both Gram positive and Gram negative bacteria (Mohammed *et al*., 2022 ; Al-Ayed *et al*., 2018). Isolation of *Candida albicans* Justification Chances of fungal infection, particularly Candidiasis, being high in chronic ulcers was proposed earlier (Shankar *et al*., 2009).

For antimicrobials susceptibility, gram-positive strains were sensitive to levofloxacin to gram-negative strains were sensitive to amikacin and ceftazidime. *Proteus mirabilis* was resistant to ceftazidime on Mueller

Hinton agar, indicating the necessity of performing susceptibility test before using this drug. *Proteus mirabilis* exhibits varying degrees of resistance to ceftazidime, and certain strains are susceptible and others highly resistant. The main resistance mechanism is the production of ESBLs which inactivate ceftazidime and other third generation cephalosporins, even in the focus of infection. A study by (Kwiecińska-Piróg *et al*., 2013) reported that 64% of *P. mirabilis* strains tested were resistant to ceftazidime, indicating a notable prevalence of resistance in certain populations. Conversely, other studies have shown that many clinical isolates remain sensitive to ceftazidime, suggesting that resistance is not universal across all strains (Li *et al*., 2023 ; Harada *et al*., 2014). And for fungi, The results of this study showed that *Candida albicans* isolates were sensitive to Caspofungin and resistant to Voriconazole. These findings are partially consistent with those reported by (Pappas *et al*., 2018), who noted that *C. albicans* generally exhibits good susceptibility to echinocandins such as Caspofungin, supporting its use as a first-line agent for invasive fungal infections. Similarly,( Pfaller *et al*., 2011) confirmed that resistance to Caspofungin among *C. albicans* isolates remains relatively low, except in certain rare strains.

In contrast, the observed resistance to Voriconazole in this study differs from earlier reports indicating that *C. albicans* is typically susceptible to azole antifungals, including Voriconazole (Pfaller & Diekema, 2007). However, more recent studies have documented increasing rates of acquired resistance, especially among isolates from patients with prior azole exposure or in environments with extensive azole use (Whaley *et al*., 2017). The resistance observed in this study may be attributed to mutations in genes or the overexpression of efflux pumps—both common mechanisms of resistance in *C. albicans*.

Bismuth oxide nanoparticles (BiONPs) synthesized with plant extracts were reported to possess great activity against a diverse panel of bacterial and fungal pathogens, and therefore indicating their potential as effective alternative antimicrobial agents.

BiONPs synthesized using *Rosmarinus officinalis* were found to exhibit high inhibitory activity against all bacterial and fungal isolates even at low concentrations (25%). These findings corroborate those of (Qabaha, 2013), who found that rosemary extract exhibited considerable inhibitory action against *Staphylococcus aureus* and *Candida albicans*, and attributed this to its high levels of phenolic compounds and antioxidants. *Rosmarinus officinalis* extract is rich in active phenolic compounds such as rosmarinic acid, carnosol, and carnosic acid, which are responsible for inhibiting microbial growth and demonstrating antioxidant activity (Nieto *et al*., 2018).

BiONPs, that synthesized from *Camellia sinensis*, was also explored against *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Proteus mirabilis*, and *Pseudomonas oryzihabitans* isolated from diabetic foot ulceration. CS- BiONPs showed a great antibacterial activity against *Staphylococcus pseudintermedius* and *Pseudomonas oryzihabitans* at concentrations 100%, 50%, and 25% , respectively. however,CS-BiONPs showed antibacterial effect against *Staphylococcus aureus* at concentrations 100% and 50%, respectively, and against *Proteus mirabilis* only at concentration 100%. CS-BiONPs did not showed any antibacterial activity at concentrations 25% against *Staphylococcus aureus* and at concentrations 50% and 25% against *Proteus mirabilis* , These results are supplemented by the outcome of (Khan *et al*., 2019), who reported that green tea extract possesses broad-spectrum antimicrobial activity against several bacteria and fungi, such as *Staphylococcus aureus* and *Candida spp*. *Camellia sinensis* extract is

rich in bioactive polyphenolic compounds, particularly catechins such as EGCG, which have shown robust antibacterial and antifungal activities primarily through inhibition of enzymatic activities of bacteria and cell wall disruption (Friedman, 2007).

BiONPs isolated from *Silybum marianum* had considerably restricted antimicrobial activity, reaching only *Staphylococcus aureus*. Our findings validate the findings of (Iqbal *et al*., 2022) where the extract exhibits some degree of antibacterial activity ; however, it is comparatively less effective than other plant extracts. This fraction contains silymarin, a flavonolignan compound analogous to silybin, which possesses moderate antimicrobial activity but is more famous for its hepatoprotective effects (Křen & Walterová, 2005).

BiONPs extracted from *Rosmarinus officinalis* and *Camellia sinensis* exhibited excellent antifungal activity against *Candida spp*., corroborating the findings reported by (Qabaha, 2013 ; Khan *et al*., 2019), who concluded antifungal properties inherent in these extracts.

Some bacteria and yeast populations have significant resistance against plant origin compounds due to a variety of complicated mechanisms:

1. Mechanisms of defense inside: The bacterial as well as the yeast cells carry efflux pumps, which push out active molecules outside the intracellular environment, which lowers their in vivo levels and restricts their activity (Alqahtani *et al*., 2023 ; Holmes *et al*., 2012). Second, such species possess enzymes for modifying or degrading plant origin compounds.

2. Biofilm formation: Most bacteria, including *Pseudomonas spp*., and fungi like *Candida albicans* form strong biofilms that inhibit the entry of

molecules from plants into their cells, and they are very hard to kill (Singh *et al*., 2022 ; Nett & Andes, 2016).

3. Complex Cellular Architecture: Gram-negative bacteria possess an outer membrane that blocks the invasion of antimicrobial molecules, while yeasts contain a mannan- and beta-glucan-enriched cell wall contributing to their resistance (Lee *et al*., 2020 ; Kalia *et al*., 2021).

4. Physiological Transformation and Adaptation: Certain yeast species possess the ability to alter their morphology from a spherical to a filamentous form in reply to a variety of stressors, thereby increasing their potential for adhesion and invasion (Mayer *et al*., 2013). Moreover, both bacterial and yeast populations can exhibit the gradual evolution of resistance following prolonged exposure to phytochemicals, either in natural ecosystems or controlled laboratory environments (Nguyen *et al*., 2021 ; da Silva Dantas *et al*., 2020).

5. Nature of the Plant Extract: The efficacy of plant extracts is based on numerous factors, including the species of the plant, the part of the plant utilized (for example, leaves or roots), the method of extraction, and the content of bioactive molecules, including phenolic compounds and flavonoids, which collectively account for their variable antimicrobial activity (Cowan, 1999).

Plant extracts are rich in a variety of bioactive compounds such as phenolics, flavonoids, organic acids, terpenoids, and tannins, which play two major roles in the green synthesis of nanoparticles:

1. Reduction:

The phytochemicals in the extract act as reducing agents, converting metal ions (e.g., $Bi^{3+}$ from bismuth nitrate) into their zero-valent,

nanoscale metallic form ($Bi^0$ ), which serves as the core for nanoparticle formation (Iravani, 2011).

2. Capping/Stabilization:

Once the nanoparticle nuclei are formed, the same or other compounds in the extract function as capping agents, stabilizing the nanoparticles and preventing their agglomeration. This helps maintain their nanoscale size and unique physicochemical properties (Ahmed *et al*., 2016).

When synthesizing nanoparticles using plant extracts, several laboratory indicators can suggest successful nanoparticle formation :

1. Color Change:

One of the earliest and simplest signs is a visible change in the solution's color (e.g., dark brown), attributed to the surface plasmon resonance (SPR) phenomenon of nanoparticles (Iravani, 2011).

2. Turbidity or Fine Precipitate Formation:

The appearance of slight turbidity or the presence of fine sediment at the bottom of the tube may indicate the formation of small-sized nanoparticles.

3. Spectroscopic and Analytical Confirmations:

Following visual confirmation, nanoparticle formation is typically validated using various analytical techniques:

• UV–Visible spectroscopy to monitor the characteristic absorption peaks of nanoparticles.

• FTIR spectroscopy to identify the functional groups involved in stabilization.

• SEM/TEM for morphological and size characterization.

• XRD to determine the crystalline structure of the synthesized nanoparticles (Sharma *et al*., 2009).

In the immunological aspect, it confirmed a significant amount of neutrophil rise against the presence of bacteria (Gram-positive and Gram-negative), conforming to scientific literature that qualifies them as innate immune system's frontline guards. Neutrophils act upon bacteria through many mechanisms, among which are:

Phagocytosis: Neutrophils phagocytize and kill bacteria with lysosomal enzymes and reactive oxygen species (ROS) (Nathan, 2006).

Release of neutrophil extracellular networks (NETs): They are fibrous networks that carry DNA and enzyme to kill bacteria and inhibit their spread (Brinkmann *et al*., 2004).

Attraction of other immune cells by secretion of cytokines such as IL-8 and TNF-α (Mantovani *et al*., 2011).

The frequency of elevated monocytes in the study suggests their function in :

- Macrophage differentiation, which phagocytose remaining bacteria after a neutrophilic attack (Italiani & Boraschi, 2014).

- Antigen Presentation, which triggers activation of T cells and converts the response into adaptive immunity (Auffray *et al*., 2009).

- Inflammatory mediator secretion like IL-1β and IL-6, which induce proliferation of other immune cells (Serbina *et al*., 2008).

As for the mechanism of action of monocytes :

Monocytes migrate to the injured tissues and differentiate into macrophages, which are mainly divided into two types:

• M1 (pro-inflammatory): These macrophages secrete inflammatory mediators such as TNF-α, IL-6, and reactive oxygen species (ROS), and are active during the acute phase of infection.

• M2 (anti-inflammatory): These macrophages secrete IL-10 and TGF-β, contributing to tissue repair and remodeling after inflammation (Kanter *et al*., 2012).

In diabetic foot ulcers, there is an imbalance between the two macrophage types, where :

• The number of M1 macrophages increases ineffectively, leading to uncontrolled chronic inflammation.

• M2 macrophages fail to perform their role in tissue repair due to insulin resistance (Olefsky & Glass, 2010).

Effect of Insulin

Normal insulin helps regulate the switch toward the M2 phenotype. However, in diabetes:

• Insulin resistance causes macrophages to remain in the pro-inflammatory M1 state.

• Hyperglycemia contributes to the disruption of PPAR-γ signaling pathways (Olefsky & Glass, 2010).

# Chapter Six

# Conclusions & Recommendations

# Conclusions and recommendations

## 6.1 Conclusions

1. The study confirms that diabetic foot ulcers (DFUs) are a serious indicator of disease progression in diabetic patients, particularly among older age groups, underscoring the need for early intervention.

2. Biochemical and immunological analyses revealed a chronic inflammatory state associated with hyperglycemia, which impairs wound healing and exacerbates infection.

3. The study identified a polymicrobial and multi-drug resistant environment in DFUs, highlighting the limited effectiveness of conventional treatments.

4. Bismuth nanoparticles synthesized using *Rosmarinus officinalis* and *Camellia sinensis* demonstrated potent antimicrobial activity, even at low concentrations, indicating their promise as alternative therapeutic agents.

5. A notable resistance was observed in some microbial isolates against both antibiotics and plant-derived compounds, emphasizing the urgent need to develop novel therapeutic strategies.

## 6.2 Recommendations

1. Early diagnosis of diabetic foot ulcers is essential to reduce amputation and mortality rates.

2. Promote the use of plant-based bismuth nanoparticles as promising alternatives to conventional antimicrobials.

3. Perform routine microbial susceptibility testing before prescribing antibiotics.

4. Enhance patient education on diabetic foot care as a key preventive strategy.

5. Expand microbial diagnostics to include anaerobic bacteria and deep tissue pathogens.

6. Use advanced techniques (e.g., molecular/genomic methods) for accurate identification of microbial species.

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