Thymol-loaded hydrogels for diabetic wound treatment

By

Maria Shafique

(Registration No: 00000362445)

Master of Science in Biomedical Sciences

Supervisor: Dr. Nosheen Fatima Rana

School of Mechanical and Manufacturing Engineering (SMME)

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2024)

Thymol-loaded hydrogels for diabetic wound treatment

By

Maria Shafique

(Registration No: 00000362445)

A thesis submitted to the National University of Sciences and Technology, Islamabad,

in partial fulfillment of the requirements for the degree of

Master of Science in

Materials Engineering

Supervisor: Dr. Nosheen Fatima Rana

Department of Biomedical Engineering & Sciences School of Mechanical & Manufacturing Engineering National University of Sciences and Technology

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2024)

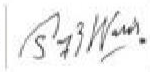
THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS/MPhil thesis written by **Regn No. 00000362445 Maria Shafique** of **School of Mechanical & Manufacturing Engineering (SMME)** has been vetted by undersigned, found complete in all respects as per NUST Statues/Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis titled. **Thymol-loaded hydrogels for diabetic wound treatment**

Signature: 

Name (Supervisor): Nosheen Fatima Rana

Date: 03 - Sep - 2024

Signature (HOD): 

Date: 03 - Sep - 2024

Signature (DEAN): 

Date: 03 - Sep - 2024

Form TH-4

National University of Sciences & Technology (NUST)

MASTER'S THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: Maria Shafique (00000362445)
Titled: Thymol-loaded hydrogels for diabetic wound treatment be accepted in partial fulfillment of the requirements for the award of MS in Biomedical Sciences degree.

Examination Committee Members

1.	Name: Aneeqa Noor	Signature:
2.	Name: Mehak Rafiq	Signature:
3.	Name: Muhammad Asim Waris	Signature:

Supervisor: Nosheen Fatima Rana Signature:

Date: 03 - Sep - 2024

Head of Department 03 - Sep - 2024

Date

COUNTERSINGED

03 - Sep - 2024

Date Dean/Principal

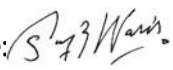
CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, entitled "Thymol –Loaded hydrogels for diabetic wound Treatment" was conducted by Mr./Ms. Maria Shafique ..... under the supervision of Dr. Nosheen Fatima Rana.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the.....SMME..... in partial fulfillment of the requirements for the degree of Master of Science in Field of ……Biomedical Sciences ……and Engineering……. Department of … National University of Sciences and Technology, Islamabad …

Student Name: Maria Shafique Signature: 

Supervisor Name: Dr Nosheen Fatima Rana Signature: 

Name of Dean/HOD: Dr Muhammad Asim Waris Signature: 

AUTHOR'S DECLARATION

I Maria Shafique hereby state that my MS thesis titled "Thymol-loaded hydrogels for diabetic wound treatment" is my own work and has not been submitted previously by me for taking any degree from National University of Sciences and Technology, Islamabad or anywhere else in the country/ world.

At any time if my statement is found to be incorrect even after I graduate, the university has the right to withdraw my MS degree.

Name of Student: Maria Shafique

Date: 03-09-24

PLAGIARISM UNDERTAKING

I solemnly declare that research work presented in the thesis titled "……Thymol-loaded hydrogels for diabetic wound treatment……" is solely my research work with no significant contribution from any other person. Small contribution/ help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and National University of Sciences and Technology (NUST), Islamabad towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS degree, the University reserves the rights to withdraw/revoke my MS degree and that HEC and NUST, Islamabad has the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized thesis.

Student Signature: _____

Name: Maria Shafique

Dedicated to the pillars of my strength, my parents, whose unwavering support and endless encouragement have been the foundation of all my achievement, to my sisters, for their constant love and motivation that have guided me through every challenge. And to my esteemed supervisor, whose wisdom, patience, and guidance have been the most important beacon on this journey—this work is a testament to your mentorship. Without each of you, this milestone would not have been possible.

ACKNOWLEDGEMENTS

All praise and thanks are due to Allah, the Most Gracious, and the Most Merciful, for granting me the strength, wisdom, and perseverance to complete this research. Without His divine guidance and blessings, this work would not have been possible.

I am deeply grateful to my parents. Thank you for your endless love, support, and encouragement. You have always believed in me, and I couldn't have done this without you. To my siblings, thank you for always being there and cheering me on.

I would like to express my deepest gratitude to my supervisor, Dr Nosheen Fatima, for guiding me every step of the way. Your advice, patience, and support made all the difference, and I truly appreciate everything you've done.

I am also deeply grateful to the members of my thesis committee, Dr Asim Waris, Dr Aneeqa Noor, and Dr Mehak Rafique for being on my thesis guidance and evaluation committee.

A special thank you to my seniors, Affra Nawaz, Iqra Shafique, and Tehreem Tanveer for their helpful advice and encouragement. Your support meant a lot to me.

To my friends, Ayesha Qureshi, Komel Tariq, Huraira Tariq, Hooreen kayani, Mariyam Saddiqa thank you for always being there for me and for cheering me on when I needed it the most. Your friendship has made this journey enjoyable.

Last but not least, to my cat Muffin, whose silent purring and comforting presence provided me with the motivation and calm I needed to persevere during this journey

TABLE OF CONTENTS

LIST OF FIGURES

Page No.

LIST OF TABLES

ABSTRACT

Diabetic wound (DW) healing remains a significant healthcare concern due to prolonged healing times and high infection rates, leading to inadequate treatment outcomes for many patients. Current therapies often fail to address the complex requirement of diabetic wounds, necessitating novel approaches. This study explores the development of a thymol-loaded hydrogel as an innovative therapeutic strategy to accelerate the wound healing and prevent infections in patients with diabetes. Thymol, naturally occurring compound with well-documented anti-inflammatory and antibacterial properties, was encapsulated in liposomes to enhance its stability and ensure sustained release at the wound site. These thymol-loaded liposomes were then incorporated into a hydrogel matrix, synthesized through cross-linking polymerization optimized for stability, controlled release, and maintaining a moist wound environment. The hydrogel's antibacterial efficacy was assessed using the well diffusion method against *Staphylococcus aureus* and *Enterococcus faecalis*. Additionally, anti-biofilm assays demonstrated the hydrogel's ability to prevent biofilm formation. In-vivo experiments conducted on a diabetic rat model revealed that wounds treated with this hydrogel exhibited significantly faster healing and improved tissue regeneration compared to untreated controls. These findings suggest that thymol-loaded hydrogel represents a promising approach for managing diabetic wounds (DW), offering both accelerated healing and effective infection control. This novel therapeutic approach holds potential for clinical application, improving outcomes for diabetic patients suffering from chronic wounds.

Keywords: diabetic wound healing, thymol loaded hydrogel, liposome encapsulation, *Staphylococcus aureus, Enterococcus faecalis*.

CHAPTER 1: INTRODUCTION

1.1. Overview of Diabetes Mellitus (DM):

Diabetes mellitus (DM), also referred to as diabetes, is a chronic and severe condition marked by continuously high levels of glucose in the blood due to inadequate production of insulin or the body's inability to properly utilize the insulin it produces. Diabetes impacts people of all age groups, genders, and geographical areas, making it a highly frequent worldwide cause of death and illness. Approximately 240 million people worldwide have undiagnosed diabetes, and approximately half of diabetic adults are ignorant of their illness. Healthcare systems worldwide are financially burdened by diabetes. The condition affects 537 million people, or 10.5% of the global 20-79 age group. The International Diabetes Federation (IDF) estimated that in 2021, over 537 million individuals, accounting for 10.5% of the global population, were affected by diabetes. The total expenditure on healthcare worldwide amounted to $966 billion. Healthcare spending is projected to surpass $1054 billion by 2045. The estimated incidence of DM is anticipated to increase to 643 million (11.3%) by 2030 and 783 million (12.2%) by 2045, which is worrisome.[1, 2]

1.2. The Diabetic wound (DW):

The management of diabetes is crucial for enhancing patient quality of life and survival. Hyperglycemia, or high blood sugar, raises the risk of comorbid disorders affecting many organs and affects the body's ability to produce new tissue. Wound healing in diabetic persons is impeded by a complex interplay of vascular, neuropathic, immunological, and metabolic variables. Hyperglycemia leads to hardening of the arteries, reduced blood flow, and poor oxygen supply to tissues[3, 4].Diabetic patients also experience alterations in their blood arteries, which diminishes the migration of leukocytes into wounds, hence increasing the likelihood of infections. Moreover, peripheral neuropathy can result in numbness and diminished pain perception, which can lead to unnoticed and untreated wounds that may develop into chronic conditions..[5]. The lower extremities are highly vulnerable to minor injuries that have the potential to develop into long-lasting lesions as a result of alterations in motor function and sympathetic reactions. These changes can cause

foot deformities, increased pressure on the soles, and dryness of the skin, resulting in the skin cracks and minor injuries. The impaired wound healing in individuals with diabetes is caused by the dysregulation of angiogenic factors such as TGF-β, FGF2, and VEGF, which hinder the formation of new blood vessels. Persistent inflammation prevents the wound from initiating to the stages of proliferation and remodelling in the healing process. Consequently, wounds in individuals with diabetes have a delayed healing process; frequently resulting in infections caused by disrupted cell circulation and diminished levels of natural growth hormones. This ultimately leads to the development of persistent non healing ulcer. In severe cases, infections necessitate limb amputation.[6]

1.3. Diabetic wound Current treatment and limitations:

Common wound-care practice for poor wound healing involves infection management, debridement, pressure relief, and moist wound bed maintenance. Chronic wound care begins with infection prevention and tissue clean-up. Due to the absence of the skin's inherent barrier, infections can easily arise, and microorganisms in the wound may slow healing. Similarly, Debridement exposes healthy tissue where wound-healing cells can multiply. Enzymatic, mechanical, and surgical debridement treatments use natural fibrin breakdown enzymes to self-activate. The latter is most effective in removing necrotic tissue and preventing its spread.[7]

Topical dressings provide moisture for healing and produce granulation tissue and epithelialization. These treatments expedite wound healing, minimise scarring, and reduce infection risk. Re-epithelization, hydration, antimicrobial, trauma prevention, and exudate wicking are common dressing benefits. Passive, active, therapeutic, or interactive. Wounds are covered by passive dressings. Interactive dressings prevent bacteria. They might be occlusive or semi-occlusive and vary in composition. Films, foams, alginates, hydrogels, hydrocolloids are popular. Bioactive dressing promotes granulation tissue with growth agents and hydration. Collagen, hyaluronic acid, chitosan, growth factors, and antimicrobials promote wound healing.[5]

Diabetes medications like insulin, metformin, some sulfonylureas, thiazolidinedione, and DPP-4 inhibitors have anti-inflammatory and other effects that may help treat chronic

wounds. These drugs polarise macrophages to heal, reduce MMPs, promote keratinocyte and fibroblast proliferation, angiogenesis, and granulation tissue development. The clinical correlation between this and enhanced wound healing yet to be shown. In some cases, as seen below, data are encouraging and warrant further study. [8]

Growth factors activate cellular and molecular responses during healing. They boost granulation tissue, inflammatory control, angiogenesis, ECM creation, remodelling, and re-epithelialization. Clinical trials have investigated PDGF, VEGF, EGF, FGF, and TGFβ1 for treating diabetic foot ulcers. Though promising, several trials showed bias and poor safety. Innovative medication delivery methods have been developed to prevent growth factor preparations like PDGF or EGF from failing due to poor formulations. Polymeric micro- and nanospheres, lipid nanoparticles, hydrogels, scaffolds, and nanofibrous structures limit growth factor release to stabilise wound protein and improve treatment. Recent approaches use degradable biomaterials or gene-mediated therapeutic delivery to provide high growth factor concentrations to the wound.[9, 10]

Treatment of poor wound healing with stem cells is intriguing. The injured area can recruit cells, angiogenize, remodel the ECM, and immunomodulation with cytokines and growth factors from transplanted stem cells. Clinical trials have shown that commercial topical treatments with adult mesenchymal stem cells (MSC) work. New induced pluripotent stem cells (iPSC) may be autologous and have low immunological rejection. Numerous preclinical studies in animal models of wound healing suggest that iPSC may soon be used as a new therapeutic tool for human wound healing[4].

Based on the information above, there are several therapeutic methods, but each has its own limitations and none seem to be enough to guarantee a successful, conclusive, and non-recurrent healing session. Therefore, new therapy options must be developed (or discovered) quickly. Although the sequence of events that occur during poor wound healing has been thoroughly characterised, pharmaceuticals that target one or more of the molecular events can be available on the market.

1.4. Nanotechnology-Based Drug Delivery Systems (NDDS):

Nanotechnology- based drug delivery systems (NDDSs) are a versatile class of drug delivery systems that can be fabricated using various biomaterials. These particles have a diameter at the nanoscale, enabling them to enhance drug stability, sustained release, and controlled release.[11].An remarkable range of NDDSs, containing therapeutic agents, has emerged and is currently being used to treat diabetic wounds. Nano drug systems (NDDSs) can be classified into liposomes, polymeric nanoparticles, inorganic nanoparticles, lipid nanoparticles, nanofibrous structures, and nano-hydrogel.[11].

1.5. Use of Liposomes as a drug carrier:

Liposomes are spherical phospholipid bilayers with an aqueous core. This bilayer of natural or synthesised phospholipids, steroids, and polymers exhibit amphipathic properties. Liposomes, produced from multilamellar vesicles, range in size from 20 nm to 5μm depends on their molecular makeup.[12].

Liposomes have shown potential in improving therapeutic outcomes for chronic wounds, especially those associated with diabetes. A research study proposed the use of glucocorticoid-loaded PS liposomes to specifically target immune cells in chronic wounds. This method expedites the process of healing and mitigates negative consequences by employing lower concentrations of medication.[13].

Diabetic wound care have been revolutionised by liposomes. They can improve patient outcomes by delivering therapeutic ingredients to the wounded site, keeping it wet, and regulating recovery. Research may make liposome-based therapeutics vital for healing complicated wounds. Liposomes give medications to the wound, speeding healing and moisturising it. Antioxidant, inflammation-modulating, and tissue-regenerative lecithin liposomes help control wounds.[14].

Hydrogels remain prominent due to their many benefits. Hydrogel-based dressings with different wound-healing properties have been extensively studied, improving wound care.[15].

1.6. Hydrogel as drug delivery systems:

Wound healing, especially diabetic wounds, is complicated and needs numerous processes, making wound management challenging to find effective solution for wound management. Researchers' interest in wound healing has led to new treatments that improve results. Many remedies have been devised, but none have treated all wounds. Hydrogels are popular for study due to their many benefits. Hydrogel-based dressings with diverse uses have shown promising wound healing results. Due to numerous advantages of Hydrogels have gained significant interest in research due to numerous advantages. Various hydrogel-based dressings with multiple purposes have been explored for wound healing, with outstanding outcomes[15].

Advanced three-dimensional hydrogels are constructed of hydrophilic polymer chains. They rapidly expand in water, generating a partly solid material.[16]. Over 90% of the hydrogel structure contains water, which keeps the wound moist and aids tissue repair[17]. Hydrogels are ideal for wound healing due to their many properties including firm adherence, shape adaptation, and mechanical protection, allow for adequate coverage and protection of the wound[18]. Hydrogel-based dressings have the benefit of being easily adjustable, which means that they can include antibacterial and antimicrobial substances, cells, biomolecules, and growth factors[19]. This augmentation is intended to accelerate the processes of wound contraction and healing. A hydrogel can be formed by cross-linking by using any hydrophilic polymer. Water-soluble polymers can exist in either a natural or synthetic form. Synthetic materials possess distinct qualities due to their highly modifiable physical properties and adhesive properties. Compared to synthetic polymers, natural polymers demonstrate superior biocompatibility and biodegradability.

Hydrogels are categorised by their synthesis method, polymer properties, ionic charge, or polymer chain linkages. The monomer content, synthesis process, and additions can dramatically affect hydrogel characteristics. Hydrogels can change from gel-like to soil-like in response to temperature, pressure, pH, ionic charge, or antigens. Once the stimulus is gone, it can return to its natural state. Unique properties make hydrogels "smart" materials. Hydrogels are used in wound healing, medication administration, cellular

treatments, tissue regeneration, medical device fabrication, and biosensor development.[20].

Hydrogels can reduce complications, accelerate wound healing, increase quality of life, and improve wound treatment. Hydrogels are ideal for deep wounds due to their non-adhesiveness, moisture retention, gas permeability, exudate absorption, biocompatibility, and patient comfort. Like dermal tissue, they have an extracellular matrix-like structure. They promote cell migration and partial tissue regeneration. Adding antibiotics, nanoparticles, stem cells, and growth hormones to hydrogels improves their properties. Hydrogels that respond to external stimuli can release drugs precisely or monitor healing [21].

1.7. Thymol:

Herbal extracts have gained popularity in recent years due to their many medical uses, particularly in wound healing. Essential oils from thymus, Origanum, and coridithymus include thymol, a naturally occurring phenolic monoterpene. Its antioxidant, anti-inflammatory, local anaesthetic, antinociceptive, cicatrising, antiseptic, and especially antibacterial and antifungal properties have garnered attention in biomedical applications.[22].

Several studies have examined how Thymol aids wound healing. During wound healing, thymol works in numerous ways. Thymol regulates reactive species like nitric oxide, pro-inflammatory cytokines like TNF-a and IL-1b, and growth factors like TGF-1b. The early stages of healing benefit from this modulation[23, 24]. Thymol promotes proliferative re-epithelialization, angiogenesis, granulation tissue formation, and collagen fibre deposition. Thymol's ability to modulate early inflammation is likely responsible for the reported advantages, as the duration of inflammatory infiltration during wound healing substantially affects the development of granulation tissue into scar tissue. A 14-day study found that Thymol increases collagen formation and replaces collagen type III with type I in wounds. Furthermore, Thymol's high antibacterial properties help cure wounds by preventing infections, especially in chronic wounds where bacterial infections are problematic.[25].

1.8. Objectives of this research:

This study will develop and test a thymol-containing hydrogel treating diabetic wounds. Pursuing these goals will achieve this:

1. The goal is to generate stable liposomes with thymol, allowing for easy absorption by the body.
2. Conduct thymol-loaded particle characterisation using several techniques, including SEM, AFM, FTIR, UV, and Zeta Potential Analysis. This analysis measures particle size, shape, surface characteristics, and chemical content.
3. Measure thymol encapsulation efficiency in liposomes for optimal medication loading and minimal leakage.
4. The goal is to study the kinetics of thymol release from liposomes in a controlled environment to determine its potential for therapeutic usage.
5. Develop a biocompatible hydrogel matrix with thymol-loaded liposomes to create a localised and sustained wound treatment system.
6. Our goal is to examine the physical and chemical properties of thymol-containing hydrogels, including structure, porosity, viscosity, and contact angle.
7. This study aims to evaluate the efficacy of thymol-loaded hydrogel against common bacteria like Staphylococcus aureus and Enterococcus faecalis, which cause diabetic wound infections.

CHAPTER 2: LITERATURE REVIEW

1.1 role of Skin:

The skin is the largest organ in the body which make up 10% of the mass of body and is essential for survival and defence. It consists of three layer; hypodermis (fat), dermis and the epidermis. Epidermis which is the most outer layer of the skin play important role in maintaining the body internal balance and provide protection from harmful microorganisms and the environment. Dermis gives tensile strength to the skin through extracellular matrix support. All the blood vessels, nerves, hair follicles and sweat glands are present in dermis. The natural state of skin is characterized by dryness and acidity with a PH level ranging from 4 to 6.8[26].

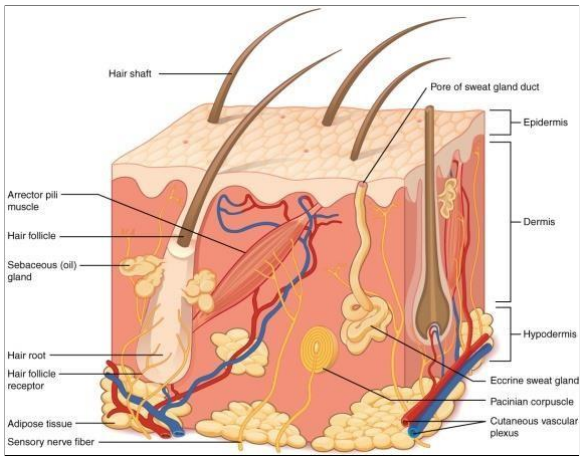


Figure 1 Error! Reference source not found.

When an individual has a severe injury to large areas of skin, he is “open” to reduced local function, which can result in dehydration and infections and sometimes even death. When the skin is wounded, multiple cell types within these three layers need to coordinate at precise stages to bring about healing[27]. A wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue. A wound may result from precise disruption of tissue by the surgeon’s knife (incision) to widespread damage of tissue (e.g. major trauma, burns). A wound may also

result from a contusion, hematoma, laceration or an abrasion. The continuity of the skin must be restored expeditiously because it plays a crucial role in maintaining homeostasis.

2.2 Wound Healing (WH):

The Wound healing is a natural physiological response to damage in bodily tissues. Even so, the process of wound healing is not simple, because it involves an intricate interaction among many types of cells, cytokines, mediators, and the vascular system. This process encompasses haemostasis, inflammation, proliferation, and culminates in the formation of completely formed scar tissue.[28]

2.3 The phases of Wound Healing:

2.3.1 Hemostasis:

After an injury, blood arteries immediately constrict to produce a clot and restrict blood flow. Platelets are one of the main factors that activate this process and can be activated when they come into contact with the sub-endothelial matrix in the wall of a blood vessel. ECM proteins including fibronectin, collagen, and von Willebrand factor link platelets to blood vessel walls. Thrombin induces platelets to release bioactive substances from granules, improving coagulation[29]. Platelets increase fibroblast and keratinocyte development and help fight germs early. Many factors will influence coagulation after blood flow stops to prevent thrombosis. Smooth muscle and endothelial cells from endothelial progenitor pools rebuild the injured arterial wall during this time[30]

2.3.2 Inflammation:

Inflammation is the main line of defence against wound infections. Mast cells and macrophages at the injury site are activated by these signals. Pro-inflammatory cytokines and chemokines released during activation draw circulating leukocytes to the site[31] Neutrophils, the first wound responders, phagocytose dead tissue and dangerous germs. Neutrophils produce ROS and antibacterial peptides. These chemicals and pathogens get caught in extracellular traps. Neutrophils are removed from the wound site by efferocytosis macrophage[32].

Macrophages, recruited like neutrophils but later due to the time they need to reach contaminated tissue, lead tissue repair. The phagocytotic cells scavenge detritus and malfunction pathogens while phenotypically shifting in response to environmental inputs. Healing antigen shifts M1 macrophages from pro-inflammatory to anti-inflammatory[33]. Growth factors are produced to induce re-epithelialization, angiogenesis, and fibroplasia after cytokine change and other events cause this flip. Macrophages stabilise and remodel blood vessels. Lack of macrophages in wound healing slows healing and increases inflammation, highlighting their importance[34] Early wound healing inflammation is reduced by T and mast cells.[35]

2.3.2 proliferation:

In the proliferative phase activated keratinocytes, fibroblasts, macrophages and endothelial cells coordinate wound closure, matrix deposition and angiogenesis..[36]. Keratinocytes switch their adhesion characteristics, start to move by secreting matrix metalloproteinase (MMPs) and concomitantly deposit new extracellular-matrical proteins. Hair follicle stem cells facilitate this process by proliferating and providing new epidermal cell a that migrate from excised appendages in superficial wounds or the epidermis edge in deeper wounds. [37, 38].

Angiogenesis, a key process at this phase is promoted by hypoxia mediated factors like vascular endothelial growth factor VEGF, (HIFs), and recruitment of epithelial cells in the wound bed to form matured stable vasculature. In angiogenesis, macrophages produce proteases and chemotactic factors that control endothelial cell behaviour and modify the nascent vasculature to avoid over vascularization [39]. Another crucial yet understudied topic is nerve fibre regeneration. Neural and immunomodulating neuropeptides affect cell proliferation and wound healing. In diabetes, substance P deregulation delays wound healing, but restoring this neuropeptide to the surface increases neurone development and wound repair. Decreased healing occurs without wound-activated glial cells, which synthesise chemotactic factors.[40]

2.3.4 Matrix Remodelling:

This process of matrix remodeling, which starts with clotting fibrin deposited in the wound and concludes over years to generate a mature scar (composed primarily type I collagen) is an unavoidable consequence of healing wounds.[41] This process involves fibroblasts replacing a blood-derived fibrin clot with hyaluronan, fibronectin, and proteoglycans to form mature collagen fibrils. These proteoglycans are essential for the assembly of mature, covalently cross-linked collagen fibrils and also regulate cell migration [42].

Nevertheless, the ECM of scar tissue never fully recovers all its original structural integrity or conformation compared to that in uninjured skin. Collagen present in scar tissue forms large parallel bundles, while in normal skin it has a basket weave formation and this is counter to the tensile strength of some scars which may reach 80% as strong as pre-wound skin[43].

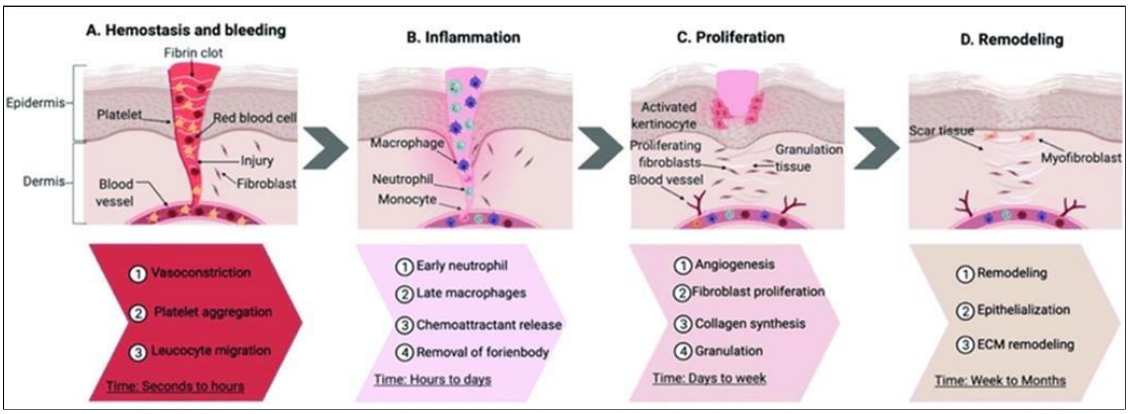


Figure 2: The stages of wound healing

2.4 Introduction to diabetic wound:

The diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin action, insulin secretion or both. Insulin insufficiency causes chronic hyperglycemia and carbohydrate, lipid, and protein metabolic problems[8]. It is one of the biggest worldwide health issues, chronic non-communicable diseases (CNCDs). It increases the risk of blindness, vascular brain disorders, renal failure, and amputations.

The IDF reported in 2021 that 537 million people, or 10.5% of the world's population, had diabetes. This ailment cost $966 billion in healthcare. Diabetes cases are expected to reach 783 million by 2045, with healthcare expenses exceeding $1054 billion. In poor and middle-income nations, nearly half of people with diabetes are unaware of their illness, with the highest prevalence of undiagnosed diabetes Mellitus (DM)[44].

Diabetes causes various pathological alterations that impair wound healing. Chronic hyperglycaemia affects vascular and impairs blood flow. Diabetics' peripheral vascular disorders and neuropathy make wound identification difficult. Diabetic wounds include increased inflammation, reduced angiogenesis, poor keratinocyte migration, and low fibroblast proliferation. These modifications promote diabetic wound consequences such infections, wound dehiscence, and chronic non-healing wounds.[45]

2.5. Impacts of Diabetes on Wound Healing:

Normal wound healing involves inflammation, proliferation, and remodelling. Diabetes affects all these healing processes[46, 47]. Normal wound healing involves inflammation, proliferation, and remodelling. Diabetes affects all these healing processes. Diabetic wounds heal more slowly than non-diabetic due to inflammation. Prolonged pro-inflammatory state delays wound healing and can cause chronic wounds.

2.5.1. Inflammation in Diabetic Wounds:

In diabetic wounds, macrophages produce excessive pro-inflammatory cytokines. furthermore, In diabetic wounds, inflammatory macrophages do not easily become anti-inflammatory[48]. Diabetic wounds produce more neutrophil extracellular traps (NETs), which promotes inflammation and slows wound healing. MiRNA is a significant molecular alteration in diabetic wounds that produces excessive inflammation[49].Micro-ribonucleic acid (miRNA) is one of the major molecular change in diabetic wounds that causes excessive inflammation (miRNA)[50]. Although healing miRNA was discovered at the same levels in diabetes and non-diabetic skin, its expression varied in damaged skin, suggesting that miRNA also plays a role in dysregulated inflammation. In diabetic wounds, transcription factor and epigenetic dysregulation increases pathological inflammation[51].

2.5.2. Angiogenesis in Diabetic Wounds:

One of the main causes of poor wound healing in diabetic wounds is inadequate angiogenesis, which can happen through a number of different processes[52]. Initially, diabetic wounds lack the essential proangiogenic elements, presumably because fewer macrophages are producing them. Furthermore, there is an up-regulation of antiangiogenic factors and a down-regulation of capillary maturation factors[53]. Matrix metalloproteinase and miRNAs, known to inhibit angiogenic genes, also have a role. Additionally, Deficient oxygen delivery and alterations in the diabetes vasculature further hinder leukocyte migration into the wound, raising the risk of infections[54].

2.5.3. Scarring in Diabetic Wounds:

Diabetes has been shown to cause a scar that heals differently from healthy scars in terms of collagen synthesis and structure. These differences lead to a scar that is less able to contract and has more collagen, which reduces the scar's tensile strength and hinders proper wound healing [62]. Part of the reason for the poor contraction of diabetic wounds is that the fibroblasts in these wounds are senescent and refractory to proliferate, which means that in order to heal, diabetic wounds rely more on granulation and re-epithelialization. This means that diabetic wounds withstand tensile forces and shear stress [63].

2.6. The pathophysiology of diabetes:

Diabetic wound healing requires a balanced interaction between inflammatory cells and biochemical mediators. Numerous elements contribute to this harmonic metastasis. Cellular macrophages produced from monocytes are the main players in this process since they are the main producers of important pro-inflammatory cytokines. These cytokines—including VEGF, TGF-β, TNF-α, IL-1β, and IL-6—are essential for both the typical healing processes associated with wound repair and the unique healing mechanisms seen in diabetic conditions. The schematic representation in the figure below provides an explanation of the detailed pathophysiology of Diabetic wound.

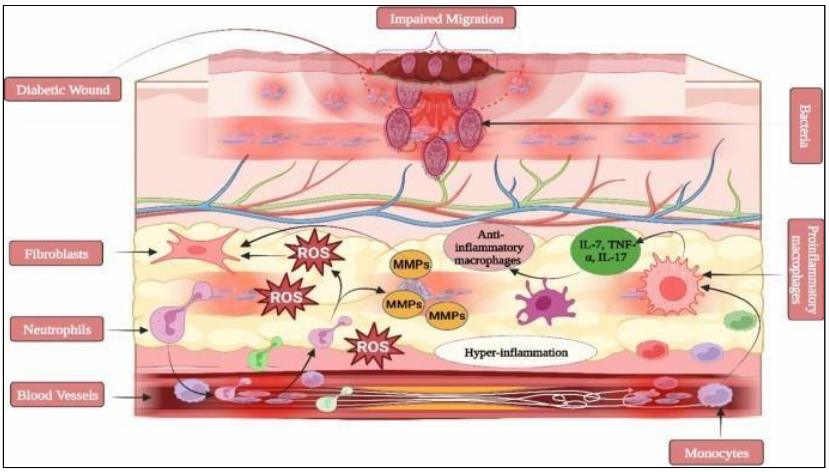


Figure 3: Understanding the pathogenesis and molecular targets for diabetic wounds[55]

A complex interaction of multiple factors can lead to problems such as retinopathy, myopathy, diabetic neuropathy (DN), peripheral vascular disease (PVD), myopathy, and nephropathy in diabetic wounds (DWs). This complex process includes deficits in the angiogenesis response, compromised macrophages and neutrophils activity, production of pro-inflammatory cytokines, and microvascular issues like atherosclerosis. Furthermore, diabetic wound-healing models show reduced fibroblast and keratinocyte migration, proliferation, and synthesis of growth factors. Moreover, there is an imbalance between ECM and MMPs, a reduction in epidermal nerve levels, a pDGF alteration, and a lack in the fibrinolysis inhibitor [56].

2.7. Complications in diabetic wound:

Diabetic wounds (DWs) significantly impact patients' daily life, morbidity, and mortality. They can be classified as delayed acute or chronic wounds, causing decreased tissue regeneration and recurrent inflammation. Diabetes also leads to recurrent inflammation, affecting the formation of fully developed granulation tissue and wound strength. Prompt and urgent treatment is crucial for all wounds, which can be external or internal. Peripheral neuropathy in diabetes individuals can lead to unrecognized external wounds, while interior injuries like ulcers and calluses carry a high risk of bacterial infection, causing tissue and skin damage. Several variables contributing to the development of DWs are outlined in Figure.[57-59].

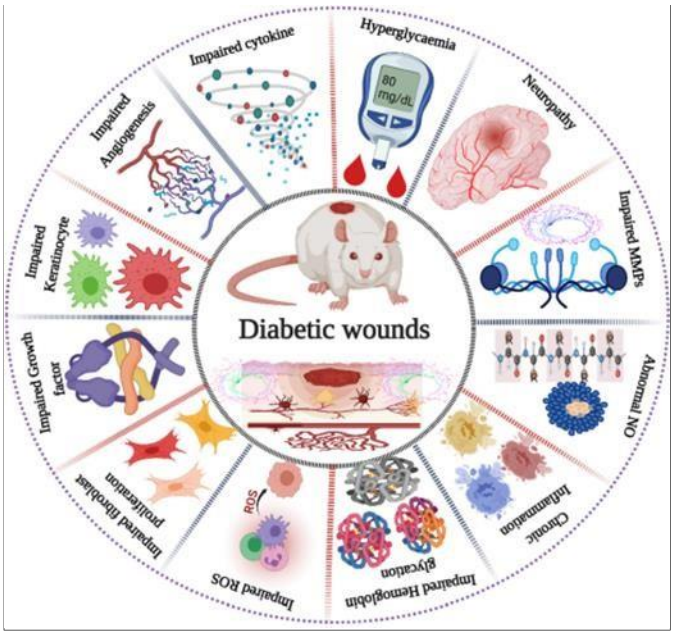


Figure 4: Causal factors of diabetic wound [60]

2.8. diabetic wound care treatment strategies:

Diabetic wound represent the most challenging and common consequence of diabetes mellitus, impacting around 25 percent of individuals with diabetes at some point in their lives. These wounds frequently exhibit inadequate healing rates, resulting in extended hospitalizations, elevated healthcare expenditures, and reduced patient quality of life. Managing diabetic wounds is challenging and frequently necessitates a multifaceted strategy. [58, 59].

2.8.1. blood glucose levels control:

Controlling blood sugar levels is crucial for managing diabetic wounds (DWs), as high glucose levels can hinder wound healing by reducing collagen production, disrupting blood vessel formation, and promoting oxidative stress. Medication, food, and exercise are essential for managing blood sugar levels in DW healthcare. Optimal wound healing requires a well-rounded diet rich in protein, vitamins, and minerals. Exercise also enhances blood flow and facilitates healing in individuals with diabetes.[61].

2.8.2. Metformin therapy:

Metformin, a type 2 diabetes drug, has been found to aid in wound healing and angiogenesis. Its anti-inflammatory effects may increase blood flow to damaged tissues, aiding in healing and preventing wound infections. In-vitro tests showed that metformin administration promoted BM-EPC function, increased tube formation, NO generation, and decreased O2 levels. It also reduced TSP-1 expression in cultured BM-EPCs. Metformin treatment may aid T2DM wound healing and angiogenesis by enhancing NO generation and reducing O2−, TSP-1 expression, and inflammation in EPCs. In a study, combined therapy formed hair follicles, enhanced dermis and epidermis regeneration, and reduced inflammatory cell infiltration and oedema compared to single drug-loaded scaffolds. Metformin and mesenchymal cells (MSCs) can also increase angiogenesis and wound healing in diabetics.[62]

2.8.3. Insulin therapy:

Insulin therapy has been shown to accelerate wound healing by regulating oxidative and inflammatory responses. It reduces reactive oxygen species, which can damage lipids, proteins, and DNA in burn wounds. Insulin treatment increases M2 macrophages and interleukin-10 levels, resulting in anti-inflammatory effects and aiding in the clearance of necrotic tissue. Dressings containing insulin enhance re-epithelialization, angiogenesis, and extracellular matrix formation, resulting in faster wound healing. HIF-1α, a crucial wound healing controller, remains constant in wound cells treated with insulin dressings. Insulin can mitigate the destabilization of HIF-1α induced by methylglyoxal (MGO), a type of advanced glycation end products (AGEs) in diabetes. Insulin enhances the activation of HIF-1α target genes, stimulating the formation of new blood vessels and the deposition of extracellular matrix, both crucial for wound healing. [63].

2.8.4. Wound debridement:

Wound debridement is a crucial aspect of diabetic wound management, involving the removal of dead or contaminated tissue to promote healing.[64]. Various techniques, including surgical, mechanical, and enzymatic debridement, are effective in this process. Surgical debridement is the most intrusive and is typically performed on wounds with a significant amount of dead tissue. [65]. Mechanical debridement involves using a dressing or irrigation to remove necrotic tissue, while enzymatic debridement involves applying topical enzymes to break down dead tissue.[66]. While surgical debridement has been linked to reduced healing time, there is insufficient evidence to support its effectiveness. Hydro-active dressings infused with polyhexamethylene biguanide have shown promising results in stimulating macrophage activation, suppressing bacterial growth, and reducing inflammation in human trials. In summary, the combination of sharp debridement, meshed skin grafts, and negative pressure wound therapy is effective in facilitating wound healing in diabetic wounds[67].

2.8.5. Off-loading pressure therapy:

Diabetes footwear has emerged as a viable alternative to traditional casts and non-removable walkers for lowering plantar pressures in individuals with diabetes. [68, 69]. This category of footwear, including shoes and insoles designed to relieve foot stress, effectively decreases plantar pressures. Specific characteristics like metatarsal additions, apertures, and arch shapes are crucial for optimal outcomes. [70]. Surgical off-loading, such as Achilles tendon release and foot reconstruction, is also a viable option for diabetic foot ulcers. Research shows that surgical off-loading can lead to improved healing and amputation rates compared to non-surgical treatments. Specialized footwear, custom orthotics, or assistive devices like wheelchairs or crutches can also be used to alleviate pressure on the wound area and facilitate healing.[71].

2.8.6. surgery:

Diabetic wound-healing surgery is a treatment for delayed wound healing in diabetic patients due to damage to blood vessels and nerves, impaired blood flow, and reduced sensitivity in the feet and legs. This can increase susceptibility to infection and delay the healing process. Surgical procedures are often reserved as the last resort when non-surgical treatments have proven ineffective. Proper wound care, including maintaining cleanliness and dryness, and regular dressing replacements, is crucial for successful wound healing. Maintaining optimal blood sugar levels also helps minimize complications.[72].

2.9. An alternative therapy for Diabetic wound's complication:

Diabetes-related wounds often have delayed and compromised healing processes, a concern that medical plants are a valuable resource for therapeutic treatments. These plants are used in both traditional and alternative medicine, with their medicinal effects including wound healing, antioxidants, fighting against microbes, reducing inflammation, and lowering blood sugar levels.[73]

Traditional Chinese medicine, an integral part of traditional Chinese medicine, is extensively used in clinical settings for wound healing.[74]. Medicinal herbs expedite the healing process through their active ingredients, which have diverse effects such as stimulating fibroblast growth, initiating growth factor production, scavenging free radicals, stopping bleeding, preventing microbe growth, enhancing collagen synthesis and strength, improving blood circulation, reducing cell damage, promoting DNA synthesis, facilitating wound contraction and healing, and stimulating keratinocyte production and movement. However, natural treatments are primarily used topically for wound dressing, with a limited number being ingested orally to address diabetic wounds [75, 76].

2.9.1. Thymol:

Thymol, also known as 2-isopropyl-5-methylphenol or 5-methyl-2-isopropylphenol, is a naturally occurring monoterpenoid phenol found in the oil of the Thymus vulgaris plant. Thymol is an isomer alongside with carvacrol and is the primary bioactive component in the oil derived from the plant.The plant in concern is a perennial, aromatic, and highly

branched woody plant of small height [77]. T. vulgaris exhibits bilabiate flowers that are white, yellow, or purple in colour and blossom in the spring. When the leaves of this plant are touched, it releases a pleasant and aromatic scent. Thyme belongs to the Origanum genus, which is closely related to oregano. For centuries, it has been employed as both a seasoning in cooking and a herb with healing properties. Nevertheless, scientists have also investigated its antioxidant and antibacterial properties.

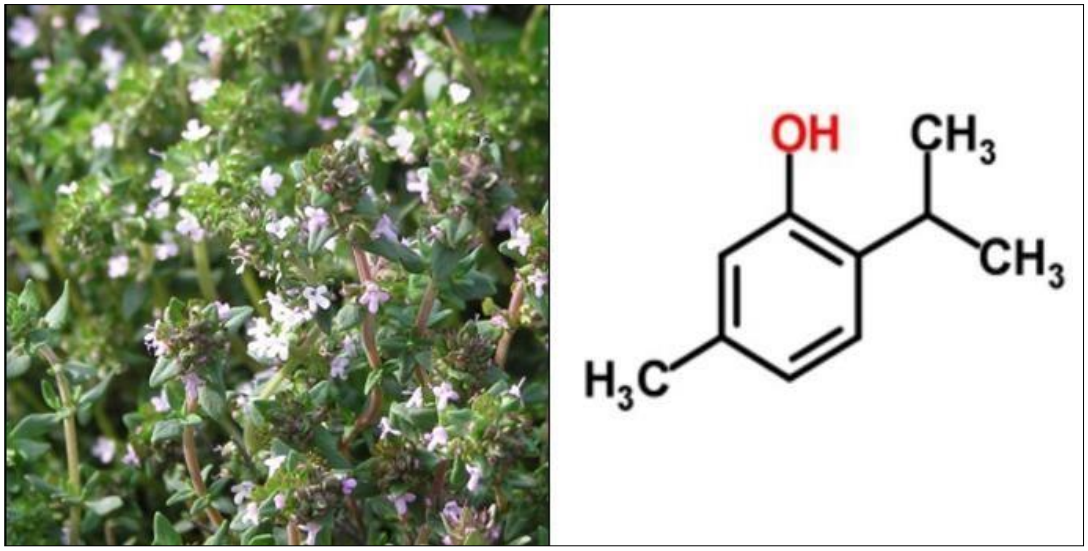


Figure 5: Thymus vulgaris plant (A) and the chemical structure of thymol, also known as 2-Isopropyl-5-methylphenol (B)

2.10. Biomedical Properties of thymol:

2.10.1. Antioxidant Properties

Thymol, a compound with significant antioxidant properties, has been extensively studied using cell lines and animal models. It effectively eliminates hydroxyl free radicals, leading to the formation of phenoxyl radicals.

These radicals form adducts that undergo dehydration, which can be accelerated in an alkaline environment. Thymol's non-toxic nature and ability to undergo redox reactions make it a promising antioxidant. Thymol also increases the concentrations of natural antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione-S-transferase (GST).[78].

It also increases the concentrations of non-enzymatic antioxidants like vitamin C, vitamin E, and reduced glutathione (GSH). Thymol demonstrates higher efficacy in terms of reducing power, DPPH, superoxide, and hydroxyl radical scavenging activities and offers defence against oxidative harm to lipids.[79].

2.10.2. Anti-Bacterial properties:

Antibiotic resistance is a growing issue in treating bacterial diseases, prompting the search for natural chemicals to counteract infections. Essential oils (EOs) with phenolic monoterpene compounds, particularly thymol, have the most potent antibacterial activities due to their chemical structure. Thiols, small and lipophobic particles, can pass through lipid barriers, making pathogens' cell membrane susceptible to EO chemicals. [80]. Thymol's antibacterial effect is attributed to its interaction with the polar region of the membrane, causing significant alterations in the membrane's structure, including destabilization of the lipid layer, loss of elasticity, and increased fluidity. This leads to increased permeability to potassium and hydrogen ions and impacts the functionality of intracellular membrane proteins, including enzymes and receptors. Thymol interacts with embedded proteins, causing alterations in the structure and function of internal and membrane proteins, causing cell membrane tension and destabilization.[81].

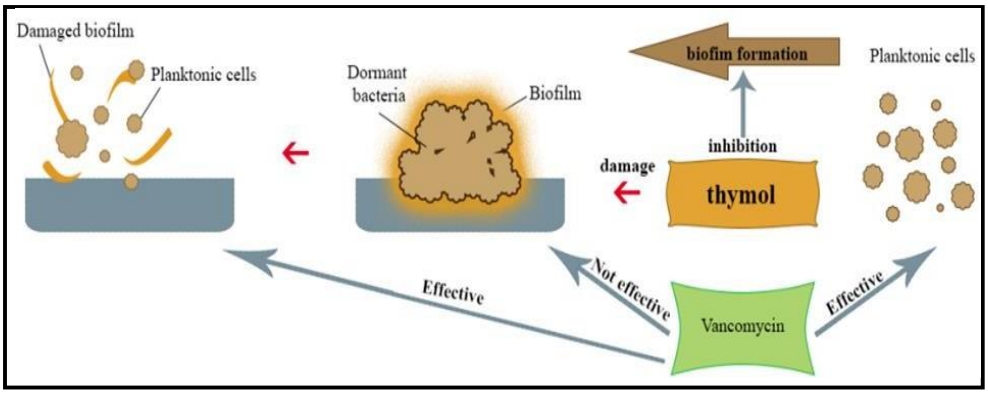


Figure 6: Potential mechanism underlying the antibiofilm activity of thymol [60]

2.10.3. Anti-fungal Properties:

Ergosterol, a unique sterol found in fungus cell membranes, is crucial for optimal growth and functioning. Thymol, a substance that influences its concentration, may have antifungal properties by affecting the metabolism of fatty acids, particularly ergosterol, within the fungal cell. This leads to increased reactive oxygen species and oxidative stress, reducing the extracellular polymer matrix and capsular polysaccharide.[82]

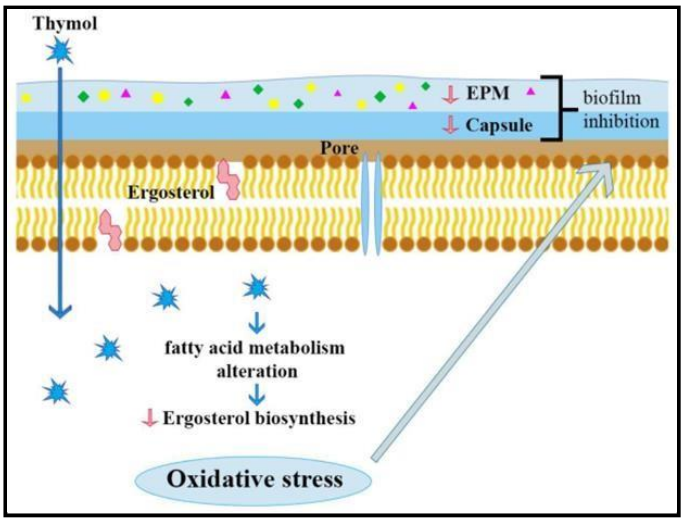


Figure 7: Mechanism underlying the possible antifungal activity of thymol[82]

Thymol treatment can inhibit ergosterol levels in cell membranes of Candida and Cryptococcus, leading to membrane damage and cell death. A study found that T. vulgaris essential oil has potent antifungal properties against various strains, with doses ranging from 0.5 to 10mg/mL. This makes it a potential substitute for amphotericin B, which is known for its significant toxicity. The oil's potency makes it a promising alternative to existing antifungal drugs[83, 84].

2.11. Role of Thymol in Diabetic Wound Treatment:

Wound treatment solutions are gaining significant attention in research and marketing. Wound dressings, which cover the wound area, protect the site, regulate moisture, and create a healing environment. Bioactive substances like growth factors, anti-inflammatory agents, antioxidants, and antibacterial agents can expedite wound healing. Thymol, a

compound with significant potential in wound healing, has been studied for its ability to promote healing. It regulates the synthesis of reactive species, pro-inflammatory cytokines, and growth factors, which play a role in the early stages of the healing process. Thymol also promotes the growth of new epithelial cells, blood vessels, connective tissue, and collagen fibers. The compound's benefits may be linked to its ability to regulate inflammation, as the duration of inflammatory infiltration influences the transformation of granulation tissue into scar tissue. Thymol therapy has been shown to result in collagenization and the potential to replace collagen type III with type I within a 14-day period. Additionally, Thymol's potent antibacterial properties can help prevent bacterial infections, especially in chronic wounds, and aid in wound healing. [85, 86]

2.12. Nanotechnology in wound treatment:

Nanotechnology is a rapidly growing field that focuses on synthesizing and developing materials with nanometre dimensions. It has been widely used for treating various diseases, including wound healing, which is a complex physiological process involving four phases: haemostasis, inflammation, proliferation, and remodelling..[87, 88].

Chronic wounds can be caused by factors like diabetes, obesity, infection, and stress, leading to prolonged healing times. Traditional drug delivery devices are ineffective in delivering therapeutic medicines to the wound site. Nanotechnology has proven to be an effective approach to addressing these issues. Nanoparticles (NPs) are among the various nano-materials that have attracted researchers due to their ability to promote wound healing. [89]. NPs can be classified into three categories: inorganic NPs, lipid-based NPs, and polymeric NPs. Inorganic NPs can be used as therapeutic compounds, while polymeric and lipid-based nanoparticles transport medications to wound sites in specific release patterns. They can also serve as carriers for delivering bioactive substances as shown below[90].

.

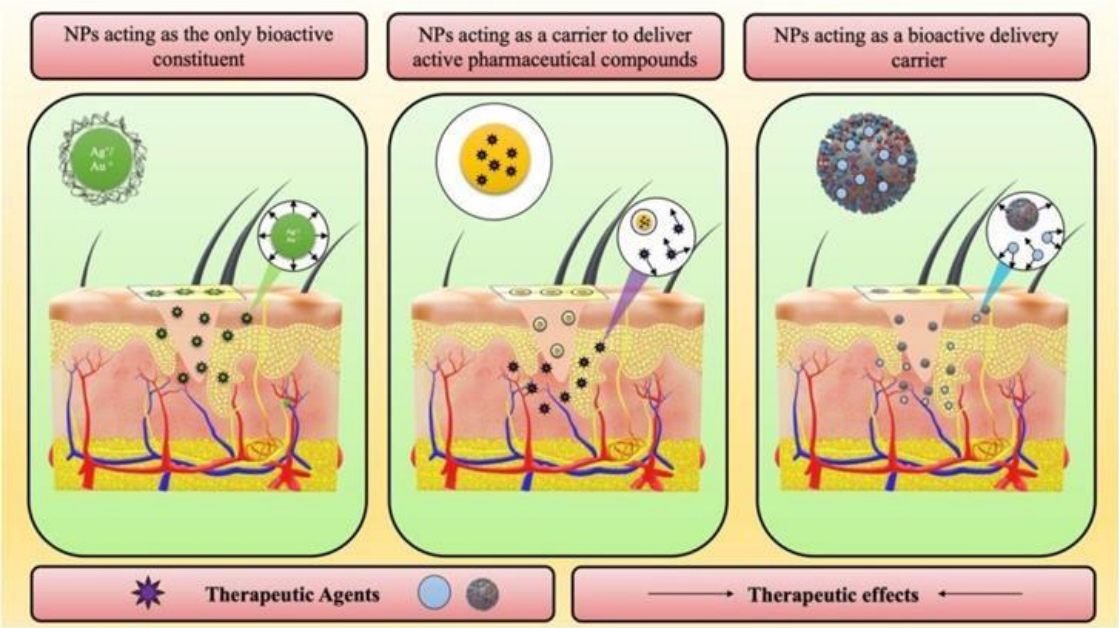


Figure 8: An illustrative depiction of the application of nanoparticles (NPs) in the process of wound healing[91]

2.12.1. Lipid-based Nanoparticles as Drug Carrier:

Lipid-based nanoparticles (LBNs) are drug-delivery devices with a lipid matrix enclosed by a surfactant film, designed to deliver drugs to specific body sites. These adaptable systems, with a size range of 1 - 100 nm, are biodegradable and biocompatible, allowing precise control of drug release, protection, and targeted delivery. They enhance the transportation of hydrophilic and hydrophobic molecules in oil-in-water nanoemulsions, minimizing potential adverse effects.

LBNs have been used in cosmetic and medical fields to improve the durability, effectiveness, and preservation of targeted drug delivery. They facilitate wound healing by promoting wound wetness and stimulating the release of growth factors, enhancing drug localization at the injury site.

LBNs are easily expanded, sterilized, and verified when combined with medication molecules, providing extended drug release, decreased dosing frequency, and enhanced patient adherence. Their exceptional therapeutic efficacy makes them suitable for a wide range of applications.[92].

2.12.2. Types of lipid –based Nanoparticles:

Lipid-based nanoparticles (LBNs) have the ability to improve wound healing and skin regeneration by controlling the release of medication, increasing solubility, and minimising degradation. Lipid-based nanoparticles (LBNs) encompass a range of different kinds, including liposomes, solid-lipid nanoparticles, nanostructured lipid carriers, lipid-polymer nanoparticles, nanoemulsions, and polymeric phospholipid micelles. Their advantages in the administration of medication and effectiveness in wound healing are illustrated in Figure 1and .[93]

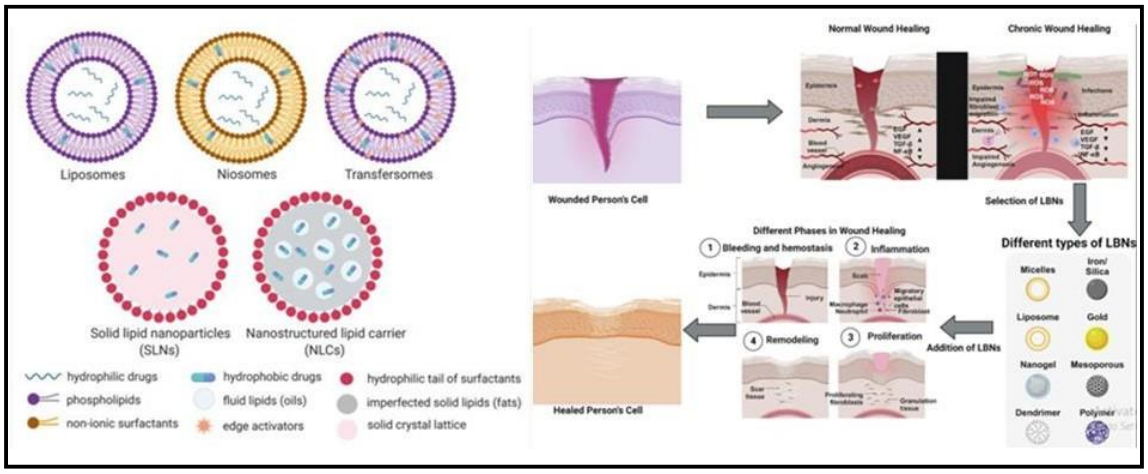


Figure 9:Various categories of lipid-based nanoparticles and depicting the use of LBNs for the treatment of both normal and chronic wounds [94]

2.12.3. Liposomes as a drug carrier:

Liposomes are drug vesicles that are formed through the self-assembly of phospholipids, resulting in the formation of a bilayer or many concentric bilayers. Extensive study has been conducted on their use as carriers for small molecule drugs, proteins, nucleic acids, and imaging agents. [95]. The thickness of the phospholipid bilayer in liposomes is between 4 and 5 nm, while the size of liposomes can range from 30 nm to micrometer. Liposomes can be administered through many ways, such as parenteral (injection), pulmonary (inhalation), oral (by mouth), transdermal (through the skin), ocular (eye), and nasal (through the nose). They possess notable attributes, such as safeguarding enclosed

substances from deterioration, prolonging the duration of medicine effectiveness, regulating the release of drugs, and exhibiting a high degree of compatibility with living organisms and safety[96]. They have the ability to specifically convey their cargo to the affected area, so minimising general adverse effects, enhancing the highest dose that can be taken, and improving therapeutic benefits.

The presence of wound infection can lead to the destabilisation of liposome membranes, resulting in the release of the enclosed medication due to the growth and metabolic components associated with pathogenic bacteria. This study provides evidence for the controlled and selective release of LBNs inside a responsive system that is activated by bacterial enzymes. This release mechanism does not require any additional components or chemical modifications of the nanocarriers, making it a promising approach for treating wound infections.[97]

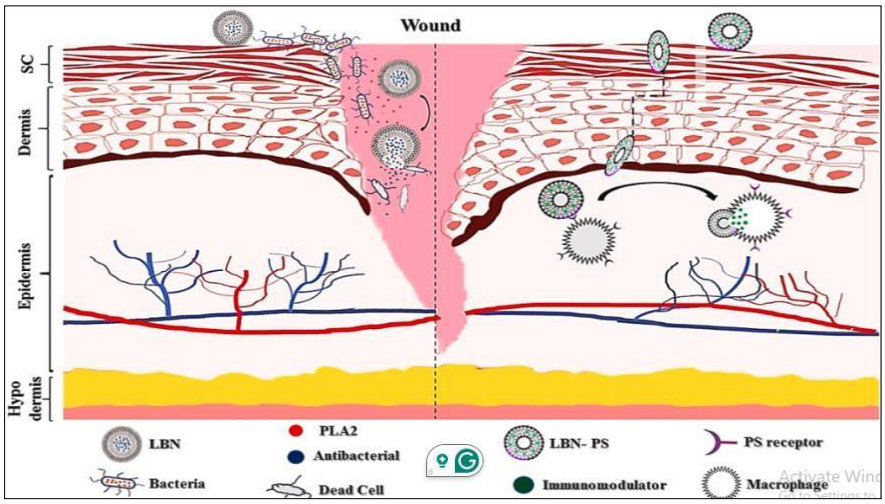


Figure 10: Visual representation of the controlled release of drug in wound conditions with the use of Lipid-Based Nanoparticles (LBNs), either for treating infections or targeting specific areas [98]

2.13. Hydrogel as a Drug delivery system:

Hydrogels are composed of a 3D structure that has the ability to absorb a significant quantity of water and expand when in contact with water, because of their hydrophilic groups, including -NH2, -COOH, -OH, -CONH2, -CONH, and -SO3H.

The network is typically composed of polymer chains that are cross-linked, occasionally through cross-linked colloidal clusters. Their flexibility and softness are a direct consequence of their capacity to absorb water. Hydrogels can be designed by chemically or physically crosslinking chains of natural or synthetic polymers. Hydrogels strongly resemble living tissue due to their high water content, soft texture, and porosity.

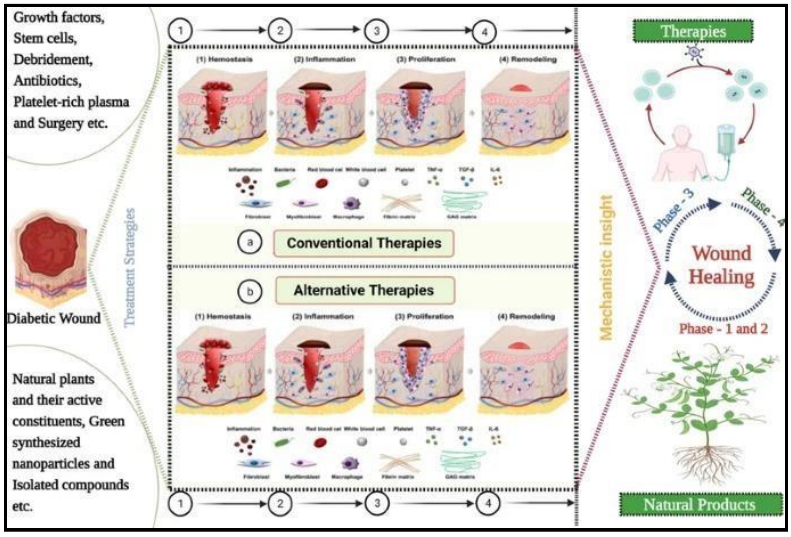


Figure 11 Hydrogel for diabetic wound healing [99]

Due to advancements in hydrogel production technology, we may anticipate the expanded use of hydrogels across several industries. Nevertheless, scientists continue to show interest in the biological applications of hydrogels, as indicated by the presence of 25,000 references to hydrogels for biomedical purposes during the last five years.

CHAPTER 03: MATERIAL AND METHODS:

3.1 Materials:

3.1.1 Chemicals and reagents:

The research utilised analytical grade reagents and chemicals obtained from Sigma-Aldrich, without any further purification and all the components were used as received without any extra purification steps.

The primary chemicals used in this research include thymol, lecithin, chloroform, ethanol, chitosan, polyvinyl alcohol (PVA), Gelatin, and acetic acid (1%). The selection of these chemicals was based on their role in the synthesis of both thymol-loaded liposomes and the hydrogel.

3.2 Methodology:

3.2.1 Liposomes synthesis:

The thin-film hydration method was used to synthesize liposomes. This method is frequently employed to encapsulate both hydrophobic and hydrophilic substances within lipid bilayers[100]. The liposome formulation was prepared by combining lecithin and cholesterol in a molar ratio of 4:1. Initially, the required amount of lecithin and cholesterol were accurately measured. The lipids were then dissolved in chloroform to form a concentration of 100 μM stock solution.

At the same time, a 200 μM solution containing the drug thymol in ethanol was prepared. A precise volume of 500 μL was measured from the drug solution and then added to the lipid solution. The lipids and drug were mixed together and then treated with sonication. The sonication was performed at a frequency of 80 MHz for 40 minutes to ensure the proper mixing and the potential encapsulation of the drug within the lipid bilayers. After subjecting the mixture to sonication, it was separated into two separate phases: a water phase and a lipid phase. Lipid phase, which included the sonicated mixture, and the water phase were heated individually in a water bath. The temperature was carefully controlled

and maintained at 60 °C, that is close to the phase transition temperature of the lecithin used in this formulation.

When the two phases reached the desired temperature, they were mixed. This mixture of both phases was then stirred continuously for 10 minutes at a speed of 90 RPM to promote the formation of a homogenous dispersion. The dispersion was again sonicated, this time at a frequency of 50 MHz for 40 minutes, to enhance the liposome structure and facilitate the encapsulation of thymol.

Following the process of sonication, the next step was the extraction of chloroform from the mixture. The process used for this purpose was rotary evaporation, which required keeping the temperature above the phase transition temperature of lecithin, specifically at 50°C, to ensure the complete removal of the solvent.

Finally, the liposome formulation was then purified to remove any remaining drug that was not encapsulated. This was achieved via mini column filtration using a 0.2 μM filter. This step ensured that the final liposome suspension contained only the drug-loaded liposomes, free from any unencapsulated thymol.

3.2.2 Synthesis of hydrogel matrix:

The thymol-loaded hydrogel was prepared by combining polyvinyl alcohol (PVA), chitosan (CS), and gelatin (Gel) in a carefully optimized ratio[101]. The preparation began by dissolving each polymer in distilled water (D.W) or a suitable solvent. The PVA (0.05 g/mL) was dissolved in distilled water, whereas CS (0.1 g/mL) was dissolved in a 1% acetic acid solution under continuous magnetic stirring at room temperature for 24 hours to ensure complete mixing. The Distilled water was used to dissolve gelatin (0.1 g/mL) with a stirring time of 2 hours.

Once the individual polymer solutions were prepared, they were mixed together in a final volume of 5 mL according to the optimized proportion of 1:2:2 for PVA: CS: Gelatin. This mixture was then subjected to additional stirring for 1 hour at room temperature to ensure a uniform blend of the polymers.

After thoroughly mixing of the polymeric solutions, the thymol-loaded liposomes, which were synthesized according to the instructions in section 1.2.1, were added to the hydrogel matrix. The liposomal suspension was slowly added into the polymer mixture while constantly stirring to ensure the equal distribution of the liposomes throughout the hydrogel. Ensuring the uniform distribution of thymol throughout the gel is essential to maintain the integrity of the liposomes.

When liposomes were completely incorporated into the polymer mixture, the resulting solution was stirred for an extra 2 hours to ensure its consistency. Subsequently, the mixture was transported to a refrigerator and kept at a low temperature for 24 hours to facilitate the gelation process, during which the hydrogel developed a durable, cross-linked network

The thymol-loaded liposome hydrogel was formed by physical interactions and spontaneous crosslinking mechanisms inside the polymeric network. After being cooled and placed in the refrigerator, the mixture transformed from a viscous solution into a semi-solid gel. This gelation process ensures a uniform distribution of thymol-loaded liposomes within the hydrogel, which is crucial for its efficacy in treating diabetic wounds by providing controlled antibacterial effects and facilitating wound healing.

3.3 Characterization:

3.3.1 Physical and Chemical Characterization

The thymol-loaded hydrogel was evaluated for its physical and chemical properties by various characterization techniques:

3.3.1.1 Scanning electron microscopy:

To observe the surface structure of the thymol loaded liposomes, empty liposomes and the hydrogel, Scanning Electron Microscopy (SEM) was used. This method yielded high-resolution images that showed the structure, size, and arrangement of the thymol-loaded liposomes, empty liposomes and these thymol loaded particle within the hydrogel matrix with more detail. The particle size values range from 0 to infinity. Particles in this area that have circularity values beyond the specified range will also be excluded. Considered the 8-

bit binary representation of the most suitable ellipse that corresponds to the observed particle (Cf. Edit. Range). The ellipse fitting algorithm was used to the image with grey levels. The ellipses found in the image have a value of 0, while the backdrop has a value of 255.

A micro-pipette is utilized for pouring a minute quantity of the material onto a cover slip, and thymol loaded liposomes, empty liposomes and particle loaded in hydrogel matrix were photographed. The JSM-6490A scanning electron microscope at the National University of Science and Technology (NUST), Islamabad was used to take the images. Through the analysis of these images, we can analyze the incorporation of the liposomes and evaluate the overall structural integrity of the liposomes and hydrogel.

3.3.1.2 Atomic force microscopy (AFM):

To examine the nanoscale topographical characteristics of the thymol loaded liposomes and hydrogel we used Atomic Force Microscopy (AFM). This technique provided extensive information on the surface roughness and texture of both thymol-loaded liposomes and hydrogel. AFM measurements facilitated the understanding of the hydrogel's mechanical properties and surface features, which are crucial for its interaction with the wound environment.

3.3.1.3 Fourier-transform Infrared Spectroscopy (FTIR):

Fourier-transform infrared spectroscopy (FTIR) was employed to determine and confirm the chemical composition of the thymol-loaded liposomes and hydrogel. This method allowed the detection of distinct functional groups linked to thymol-loaded liposomes and the hydrogel components. The FTIR spectra were examined to confirm the successful incorporation of thymol into the hydrogel and ensure that the encapsulation method didn't affect the chemical composition of thymol.

Before doing FTIR analysis, the samples were dried in the air. The FTIR spectra were obtained using a FTIR spectrophotometer covering a wavelength of 4000 to 400 cm−1. An extensive FTIR spectral study was performed on all the components included in the

formulation, which include thymol, empty liposomes, thymol loaded liposomes, chitosan, polyvinyl alcohol and gelatin.

Zeta Potential:

Zeta potential analysis was performed by using dynamic light scattering (DLS) using Nanotrac Wave II (Microtrac® Systems) to assess the stability of the hydrogel dispersion in aqueous solutions. This method provided information on the, stability, average size and surface charge of the liposomes loaded with thymol, which is essential for understanding their stability and interaction in the released medium.

A 1:100 dilutions of each formulation was prepared (using distilled water), and then the particle size was measured at 25°C temperature. The mean diameter ± standard deviation values for each sample were derived from six determinations. A minimum of three reading were noted to find the average particle size and zeta potential. Stability of the thymol loaded and empty liposomes were assessed by measuring their particle size and surface charge using a zeta potential analyzer. A high zeta potential indicates excellent stability and dispersion of the thymol loaded particles.

3.3.1.5 UV-Analysis:

Uv-Vis spectroscopy was used to measures the extent to which a sample absorbs light when exposed to light radiation, and the amount of absorption is determined by the intensity of the transmitted light. Essentially, the incident laser beam is divided into two separate components. A part of the light passes through the cuvette that is filled with the sample to be analysed, while the other part passes through a reference cuvette that only contains the solvent. Measurements of absorbance can be obtained at particular wavelengths or throughout a spectrum, producing an absorbance profile across different wavelengths. The highest level of absorption, known as the lambda max, is observed at a specific wavelength. This approach is crucial for assessing electronic transitions within molecules and follows the principles of the Beer-Lambert Law. According to this law, absorbance (A) is the result of multiplying molar absorptivity (E), the concentration of the solute (c), and the route length (L) of the cuvette:

$$A = E - C - L$$

The molar absorptivity, which measures the ability of substances to absorb light, is directly proportional to the molar concentration in the cuvette containing the sample. The correlation between these variables enables UV-Vis spectroscopy to work as an effective tool for quantitative analysis. The spectrophotometer was utilised to document the UV-Vis absorption spectra of both empty nanoparticles and nanoparticles loaded with thymol. The measurements were conducted over a wavelength range of 200-600 nm with a resolution of 1 nm, utilising distilled water as the reference medium. A comparative analysis was performed by acquiring spectra for the thymol medication, thymol-loaded nanoparticles, and empty nanoparticles.

3.4 Drug encapsulation efficiency

For the optimization of the encapsulation of thymol within liposomes, it was essential to determine the concentration of thymol that allows for maximum encapsulation efficiency. This process began with the preparation of several dilutions of thymol that cover a wide range of concentration. The dilutions were subsequently analysed using a UV-Visible spectrophotometer, scanning wavelengths ranging from 200 to 450 nanometer. The goal of this was to acquire absorbance measurements that could be utilised to construct a standard curve. A standard curve was formed by plotting the absorbance values against the known concentrations of thymol. The curve exhibited a direct correlation between concentration and absorbance, which is essential for accurate measurement of thymol in subsequent steps. The linear relationship of the standard curve ensured that the absorbance values of unknown samples could be directly linked to the concentration of thymol, so providing a reliable method for quantification.

Following the establishment of the standard curve, the next step involved determining the amount of unencapsulated thymol present in the liposomes. In order to accomplish this, liposome samples containing thymol were prepared and then subjected to centrifugation. The centrifugation was performed at a speed of 4500 revolutions per minute for a duration of 1 hour. This procedure enabled the separation of liposomes from the unentrapped, free thymol that was present in the supernatant.

After centrifugation, the supernatant that remained on the top containing the unencapsulated thymol was carefully collected and analyzed using the UV-Vis spectrophotometer. The absorbance values from these supernatant samples were then compared to the standard curve to determine the exact concentration of unentrapped thymol.[102]

The encapsulation effectiveness of thymol within the liposomes was determined using the following formula:

$$Encapsulation\ Efficiency(\%) = (Total\ drug - Unentrapped\ \frac{drug}{total} \times 100)$$

In this formula, "Total thymol" indicates to the initial amount of thymol added during the liposome preparation, while "Unentrapped thymol" denotes the amount of thymol that remained in the supernatant and did not become encapsulated within the liposomal structure.

This calculation allowed for the determination of the percentage of thymol successfully encapsulated within the liposomes. The Higher encapsulation efficiency indicates a more effective liposome formulation, where a larger proportion of the thymol is retained within the liposomal bilayer. This experiment contributed in identifying the most suitable thymol concentration for liposome encapsulation, which contribute to the overall optimization of the liposome-based drug delivery system.

3.5 viscosity:

Viscosity is a characteristic of a fluid that is used to describe its internal resistance. Although viscosity testing is commonly used in engineering systems to assess fluids, it becomes essential when studying hydrogels to evaluate the rheological characteristics of these materials. The rheological examination is crucial as it yields information that aids in making decisions regarding the further processing or storage of the fluid. A 20ml sample of hydrogel containing drug and another sample of hydrogel with empty particles were made. The viscosity measurement was conducted by the USPCAS-E.

3.6 Antibiofilm Assay:

The study evaluated the anti-biofilm activity of *S. aureus and E. faecalis* using a microtiter plate spectroscopic experiment. The cultures were created using 5 millilitres of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. After incubation, 100 µL of each diluted culture was added to each well of a 96-well plate. The test wells were exposed to compounds at the minimum inhibitory concentration (MIC) and incubated for 24 hours. The next day, autoclaved distilled water was used to clean the wells, followed by 250 µL of crystal violet solution (0.1% w/v) and incubated for 15 minutes. The wells were then rinsed with distilled water and air-dried. 300 µL of 95% ethanol was added to each well, and the lid was sealed for 15 minutes. A volume of 150 µL of crystal violet and ethanol solution was transferred to a new 96-well plate, and the optical density at 630 nm was measured using a Multiskyskan Sky Microplate Spectrophotometer. The percentage of biofilm inhibitory activity was calculated using the method $[(C - B) - (T - B)/(C - B)] * 100\%$. A positive control was prepared using ethanol (96%).

3.7 Antibacterial activity:

The agar well diffusion technique is a widely used method in microbiology to evaluate the antibacterial properties of various substances, including drugs, botanical extracts, and artificial compounds. This technique involves creating wells in a solid agar medium and introducing a layer of microorganisms, usually bacteria, into it. The sample to be examined is then placed into the designated wells and allowed to diffuse into the agar medium. If the substance has antimicrobial properties, it will inhibit bacterial growth in the agar around the well. The antibacterial efficacy of a chemical is determined by the magnitude of the inhibition zone, which is the transparent region surrounding the well where microbial growth is limited. The well diffusion method is a valuable tool in microbiological research and the development of antimicrobial medications, providing a simple yet effective approach to evaluate and compare the antibacterial characteristics of different substances. It is commonly used in research, clinical laboratories, and pharmaceutical development for assessing the effectiveness of antimicrobial medicines against bacterial strains. It is a straightforward and cost-effective approach for the initial evaluation of prospective antibacterial drugs.

3.9 Development of Model:

The study assessed the efficacy of thymol-loaded liposomes in a hydrogel matrix in Wistar rats with alloxan-induced diabetes, using in vivo experiments. The work was conducted according to the ethical guidelines and received approval from the institutional review board.

3.9.1 Animal Model and Grouping

Twelve female Wistar rats, aged 8 weeks, were acquired from (ASAB) NUST. Upon arrival, the rats were introduced into a meticulously controlled environment with a temperature maintained at 27 ± 2 ℃ and a light-dark cycle of 12 hours each. Throughout the research, the rats were supplied with tap water and a standardised diet to guarantee they obtained adequate nourishment and hydration.

Prior to starting the experimental procedures, the rats were given a period of 7 days to adapt to their new surroundings. After the rats had adapted to their environment, they were randomly assigned to four groups and each group was placed in its own cage.

i. Control Diabetic Group (n = 3): Rats in this group were intentionally induced with diabetes but did not receive any type of treatment or intervention.
ii. The group of rats with diabetes and untreated wound infections: (n=3)
iii. The Diabetic group with Wound Infection treated with Thymol-Loaded Hydrogel (n =3) comprised of rats that were initially induced with diabetes, subsequently infected with a wound, and lastly subjected to treatment using thymol-loaded liposomes incorporated into a hydrogel.
iv. In the Diabetic group with Wound Infection treated with Empty Liposomes in Hydrogel Group (n = 3), rats were deliberately induced with diabetes, later infected with a wound, and then administered a hydrogel containing empty liposomes, which served as control

3.9.2 Induction of Diabetes

On the seventh day, diabetes was induced in all the rats by administering intraperitoneal injections of alloxan monohydrate at a dose of 150 mg/kg body weight. Alloxan, a substance that causes diabetes, specifically targets and destroys the beta cells in the pancreas that produce insulin, resulting in high blood sugar levels. The confirmation of diabetes induction was achieved through the measurement of blood glucose levels.

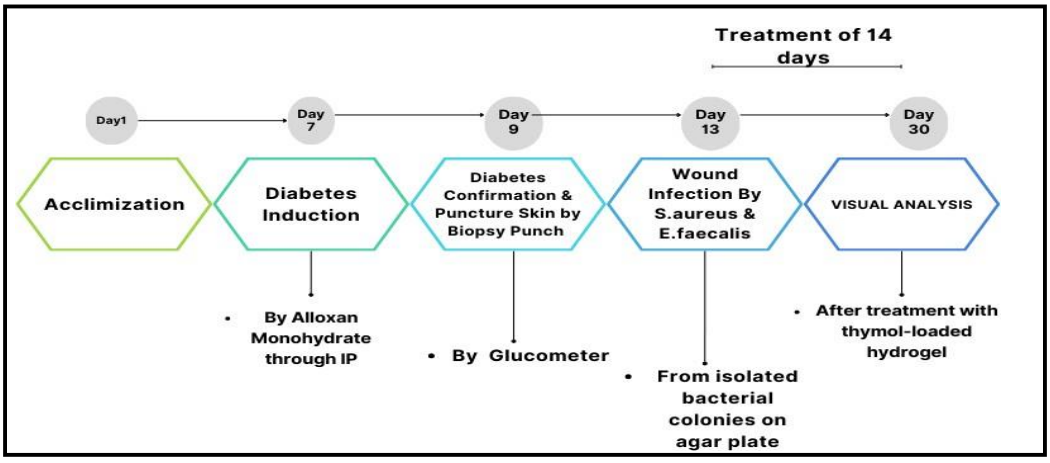
3.9.3 Induction of Wound Infection

To create a wound infection, the rats were injected with two bacterial strains, namely Staphylococcus aureus and Enterococcus faecalis. On the ninth day, a deliberate wound was made on the rats' dorsal skin using a biopsy punch. The wound site was subsequently infected with a mixture of *S.aureus* and *E.faecalis* at two different time intervals, with a 30-minute gap, for two consecutive days. This approach guaranteed the development of a long-lasting bacterial infection at the site of the wound.

Throughout the infection period, the rats were given cariogenic diet water containing 5% sucrose to increase blood sugar levels and facilitate the development of diabetes.

3.9.4 Treatment

After one week of the induction of the wound infection, the rats were treated based on their assigned groups



CHAPTER 4: RESULTS AND DISCUSSION

4.1 Synthesis of thymol loaded liposomes

Thymol loaded liposomes prepared through thin film hydration method, exhibit characteristic appearance that reflects the precision and care involved in their preparation. Following the process of rotary evaporation, the final liposome suspension appears as translucent or slightly opaque solution, suggesting a well-dispersed and stable suspension. The slight yellowish tint in the suspension is due to the natural colour of thymol and lecithin indicating a stable product without any degradation. The uniform appearance with no phase separation after filtration confirm the liposome's stability, ensuring that the final product is free from unencapsulated thymol and reading for drug delivery application.

Figure 12: the mixing of lipid solution and thymol(A) thick suspension after mixing (B)rotary evaporation(C) liposomes after filtration (D)

The finding from the preparation of thymol loaded liposomes using thin film hydration method provide valuable insight in formation of liposomes process and its impact on efficacy and stability. The translucent and opaque nature of liposomes suggest that liposomes are formed properly and well dispersed in aqueous medium. This dispersion is essential as poorly dispersed liposomes could lead to aggregation and hinder the therapeutic effect of thymol.

The tint color in the suspension is due to the natural coloring of thymol and lecithin which show confirm that thymol is properly encapsulated without undergoing degradation process during the liposomes preparation.

The absence of free thymol after filtration shows that encapsulation efficiency is high which minimizes the need for further purification step and minimizing the need of further steps making the formulation viable for applications.

4.2 Synthesis of hydrogel matrix

The final appearance of thymol loaded hydrogel prepared by mixing polyvinyl alcohol, Chitosan and Gelatin is a white semi solid gel that is uniform and consistent throughout. Initially, the mixture was viscous solution but after the 24 hr. cooling period in the refrigerator, it solidifies into a stable semi-solid gel. The gelation confirms the successful crosslinking with the hydrogel ensuring the equal distribution of liposomes in the gel matrix, which is crucial for its application in treating diabetic wounds.

Figure 13: the final hydrogel formulation after 24hrs cooling

The final look of the thymol loaded gel after the cooling process indicate the successful cross linking of chitosan, polyvinyl alcohol and gelatin. The stability and consistency of the gel confirms that liposomes are well distributed and provide a controlled and sustain

release of the drug (thymol) to the wound site which is crucial for treating diabetic wound where a sustained antimicrobial presence is needed to heal the wound and reduce the infection.

4.3 Physical Characterization of thymol loaded liposomes and hydrogel matrix:

Successful synthesis of thymol encapsulated in liposomes and hydrogel was confirmed by physical characterization

4.3.1 Uv-Analysis:

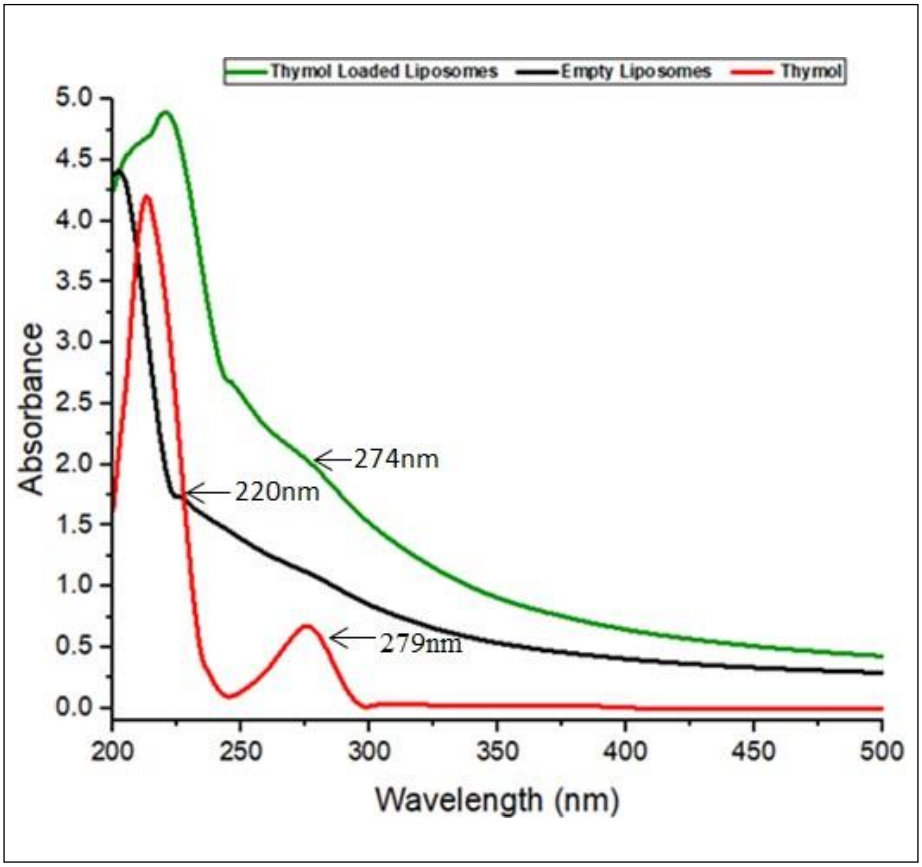


Figure 14: UV analysis of thymol, empty liposomes and thymol loaded liposomes

The figure displays the UV spectra of liposomes loaded with thymol, empty liposomes and thymol alone. Thymol exhibited an absorbance peak at 279nm, while empty liposomes

displayed a peak at 220nm. Thymol loaded liposomes demonstrated a minor shift in absorbance, indicating the effective encapsulation of thymol within the liposomes.

The UV analysis of thymol reveals an absorbance peak at a wavelength of 279nm. This peak serves as a benchmark for verifying the chemical integrity and post-encapsulation activity of thymol.

The empty liposomes exhibited a peak at a wavelength of 220nm, indicating the absorption of lipids in the liposomes preparation. The presence of this prominent peak in the empty liposomes indicates that the structure of the liposomes is intact and does not affect the absorption of thymol. Furthermore, it guarantees that the liposomes, when administered to the wound site, will not have any negative impact on the bioactivity of thymol. The presence of thymol within the liposomes was confirmed by a small change in absorbance near 279nm, indicating effective encapsulation. This peak is significant as it demonstrates that thymol is not only combined with the liposomes, but also enclosed within the lipid bilayer or aqueous core of the liposomes. Ultraviolet analysis verifies the successful encapsulation of thymol within the liposomes, which is a crucial step to guarantee the efficacy of the formulation in treating diabetic wounds. The implementation of regulated release mechanisms effectively mitigated the possible harm to cells while simultaneously sustaining the desired therapeutic concentration of thymol, so significantly augmenting the overall suitability of the formulation for clinical utilisation.

4.3.2 Fourier Transform Infrared Spectroscopy Analysis:

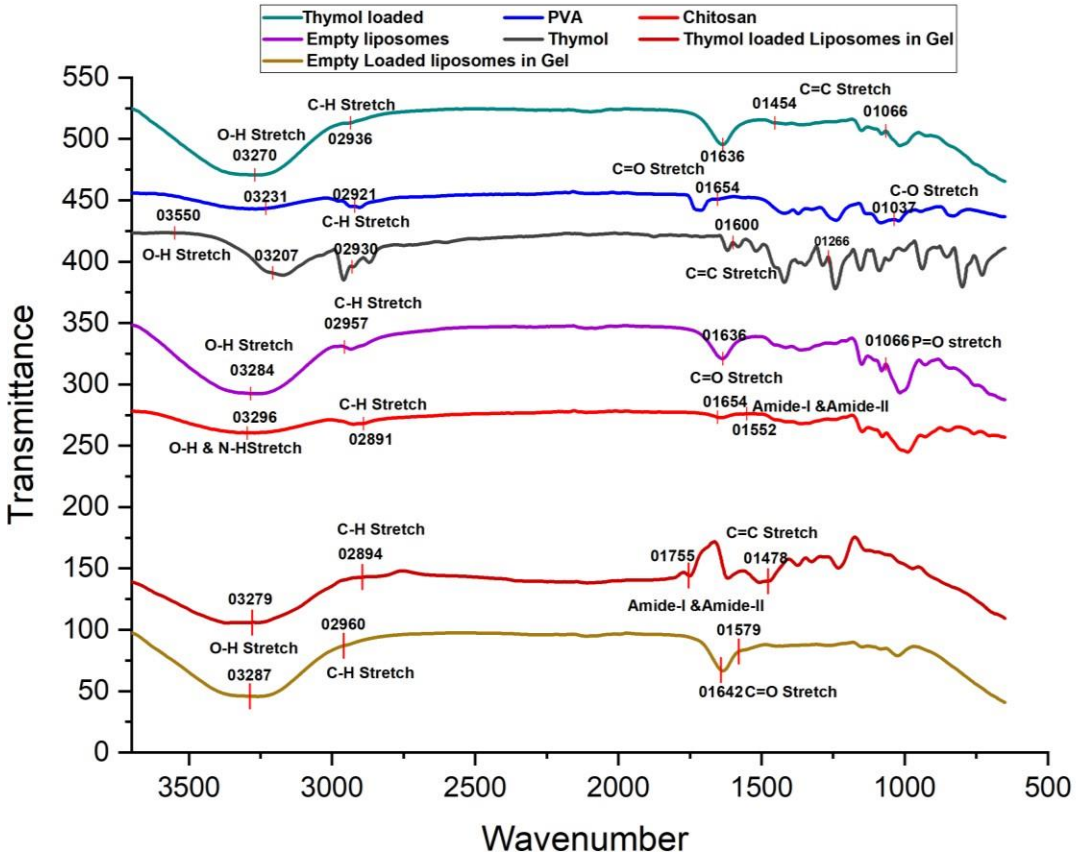


Figure 15: FTIR analysis of thymol, empty liposomes , thymol loaded liposomes, thymol loaded liposomes in gel, empty liposomes in gel, chitosan and Polyvinyl alcohol

The FTIR analysis identified the distinctive peak of different components, offering valuable information about their chemical structure and interaction. Peaks at approximately 3300 cm^{-1} suggest the existence of a hydroxyl group. The presence of the aromatic C=C stretching in thymol, typically occurring between 1450-1600 cm^{-1}, indicates that the aromatic structure of thymol remains unchanged throughout encapsulation. The presence of C-H stretching at around 2900 cm^{-1} in all components suggests the presence of an alkyl chain. Peaks in the range of 1000-1100 cm^{-1} suggest the presence of C-O stretching. These peaks are also observed in the gel containing blank liposomes. The high peak observed at approximately 1650 cm^{-1} indicates the presence of a carbonyl group (C=O), namely in PVA and chitosan. The existence of amide bands, which are characteristic of chitosan, was

confirmed in the gel spectra. A slight change in the highest point's location indicated a weak connection between thymol, liposomes, and the gel matrix. This suggests that the functional groups are still present, assuring the stability of thymol in the formulation.

The FTIR analysis verifies the effective encapsulation of thymol within the hydrogel and demonstrates the stability of the formulations. The weak contact between the components and the preservation of functional groups validate the hydrogel's ability to provide a controlled release of thymol, hence enhancing its efficacy for wound healing applications.

4.3.3 Scanning Electron Microscopy (SEM):

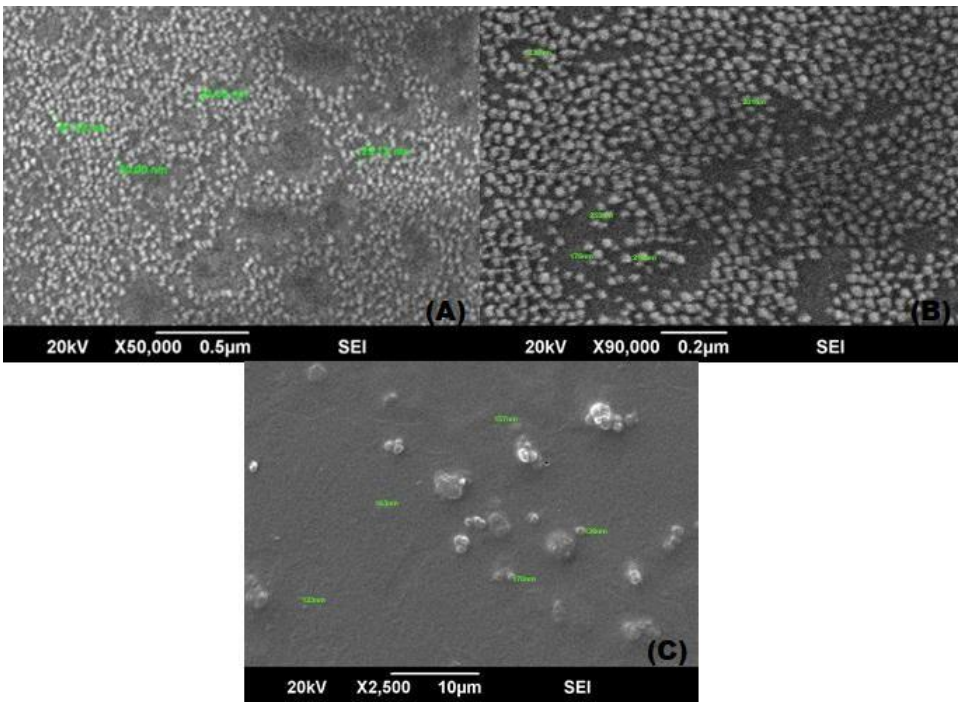


Figure 16 SEM images of Empty liposomes (A), Thymol loaded Liposomes (B) and thymol loaded hydrogel (C)

The Scanning Electron Microscopy (SEM) analysis revealed distinct morphological characteristics of both Empty and thymol-loaded liposomes (figure 21). This analysis showed that empty liposomes possess a smooth and spherical morphology with an average size of 29nm whereas thymol-loaded liposomes showed a slightly irregular surface of average size 255nm indicating successful drug encapsulation. A noticeable increase in the average size of liposomes was observed after the thymol loading, reflecting the impact of

thymol encapsulation on liposomes. Furthermore, the SEM image of hydrogel matrix containing thymol loaded liposomes showed a well distribution of liposomes within the matrix.

After conducting an investigation using scanning electron microscopy (SEM), it was shown that empty liposomes and liposomes loaded with thymol exhibited significant morphological properties (figure 12). Based on the findings of this investigation, it was determined that empty liposomes have a smooth and spherical morphology with an average size of 29nm, but thymol-loaded liposomes displayed a surface that was somewhat uneven with an average size of 200nm, indicating that the drug was successfully encapsulated. After the thymol loading, there was a noticeable increase in the average size of the liposomes, which is a reflection of the effect that thymol encapsulation has on liposomes. In addition, the scanning electron microscopy (SEM) image of the hydrogel matrix that contained thymol-loaded liposomes demonstrated that the liposomes were distributed evenly throughout the matrix.

4.3.4 Zeta Potential analysis:

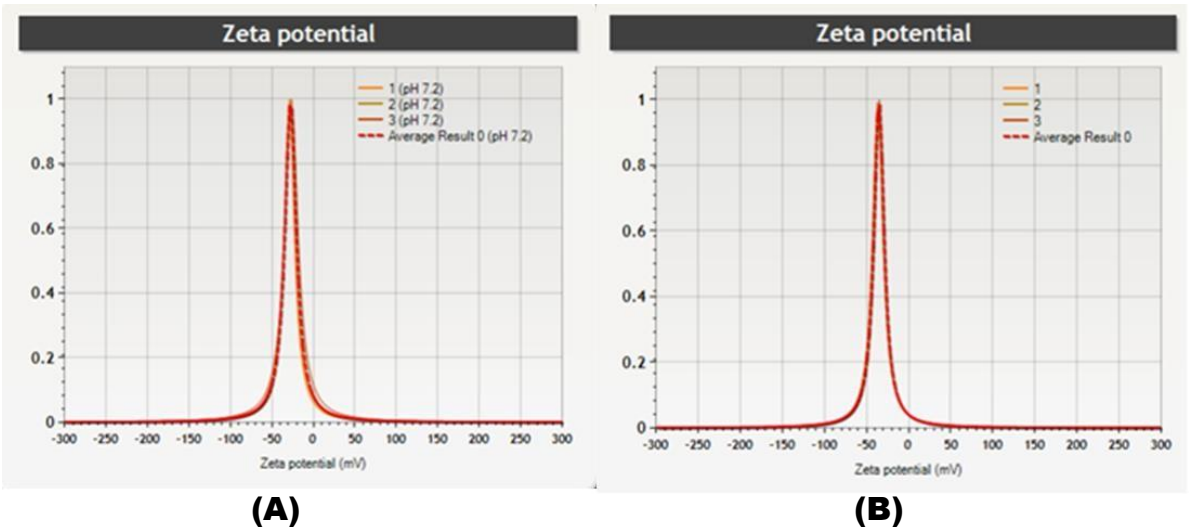


Figure 17: : Zeta potential of Empty Liposomes (A) and Thymol-loaded liposomes (B)

The zeta potential measurements showed that empty liposomes had an average zeta potential of -27.99mv indicating the moderate stability. Thymol loaded liposomes on the other hand demonstrated a higher average zeta potential of -35. 83mv.This increase in negative charge implies that thymol loading enhances the stability of liposomes through improved electrostatic repulsion, resulting in a more stable colloidal dispersion.

In short, the results of the zeta analysis demonstrated that thymol-loaded liposomes are more stable and produce less aggregates over the course of time. When it comes to colloidal dispersion, stability is of the utmost importance because aggregation can lead to irregular drug distribution, decreased bioavailability, and a shorter shelf life for the formulation. It may be deduced from the stable thymol loaded liposomes that the drugs retain an even distribution throughout the formulation, which enables the consistent release at the wound site.

4.3.5 Atomic force Microscopy

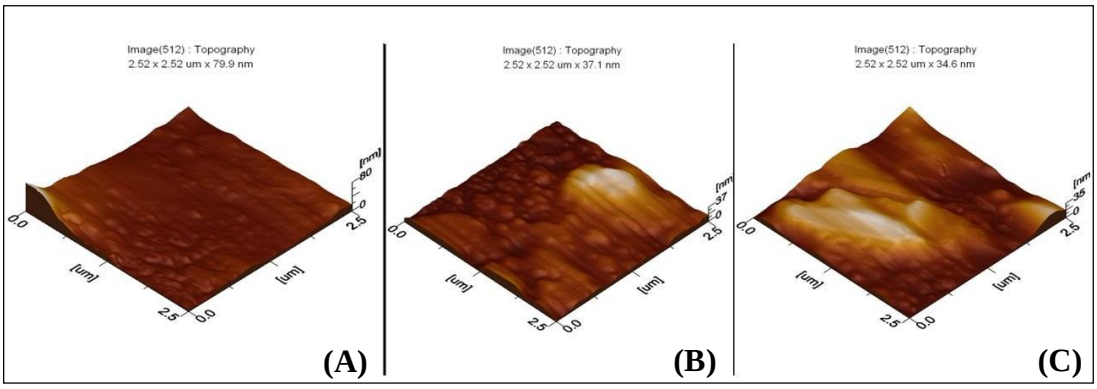


Figure 18: AFM analysis of thymol-loaded liposomes (A), thymol loaded hydrogel (A) and empty liposomes(C)

The AFM analysis of empty liposomes, thymol loaded liposomes and hydrogel with thymol loaded liposomes revealed the characteristics of surface morphology. Empty liposomes ware observed to have a relatively smooth surface with the height variation (roughness) of 34.6nm. The surface roughness increased to 79.9nm for thymol loaded liposomes indicating the successful encapsulation of thymol. The hydrogel matrix containing thymol loaded liposomes showed an intermediate roughness of 37.1nm reflecting the presence of loaded liposomes within the hydrogel matrix. These results confirmed that encapsulation process was effectively carried out with clear difference in surface topography.

4.3.6 Drug Encapsulation:

The Encapsulation efficiency of thymol loaded hydrogel was calculated to be 87% reflecting a high degree of encapsulation of thymol within the liposomes. By Using a standard curve equation, with an R^2 value of 0.9994.

$$Y = 0.1959x - 0.0365$$

The concentration of un-encapsulated drug was measured by UV analysis of 1st supernatant after centrifugation at the absorbance of 274nm for thymol.

Absorbance (**274nm**) = **1.67785**

X= Concentration of unknown

Y= Absorbance value at specific points

By substituting the value of absorbance at 274nm, the amount of un-encapsulated drug =**0.12214mg/ml**

The total drug was 1mg/ml and the absorbance of total drug at **274nm** =**1.67785**

The Entrapment Efficiency was calculated by formula;

EE% = (Total Drug – Unencapsulated Drug) / (Total drug) × 100

EE= **87%**

4.3.6 Antibiofilm Assay:

The biofilm percentage was calculated using the formula,

$$(C - B) - \frac{(T-B)}{(C-B)} \times 100$$

Where C represents the absorbance of control (biofilm without treatment), b denotes the absorbance of blank and T indicate the absorbance of test samples. These finding concluded that the thymol loaded with liposomes displayed a significant level of effectiveness with the range of 80-90% of suppression of bacterial growth and formation of biofilm as shown in figure below.

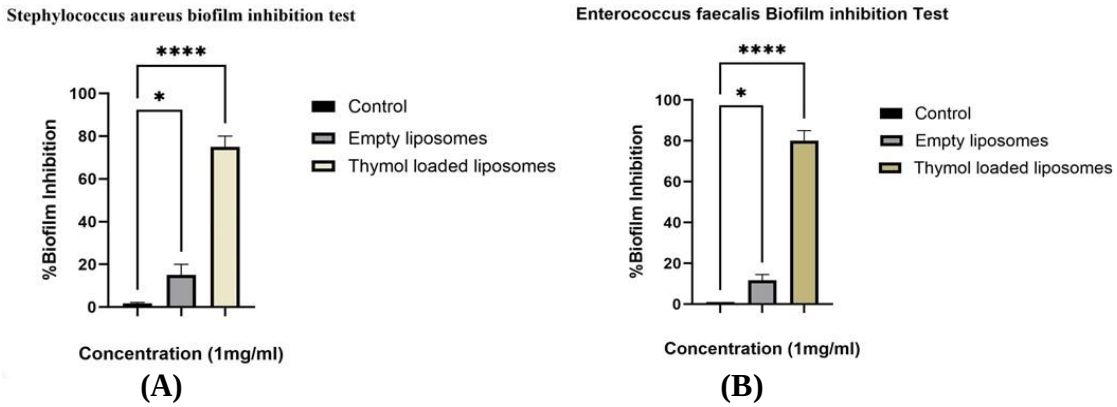


Figure 19 antibiofilm inhibition test of Control, Empty liposomes, and Thymol-loaded liposomes against S.aureus (A) Antibiofilm inhibition test of control, Empty liposomes and Thymol-loaded liposomes against E.faecalis

To illustrate the percentage of biofilm activity against S. aureus and E. faecalis, the bar graph displays the percentage of biofilm activity of control, thymol loaded liposomes, and empty liposomes. The graph displays one asterisk and four asterisks, indicating that the p value less than 0.05, suggests that the data is statistically significant. P value less than 0.05, denoted by a single asterisk, and if the p value equal to 0.01, denoted by three asterisks. This indicates that the p value is statistically significant, and there is a significant difference between the means of the three groups. This leads to the rejection of the null hypothesis, which states that there is no significant difference between the means of the three groups.

4.3.7 Viscosity

Viscosity measurement of gel matrix with thymol loaded liposomes was conducted to evaluate its rheological properties. The results showed that the viscosity of thymol loaded liposomes in gel increases (403cp) as compared to unloaded gel (201cp) indicating that the incorporation of liposomes contributed to thicker and more viscous gel formulation. This change in viscosity is advantageous for controlled release applications.

Table 1.1: viscosity measurement of empty liposomes and thymol loaded liposomes

	Viscosity (cp)	Acceleration (±cp)	Temperature(°C)
Empty liposomes loaded gel	201	12.80	37
Thymol loaded gel	403	12.8	37

4.3.9 Antibacterial Activity:

For the purpose of confirming the antibacterial efficiency of thymol-loaded liposomes, pure thymol solution, and empty liposomes (as a control), the effectiveness of these three types of liposomes was evaluated against Gram-positive bacterial strains, specifically S. aureus and E. faecalis. Following the completion of the experiment, it was found that the pure thymol solution as well as the nanoparticle loaded with thymol diffused into the agar and efficiently suppressed the development of bacteria. When compared to the concentration of pure thymol solution, which was 10 mg/ml, the concentration of empty nanoparticles and nanoparticles filled with thymol was 0.05 mg/ml. As a result, the findings indicate that a tiny quantity of liposomes loaded with thymol can be just as efficient as a solution of pure thymol in combating the bacterial strains being investigated. The most important findings from our research demonstrate that using of thymol loaded liposomes resulted in more favourable results when compared to the use of thymol on alone. The results are presented in the figure that can be found below, which is a bar graph that shows thymol, liposomes loaded with thymol, and empty liposomes.

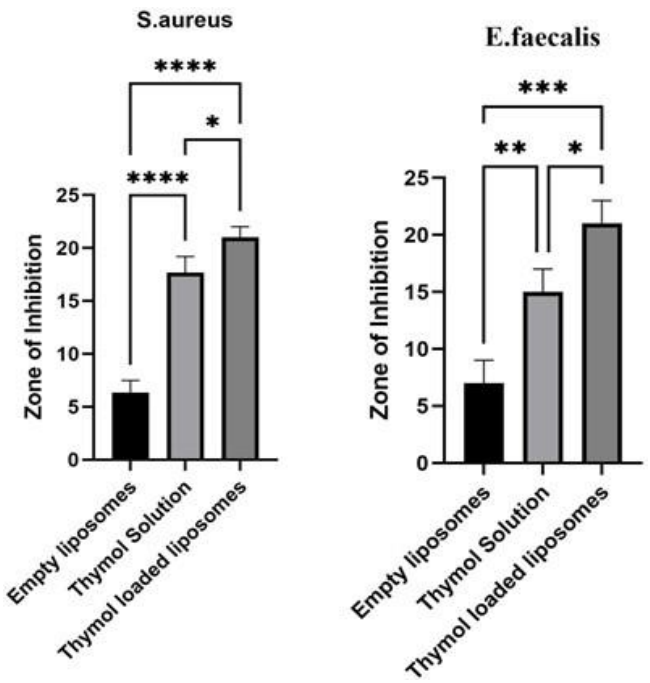


Figure 20 The antibacterial Activity of thymol solution, Empty liposomes, thymol loaded liposomes against S.aureus faecalis and the antibacterial activity of Empty Liposomes, thymol-loaded liposomes and pure thymol

Graph showed that thymol loaded liposomes exhibit superior antibacterial effect compared to thymol solution and empty liposomes. Thymol loaded liposomes give zone of 20mm against *S.aureus* and 19mm against *E.faecalis* whereas thymol solution give 18mm and 15mm respectively which suggests that thymol loaded give more effective antibacterial activity even at lower concentrations. This ability of thymol loaded particles is more effective to control the bacterial growth and improve infection management and accelerate wound recovery in diabetic wound healing. The sustained release of thymol loaded particles from gel ensure prolonged antibacterial effects and improve overall therapeutic outcomes

4.3.10 In-vivo Results:

Figure 21: grouping of rats

Figure 22: diabetes induction by Alloxan

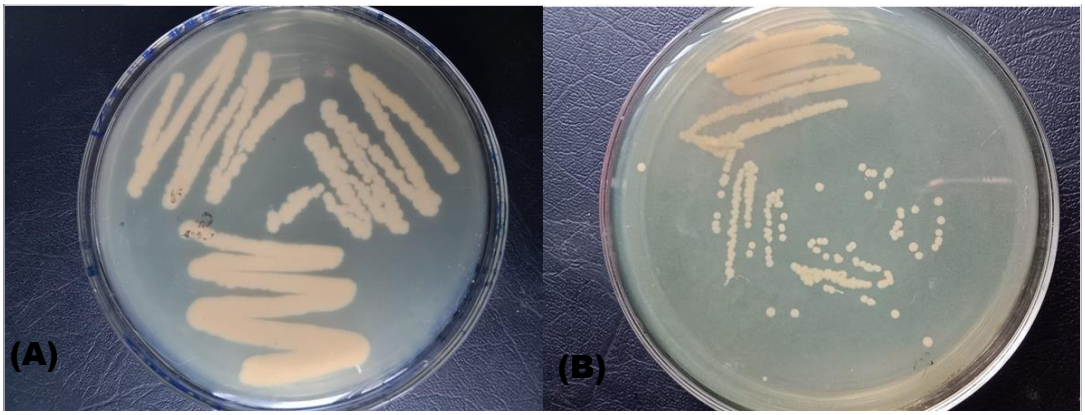


Figure 23: Isolated colonies of S.aureus (A) and E.faecalis (B)

Figure 24 wound healing after thymol loaded hydrogel treatment

i. Group with thymol loaded hydrogel treatment:

Gel containing thymol loaded liposomes applied directly to the wound site of rats.

ii. Group with empty liposome loaded hydrogel treatment:

Hydrogel containing empty liposomes serving as control also applied directly to the wound site to assess the effect of liposomes structure and hydrogel without the active drug.

After the treat of one week, anesthesia was given to rats to minimize the pain suffering and following anesthesia the rats are humanely sacrificed and then the wound tissue was collected for further visual analysis.

CHAPTER 5: CONCUSION AND FUTURE PROSPECTS

Thymol-loaded hydrogel is a promising advancement in diabetic wound treatment, addressing the unique challenges of managing chronic wounds. The hydrogel's enhanced hydrophilicity promotes a moist healing environment, facilitating faster tissue regeneration. In vivo studies on diabetic rats have shown its efficacy in real-world applications, accelerating wound closure and improving the quality of newly formed tissue. This validates the hydrogel's therapeutic potential and supports its translation into clinical settings.

Future research should explore the long-term performance and durability of the hydrogel, optimize thymol concentration and release rates, evaluate its effectiveness across various diabetic wound models and patient demographics, and compare it with existing wound care treatments. Assessment of the scalability of the manufacturing process, cost-effectiveness for widespread clinical use, potential side effects, and patient feedback will be crucial for refining the hydrogel for optimal safety and efficacy.

The promising results of this study highlight the potential of the thymol-loaded hydrogel to advance diabetic wound care, with on-going research expected to support its successful integration into clinical practice, leading to improved patient outcomes and enhanced quality of life.

REFERENCES

1. Hossain, M.J., M. Al-Mamun, and M.R. Islam, *Diabetes mellitus, the fastest growing global public health concern: Early detection should be focused.* Health Science Reports, 2024. **7**(3): p. e2004.

2. Lancet, T., *Diabetes: a defining disease of the 21st century*. 2023. p. 2087.

3. Maffi, P. and A. Secchi, *The burden of diabetes: emerging data.* Management of Diabetic Retinopathy, 2017. **60**: p. 1-5.

4. Dinh, T., S. Elder, and A. Veves, *Delayed wound healing in diabetes: Considering future treatments.* Diabetes Management, 2011. **1**(5): p. 509.

5. Greenhalgh, D.G., *Wound healing and diabetes mellitus.* Clinics in plastic surgery, 2003. **30**(1): p. 37-45.

6. Ji, J.-Y., D.-Y. Ren, and Y.-Z. Weng, *Efficiency of multifunctional antibacterial hydrogels for chronic wound healing in diabetes: a comprehensive review.* International journal of nanomedicine, 2022. **17**: p. 3163.

7. Han, G. and R. Ceilley, *Chronic wound healing: a review of current management and treatments.* Advances in therapy, 2017. **34**: p. 599-610.

8. Aynalem, S.B. and A.J. Zeleke, *Prevalence of diabetes mellitus and its risk factors among individuals aged 15 years and above in Mizan-Aman town, Southwest Ethiopia, 2016: a cross sectional study.* International journal of endocrinology, 2018. **2018**(1): p. 9317987.

9. Gainza, G., et al., *Advances in drug delivery systems (DDSs) to release growth factors for wound healing and skin regeneration.* Nanomedicine: Nanotechnology, Biology and Medicine, 2015. **11**(6): p. 1551-1573.

10. Laiva, A.L., F.J. O'Brien, and M.B. Keogh, *Innovations in gene and growth factor delivery systems for diabetic wound healing.* Journal of Tissue Engineering and Regenerative Medicine, 2018. **12**(1): p. e296-e312.

11. Qamar, Z., et al., *Nano-based drug delivery system: recent strategies for the treatment of ocular disease and future perspective.* Recent Patents on Drug Delivery & Formulation, 2019. **13**(4): p. 246-254.

12. Manna, S., et al., *Probing the mechanism of bupivacaine drug release from multivesicular liposomes.* Journal of controlled release, 2019. **294**: p. 279-287.

13. Patra, J.K., et al., *Nano based drug delivery systems: recent developments and future prospects.* Journal of nanobiotechnology, 2018. **16**: p. 1-33.

14. Wang, W., et al., *Nano-drug delivery systems in wound treatment and skin regeneration.* Journal of nanobiotechnology, 2019. **17**(1): p. 82.

15. Zhang, W., et al., *Hydrogel-based dressings designed to facilitate wound healing.* Materials Advances, 2024. **5**(4): p. 1364-1394.

16. Ahmed, E.M., *Hydrogel: Preparation, characterization, and applications: A review.* Journal of advanced research, 2015. **6**(2): p. 105-121.

17. Xiang, J., L. Shen, and Y. Hong, *Status and future scope of hydrogels in wound healing: Synthesis, materials and evaluation.* European Polymer Journal, 2020. **130**: p. 109609.

18. Fan, F., S. Saha, and D. Hanjaya-Putra, *Biomimetic hydrogels to promote wound healing.* Frontiers in Bioengineering and Biotechnology, 2021. **9**: p. 718377.

19. Sheokand, B., et al., *Natural polymers used in the dressing materials for wound healing: Past, present and future.* Journal of Polymer Science, 2023. **61**(14): p. 1389-1414.

20. Singh, T.R.R., G. Laverty, and R. Donnelly, *Hydrogels: design, synthesis and application in drug delivery and regenerative medicine*. 2018: CRC Press.

21. Francesko, A., P. Petkova, and T. Tzanov, *Hydrogel dressings for advanced wound management.* Current medicinal chemistry, 2018. **25**(41): p. 5782-5797.

22. Burt, S., *Essential oils: their antibacterial properties and potential applications in foods—a review.* International journal of food microbiology, 2004. **94**(3): p. 223-253.

23. Rane, B.R., et al., *Novel Approaches in Herbal Formulations*, in *Enhancing the Therapeutic Efficacy of Herbal Formulations*. 2021, IGI Global. p. 43-68.

24. Müller, R., M. Radtke, and S. Wissing, *Nanostructured lipid matrices for improved microencapsulation of drugs.* International journal of pharmaceutics, 2002. **242**(1-2): p. 121-128.

25. Riella, K., et al., *Anti-inflammatory and cicatrizing activities of thymol, a monoterpene of the essential oil from Lippia gracilis, in rodents.* Journal of Ethnopharmacology, 2012. **143**(2): p. 656-663.

26. Borena, B.M., Martens, A., Broeckx, S. Y., Meyer, E., Chiers, K., Duchateau, L., & Spaas, J. H., *Regenerative Skin Wound Healing in Mammals: State-of-the-Art on Growth Factor and Stem Cell Based Treatments.* Cellular Physiology and Biochemistry 1 May, 2016: p. 36 (1): 1–23.

27. Tottoli, E.M., Rossella Dorati, Ida Genta, Enrica Chiesa, Silvia Pisani, and Bice Conti, *"Skin Wound Healing Process and New Emerging Technologies for Skin Wound Care and Regeneration"*. Pharmaceutics 2020: p. 12.

28. Potekaev NN, B.O., Medvedev GV, Pushkin DV, Petrova MM, Petrov AV, Dmitrenko DV, Karpova EI, Demina OM, Shnayder NA., *The Role of Extracellular Matrix in Skin Wound Healing.* Journal of Clinical Medicine., 2021: p. 10(24):5947.

29. Takeo, M., Lee, W., & Ito, M., *Wound healing and skin regeneration.* Cold Spring Harbor perspectives in medicine,, 2015: p. 5(1).

30. Zaidi, A., & Green, L., *Physiology of haemostasis.* Anaesthesia & Intensive Care Medicine,, 2019: p. 152-158.

31. Chen, L., & DiPietro, L. A., *Toll-like receptor function in acute wounds.* Advances in wound care, 2017: p. 344-355.

32. Bui, T.M., Wiesolek, H. L., & Sumagin, R, *ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis.* Journal of Leucocyte Biology,, 2020: p. 787-799.

33. Wilkinson, H.N., & Hardman, M. J., *Wound healing: cellular mechanisms and pathological outcomes.* Open biology, 2020: p. 200-223.

34. Hu, W., Shang, R., Yang, J., Chen, C., Liu, Z., Liang, G., ... & Luo, G., *Skin γδ T cells and their function in wound healing.* Frontiers in Immunology, 2022: p. 875076.

35. Legrand, J.M., & Martino, M. M., *Growth factor and cytokine delivery systems for wound healing.* Cold Spring Harbor perspectives in biology,, 2022: p. 41234.

36. Wilkinson, H.N., & Hardman, M. J., *Senescence in wound repair: emerging strategies to target chronic healing wounds.* Frontiers in cell and developmental biology, 2020: p. 773.

37. Huang, S., Kuri, P., Aubert, Y., Brewster, M., Li, N., Farrelly, O., ... & Rompolas, P., *Lgr6 marks epidermal stem cells with a nerve-dependent role in wound re-epithelialization.* Cell Stem Cell, 2021: p. 1582-1596.

38. Potekaev, N.N., Borzykh, O. B., Medvedev, G. V., Pushkin, D. V., Petrova, M. M., Petrov, A. V., ... & Shnayder, N. A., *The role of extracellular matrix in skin wound healing.* Journal of Clinical Medicine,, 2021: p. 5947.

39. Chakroborty, D., Goswami, S., Basu, S., & Sarkar, C., *Catecholamines in the regulation of angiogenesis in cutaneous wound healing.* FASEB journal: official publication of the Federation of American Societies for Experimental Biology,, 2020: p. 14093.

40. Yu, J., Nam, D., & Park, K. S., *Substance P enhances cellular migration and inhibits senescence in human dermal fibroblasts under hyperglycemic conditions.* Biochemical and Biophysical Research Communications, 2020: p. 917-923.

41. Singer, A.J., *Healing mechanisms in cutaneous wounds: tipping the balance.* Tissue Engineering Part B: Reviews, 2022: p. 1151-1167.

42. Gardeazabal, L., & Izeta, A., *Elastin and collagen fibres in cutaneous wound healing.* Experimental Dermatology, 2024: p. 5052.

43. Opneja, A., Kapoor, S., & Stavrou, E. X., *Contribution of platelets, the coagulation and fibrinolytic systems to cutaneous wound healing.* Thrombosis research, 2019: p. 56-63.

44. Hossain, M.J., Al-Mamun, M., & Islam, M. R, *Diabetes mellitus, the fastest growing global public health concern: Early detection should be focused.* Health Science Reports,, 2024: p. 2004.

45. Gorecka, J., Kostiuk, V., Fereydooni, A., Gonzalez, L., Luo, J., Dash, B., ... & Dardik, A., *The potential and limitations of induced pluripotent stem cells to achieve wound healing.* Stem cell research & therapy, 2019: p. 1-10.

46. Dasari, N., Jiang, A., Skochdopole, A., Chung, J., Reece, E. M., Vorstenbosch, J., & Winocour, *Updates in diabetic wound healing, inflammation, and scarring.* In Seminars in plastic surgery, 2021: p. 153-158.

47. Zhao, R., Liang, H., Clarke, E., Jackson, C., & Xue, M., *Inflammation in chronic wounds.* International journal of molecular sciences, 2016: p. 2085.

48. Boniakowski, A.E., Kimball, A. S., Jacobs, B. N., Kunkel, S. L., & Gallagher, K. A, *Macrophage-mediated inflammation in normal and diabetic wound healing.* The Journal of Immunology, 2017: p. 17-24.

49. Rosique, R.G., Rosique, M. J., & Farina Junior, J. A., *Curbing inflammation in skin wound healing: a review.* International journal of inflammation,, 2015: p. 316235.

50. Davis, F.M., Kimball, A., Boniakowski, A., & Gallagher, K., *Dysfunctional wound healing in diabetic foot ulcers: new crossroads.* Current diabetes reports, 2018: p. 1-8.

51. Den Dekker, A., Davis, F. M., Kunkel, S. L., & Gallagher, K. A., *Targeting epigenetic mechanisms in diabetic wound healing.* Translational Research, 2019: p. 39-50.

52. Demidova-Rice, T.N., Durham, J. T., & Herman, I. M., *Wound healing angiogenesis: innovations and challenges in acute and chronic wound healing.* Advances in wound care, 2012: p. 17-22.

53. Okonkwo, U.A., & DiPietro, L. A., *Diabetes and wound angiogenesis.* International journal of molecular sciences, 2017: p. 1419.

54. Greenhalgh, D.G., *Wound healing and diabetes mellitus.* Clinics in plastic surgery, 2003: p. 37-45.

55. Yadav, J.P., et al., *Insights into the mechanisms of diabetic wounds: pathophysiology, molecular targets, and treatment strategies through conventional and alternative therapies.* Inflammopharmacology, 2024. **32**(1): p. 149-228.

56. Yadav, J.P., Patel, D. K., Dubey, N. K., Mishra, M. K., Verma, A., Grishina, M., ... & Pathak, P., *Wound healing and antioxidant potential of Neolamarckia cadamba in streptozotocin-nicotinamide induced diabetic rats.* Phytomedicine Plus, 2022: p. 100274.

57. Singh, S.K., Dwivedi, S. D., Yadav, K., Shah, K., Chauhan, N. S., Pradhan, M., ... & Singh, D., *Novel biotherapeutics targeting biomolecular and cellular approaches in diabetic wound healing.* Biomedicines, 2023: p. 613.

58. Petkovic, M., Sørensen, A. E., Leal, E. C., Carvalho, E., & Dalgaard, L. T, *Mechanistic actions of microRNAs in diabetic wound healing.* Cells, 2020: p. 2228.

59. Fang, W.C., & Lan, C. C. E, *The epidermal keratinocyte as a therapeutic target for management of diabetic wounds.* International Journal of Molecular Sciences, 2023: p. 4290.

60. Yang, T., et al., *An update on chronic complications of diabetes mellitus: from molecular mechanisms to therapeutic strategies with a focus on metabolic memory.* Molecular Medicine, 2024. **30**(1): p. 71.

61. Wang, Q., Luo, Z., Wu, Y. L., & Li, Z., *Recent Advances in Enzyme-Based Biomaterials Toward Diabetic Wound Healing.* . Advanced NanoBiomed Research, 2023: p. 2200110.

62. Cam, M.E., Ertas, B., Alenezi, H., Hazar-Yavuz, A. N., Cesur, S., Ozcan, G. S., ... & Edirisinghe, M., *Accelerated diabetic wound healing by topical application of combination oral antidiabetic agents-loaded nanofibrous scaffolds: An in vitro and in vivo evaluation study.* Materials Science and Engineering, 2021: p. 111586.

63. Chen, X., Liu, Y., & Zhang, X., *Topical insulin application improves healing by regulating the wound inflammatory response.* Wound Repair and Regeneration, 2012: p. 425-434.

64. Bellingeri, A., Falciani, F., Traspedini, P., Moscatelli, A., Russo, A., Tino, G., ... & Peghetti, A, *Effect of a wound cleansing solution on wound bed preparation and inflammation in chronic wounds: a single-blind RCT.* Journal of Wound Care, 2016: p. 160-168.

65. Lázaro-Martínez, J.L., Álvaro-Afonso, F. J., Sevillano-Fernández, D., García-Álvarez, Y., Sanz-Corbalan, I., & García-Morales, E, *Cellular proliferation, dermal repair, and microbiological effectiveness of ultrasound-assisted wound debridement (UAW) versus standard wound treatment in complicated diabetic foot ulcers (DFU): an open-label randomized controlled trial.* Journal of Clinical Medicine, 2020: p. 4032.

66. Elraiyah, T., Domecq, J. P., Prutsky, G., Tsapas, A., Nabhan, M., Frykberg, R. G., ... & Murad, M. H, *A systematic review and meta-analysis of débridement methods for chronic diabetic foot ulcers.* Journal of vascular surgery, 2016: p. 37-45.

67. Johani, K., Malone, M., Jensen, S. O., Dickson, H. G., Gosbell, I. B., Hu, H., ... & Vickery, K, *Evaluation of short exposure times of antimicrobial wound solutions against microbial biofilms: from in vitro to in vivo.* Journal of Antimicrobial Chemotherapy, 2018: p. 494-502.

68. van Netten, J.J., Lazzarini, P. A., Armstrong, D. G., Bus, S. A., Fitridge, R., Harding, K., ... & Wraight, P. R, *Diabetic Foot Australia guideline on footwear for people with diabetes.* Journal of Foot and Ankle Research, 2018: p. 2.

69. Collings, R., Freeman, J., Latour, J. M., & Paton, J, *Footwear and insole design features for offloading the diabetic at risk foot—A systematic review and meta-analyses.* Endocrinology, diabetes & metabolism, 2021: p. 0-132.

70. Ahluwalia, R., Maffulli, N., Lázaro-Martínez, J. L., Kirketerp-Møller, K., & Reichert, I., *Diabetic foot off loading and ulcer remission: exploring surgical off-loading.* The Surgeon, 2021: p. 526-535.

71. Yammine, K., & Assi, C., *Surgery versus nonsurgical methods in treating neuropathic plantar forefoot ulcers: a meta-analysis of comparative studies.* The International Journal of Lower Extremity Wounds, 2022: p. 7-17.

72. Rezvani Ghomi, E., Khalili, S., Nouri Khorasani, S., Esmaeely Neisiany, R., & Ramakrishna, S., *Wound dressings: Current advances and future directions.* Journal of Applied Polymer Science, 2019: p. 47-738.

73. Ansari, P., Akther, S., Hannan, J. M. A., Seidel, V., Nujat, N. J., & Abdel-Wahab, Y. H., *Pharmacologically active phytomolecules isolated from traditional antidiabetic plants and their therapeutic role for the management of diabetes mellitus.* Molecules, 2022: p. 4278.

74. Oguntibeju, O.O., *Medicinal plants and their effects on diabetic wound healing.* Veterinary world, 2019: p. 653.

75. Wang, Y., Cao, H. J., Wang, L. Q., Lu, C. L., Yan, Y. Q., Lu, H., ... & Liu, J. P, *The effects of Chinese herbal medicines for treating diabetic foot ulcers: A systematic review of 49 randomized controlled trials.* Complementary Therapies in Medicine, 2019: p. 32-43.

76. Spampinato, S.F., Caruso, G. I., De Pasquale, R., Sortino, M. A., & Merlo, S., *The treatment of impaired wound healing in diabetes: looking among old drugs.* Pharmaceuticals, 2020: p. 60.

77. Escobar, A., Perez, M., Romanelli, G., & Blustein, G., *Thymol bioactivity: A review focusing on practical applications.* Arabian Journal of Chemistry, 2020: p. 9243-9269.

78. Venu, S., Naik, D. B., Sarkar, S. K., Aravind, U. K., Nijamudheen, A., & Aravindakumar, C. T., *Oxidation reactions of thymol: a pulse radiolysis and theoretical study.* The Journal of Physical Chemistry A, 2013: p. 291-299.

79. Nagoor Meeran, M.F., & Stanely Mainzen Prince, P., *Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol-induced myocardial infarcted rats.* . Journal of Biochemical and Molecular Toxicology, 2012: p. 368-373.

80. Kryvtsova, M.V., Salamon, I., Koscova, J., Bucko, D., & Spivak, M., *Antimicrobial, antibiofilm and biochemichal properties of Thymus vulgaris essential oil against clinical isolates of opportunistic infections.* Biosystems Diversity, 2019: p. 270-275.

81. Nazar, F.N., Videla, E. A., & Marin, R. H., *Thymol supplementation effects on adrenocortical, immune and biochemical variables recovery in Japanese quail after exposure to chronic heat stress.* Animal,, 2019: p. 318-325.

82. Kowalczyk, A., et al., *Thymol and thyme essential oil—new insights into selected therapeutic applications.* Molecules, 2020. **25**(18): p. 4125.

83. Mota, K.S.D.L., Pereira, F. D. O., De Oliveira, W. A., Lima, I. O., & Lima, E. D. O., *Antifungal activity of Thymus vulgaris L. essential oil and its constituent phytochemicals against Rhizopus oryzae: interaction with ergosterol.* Molecules, 2012: p. 14418-14433.

84. Kumari, P., Arora, N., Chatrath, A., Gangwar, R., Pruthi, V., Poluri, K. M., & Prasad, R., *Delineating the biofilm inhibition mechanisms of phenolic and aldehydic terpenes against Cryptococcus neoformans.* ACS omega, 2019: p. 17634-17648.

85. Boateng, J.S., Matthews, K. H., Stevens, H. N., & Eccleston, G. M., *Wound healing dressings and drug delivery systems: a review.* Journal of pharmaceutical sciences,, 2008: p. 2892-2923.

86. Rane, B.R., Patil, A. K., Keservani, R. K., Jain, A. S., & Kesharwani, R. K., *Novel Approaches in Herbal Formulations.* In Enhancing the Therapeutic Efficacy of Herbal Formulations 2021: p. 43-68.

87. Ramos, A.P., Cruz, M. A., Tovani, C. B., & Ciancaglini, P., *Biomedical applications of nanotechnology.* Biophysical reviews, 2017: p. 79-89.

88. Wang, W., Lu, K. J., Yu, C. H., Huang, Q. L., & Du, Y. Z., *Nano-drug delivery systems in wound treatment and skin regeneration.* . Journal of nanobiotechnology, 2019: p. 82.

89. Guo, S.A., & DiPietro, L. A., *Factors affecting wound healing.* Journal of dental research, 2010: p. 219-229.

90. Pachuau, L., *Recent developments in novel drug delivery systems for wound healing.* Expert opinion on drug delivery, 2015: p. 1895-1909.

91. Loo, H.L., et al., *Application of chitosan-based nanoparticles in skin wound healing.* Asian Journal of Pharmaceutical Sciences, 2022. **17**(3): p. 299-332.

92. Khan, I., Saeed, K., & Khan, I., *Nanoparticles: Properties, applications and toxicities.* . Arabian journal of chemistry,, 2019: p. 908-931.

93. Dhiman, N., Awasthi, R., Sharma, B., Kharkwal, H., & Kulkarni, G. T., *Lipid nanoparticles as carriers for bioactive delivery.* Frontiers in chemistry, 2021: p. 580118.

94. Naziris, N. and C. Demetzos, *Lipid nanoparticles as platforms for theranostic purposes: recent advances in the field.* Journal of Nanotheranostics, 2022. **3**(2): p. 86-101.

95. Mazur, F., Bally, M., Städler, B., & Chandrawati, R., *Liposomes and lipid bilayers in biosensors.* Advances in colloid and interface science,, 2017: p. 88-99.

96. dos Santos Rodrigues, B., Banerjee, A., Kanekiyo, T., & Singh, J., *Functionalized liposomal nanoparticles for efficient gene delivery system to neuronal cell transfection.* International journal of pharmaceutics, 2019: p. 717-730.

97. Bhardwaj, H., Khute, S., Sahu, R., & Jangde, R. K., *Advanced drug delivery system for management of chronic diabetes wound healing.* Current Drug Targets, 2023: p. 1239-1259.

98. Partoazar, A., Kianvash, N., & Goudarzi, R., *New concepts in wound targeting through liposome-based nanocarriers (LBNs).* . Journal of Drug Delivery Science and Technology, 2022: p. 103878.

99. Zhou, W., et al., *Glucose and MMP-9 dual-responsive hydrogel with temperature sensitive self-adaptive shape and controlled drug release accelerates diabetic wound healing.* Bioactive materials, 2022. **17**: p. 1-17.

100. Farooq, A., Iqbal, A., Rana, N. F., Fatima, M., Maryam, T., Batool, F., ... & Alrdahe, S. S. (2022), *A Novel Sprague-Dawley Rat Model Presents Improved*

NASH/NAFLD Symptoms with PEG Coated Vitexin Liposomes. International Journal of Molecular Sciences, 2022: p. 3131.

101. Torabiardekani, N., Karami, F., Khorram, M., Zare, A., Kamkar, M., Zomorodian, K., & Zareshahrabadi, Z., *Encapsulation of Zataria multiflora essential oil in polyvinyl alcohol/chitosan/gelatin thermo-responsive hydrogel: synthesis, physico-chemical properties, and biological investigations.* International journal of biological macromolecules,, 2023: p. 243, 125073.

102. Nii, T., & Ishii, F., *Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method.* International journal of pharmaceutics, (2005): p. 298(1), 198-205.