PREPARATION AND CHARACTERIZATION OF METRONIDAZOLE-LOADED FILM CONSISTING OF CHITOSAN AND ASCORBIC ACID FOR TOPICAL APPLICATION

BY

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ABSTRACT

This study presents the formulation and optimization of chitosan-based topical films incorporating ascorbic acid and metronidazole, designed as a biocompatible wound dressing with dual therapeutic action. Glycerol was used as a plasticizer to improve film flexibility and mechanical integrity. The films were fabricated using a solvent casting technique, wherein ascorbic acid functioned dually as a solvent and a stabilizing excipient—eliminating the need for conventional acidic solubilizers.

A Box–Behnken design was applied to systematically investigate the effects of chitosan, ascorbic acid, and glycerol concentrations on critical film parameters, including tensile strength, elongation at break, and surface pH. The optimized formulation was characterized through a series of analytical techniques, including Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and powder X-ray diffraction (PXRD), to evaluate its structural,

thermal, and molecular properties. Additionally, in vitro studies assessed the film's swelling behavior, drug content, and release profiles.

Results demonstrated that both glycerol and ascorbic acid significantly enhanced the film's mechanical performance via plasticization, while FTIR and thermal analyses confirmed molecular interactions and altered crystallinity within the matrix. The optimized film exhibited uniform morphology, pH compatibility for skin application, and a biphasic co-release of the incorporated bioactives—supporting its suitability for wound healing applications.

Keywords: Chitosan, Ascorbic Acid, Metronidazole, Films, Topical Delivery

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Bilawal Khan

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Terms
API	Active Pharmaceutical Ingredient
AgSD	Silver Sulfadiazine
BBD	Box-Behnken Design
BDS	Base Deactivated Silica
BeLSEs	Bioengineered Living Skin Equivalents
BFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CDPE	β-Cyclodextrin Polyester
CI	Confidence Interval
CuKα	Copper K-alpha Radiation
DAD	Diode Array Detector
DD	Degree of Deacetylation
DSC	Differential Scanning Calorimetry
E. Coli	*Escherichia Coli*
EGF	Epidermal Growth Factor
ECM	Extracellular Matrix
EDX	Energy Dispersive X-ray Spectroscopy
EMA	European Medicines Agency
ES	Electrical Stimulation
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
FFs	Film-forming solutions
GFs	Growth Factors
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
HA	Hyaluronic Acid
HBOT	Hyperbaric Oxygen Therapy
HPLC	High-Performance Liquid Chromatography
ICH	International Council for Harmonization

IGF-1	Insulin-Like Growth Factor-1
IL-1β	Interleukin-1 Beta
LL37	Cathelicidin Family of Antimicrobial Peptides
miRNAs	MicroRNA
MMPs	Matrix Metalloproteinases
MPa	Megapascal
MWCO	Molecular Weight Cut-Off
NPWT	Negative Pressure Wound Therapy
NLCs	Nanostructured Lipid Carriers
NH_2	Amino Group
NH_3^+	Ammonium Group
OR	Odds Ratio
PAA	Polyacrylic Acid
PEC	Polyelectrolyte Complexes
PEG	Polyethylene Glycol
PDGF	Platelet-Derived Growth Factor
PDGF-A	Platelet-Derived Growth Factor
PDGF-BB	Platelet-Derived Growth Factor-BB
PLA	Polylactic Acid
PGA	Polyglycolic Acid
PRP	Platelet-Rich Plasma
PSA	Pressure-Sensitive Adhesive
PVA	Polyvinyl Alcohol
PXRD	Powder X-Ray Diffraction
rhEGF	Recombinant Human Epidermal Growth Factor
SA	Sodium Alginate
S. Aureus	*Staphylococcus Aureus*
EDX-SEM	Energy-Dispersive X-ray Spectroscopy-Scanning Electron Microscopy
SI	Swelling Index
SLNs	Solid Lipid Nanoparticles
SWF	Simulated Wound Fluid

Tregs	Regulatory T cells
TGF-β	Transforming Growth Factor-Beta
TNF-α	Tumor Necrosis Factor-Alpha
UFLC	Ultra-Fast Liquid Chromatography
USP	United States Pharmacopeia
UTS	Ultimate Tensile Strength
VSCs	Volatile Sulfur Compounds
α2β1	Alpha-2 Beta-1 Integrin
αIIbβ3	Alpha-IIb Beta-3 Integrin
α-MSH	Neuropeptide α-Melanocyte-Stimulating Hormone
2FI	Two-Factor Interaction Model
3D	3-Dimensional

CHAPTER 1
INTRODUCTION

This chapter introduces the study on the preparation and characterization of metronidazole films consisting of chitosan and ascorbic acid for topical applications. It emphasizes the unique properties and advantages of the materials used—chitosan, a biopolymer known for its biocompatibility and wound-healing potential, and ascorbic acid, an essential antioxidant that promotes tissue repair. The chapter highlights the integration of metronidazole as an antimicrobial agent to enhance the therapeutic efficacy of the films. Existing knowledge gaps regarding the combined application of these materials are discussed, along with the motivations driving this research. The chapter establishes the objectives and significance of the study, focusing on how this novel formulation can advance the field of topical therapeutics by providing an innovative and effective wound care system.

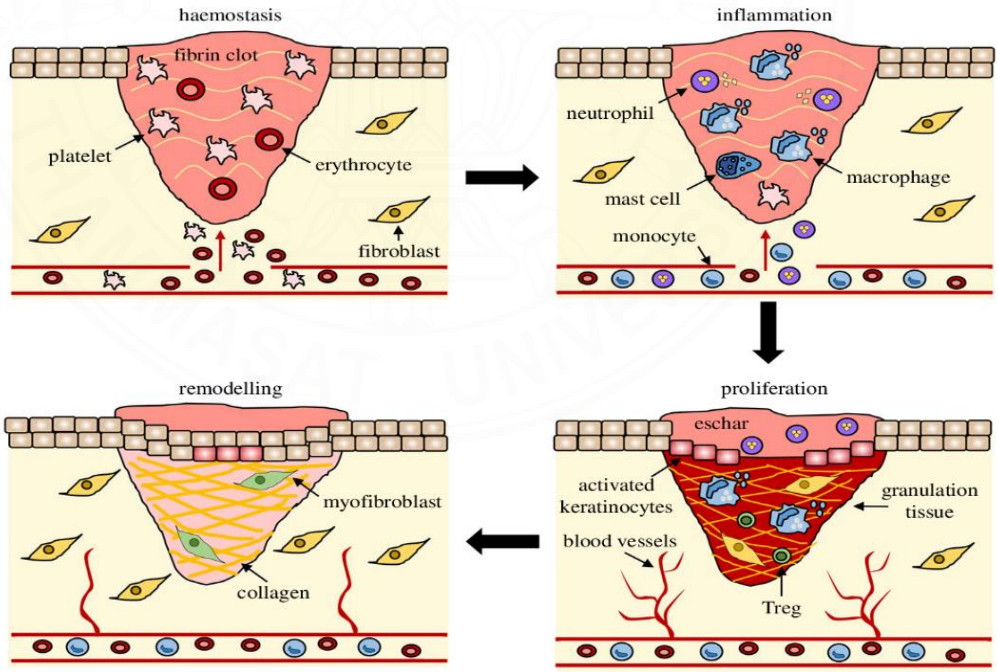
1.1 Background of Study

An intact outer layer is one of the most essential traits shared by basic bacteria and complex multicellular organisms. The largest organ in the human body, the skin, serves various purposes. The skin protects internal organs from potential environmental threats (Boateng et al., 2008). The skin's epidermis contains many vital organs, including apocrine glands, eccrine sweat glands, and hair follicles containing pilosebaceous units (Zeng et al., 2022). Due to its environmental exposure, skin is vulnerable to various external factors, which can result in multiple skin damages and injuries. A sequence of physiological processes can heal cuts and injuries because the skin has exceptional regeneration capabilities. However, this regenerative ability is occasionally compromised, causing wounds to heal slowly and putting patients' health at serious risk. Extensive burns and chronic wounds are costly and time-consuming to treat since they are prone to infection and frequently need surgery. An enormous amount of stress is placed on the healthcare system by compromised wound healing. An estimated 4.5 million Americans require care for chronic wounds, and the annual cost of managing chronic wounds is projected to be over $25 billion (Kim et al., 2019).

Though wound healing is a dynamic, intricate biological process necessary to repair damaged tissues, problems like infection, chronic inflammation, and delayed healing continue to exist, necessitating the development of novel remedies. "Disruption of normal anatomic structure and function" is the Wound Healing Society's definition of a wound, which is a break or defect in the skin brought on by thermal, physical, or physiological forces (Lazarus et al., 1994).

Figure 1.1

Hemostasis, in which platelets build an early fibrin matrix and form a plug to stop bleeding, is the first step in wound repair. Inflammation follows, which gets rid of debris and keeps infections at bay. Histamine from mast cells attracts neutrophils, which come first. Monocytes then change into tissue macrophages to remove any last bits of debris. During the proliferative phase, fibroblasts replace the fibrin clot with granulation tissue, keratinocytes migrate to seal the wound, and angiogenesis creates new blood vessels. This process is significantly aided by macrophages and regulatory T cells (Tregs). Ultimately, the remodelling phase results in tissue healing as fibroblasts continue to modify the matrix, blood vessels recede, and myofibroblasts assist in contracting the wound.



Note: From Wilkinson, H. N., & Hardman, M. J. (2020). Wound healing: cellular mechanisms and pathological outcomes. *Open Biology, 10*.

Acute and chronic wounds are categorized based on the degree of tissue damage and the healing process. Acute wounds, typically resulting from mechanical

injuries, abrasions, surgical incisions, or burns, heal within 8 to 12 weeks, often with minimal scarring (Percival, 2002). Chronic wounds, on the other hand, which do not heal within this time and frequently recur, are commonly associated with underlying medical disorders such as diabetes, obesity, and persistent infections, in addition to receiving insufficient care (Harding et al., 2002; Saghazadeh et al., 2018). These persistent issues disrupt the normal sequence of healing stages. The four main phases of wound healing are remodelling, proliferation, inflammation, and hemostasis. Figure 1.1 details all four healing stages.

Natural microflora, a delicate ecology of bacteria that usually coexist peacefully to preserve skin health, is found in human skin. However, injuries can upset this equilibrium, resulting in infections and microbial overgrowth that slow wound healing by triggering a series of tissue-damaging and inflammatory reactions. Increased pro-inflammatory cytokines and matrix metalloproteases (MMPs) cause tissue breakdown, inflammatory mediator synthesis, and toxin release in infected wounds, which impede the healing process's progression to the proliferative phase. Chronic lesions like diabetic foot ulcers or venous leg ulcers are especially challenging to treat because systemic antibiotic treatment is hampered by poor peripheral circulation and underlying conditions like diabetes. This emphasizes how urgently localized treatments that address the wound microenvironment directly, including topical applications, are needed. Topical administration systems effectively address infection and modulate the local inflammatory response, thereby maintaining therapeutic concentrations at the site, reducing systemic side effects, and improving wound healing.

Antimicrobial medicines, which treat infections and foster an environment that promotes tissue regeneration, are now essential in contemporary wound care. These days, advanced wound dressings with antimicrobial qualities offer localized distribution that guarantees high concentrations of active ingredients at the wound site. The systemic adverse effects of oral or intravenous antibiotics, like nausea, diarrhoea, and allergic responses, are reduced by this targeted strategy. In general, these dressings are classified as antibiotics and antiseptics (Boateng et al., 2016). Although antiseptic dressings have broad-spectrum effectiveness against bacteria, fungi, and viruses, they must be used carefully to prevent cytotoxicity. Antibiotic dressings, on the other hand, minimize damage to host cells while managing exudate, creating a moist wound

environment, and specifically targeting microorganisms (Lipsky & Hoey, 2009). These dressings avoid the drawbacks of systemic antibiotics by administering localized, regulated dosages, especially when it comes to treating resistant germs to drugs and facilitating efficient therapy. A significant advance in wound care is the creation of antimicrobial wound dressings, which balance cost-effectiveness, safety, and efficacy to satisfy clinical requirements. Therefore, these dressings significantly advance in treating individuals with infected chronic wounds.

Metronidazole, a nitroimidazole-class antimicrobial agent, has been a cornerstone in the treatment of anaerobic infections for over four decades due to its broad-spectrum efficacy against obligate anaerobes and protozoa (Dingsdag & Hunter, 2018). Its five-membered imidazole ring, featuring a nitro group at position five and a hydroxyl-ethyl side chain at position one, is essential to its mechanism of action. Under anaerobic conditions, the nitro group undergoes enzymatic reduction, generating cytotoxic free radicals that disrupt Deoxyribonucleic acid (DNA) synthesis, induce strand breakage, and lead to bacterial cell death (Edwards, 1979). This selective activation in hypoxic environments, such as infected chronic wounds, makes metronidazole particularly valuable in wound care, where anaerobic bacteria proliferate and contribute to delayed healing and malodor.

Chronic wounds, such as diabetic foot ulcers, pressure sores, venous leg ulcers, ischemic wounds, and fungating tumors, often become colonized or infected with anaerobic bacteria, leading to persistent inflammation, necrosis, and delayed epithelialization (Peng & Dai, 2020). One of the most distressing complications associated with chronic wounds is malodor, primarily resulting from the metabolism of anaerobic bacteria, which produce volatile sulfur compounds (VSCs) like hydrogen sulfide, dimethyl sulfide, and methyl mercaptan (Ito et al., 2023). These foul-smelling byproducts significantly impact patients' quality of life, leading to social isolation, psychological distress, and reduced self-esteem (Castro & Santos, 2015). Malodors are particularly prevalent in necrotic wounds, where anaerobic species such as *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Prevotella*, and *Clostridium perfringens* thrive in low-oxygen conditions and break down sulfur-containing amino acids into putrefactive gases. Addressing wound malodor is, therefore, a key objective in the management of

chronic wounds, necessitating effective antimicrobial strategies that target both infection control and odor reduction (Paul & Pieper, 2008).

Metronidazole's dual action—antimicrobial and anti-inflammatory—makes it an ideal candidate for incorporation into a topical wound-healing system. As a highly potent anaerobic bactericidal agent, metronidazole effectively eliminates malodorous bacterial populations, leading to rapid and sustained odor reduction in infected wounds (Freeman et al., 1997). This property is particularly beneficial for patients with fungating tumors, diabetic foot ulcers, or ischemic wounds, where anaerobic bacterial overgrowth results in putrid-smelling exudates. Multiple clinical studies have demonstrated that topical metronidazole application significantly reduces wound malodor within 48 hours, with effects persisting for several days post-application, highlighting its strong therapeutic potential (Castro & Santos, 2015; Patel et al., 2018; Paul & Pieper, 2008; Peng & Dai, 2020; Watanabe et al., 2016).

Beyond its antimicrobial efficacy, metronidazole exhibits anti-inflammatory properties, modulating the immune response in chronic wounds. By suppressing neutrophil recruitment and inhibiting excessive cytokine production, it helps in controlling wound inflammation, which is a significant barrier to effective healing (Suárez et al., 2024). Chronic wounds often remain stuck in a prolonged inflammatory phase, characterized by excessive MMPs activity and delayed tissue formation, further complicating the healing process. Metronidazole's ability to reduce inflammation while eradicating anaerobic pathogens positions it as a critical agent for promoting tissue repair and epithelialization (Schilrreff & Alexiev, 2022).

Systemic administration of metronidazole, although effective, is often associated with significant side effects, including gastrointestinal disturbances, neurotoxicity, and potential drug interactions (Hernández Ceruelos et al., 2019). Topical application, on the other hand, ensures localized drug action, providing high concentrations at the wound site while minimizing systemic absorption and adverse effects. This makes topical metronidazole particularly suitable for long-term wound management, especially in patients with comorbidities that may limit the use of systemic antibiotics.

Given these properties, metronidazole has been chosen as the model drug for incorporation into a bioadhesive wound-healing film designed to provide targeted

drug release, enhanced retention at the wound site, and improved therapeutic efficacy. By ensuring direct contact with the wound surface, this advanced drug delivery system aims to prevent secondary infections, control inflammation, and optimize the wound environment, leading to accelerated epithelialization and enhanced tissue regeneration (Löfmark et al., 2010). This formulation will ultimately contribute to faster and more effective wound healing.

Polymeric films offer a means of delivering metronidazole directly to the infection, ensuring sustained antimicrobial activity and minimizing systemic exposure. Incorporating metronidazole into innovative delivery systems is an essential advancement in wound care, in line with contemporary treatment approaches emphasizing patient-centered care, safety, and efficacy. Glycerol, a trihydroxy alcohol, is widely used as a plasticizer to increase flexibility and decrease the fragility of chitosan-based films significantly. Beyond its plasticization capacity, glycerol is widely prized for its non-toxic, biocompatible, and biodegradable qualities, making it a popular option for biomedical and ecological applications. In addition to ensuring uniform integration and optimal film performance, its hygroscopic property helps preserve the film's moisture level, reducing brittleness over time. It is compatible with polysaccharides such as chitosan. The importance of glycerol in enhancing the mechanical and functional characteristics of polymeric films is highlighted by these characteristics (Smith et al., 2021).

Numerous natural and artificial polymers have been investigated for use as wound dressings. Because of their durability and versatility, synthetic materials such as polyurethane (Khodabakhshi et al., 2019) and polyethylene glycol (Borges-Vilches et al., 2021) are commonly utilized. Natural polymers, such as Alginate (Ahmed et al., 2021; Borges-Vilches et al., 2021; Firlar et al., 2022; Khodabakhshi et al., 2019; Varaprasad et al., 2020), Chitosan (Boateng et al., 2023; Genesi et al., 2023), Collagen (Wang et al., 2023), Hyaluronic acid (Catanzano et al., 2017), and Bacterial Cellulose (Amorim et al., 2024), are also popular choices. These natural materials offer several desirable properties, including high biocompatibility and biodegradability, which make them safe and eco-friendly. They also often display excellent moisture retention, a vital characteristic for promoting successful tissue regeneration. Because natural polymer-based films, such as chitosan, may interact with cells and cellular enzymes and are

biodegradable, they are attractive for drug delivery methods because they stimulate tissue growth (Li et al., 2020).

The second most prevalent natural polysaccharide, chitin, is the source of the polymer chitosan. Insect exoskeletons and crab shells are the primary sources of chitin, whereas chitosan is mainly produced by deacetylating chitin. Furthermore, the cell walls of some fungus, such as *Mucor rouxii*, can also provide chitosan (Mi et al., 2001). Chitosan is a polymer composed of N-Acetyl-2-Amino-2-Deoxy-D-Glucosamine and 2-Amino-2-Deoxy-D-Glucosamine monomers joined by β-(1-4) glycosidic linkages. Its polyamine structure makes it highly sensitive to pH, allowing it to dissolve in acidic conditions, acquire a positive charge, and exhibit bioadhesive properties (Demir et al., 2010). Solubility, crystallinity, biodegradability, and several biological activities such as antiallergenic, anticoagulant, antioxidant, biocompatible, hemostatic, analgesic, anticholesterolemic, antitumor, antibacterial, and bactericidal properties are some of the well-known properties of chitosan (Boateng et al., 2023; Demir et al., 2010; Genesi et al., 2023; Li et al., 2020; Wittaya-areekul & Prahsarn, 2006).

Chitosan's amino group (NH_2) becomes protonated to NH_3^+ when it encounters an acidic environment, allowing for electrostatic interactions with anionic groups. It is appropriate to create films based on chitosan because of this characteristic. The chitosan's molecular weight and degree of deacetylation affect these films' mechanical strength and barrier qualities (Zeng et al., 2022). Although Chitosan films are thin, they have important qualities like hardness, elasticity, strength, and flexibility, making them ideal for various uses. Chitosan is used in cosmetics, food, pharmaceuticals, and biomedicine (Mi et al., 2001). The Food and Drug Administration has authorized chitosan-based wound dressings (FDA) (Wedmore et al., 2006).

In acidic solutions, the amino groups in chitosan get protonated, which gives it a positive charge when solubilized in some acidic solvents like acetic acid, lactic acid, citric acid, and hydrochloric acid (Qiao et al., 2021). Although acetic acid is the conventional solvent for dissolving chitosan, ascorbic acid has emerged in food science and cosmetics as a noteworthy alternative, esteemed for its enhanced biocompatibility and reduced toxicity (Liping et al., 2020; Tan et al., 2019). Despite these advantages, its application in pharmaceutical sciences, particularly in drug

delivery systems, has yet to be fully explored. With its antioxidant properties and excellent compatibility with biological systems, ascorbic acid offers a promising avenue for developing safer, more effective chitosan-based drug delivery systems, potentially enhancing both therapeutic outcomes and patient compliance.

Ascorbic acid, an important water-soluble vitamin, is essential in human nutrition (Patel et al., 2018). Ascorbic acid is a well-known antioxidant with many uses in the food, cosmetic, and pharmaceutical industries. It can scavenge free radicals, increase collagen manufacturing, reduce melanin, give photo-protection, and boost immunity. As a potent antioxidant, ascorbic acid promotes collagen synthesis, fibroblast migration, and growth, all essential for wound healing (Moores, 2013). Since humans cannot produce ascorbic acid naturally, it must be obtained from dietary sources such as citrus fruits and vegetables (Meng et al., 2017). Combining ascorbic acid with chitosan, a biopolymer widely recognized for its biocompatibility and biodegradability, offers significant pharmaceutical advantages. This formulation not only enhances the stability of ascorbic acid but also facilitates the solubility of chitosan in aqueous environments by forming water-soluble chitosan ascorbate, which is particularly beneficial for pharmaceutical applications such as drug delivery systems. Organic solvents like acetic acid, which might cause toxicity or other problems, are unnecessary when using ascorbic acid as a solvent for chitosan. Additionally, ascorbic acid and chitosan work synergistically to produce a dual antioxidant action that improves the formulation's effectiveness (Ghahremani-Nasab et al., 2023).

One of the most significant problems with topical products, such as creams and gels, is that they don't stay in place for long, which makes it hard to keep the right amount of medication on the skin's surface. Patient annoyance and decreased compliance may result from these items' frequent messiness and propensity to leak (Dobaria et al., 2009). Additionally, traditional dressings like gauze and cotton wool have drawbacks since they cannot sustain the moist environment necessary for the best possible skin health and recovery (Boateng et al., 2008). Advanced topical dressings, on the other hand, like film-based formulations, have been created to address these problems by establishing a moist, regulated environment that promotes skin healing (Hanna & Giacopelli, 1997; Wittaya-areekul & Prahsarn, 2006). These advanced systems also facilitate gas exchange, protect against contamination and physical

damage, and prevent excessive moisture buildup or drying that could damage the skin. Furthermore, they are designed for easy removal without causing additional irritation or trauma to the skin (Boateng et al., 2008; Hanna & Giacopelli, 1997; Wittaya-areekul & Prahsarn, 2006). Even though synthetic polymers and advanced dressings have shown promise in resolving these issues, issues like biocompatibility and biodegradability still exist. Because of their bioactivity, natural polymers like chitosan are especially appealing for topical preparations; nevertheless, their limited practical applicability in this context is caused by their poor solubility and dependence on hazardous cross-linking agents. Furthermore, because systemic antibiotics frequently ineffectively target the skin, topical treatments continue to struggle with the need for efficient, targeted antimicrobial treatment. Even though contemporary antimicrobial medicines have advanced, balancing long-term use, safety, and efficacy is challenging. Combining chitosan with ascorbic acid in topical preparations is a viable way to address these issues. Ascorbic acid has potent antioxidant qualities, promotes collagen synthesis, and acts as a solvent for chitosan, which may increase the formulation's overall efficacy. Both increased therapeutic advantages and bioactivity may result from this combination. Furthermore, adding metronidazole to this formulation can release therapeutic substances gradually and under control, promoting tissue regeneration and providing efficient infection control. Despite the potential of multifunctional topical formulations that include these ingredients, their use has not yet been investigated. This study aims to develop and analyze metronidazole-loaded chitosan-ascorbic acid films with an emphasis on their potential for topical applications to provide a local and targeted delivery of active pharmaceutical ingredients (APIs) to the site of action.

1.2 Statement of the Problem

Wound healing remains a significant clinical issue, particularly in chronic wounds that are often associated with peripheral circulation impairment and diabetes. Even with advancements in wound care technologies, standard topical therapies—such as creams, gels, and traditional dressings—often fall short of meeting the complex needs of wound treatment. These formulations usually don't stay in contact with the wound site for long, which results in lower therapeutic results. Furthermore, although they work well in some situations, standard dressings can't always create a moist

environment for optimal tissue regeneration. They tend to come loose during the healing process, which prolongs recovery and raises the risk of infection.

In addition to these drawbacks, infections often exacerbate chronic wounds, causing unpleasant smells, heightened inflammation, and slowed healing. Poor tissue penetration makes standard systemic antibiotic therapies frequently ineffective, particularly in patients with vascular diseases that are impaired. Although contemporary antimicrobial dressings aim to prevent infection, they struggle to balance antimicrobial efficacy, biocompatibility, and long-term safety. Furthermore, many of the components used in wound care products nowadays are synthetic, which may cause problems with biodegradability and long-term compatibility with the healing tissue. These elements highlight the necessity for better topical treatments that can safely and sustainably promote wound healing, reduce odor, and manage infections.

Chitosan is a naturally occurring polymer that has drawn much interest as a possible component for wound dressings because of its inherent antibacterial qualities, biocompatibility, and biodegradability. Its poor water solubility, which necessitates acidic conditions for dissolution, sometimes limits its practical application. Although ascorbic acid can create an acidic environment in aqueous solutions, the concentration of ascorbic acid, the molecular weight and degree of deacetylation of chitosan, and the stability of ascorbic acid throughout the process all affect how well it dissolves chitosan. Metronidazole is a well-known antibacterial that works exceptionally well against anaerobic bacteria frequently linked to infected wounds and their foul smells. A topical formulation of metronidazole may control infections locally without having the systemic adverse effects of traditional antibiotic therapies. Creating a stable, bioactive, and efficient wound-healing film that combines metronidazole, ascorbic acid, and chitosan is still a relatively untapped topic in wound care. This work aims to close this gap by creating chitosan-ascorbic acid films loaded with metronidazole for topical wound healing applications.

1.3 Research Objectives and Scope

The main goals of this study are to develop and characterize metronidazole films that contain chitosan and ascorbic acid for topical use. This study aims to:

- Fabricate metronidazole loaded film consisting of chitosan and ascorbic acid.
- To characterize the mechanophysical properties of the film.
- To assess the release characteristics of metronidazole and ascorbic acid.

1.4 Significance of Study

The proposed study aims to contribute to the development of advanced topical drug delivery systems by formulating and characterizing chitosan-ascorbic acid films loaded with metronidazole. The need for effective drug delivery systems for topical applications, particularly in infection-prone wounds, necessitates innovative formulation strategies. This study focuses on:

- **Innovative Topical Application:** A tailored approach to wound care is offered by creating a bioactive film composed of chitosan, ascorbic acid, and metronidazole for direct topical application.
- **Comprehensive Characterization**: Advanced analytical techniques, including Fourier Transform Infrared Spectroscopy (FTIR), Powder X-ray Diffraction (PXRD), and Differential Scanning Calorimetry (DSC), will be employed to investigate the films' molecular interactions, crystallinity, and thermal stability. These insights are crucial for understanding the behavior of the materials and ensuring the films' suitability for topical application. The study will also assess swelling behavior and release profiles to optimize the films for controlled and sustained drug delivery.
- **Advancing Chitosan-Based Drug Delivery Systems:** This research contributes to the broader field of drug delivery by providing valuable insights into the formulation and optimization of chitosan-based films. By exploring the interactions between chitosan, ascorbic acid, and metronidazole, the study advances knowledge on the design of multifunctional materials for topical applications.

CHAPTER 2
LITERATURE REVIEW

The development of topical drug delivery systems largely depends on incorporating novel formulation techniques and materials to address issues such as low bioavailability, unpredictable release patterns, and poor drug stability. Chitosan and other natural biopolymers, when paired with bioactive substances like metronidazole and ascorbic acid, have shown great promise in creating efficient localized therapy systems. This chapter examines the function of cutting-edge materials and their revolutionary potential in topical medication delivery, concentrating on metronidazole, chitosan, and ascorbic acid. The chapter also explores the various applications of these substances in film-based formulations, emphasizing their stability, efficacy, and compatibility in developing controlled release systems. To give a thorough grasp of the science underlying Chitosan-based topical drug delivery systems and their prospective uses in enhancing therapeutic results, this literature review will examine these materials and their formulation techniques in detail.

2.1 Challenges and Innovations in Topical Drug Delivery Systems

In healthy people, the process of wound closure is intricate and tightly controlled. Deficits in this process, however, lead to the occurrence of chronic wounds, which impact an estimated 40 million individuals worldwide. This expanding health issue has escalated to epidemic proportions, placing a significant financial burden on both national economies and healthcare systems. According to the 2016 "Global Wound Care Market" research, the sector was worth about $18 billion. Furthermore, they predicted that by 2023, this amount would rise to approximately $26 billion worldwide (Weller et al., 2020). Systemic therapy for wound healing has been the subject of several investigations, highlighting its advantages and disadvantages. Antiseptic techniques are a more practical choice for treating or stopping bacterial development in wounds since systemic antibiotics, frequently used to control symptoms and prevent infections, often find it difficult to successfully penetrate wound biofilms (Daeschlein, 2013). Research on systemic delivery of peptides and antibodies, like infliximab (anti-TNF-α), has demonstrated potential in treating ulcerating necrobiosis lipoidica

(Basoulis et al., 2016). In contrast, the neuropeptide α-melanocyte-stimulating hormone has restorative advantages, such as better collagen organization and decreased scar formation (de Souza et al., 2015). Similarly, the exenatide hormone administered systemically has improved fibroblast functions and has had a good effect on metabolic, inflammatory, and healing markers (Wolak et al., 2019). Additionally, amino acids such as proline and N-acetyl cysteine have effectively accelerated wound healing, frequently surpassing their local use (Aydin et al., 2019). However, the difficulties in delivering targeted tissue and the possibility of off-target negative effects limit these systemic techniques. Therefore, the best way to treat chronic wounds is increasingly acknowledged as the focused delivery of bioactive chemicals directly to the wound site.

According to O'Meara et al.'s review, *"Antibiotics and antiseptics for venous leg ulcers,"* topical medications are widely used in wound care, especially for chronic wounds that are frequently colonized or infected by bacteria. They draw attention to how systemic antibiotics have a limited ability to penetrate wound biofilms, which makes topical treatments more successful. Antibiotics like gentamicin, tetracycline, and mupirocin, as well as antiseptics like iodine, chlorhexidine, and silver, are often used substances and have been shown to have significant advantages. Furthermore, natural substances with regenerative, angiogenic, and antimicrobial qualities—like honey, curcumin, aloe vera, and rosemary oil—have also shown promise in the treatment of wound healing (O'Meara et al., 2010).

Growth factors (GFs), which are physiologically active peptides that control cell migration, differentiation, and development, are essential for wound healing at every stage. GFs have demonstrated considerable skin healing advantages without noticeable negative effects when applied topically, such as gels, lotions, or injections. The following GFs are frequently researched: transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GMCSF), basic fibroblast growth factor (BFGF), and epidermal growth factor (EGF) (Nour et al., 2019). Notably, PDGF-BB (Regranex®) is food and drug administration (FDA) (1997) and European Medicines Agency (EMA) (2003) approved for wound healing, while BFGF (Fiblast®) has been used clinically in Japan since 2001 for pressure ulcers (Otero-Viñas & Falanga, 2016). Autologous platelet-rich plasma (PRP), rich in GFs, has also demonstrated promising results in enhancing

healing rates. However, sustained release systems are essential for effective wound repair due to their short half-life and susceptibility to enzymatic degradation (Nour et al., 2019).

Therapeutics can be delivered locally and sustainably via encapsulation into micro and nanocarriers, frequently using core-shell architectures with the therapeutic substance at the center. For example, liposomes have been investigated as protective carriers for treating persistent wounds. By encouraging angiogenesis, a liposome containing BFGF and a silk fibroin hydrogel core was created by Xu et al. to speed up wound healing in rats (Xu et al., 2017). Transfersomes, a new generation of ultra-flexible lipid vesicles, offer enhanced stability and penetration through the skin's stratum corneum. Choi et al. dramatically increased wound closure rates in diabetic mice by conjugating protamine to the N-termini of growth factors and complexing them with hyaluronic acid (HA) using transfersomes (Choi et al., 2017). Similarly, transfersomes loaded with baicalin demonstrated complete skin restoration in a mice model by reducing inflammation markers like IL-1β (interleukin-1 Beta) and TNF-α (tumor necrosis factor-alpha) (Manconi et al., 2018).

Lipid, polymeric, and inorganic nanoparticles have also been thoroughly investigated for wound healing. Antibiotics and antimicrobial peptides, such as amphotericin B, norfloxacin, and LL37, have been delivered using polymeric nanoparticles, which have antibacterial action and improve healing (Chereddy et al., 2014; Dave et al., 2017; Sanchez et al., 2014). Inorganic nanoparticles (e.g., copper, zinc oxide, silver, titanium dioxide) and graphene oxide are employed for their antimicrobial properties and ability to penetrate skin cells (Bui et al., 2017). Lipid-based carriers, such as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs), protect growth factors from enzymatic degradation and ensure sustained drug release. Notably, recombinant human epidermal growth factor (rhEGF)-loaded SLNs and NLCs improved wound healing in diabetic mice (Gainza et al., 2015) and a porcine wound model by increasing wound closure, collagen deposition, microvasculature formation, and fibroblast proliferation (Gainza et al., 2014). These findings highlight the potential of advanced nanocarriers for wound management.

The review *"Delivery systems of current biologicals for the treatment of chronic cutaneous wounds and severe burns"* by Xue et al. (2018) covered new

approaches to boosting the presence of growth factors and cytokines in wound healing. The delivery of genes encoding these mediators is one such tactic, accomplished by transferring genetic material using viral or non-viral vectors, frequently by intralesional injections. The review also emphasized the function of miRNAs (micro ribonucleic acids), such as miR-99, miR-126, miR-132, miR-155, miR-184, miR-198, miR-203, miR-205, and miR-210, which control the migration, differentiation, and proliferation of skin cells like keratinocytes and endothelial cells, thereby facilitating more effective wound healing (Xue et al., 2018).

Local therapy is the most popular method for managing chronic wounds because of its focused impact and minimal systemic adverse effects. Physical therapies that promote angiogenesis and collagen production and reduce inflammation have significantly improved wound healing. These include debridement (Falanga et al., 2008), compression therapy (Nelson & Bell-Syer, 2014), negative pressure wound therapy (NPWT) (Lalezari et al., 2017), electrical stimulation (ES) (Kloth, 2014) Hyperbaric oxygen therapy (HBOT) (Thom, 2009), photobiomodulation, and ultrasounds (Cooper & Bachoo, 2018). However, many of these approaches lack solid data or defined standards, so further study is needed before they can be widely used.

Dressing therapies are crucial for wound healing because they regulate drug release, moisture retention, wound protection, and support for cell migration, extracellular matrix deposition, and tissue regeneration. They are made from synthetic materials, such as polyglycolic acid (PGA), polylactic acid (PLA), polyvinyl alcohol (PVA), and natural components, such as hyaluronan, collagen, and chitosan. Films, non-cellular scaffolds, bioengineered living skin equivalents (BeLSEs), and scaffolds containing stem cells are the four types of treatments that have been made possible by significant advancements in non-cellular scaffold-based procedures (Las Heras et al., 2020).

Broussard et al., in their review article *"Wound dressings: selecting the most appropriate type,"* described films as thin, elastic, and transparent sheets of biopolymers that allow gas and water vapor permeability while blocking fluids and microorganisms. They highlighted key advantages, including wound visibility, possibilities for drug loading, and adaptability as primary or secondary dressings. However, they also pointed out that a limited swelling capacity can lead to an

accumulation of excess exudate and make wound treatment more difficult (Broussard & Powers, 2013).

In their work *"Development and in vitro evaluation of chitosan–polysaccharides composite wound dressings,"* Wittaya-areekul et al. created chitosan-based films that were combined with dextran and cornstarch to increase their strength and flexibility and with polypropylene glycol to improve their elasticity. Including cornstarch and dextran caused the film's high liquid adsorption to diminish, but their vapor and oxygen permeability increased. The mixtures increased elasticity, but bio adhesiveness remained constant across all formulations. Blending chitosan with polysaccharides and polypropylene glycol improves the quality of wound dressings, increasing their efficacy for wound care, according to the study's findings. (Wittaya-areekul & Prahsarn, 2006).

The study *"Collagen hydrogel with multiple antimicrobial mechanisms as anti-bacterial wound dressing"* by Wang et al. created a hydrogel that uses recombinant collagen, oxygen-carrying liposomes, photothermal and photodynamic therapies, and antimicrobial peptides to fight multidrug-resistant bacteria and encourage wound healing. The hydrogel offered a multipurpose method of treating chronic wounds by efficiently addressing hypoxia, boosting antibacterial activity, and promoting tissue healing (Wang et al., 2023).

In *"Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing,"* Mi et al. generated an asymmetric chitosan membrane using the phase-inversion immersion-precipitation process. The membrane's microporous sublayer allows liquids to drain, while its dense top layer protects against microbes. Its characteristics, which include hemostatic effects, superior oxygen permeability, and regulated water loss, encourage quick wound healing. Histological examination revealed ordered collagen deposition and improved epithelialization, suggesting its potential as a successful wound dressing (Mi et al., 2001).

Chitosan films that contain copaiba oil, aloe vera, or both were created by Genesi et al. in their paper *"Aloe vera and copaiba oleoresin-loaded chitosan films for wound dressings: microbial permeation, cytotoxicity, and in vivo proof of concept,"* to improve the effectiveness of wound healing. At ideal concentrations (2% chitosan with 0.5% aloe vera and/or 0.5% copaiba oleoresin), the casting-prepared films showed no

cytotoxicity or microbiological penetration. Films infused with copaiba and aloe vera enhanced cell proliferation and demonstrated better vascular development and epithelialization in vivo tests than commercial dressings. This study emphasizes how chitosan, aloe vera, and copaiba oil can work in concert to promote wound healing (Genesi et al., 2023).

2.2 Applications of Chitosan, Ascorbic Acid, and Metronidazole in Topical Drug Delivery

The four distinct phases of the wound-healing process—hemostasis, inflammation, proliferation, and remodeling—all benefit from using chitosan and its derivatives. Chitosan effectively contributes to wound closure and tissue regeneration by promoting bacterial clearance during the inflammatory phase, accelerating granulation tissue formation during the proliferation stage, and facilitating hemostasis through platelet aggregation and fibrin stabilization. This highlights chitosan's potential as a biomaterial in wound healing applications (Feng et al., 2021).

According to Leonhardt et al., *"Absorbable Hemostatic Hydrogels Comprising Composites of Sacrificial Templates and Honeycomb-Like Nanofibrous Mats of Chitosan"*, chitosan plays a crucial part in various hemostatic applications. Utilizing β-cyclodextrin polyester (β-CD) hydrogels as biodegradable templates, the research creates honeycomb-like chitosan mats made of nanofibers (9.2 ± 3.7 nm in diameter), greatly expanding the material's surface area for improved hemostatic effectiveness. These hydrogels based on chitosan outperform commercial absorbable dressings in vivo, resulting in decreased blood loss, quicker hemostasis, and increased biocompatibility. According to the research, chitosan's unique qualities—such as its nanostructured shape and biocompatibility—are essential for enhancing clot formation and promoting wound healing (Leonhardt et al., 2019).

In their work *"The Modulation of Platelet Adhesion and Activation by Chitosan Through Plasma and Extracellular Matrix Proteins,"* Lord et al. shows that chitosan facilitates wound healing by improving platelet adhesion and activation, which are essential processes in the creation of thrombi. The alpha 2 beta 1 Protein (α2β1) integrins mediate collagen attachment, while plasma and extracellular matrix proteins mostly use αIIbβ3 integrins to mediate these effects. With the help of proteins like

fibrinogen and perlecan, chitosan also raises platelet activation indicators, including αIIbβ3 and P-selectin. Similar to chitosan or collagen alone, collagen-coated chitosan stimulates platelets, demonstrating chitosan's function in promoting clot formation and wound closure (Lord et al., 2011).

Wounds create an environment that is favorable for the growth of bacteria, and wound healing includes processes such as hemostasis, inflammation, and re-epithelialization. The importance of antibacterial wound dressings stems from the immune system's failure to eliminate microorganisms, which result in bacterial infections. By chelating metallic cations, breaking down bacterial cell walls and membranes, interacting with intracellular targets, and depositing on bacteria, chitosan, commonly used to treat wounds, has antibacterial qualities. These steps increase how well it works to stop infection and promote recovery (Li, Bai, et al., 2019). Bacteria are classified as Gram-negative or Gram-positive based on their Gram stain. Gram-negative bacteria have a cell wall comprising a peptidoglycan layer and an outer membrane. The outer membrane contains lipopolysaccharides and phospholipids, which give the bacteria a negative charge. Gram-positive bacteria have a cell wall made of peptidoglycans and teichoic acids, also carrying a negative charge due to carboxyl and phosphate groups in the teichoic acids. The NH_2 groups of high molecular weight chitosan are protonated to create NH_3^+ cations when they dissolve in acidic liquids. By interacting electrostatically with the negatively charged elements on bacterial cell membranes, these cations upset the equilibrium between the production and disintegration of cell walls. This imbalance causes membrane deformation, rupturing under osmotic pressure, and leading to cell lysis (Feng et al., 2021).

Ascorbic acid (vitamin C) enhances wound healing by promoting collagen synthesis and reducing oxidative stress. *"The Effects of Vitamin C on Wound Healing—Systematic Review*" by Thevi et al. (2024) found that vitamin C supplementation significantly improved recovery, with a 3.94-fold higher likelihood of faster healing compared to a placebo (OR 3.99, 95% CI 2.06 to 7.73). While it accelerates tissue regeneration, it does not significantly affect pain levels. The evidence is of good quality, but more large-scale trials are needed. Vitamin C is a safe, cost-effective adjunct for wound healing, particularly in patients with delayed recovery or weakened immune systems (Thevi et al., 2024).

A comprehensive review by Bechara et al. (2022), titled "*A Systematic Review on the Role of Vitamin C in Tissue Healing,"* analyzed 18 studies examining the association between vitamin C supplementation and healing outcomes. The findings highlighted that vitamin C improved healing, particularly in pressure ulcers, although small sample sizes, combined nutritional interventions, and lack of baseline vitamin C measurements limited the studies. Larger-scale research focusing solely on vitamin C is needed to clarify its role in other wound types. However, to promote healing, vitamin C supplementation is advised for patients with pressure ulcers (Bechara et al., 2022). Ascorbic acid, another name for vitamin C, is necessary to produce collagen and for wound healing.

Strong antibacterial qualities make metronidazole an important antibiotic for wound healing, as it works against aerobic and anaerobic bacteria. It is a recommended treatment for anaerobic infections because it is effective against gram-positive and gram-negative bacteria. The effectiveness of metronidazole-loaded poly (3-hydroxybutyrate)/gelatin nanofibrous scaffolds in promoting wound healing is demonstrated in the study *"Metronidazole Topically Immobilized Electrospun Nanofibrous Scaffold: Novel Secondary Intention Wound Healing Accelerator"* by El-Shanshory et al. These scaffolds demonstrated rapid tissue regeneration, antibacterial action, and sustained drug release. In vivo tests showed that they were better at promoting the creation of granulation tissue and reducing inflammation, with 99.83% of wounds closing in 14 days as opposed to 87.39% with sterile fabric. Metronidazole administration by nanofibers targets local infection and promotes tissue regeneration, providing a promising method for quick and efficient wound healing (El-Shanshory et al., 2022).

The beneficial effect of metronidazole in enhancing wound care is highlighted in the study *"Effect of Metronidazole Combined with Autolytic Debridement for the Management of Malignant Wound Malodor"* by Peng et al. When compared to silver sulfadiazine-based therapy, the combination of metronidazole and autolytic debridement dramatically decreased malodor in patients with malignant wounds, with better results shown on days 3 and 12 after treatment. This method improved the patient's quality of life by reducing the stigma brought on by wound odor

in addition to controlling infection. These results show how well metronidazole improves wound care and deal with odor-related issues (Peng & Dai, 2020).

Sarheed et al. created sodium alginate hydrogels containing metronidazole for wound healing in their study *"An Investigation and Characterization on Alginate Hydrogel Dressing Loaded with Metronidazole Prepared by Combined Inotropic Gelation and Freeze-Thawing Cycles for Controlled Release."* They used Ca^{+2} cross-linking and freeze-thawing cycles to improve swelling and prolonged drug release. By decreasing wound size and encouraging epithelialization, the tailored hydrogel (6% sodium alginate, 1% metronidazole) enhanced wound healing in vivo, surpassing controls and traditional therapies. These results support the use of metronidazole in formulations based on biopolymers by demonstrating its effectiveness in antimicrobial wound dressings (Sarheed et al., 2015).

2.3 Development and Characterization of Chitosan-Based Films for Topical Applications

Adhesives, membranes, and polymeric films have become effective drug-delivery methods that transport therapeutic substances straight to the target tissues. These systems offer targeted treatment with the potential for both topical and systemic effects, and they can be applied to the skin or mucosal surfaces, such as the oral, vaginal, and ocular canals. Polymeric films have the following benefits: regulated release, minimal variations in plasma drug concentration, low toxicity, and avoidance of pre-systemic metabolism (Al Hanbali et al., 2019). Films' ability to stick to mucosal tissues, known as mucoadhesive qualities, is beneficial for drug administration since it improves medication release at the site of injury and serves as a barrier to stop the growth of microorganisms (Kianfar et al., 2012; Sizílio et al., 2018). Chitosan is a unique natural biopolymer ideal for developing topical drug delivery systems because of its film-forming, biodegradable, and biocompatible properties. Active ingredients like ascorbic acid and metronidazole, essential for wound healing and other therapeutic uses, can be included in chitosan-based films. Excipients like plasticizers (like glycerol) to enhance mechanical qualities, surfactants for solubilization, and preservatives to stop microbiological contamination during use and storage are frequently used to formulate these films (Irfan et al., 2016). The polymer concentration, plasticizer type, and drug

content must be carefully optimized during the chitosan-based film manufacturing process to achieve the intended release profile and film stability. Tests for thickness, homogeneity, moisture content, and tensile strength are among the physicochemical analyses carried out to guarantee the caliber and functionality of these films. An in vitro release experiment is also necessary to evaluate the drug release kinetics and the film's capacity to transport the active ingredient to the intended location (Love, 2010). The formulation techniques for chitosan-based films are thoroughly examined in this section, with particular attention paid to the films' potential for localized drug administration and their contribution to the development of topical medicinal applications.

Several production methods can be used to create polymeric films for topical treatments, each with unique benefits and drawbacks. Despite difficulties with pore size uniformity, mechanical strength, and scalability, electrospinning, which is based on the electrohydrodynamic process, produces nanofibers with high porosity, homogeneity, and mechanical strength that are appropriate for pharmaceutical and biomedical applications (Silva et al., 2019). While solvent toxicity and mechanical limitations are significant disadvantages, Electrospraying allows the creation of thin films or particles through liquid atomization, offering advantages like controlled film thickness and adaptability. (Wang et al., 2019). Due to its high cost and issues with thermal stability, hot-melt extrusion, which has been used since the 1980s, improves medication solubility and bioavailability and allows for continuous processing without the need for solvents (Stanković et al., 2015). Finally, 3D bioprinting is a cutting-edge method that creates biopolymer-based films for tissue engineering. It provides accurate architectural designs but is limited by high production costs, long lead times, and viscosity-related issues (Tsegay et al., 2022).

A common and scalable approach for creating thin films is solution casting, which involves solvent evaporation to harden a liquid solution. A uniform mixture of ingredients is made in an appropriate solvent; then, the mixture is poured into a mold. The solvent is then evaporated under carefully regulated circumstances to harden the film, which is removed for additional use. Its accurate control over film thickness and homogeneity makes this technology easy to use, affordable, and versatile, making it appropriate for industrial and laboratory settings. Despite certain disadvantages, like

the possibility of solvent residue and phase separation from too much plasticizer, it is still better than other techniques because of its simplicity, adaptability, and capacity to create films with a variety of qualities (Anbukarasu et al., 2015; Notario-Pérez et al., 2020). These benefits have led us to use the solution casting technique in our study to create better films with exact thickness and desired properties for wound healing applications. Figure 2.1 highlights the steps involved in preparing films using the solution casting method.

Figure 2.1

Solution Casting Method: Procedure for producing polymeric films using the solution casting method step-by-step: The process involves preparing the polymeric suspension by mixing it under standard conditions using a magnetic stirring bar, pouring it into a Petri dish and carefully weighing it to ensure uniformity, evaporating the solvent in a drying oven to solidify the film; and then removing the completed polymeric film as the end product.

Note: From Riccio, B. V. F., Silvestre, A. L. P., Meneguin, A. B., Ribeiro, T. d. C., Klosowski, A. B., Ferrari, P. C., & Chorilli, M. (2022). Exploiting Polymeric Films as a Multipurpose Drug Delivery System: a Review. *AAPS PharmSciTech*, *23*(7), 269.

Layek et al. (2019) explored the "*Design, Development, and Characterization of Imiquimod-Loaded Chitosan Films for Topical Delivery"* to address the shortcomings of the currently available Aldara™ cream, which treats superficial basal cell carcinoma and contains 5% w/w imiquimod. The trial brought problems with the cream's formulation, including low patient compliance, inconsistent

dosage, and insufficient drug release. Propylene glycol was used as a plasticizer to create imiquimod-loaded chitosan films to solve these problems. These films maintained imiquimod's physical integrity while displaying outstanding drug content consistency and acceptable physicochemical qualities appropriate for wound dressings. The films achieved sustained drug release over seven days while maintaining imiquimod's bioactivity, as evidenced by in vitro growth inhibition assays. Furthermore, the chitosan-based films demonstrated significantly higher skin drug accumulation than the commercial cream, emphasizing their potential as an improved topical delivery system (Layek et al., 2019).

Silva et al. (2008) investigated *"Films Based on Chitosan Polyelectrolyte Complexes for Skin Drug Delivery: Development and Characterization,"* aiming to optimize chitosan-based polyelectrolyte complexes (PEC) for skin applications. Chitosan and polyacrylic acid (PAA) polymers were used in the study, along with various crosslinkers and densities, to create films with the best possible functional qualities, such as flexibility, resistance, water vapor transmission rate, and bioadhesion. The films were developed by controlling pH to maximize polymer interaction, with film formation occurring near the pKa of the components. Furthermore, the impact of additives such as glycerol, polyethylene glycol 200 (PEG200), Hydrovance, and trehalose was investigated; glycerol improved resistance, flexibility, and the rate at which water vapor was transmitted, with the most significant effect occurring at 30%. A pressure-sensitive adhesive (PSA) was added, which significantly improved bioadhesion while having little impact on the mechanical characteristics of the films. The optimized PEC films with adhesive demonstrated excellent characteristics for skin application, positioning them as promising candidates for the incorporation of drugs for topical and transdermal delivery (Silva et al., 2008).

The *"Development of Chitosan/Silver Sulfadiazine/Zeolite Composite Films for Wound Dressing"* was examined by Yassue-Cordeiro et al. (2019) to offer a novel substitute for the traditional silver sulfadiazine (AgSD) creams and gauzes that are frequently used for burn treatment. To mitigate the cytotoxicity of AgSD, the study used zeolite as a sustained-release drug delivery method. Comparing composite films with AgSD-impregnated zeolite to their non-impregnated counterparts, the former showed notable changes in their physicochemical characteristics, such as FTIR spectra,

XRD diffraction patterns, and Energy Dispersive X-ray Spectroscopy-Scanning Electron Microscopy (EDX-SEM) studies. In addition to being opaque and stiffer, the resultant films showed improved antibacterial efficacy against gram-negative bacteria and *Candida albicans*. According to the study, these films might be employed in a concentration-dependent manner without causing harm, even though cytotoxicity was noted in fibroblast experiments. This study highlights the potential of composite films based on chitosan as cutting-edge materials for wound dressings with enhanced antibacterial activity and regulated medication release properties (Hissae Yassue-Cordeiro et al., 2019).

The *"Mechanical, Structural, and Physical Aspects of Chitosan-Based Films as Antimicrobial Dressings"* were investigated by Escárcega-Galaz et al. (2018) to evaluate their suitability for aiding in healing skin ulcers. The study compared films manufactured with a glycerol-honey mixture and films made with pure chitosan, looking at their mechanical, physical, and microbiological properties. The films demonstrated excellent resistance to breaking and a distinct correlation between chitosan content and ripping force. They lacked cracks and fissures and were homogeneous, smooth, porous, and translucent. When glycerol and honey were added to honey-formulated films, the elongation percentage and contact area rose to 44%. Only direct contact has antibacterial activity, particularly against *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. Because of their beneficial structural, antibacterial, and morphological properties, the films are a good option for use in dressings meant to cure skin ulcers (Escárcega-Galaz et al., 2018).

Building on the knowledge gained from the literature, the use of chitosan, ascorbic acid, and metronidazole in formulating a wound-healing film is justified by their synergistic potential and complementary qualities. A natural biopolymer with superior hemostatic activity, biocompatibility, and built-in antibacterial qualities, chitosan is a perfect scaffold for wound healing applications. In addition to accelerating tissue healing, promoting collagen formation, and reducing oxidative stress, ascorbic acid offers a less hazardous substitute for the organic acids typically used to dissolve chitosan, potentially lowering toxicity hazards—additionally, metronidazole, a potent antibacterial drug that works against anaerobic bacteria frequently linked to infected wounds. The next chapter discusses the development and description of a novel wound-

healing film, which is made possible by this approach, filling the knowledge gap in literature.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Experimental

This section provides a comprehensive explanation of the procedure employed to fabricate metronidazole-loaded films incorporating ascorbic acid and chitosan. It details the systematic approach taken to develop films specifically tailored for topical application. Overall, this section upholds scientific rigor in documenting the formulation and fabrication of drug-loaded polymeric films.

3.1.1 Materials

Materials used in this investigation include ascorbic acid, low molecular weight chitosan (degree of deacetylation 90%), glycerol, metronidazole (model drug), Milli-Q water, and chemicals for pH adjustment and buffer preparations. Ascorbic acid and chitosan were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Metronidazole and glycerol were supplied by PC Drug Center Co., Ltd. (Bangkok, Thailand). Bovine serum albumin (BSA) was obtained from Fisher BioReagents (Thermo Fisher Scientific, MA, USA). HPLC-grade methanol was obtained from Macron Fine Chemicals (Avantor Performance Materials, PA, USA). Cellulose membranes (SnakeSkinTM dialysis membrane, MWCO = 10 kDa) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Simulated Wound Fluid (SWF) (pH 7.4) was made following the method (G. T. Voss et al., 2020). Water utilized in this study was purified using a Milli-Q® Advantage A10 Water Purification System (Merck KGaA, Darmstadt, Germany). All other chemicals used were of analytical grade, ensuring the accuracy and consistency of experimental conditions.

3.1.2 Experimental Design for Optimization

The experimental design and data analysis used Design-Expert® software (version 13; Stat-Ease, Inc., Minneapolis, USA). In this study, a Box–Behnken design (BBD) was employed to evaluate the effects of chitosan (A), ascorbic acid (B), and glycerol (C) on the mechanical properties—specifically, ultimate tensile strength (Y1) and elongation at break (Y2)—as well as surface pH (Y3) of the prepared films. Factors A and B were expressed as % w/w relative to the total weight of the film-casting solution, while factor C was expressed as a percentage by weight based on chitosan

content. As presented in Table 3.1, the independent factors were examined at three levels: high (+1), medium (0), and low (-1).

Table 3.1

Factors and their corresponding levels used in the Box–Behnken design for the experimental optimization study.

Factors	Levels		
	Low (-1)	Medium (0)	High (+1)
Chitosan (A)	3	3.5	4
Ascorbic Acid (B)	3	4.5	6
Glycerol (C)	20	25	30

Table 3.2

BBD (Experimental design) matrix detailing the composition of films (F1–F17). Metronidazole is fixed at 0.5% w/w across all formulations, while the total weight of the film-forming solution is maintained at 20 g, with 15 g allocated for casting in Petri dishes.

Formulations	Standardized levels of independent variables		
	Chitosan (A)	Ascorbic Acid (B)	Glycerol (C)
F1	-1	-1	0
F2	0	+1	+1
F3	0	0	0
F4	+1	0	-1
F5	-1	0	-1
F6	-1	0	+1
F7	0	0	0
F8	0	0	0
F9	0	+1	-1
F10	0	0	0
F11	-1	+1	0
F12	0	-1	+1
F13	0	-1	-1

Table 3.2

BBD (Experimental design) matrix (Cont.)

F14	0	0	0
F15	+1	0	+1
F16	+1	+1	0
F17	+1	-1	0

As shown in Table 3.2, a total of 17 experimental formulations were designed, including five center points to evaluate experimental reproducibility and estimate pure error. Each response was measured in triplicate for every experimental run to minimize variability and enhance the accuracy of the data; the average values of these replicates were then used to construct predictive models describing the relationship between the independent variables and the measured responses. To assess the statistical validity and adequacy of the models, analysis of variance (ANOVA) was performed, with a p-value less than 0.05 indicating statistical significance. Additionally, model performance was evaluated using key statistical parameters: the coefficient of determination (R^2), which indicates how well the model explains variability in the data; the adjusted R^2, which accounts for the number of predictors and model complexity; and the predicted R^2, which estimates the model's ability to predict new observations (Brereton, 2019; Gao, 2024; Ng et al., 2018). A non-significant lack-of-fit ($p > 0.05$) was also required, indicating that the model sufficiently captured the experimental variability without significant unexplained error, thereby confirming the model's suitability for analyzing and optimizing the formulation variables.

3.1.3 Preparation of Films

The film samples were prepared using a film-casting method. The contents of the three constituents in the film-casting solution were varied according to the experimental design described in the previous section. A consistent process was used to prepare the films to ensure consistency and reproducibility. Ascorbic acid was first dissolved in Milli-Q water while constantly swirling at 600 rpm for 15 minutes. Chitosan was added gradually and stirred at the same speed for three hours to ensure complete dissolution. Metronidazole was added to the mixture and agitated for a further hour at 600 rpm. Glycerol was then added as a plasticizer, and the mixture was stirred for 30 minutes at 200 rpm to achieve homogeneity. To eliminate any trapped air

bubbles, the solution rests at room temperature for an hour before casting. After that, 15 grams of the prepared solution were poured into a Petri dish, and it was equally distributed to create a film. After 18 hours of drying at 35°C in an oven, the films were removed and stored for further characterization.

3.2 Characterization of Films

This section comprehensively evaluates the developed films to assess their mechanical, physicochemical, and structural properties—key parameters for their potential application in wound healing. A total of seventeen formulations were systematically investigated, focusing on three critical response variables: ultimate tensile strength (Y_1), elongation at break (Y_2), and surface pH (Y_3). The formulation variables included chitosan concentration (A), ascorbic acid concentration (B), and glycerol concentration (C). A BBD was employed to explore the influence of independent variables and to identify optimal formulation conditions.

Following statistical optimization, the selected formulation was subjected to in-depth characterization techniques, including DSC to evaluate thermal behavior, PXRD to determine crystallinity, and FTIR to elucidate molecular interactions. In addition, the optimized film was further analyzed for its swelling capacity and cumulative drug release profile, which are critical to confirm its functional suitability.

3.2.1 Thickness and Density Measurement

A handheld Starrett 733.1 digital micrometer (Starrett, USA) was used to measure the thickness of the films at 3 randomly chosen locations on each film. These measurements were performed in triplicate, and the average value was recorded as the film's thickness. The density of the films, expressed as mass per unit volume (g/cm³), was calculated using the formula:

$$Density = Mass / Volume \quad (3.1)$$

where the volume of the film was estimated by multiplying its thickness by its surface area, using the formula:

$$Volume = Thickness \times Area \quad (3.2)$$

All measurements were conducted in triplicate, and the average values were used for analysis (Tan et al., 2019).

3.2.2 Mechanical Strength Analysis

The mechanical properties of the films, specifically the ultimate tensile strength and elongation at break, were assessed using a TA.XT plus texture analyzer (Stable Micro Systems, UK) operated in tensile mode. Film samples were cut into rectangular strips measuring 1 × 5 cm. To ensure consistent gripping and minimize slippage, the 1 × 1 cm^2 sections at both ends of each strip were secured with clamps, resulting in an effective testing area of 1 × 3 cm^2. The tensile test was performed at a constant extension rate of 0.5 mm/s (Vuddanda et al., 2017). All measurements were conducted in triplicate.

3.2.3 Surface pH Measurement

The surface pH of the films was determined following a previously described methodology (M M Agwa & Elmotasem, 2022) with slight modifications to optimize measurement accuracy. Briefly, film samples (1 × 1 cm^2) were placed individually into clean glass tubes containing 5 ml of Milli-Q water and allowed to swell at room temperature for one hour. After the swelling period, the system was left to equilibrate for an additional 1 minute. Subsequently, the surface pH was measured using a calibrated pH meter equipped with a combination glass electrode. The electrode was carefully positioned in gentle contact with the surface of the hydrated film to avoid any disruption or damage to the sample. Measurements were performed in triplicate, and the mean pH value was calculated.

3.2.4 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR spectroscopy was employed to characterize the chemical structure of the prepared films and to confirm the presence of functional groups associated with the incorporated components. FTIR spectra were acquired using a Bruker Alpha II FTIR spectrometer (Bruker, Germany) equipped with a single-reflection diamond attenuated total reflection (ATR) accessory. Spectral data were collected in ATR mode across the wavenumber range of 4000–600 cm^{-1}, with each spectrum obtained by averaging 32 scans at a resolution of 2 cm^{-1}. Spectra were recorded for blank films and films loaded with metronidazole to assess possible interactions between the polymer matrix and the active pharmaceutical ingredient. For comparative analysis, reference spectra of pure metronidazole, ascorbic acid, and chitosan were obtained under identical conditions. This comprehensive analysis

enabled the identification of characteristic functional groups and potential shifts or changes in peak intensities indicative of molecular interactions or chemical modifications within the film matrix (Guilherme T. Voss et al., 2020).

3.2.5 Differential Scanning Calorimetry (DSC) Analysis

DSC was conducted to examine the thermal transitions of individual components, chitosan, ascorbic acid, metronidazole, and the optimized film formulation. The analysis was performed using a Mettler Toledo DSC 3+ instrument (Mettler-Toledo, France), calibrated with indium. Samples, including pure compounds and the optimized film, were hermetically sealed in aluminum pans and subjected to a controlled heating rate of 10 °C/min under a nitrogen purge flow of 50 mL/min. The resulting thermograms enabled direct comparison of thermal behavior, including melting points and degradation temperatures, to assess changes in physical changes within the film matrix.

3.2.6 Powder X-ray Diffraction (PXRD) Analysis

The physical state—crystalline or amorphous—of the developed films, both with and without the incorporation of metronidazole, was analyzed using PXRD. In addition, the individual starting materials, including chitosan, ascorbic acid, and metronidazole, were also characterized to assess their structural contributions. The procedure was adapted with minor modifications from the method described by Jillani et al. (Jillani et al., 2022).

PXRD patterns were obtained using a Rigaku MiniFlex II diffractometer (Rigaku Corporation, Tokyo, Japan) equipped with a CuKα radiation source ($\lambda = 1.5406$ Å), operating at 40 kV and 15 mA. Film samples were carefully cut into 2 cm^2 and mounted onto square sample holders. The scanning was carried out over a 2θ range of 3° to 50°, with a step size of 0.1°, a counting time of 0.1 seconds per step, and a scan speed of 6°/minute. This analysis enabled the identification of characteristic crystalline peaks or halo patterns, allowing the evaluation of any potential changes in the material's physical state upon film formation and drug incorporation.

3.2.7 Swelling Index Analysis

The swelling behavior of the film samples was evaluated by monitoring the weight change of the films upon immersion in simulated wound fluid over specific time intervals, following methods adapted from previously reported

studies (Silva et al., 2023; G. T. Voss et al., 2020). Film specimens, cut into 1 × 1 cm^2 squares from the optimized formulation, were first dried in a hot air oven and weighed to determine their initial dry weight (W_1). Each film sample was then placed in a 3.5 cm Petri dish containing 5 ml of SWF, prepared according to the composition described by Voss et al. (G. T. Voss et al., 2020). The SWF consisted of 2% w/v bovine serum albumin (BSA), 0.02 M calcium chloride ($CaCl_2$), and 0.4 M sodium chloride (NaCl), adjusted to pH 7.4 to simulate physiological wound conditions. At predetermined time points, the swollen films were carefully removed from the SWF, and excess surface fluid was gently blotted using filter paper. The swollen films were then reweighed to obtain the wet weight (W_2). The swelling capacity, expressed as the swelling degree or swelling index (in percentage), was calculated using the following equation (Ghorbani et al., 2022; Silva et al., 2023; G. T. Voss et al., 2020):

$$Swelling\ Index\ (\%) = \frac{(W_2 - W_1)}{W_1} \times 100 \qquad (3.3)$$

All measurements were performed in triplicate, and the average values were used for analysis.

3.2.8 High-Performance Liquid Chromatography (HPLC) Analysis

A Shimadzu UFLC system (Shimadzu, Kyoto, Japan) was employed, equipped with an auto-sampler and a diode array detector (DAD). Chromatographic separation was achieved using a Hypersil BDS C18 column (250 mm length × 4.6 mm internal diameter, with a particle size of 5 µm), which offers high resolution and reproducibility for pharmaceutical compounds. The mobile phase consisted of a mixture of methanol and 0.05 M phosphate buffer (pH 6.2) in a 70:30 (v/v) ratio. The phosphate buffer was prepared by mixing 50 ml of monobasic potassium phosphate solution with 8.1 ml of 0.2 M sodium hydroxide (NaOH), and the final volume was adjusted to 200 ml using Milli-Q water.

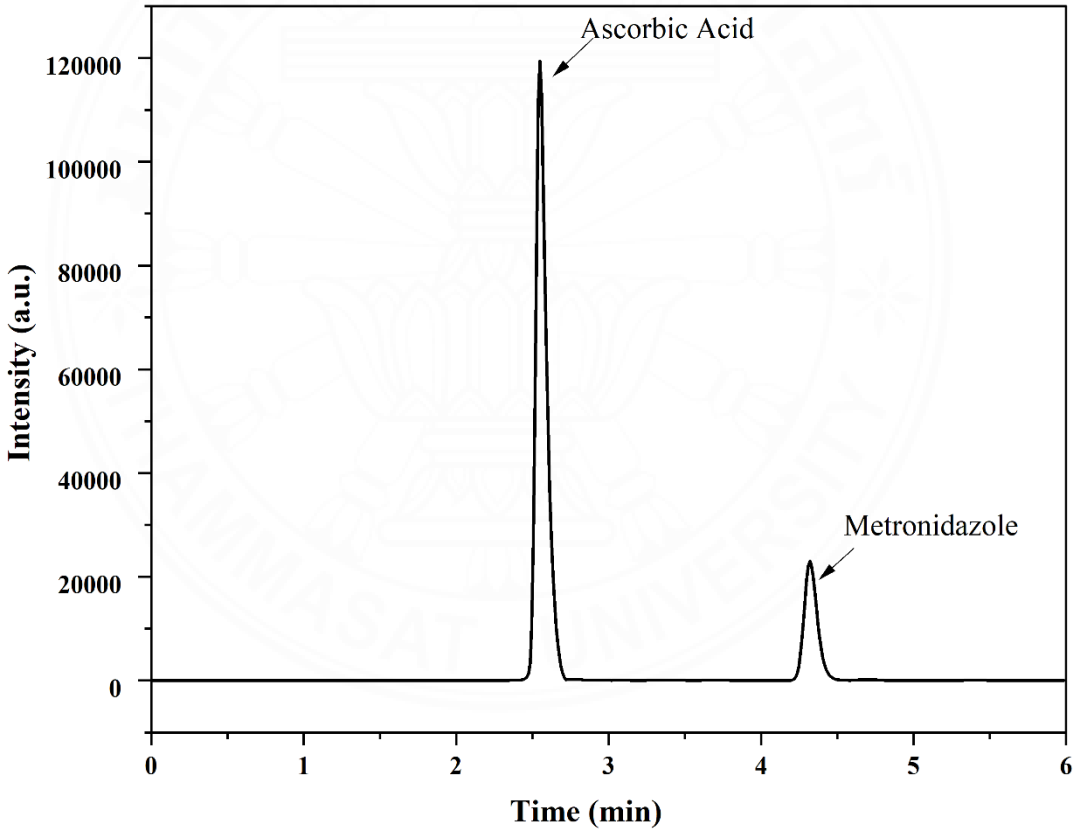
The flow rate was maintained at 1.0 ml/min, ensuring efficient separation of both analytes. Detection was performed at 254 nm, a wavelength appropriate for the simultaneous analysis of ascorbic acid and metronidazole. This method was adapted with modifications based on a previously reported protocol (Asare-Nkansah et al., 2014). Each sample was filtered using a 0.22 µm filter before injection, and a 20 µl injection volume was used for every run. Calibration curves were

constructed for ascorbic acid and metronidazole using standard solutions across an appropriate concentration range, showing linearity with correlation coefficients (R^2) greater than 0.99 for both analytes. All analyses were performed in triplicate to ensure reproducibility.

A representative chromatogram of a standard mixture of ascorbic acid and metronidazole was generated using the HPLC method. The chromatographic profile demonstrated effective separation, with sharp, well-resolved peaks at retention times of approximately 2.6 minutes for ascorbic acid and 4.4 minutes for metronidazole.

Figure 3.1

The representative chromatogram obtained using HPLC.



3.2.9 In Vitro Drug Release Studies

The release profiles of metronidazole and ascorbic acid from the film formulations were evaluated using a modified Franz-type diffusion cell setup, based on the method described by Sampaopan et al. (Sampaopan & Suksaeree, 2022) with slight modifications. Each film sample was carefully cut into a 1.5 cm diameter circle. These films were placed on a cellulose membrane and positioned in the receptor chamber

filled with 15 ml of release medium (SWF without BSA). The diffusion cell was maintained at a constant temperature of 37 ± 0.5°C using a water jacket, while the receptor chamber was continuously stirred with a magnetic stirrer to ensure uniform diffusion. At predetermined intervals (0.5, 1, 1.5, 2, 3, 4, 6 h), 0.5 ml of the release medium was withdrawn and replaced with an equal volume of fresh SWF with BSA to maintain sink conditions (Ahmad et al., 2020). The concentrations of metronidazole and ascorbic acid in the receptor medium were quantified using high-performance liquid chromatography as explained in section 3.2.8. Each experiment was performed in triplicate.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Thickness and Density Measurement

The thickness and density (Table 4.1) of the metronidazole-loaded chitosan films, formulated with ascorbic acid, were systematically evaluated to determine their uniformity and structural consistency, critical parameters for topical drug delivery performance.

Table 4.1

The table summarizes the thickness (in cm) and density (in g/cm³) values for 17 distinct formulations (F1 to F17). For each formulation, both the average and standard deviation (SD) of thickness and density are reported.

Formulation	Thickness (cm)		Density (g/cm³)	
	Average	SD	Average	SD
F1	0.0128	0.0002	1.1803	0.0228
F2	0.0173	0.0026	1.2477	0.0254
F3	0.0153	0.0004	1.2197	0.0268
F4	0.0171	0.0010	1.3183	0.0275
F5	0.0123	0.0012	1.1087	0.0320
F6	0.0148	0.0016	1.1224	0.0564
F7	0.0150	0.0014	1.2080	0.0509
F8	0.0155	0.0010	1.2094	0.0241
F9	0.0143	0.0008	1.2537	0.0418
F10	0.0151	0.0005	1.2158	0.0779
F11	0.0133	0.0012	1.1997	0.0607
F12	0.0156	0.0011	1.1447	0.0192
F13	0.0141	0.0012	1.2280	0.0514
F14	0.0156	0.0013	1.2177	0.0236
F15	0.0179	0.0024	1.2557	0.0440
F16	0.0176	0.0013	1.2673	0.0165
F17	0.0168	0.0012	1.2683	0.0531

The film thickness was found to range between 0.0123 ± 0.0012 cm and 0.0179 ± 0.0024 cm, while the density values varied from 1.1087 ± 0.0320 g/cm³ to 1.3183 ± 0.0275 g/cm³. These results fall within the reported ranges for similar chitosan-based wound healing films in previous studies, such as chitosan film containing fucoidan (thickness 0.00297–0.0269 cm) and chitosan based films incorporating essential oil nanoemulsions (thickness 0.0113–0.0197 cm; density 0.62–1.24 g/cm³), highlighting the relevance and applicability of the developed formulations for topical applications (Elshamy et al., 2021; Sezer et al., 2007).

4.2 Model-Based Optimization Results

In this study, the BBD was employed as a systematic and efficient statistical tool to evaluate the influence of three independent formulation variables—chitosan concentration (A), ascorbic acid content (B), and glycerol level (C)—on key response parameters of the developed films. Specifically, the design aimed to investigate their effects on mechanical properties, including ultimate tensile strength (Y_1) and elongation at break (Y_2), as well as the pH (Y_3) of the resulting films. The BBD approach allows for the simultaneous assessment of the selected variables' linear, interactive, and quadratic effects, thereby facilitating a comprehensive understanding of how these formulation components influence the physico-mechanical and chemical attributes of the films (Beg & Akhter, 2021). This experimental design enhances the interpretability of complex interactions among variables and optimizes the formulation with a reduced number of experimental runs compared to full factorial designs.

The intercept in the model equations (in the next sections) derived from the contour plot analysis represents the estimated response value when all independent variables are set at their coded mid-levels (zero). It serves as a reference point or baseline, facilitating comparisons to evaluate how deviations from this central position influence the measured responses. By anchoring the model at this midpoint, researchers can systematically explore the effects of formulation variables on targeted responses within a defined experimental space (Shrivastava & Daharwal, 2022).

Building on this, different models are used to check how the formulation variables influence each response. These models include linear, two-factor interaction (2FI), and quadratic terms. Linear terms (A, B, C) describe simple, proportional

relationships between individual factors, such as chitosan concentration (A), ascorbic acid content (B), and glycerol level (C), and the response. Two-factor interaction (2FI) terms (AB, AC, BC) reflect the combined influence of two variables acting together, highlighting how their simultaneous variation may yield effects different from those predicted by their individual contributions. Quadratic terms (A^2, B^2, C^2) account for curvature in the response surface, indicating the presence of nonlinear relationships and revealing optimal or threshold levels for each factor. These terms collectively enhance the model's predictive accuracy and are essential for interpreting response trends and guiding the rational optimization of formulation parameters.

4.2.1 Influence of Formulation Variables on Ultimate Tensile Strength (Y_1)

The regression analysis data indicated that the influence of the formulation components on the ultimate tensile strength (UTS) was best described by a response surface two-factor interaction (2FI) model. This model exhibited a high coefficient of determination (R^2) of 0.9377 and a sequential p-value of 0.0009, suggesting strong statistical significance. Furthermore, the lack-of-fit test yielded a p-value of 0.0864, which is not statistically significant, confirming that the model adequately fits the experimental data.

Accordingly, the equation for ultimate tensile strength (Y_1) based on the coded variables is as follows:

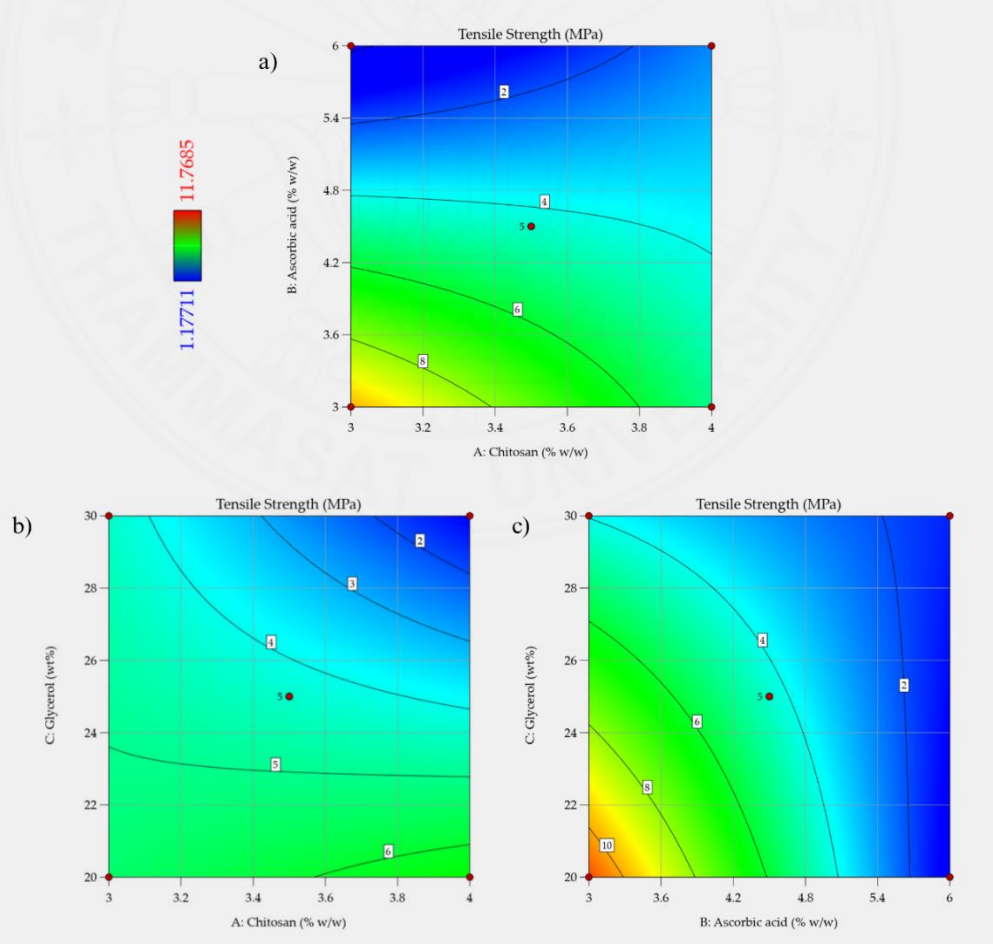
$$Y_1 = 4.34 - 0.5228\mathrm{A} - 3.12\mathrm{B} - 1.59\mathrm{C} + 1.91\mathrm{AB} - 1.08\mathrm{AC} + 1.92\mathrm{BC} \qquad (4.1)$$

The model's adjusted R^2 value of 0.9003 and predicted R^2 value of 0.7292 exhibited a difference of less than 0.2, which is within acceptable limits and suggests a good level of consistency between the model's predictive capability and its fit to the data (Abla et al., 2023; Kumar, 2020). All interaction terms (AB, AC, BC) were statistically significant ($p < 0.05$), confirming that the combined influence of the variables plays a critical role in determining the ultimate tensile strength. Interaction effects are substantial in response surface methodology as they describe how the outcome variable changes when two independent factors vary simultaneously (Fernandes et al., 2023).

Regarding the individual effects of the components, both glycerol and ascorbic acid significantly influenced the ultimate tensile strength ($p < 0.05$), whereas chitosan's main impact was not statistically significant ($p = 0.1487$). The contour plots in Figure 4.1 visually support these findings, illustrating the predominant effects of ascorbic acid and glycerol on the ultimate tensile strength.

Figure 4.1

Contour plots illustrating the effects of formulation variables on the ultimate tensile strength (UTS) of chitosan-based films. (a) Interaction between chitosan and ascorbic acid at a fixed mid-level of glycerol (25% w/w), showing reduced tensile strength with increasing ascorbic acid. (b) Interaction between chitosan and glycerol at a constant mid-level of ascorbic acid (4.5% w/w), where UTS decreases with higher glycerol content. (c) Interaction between ascorbic acid and glycerol at a fixed chitosan level (3.5% w/w), indicating maximal UTS at lower concentrations of both components. Color gradients represent UTS values (MPa), with contour lines and data points reflecting model predictions and experimental observations.



Regarding the individual effects of the components, both glycerol and ascorbic acid significantly influenced the ultimate tensile strength ($p < 0.05$), whereas chitosan's main impact was not statistically significant ($p = 0.1487$). The contour plots in Figure 4.1 visually support these findings, illustrating the predominant effects of ascorbic acid and glycerol on the ultimate tensile strength.

Specifically, increasing the concentration of ascorbic acid led to a marked decrease in the ultimate tensile strength of the films. This negative correlation is consistent with previous literature, which reports that the incorporation of ascorbic acid into biopolymer-based films compromises tensile strength (Janjarasskul et al., 2011; Kowalczyk, 2016). It is proposed that ascorbic acid may act as a plasticizer, disrupting intermolecular hydrogen bonding within the polymer matrix and thereby diminishing the structural cohesion of the polymer network (Janjarasskul et al., 2011; Kowalczyk, 2016). Similarly, elevating the proportion of glycerol relative to chitosan also significantly reduced the ultimate tensile strength. Glycerol's plasticizing effect, attributed to its ability to form hydrogen bonds with polymer chains, interferes with the intrinsic interactions between these chains (Edikresnha et al., 2019; Matta et al., 2011). This disruption facilitates greater molecular mobility and chain slippage under tensile stress, ultimately lowering the mechanical resistance of the films.

In summary, both ascorbic acid and glycerol appear to diminish the ultimate tensile strength primarily through plasticization mechanisms. While this reduces tensile strength, it will likely enhance material flexibility—a phenomenon further discussed in the context of elongation at break measurements.

4.2.2 Influence of Formulation Variables on Elongation at Break (Y_2)

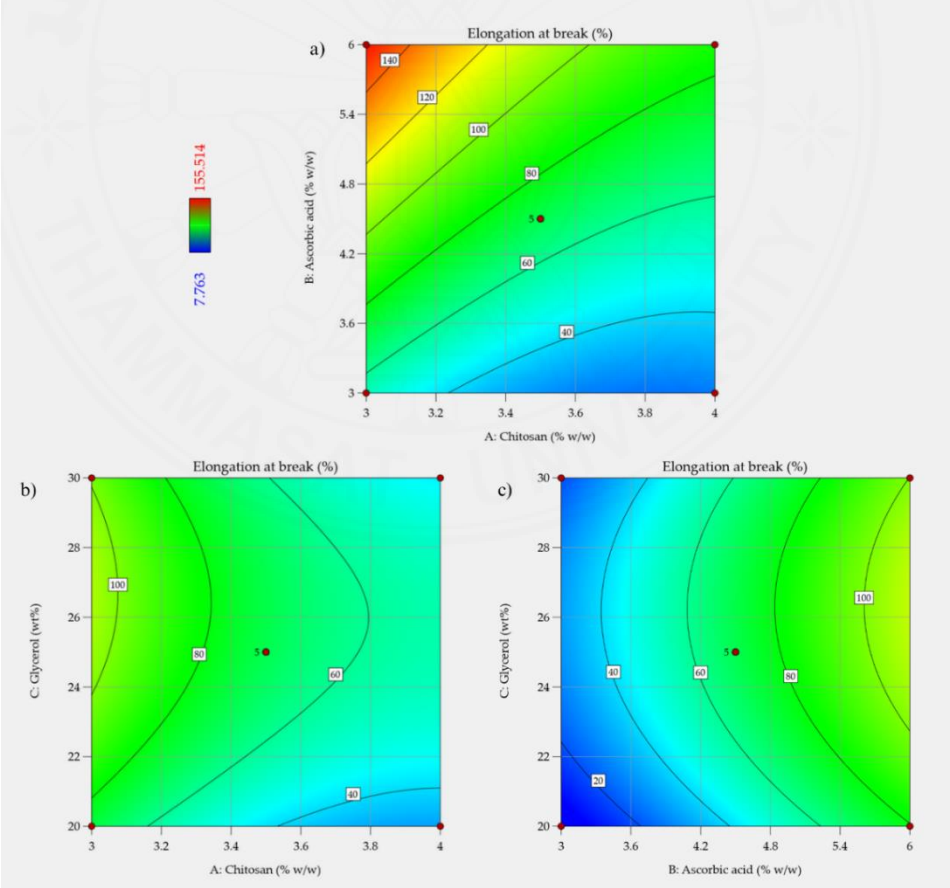
A quadratic regression model best described the elongation at break response (Y_2), which demonstrated strong statistical adequacy. The coefficient of determination (R^2) was 0.9722, indicating a high degree of fit. Furthermore, the model's sequential p-value was 0.0131, suggesting statistical significance. In contrast, the lack-of-fit p-value of 0.4495 indicated that the model adequately captured the variability in the experimental data without significant deviation. The adjusted R^2 value of 0.9365 and the predicted R^2 value of 0.7762 were in reasonable concordance, reflecting both the model's good fit and predictive capability.

Thus, the equation for elongation at break (Y_2) in terms of coded variables is expressed as:

$$Y_2 = 69.85 - 24.13\text{A} + 39.55\text{B} + 9.63\text{C} - 9.91\text{AB} - 3.66\text{AC} + 0.8643\text{BC} + 10.43A^2 - 0.7586B^2 - 18.94C^2 \quad (4.2)$$

Figure 4.2

Contour plots illustrating the effects of formulation components on the elongation at break (%) of chitosan-based films. (a) Interaction between chitosan and ascorbic acid at a fixed mid-level of glycerol (25% w/w), showing increased elongation with higher ascorbic acid and lower chitosan levels. (b) Interaction between chitosan and glycerol at a constant mid-level of ascorbic acid (4.5% w/w), indicating that elongation improves with higher glycerol content and lower chitosan concentration. (c) Interaction between ascorbic acid and glycerol at a fixed chitosan level (3.5% w/w), demonstrating enhanced elongation at higher levels of both ascorbic acid and glycerol. Color gradients % represent elongation at break, with contour lines and experimental points indicating model predictions and observed values.



According to the regression coefficients, all three independent variables—A, B, and C—exerted statistically significant effects on elongation at break,

confirming their relevance within the model. Among the quadratic terms, only C^2 was statistically significant, indicating a nonlinear relationship between glycerol content and film flexibility. Analysis of the linear coefficients in terms of the coded variables and examination of the contour plots (Figure 4.2) revealed that chitosan content negatively influenced elongation at break. In contrast, both ascorbic acid and glycerol had a positive impact on this mechanical property.

The presence of these agents, ascorbic acid and glycerol, disrupts intermolecular forces between the polymer chains in the matrix, thereby enhancing chain mobility and imparting greater flexibility to the films. This plasticization effect results in improved ductility, as demonstrated by the increased deformation before breakage and a concurrent decrease in tensile strength, consistent with previous findings (Matta et al., 2011).

The observed decrease in elongation at break with increasing chitosan concentration is consistent with the known characteristics of chitosan-based films, which are typically brittle and possess low flexibility. This mechanical behavior is primarily attributed to strong intra- and intermolecular hydrogen bonding interactions, particularly among hydroxyl groups and between hydroxyl and amine functional groups (Wang et al., 2017). As the concentration of chitosan increases, these interactions become more pronounced, leading to tighter polymer chain packing and reduced molecular mobility, ultimately diminishing the film's capacity to elongate under stress (Son Tae-won, 2006).

4.2.3 Influence of Formulation Variables on Surface pH (Y_3)

The quadratic model was the most suitable for describing the influence of formulation variables on the surface pH. Statistically significant contributors to the model included all primary (linear) factors—chitosan (A), ascorbic acid (B), and glycerol (C)—as well as two interaction terms (AB and BC), and the squared terms of A^2 and B^2. The equation for surface pH (Y_3) is:

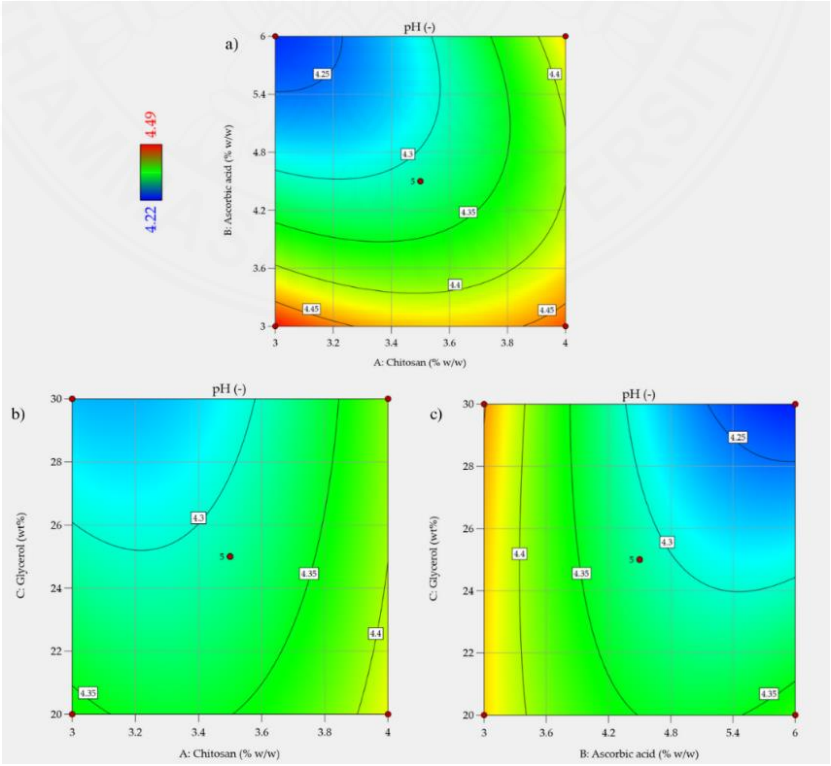
$$Y_3 = 4.31 + 0.045\mathrm{A} - 0.0725\mathrm{B} - 0.03\mathrm{C} + 0.055\mathrm{AB} + 0.015\mathrm{A} - 0.04\mathrm{B} + 0.0405A^2 + 0.0505B^2 + 0.0055C^2 \quad (4.3)$$

The contour plots (Figure 4.3) effectively illustrate the influence of chitosan, ascorbic acid, and glycerol on the pH of the films. Examining the individual

effects of the components, it was observed that an increase in chitosan concentration corresponded to an elevation in pH. Conversely, elevating the levels of ascorbic acid or the proportion of glycerol relative to the total polymer content decreased pH. The role of chitosan in modulating the pH is attributed to its intrinsic basicity (do Amaral Sobral et al., 2022). The amino groups in chitosan can interact with acidic species in the formulation, thereby altering the ionic equilibrium and consequently affecting the pH of the final film. Specifically, as the chitosan concentration increases, its amine groups may become protonated, binding available hydrogen ions and reducing their free concentration in the matrix. This mechanism leads to a moderate increase in pH.

Figure 4.3

Contour plots showing the influence of formulation components on the pH of chitosan-based films. (a) Interaction between chitosan and ascorbic acid at a fixed mid-level of glycerol (25% w/w), with pH slightly decreasing at lower chitosan and higher ascorbic acid levels. (b) Interaction between chitosan and glycerol at a constant mid-level of ascorbic acid (4.5% w/w), where pH remains relatively stable across the tested range. (c) Interaction between ascorbic acid and glycerol at a fixed chitosan level (3.5% w/w), indicating a slight decline in pH with increasing ascorbic acid. Color gradients represent pH values, with contour lines and data points illustrating model predictions and experimental values.



Ascorbic acid, a weak organic acid, exerted the opposite effect by lowering the pH in a concentration-dependent manner. This is consistent with its acidic character and tendency to donate protons within the polymeric matrix. Although glycerol also demonstrated a statistically significant effect on pH, its influence was less pronounced than that of chitosan and ascorbic acid. Glycerol, a polyhydric alcohol, may modulate pH indirectly through its interactions with other film constituents. It has been proposed that glycerol can engage in hydrogen bonding with chitosan, potentially displacing acetic acid from the polymeric network. This liberated acetic acid may then evaporate during the film drying process (Brown et al., 2001). However, unlike acetic acid, ascorbic acid remains entrapped within the film matrix in the present study. Consequently, increasing glycerol content may facilitate ascorbic acid retention, thereby slightly reducing pH.

4.2.4 Optimized Formulation for Film Development

The design of polymeric films for use in wound dressings requires careful consideration of their mechanical properties, as these directly influence their functional performance on the skin. For wound dressing to be effective, it must be capable of shielding the injured area from external mechanical forces while maintaining a degree of flexibility that allows it to conform to the natural movements of the skin without causing restriction or discomfort. To ensure such compatibility, the mechanical characteristics of the film should be approximately those of intact human skin. The mechanical response of human skin is known to be influenced by several factors, including age, sex, and the anatomical location from which the sample is derived. According to previous studies, human skin typically exhibits tensile strength values in the range of 2.5 to 30 MPa, while its elongation at break can vary significantly, generally falling between 10% and 115% (Adekogbe & Ghanem, 2005; Liu et al., 2022). Additionally, the ultimate strain capacity of healthy skin has been reported to average around 100% (Adekogbe & Ghanem, 2005). Given these parameters, materials intended for wound care applications must fall within a suitable range—specifically, an ultimate tensile strength between 4 and 30 MPa is considered optimal for ensuring adequate mechanical integrity without sacrificing user comfort or film flexibility (Kim et al., 2016).

In addition to mechanical characteristics, the surface pH of topical film formulations is another critical parameter, particularly in the context of wound healing. A neutral to alkaline wound environment can promote microbial colonization and infection; thus, maintaining a slightly acidic pH at the wound site is beneficial for both antimicrobial defense and tissue regeneration (Patil & Wairkar, 2024; Sim et al., 2022). Films with a mildly acidic surface pH can help suppress bacterial proliferation and simultaneously enhance fibroblast activity, thereby supporting the wound healing process (Li, Ma, et al., 2019). For topical applications, an optimal pH range between 4.1 and 5.8 is recommended to minimize the risk of skin irritation while supporting dermal compatibility (Carra et al., 2024).

To arrive at a formulation that meets the required performance benchmarks, a comprehensive statistical analysis was first conducted using ANOVA. This analysis enabled the identification of significant variables and their interactions affecting key response parameters. Following the identification of influential factors, a numerical optimization technique was applied to determine the most suitable composition of the casting solution. This process focused on achieving target values for three critical film attributes: ultimate tensile strength within the 4–30 MPa range, elongation at break between 90% and 115%, and a surface pH falling within the desirable range of 4.1 to 5.8.

The optimization was guided by a desirability function approach, which is a widely used method for multi-response optimization. In this framework, each individual response is converted into a dimensionless desirability value ranging from 0 (highly undesirable) to 1 (highly desirable). These individual desirabilities are then combined into an overall composite desirability index, which reflects how closely a given formulation aligns with all target specifications simultaneously (Mori et al., 2020). A higher composite desirability score indicates a more optimal balance across all measured attributes.

Based on this approach, the formulation designated as F6 was identified as the most favorable among all tested combinations. This formulation achieved the highest composite desirability score, indicating that it best met the mechanical and physicochemical criteria established for wound dressing applications. The coded levels associated with formulation F6 were -1 for factor A, 0 for factor B,

and +1 for factor C. When translated into actual component concentrations, this corresponded to a formulation containing 3.0% w/w chitosan, 4.5% w/w ascorbic acid, and 30.0 wt% glycerol, with the glycerol content calculated relative to the amount of chitosan. This composition demonstrated an ideal combination of flexibility, tensile strength, and surface pH, satisfying all the predefined thresholds essential for safe and effective topical application. To visually demonstrate the outcome of this optimization process, Figure 4.4 presents an example image of the finalized formulation F6 film. As shown, the optimized film exhibits a remarkably uniform appearance.

Figure 4.4

Figure 4.4 displays a representative image of the optimized F6 film, precisely cut into a square shape.

As a result of these findings, formulation F6 was selected as the optimized film formulation. It was subsequently subjected to further experimental evaluation, including structural, thermal, crystallinity, swelling, and drug release characterization, to confirm its suitability for use in wound healing applications and to ensure consistency in its performance profile.

4.3 Characterization of the Optimized Film Formulation

4.3.1 Fourier-Transform Infrared (FTIR) Spectroscopy Analysis

Fourier-transform infrared (FTIR) spectroscopy was employed to characterize the chemical structure of the pure components and the optimized film formulation (F6), with spectral analysis conducted over the full wavenumber range of 4000 to 600 cm^{-1}. The resulting spectra are presented in Figure 4.5, which depicts the

vibrational features of metronidazole, ascorbic acid, chitosan, glycerol, and the composite film.

The FTIR spectrum of metronidazole (Figure 4.5a) displays a broad peak at 3207 cm^{-1}, corresponding to the O–H stretching vibration. A neighboring peak at 3100 cm^{-1} is attributed to the C=C–H stretching of the imidazole ring. Furthermore, a distinct absorption band at 1533 cm^{-1} is assigned to the asymmetric N=O stretching vibration, characteristic of the nitro group present in the compound (Cirri et al., 2021; Utomo et al., 2023).

In the case of ascorbic acid (Figure 4.5b), multiple strong (3524 cm^{-1}, 3406 cm^{-1}, 3309 cm^{-1}, and 3207 cm^{-1}) and broad bands are observed between 3525–3212 cm^{-1}, representing O–H stretching vibrations due to hydroxyl groups engaged in hydrogen bonding. The C–H stretching mode appears at 2916 cm^{-1}. Notably, a prominent peak at 1752 cm^{-1} corresponds to the C=O stretching of the five-membered lactone ring, while a secondary band at 1651 cm^{-1} signifies C=C stretching within the molecule (Sreeja et al., 2015; Yohannan Panicker et al., 2006).

The spectrum of chitosan (Figure 4.5c) is characterized by a broad absorption above 3000 cm^{-1}, arising from overlapping O–H and N–H stretching vibrations indicative of its polyhydroxy and amino structure. C–H stretching bands are detected at 3000 cm^{-1} and 2867 cm^{-1} (Leceta et al., 2013; Liu et al., 2014). Additionally, three characteristic amide-related absorptions are identified: 1646 cm^{-1} (amide I), 1586 cm^{-1} (amide II), and 1374 cm^{-1} (amide III), confirming the presence of residual N-acetylated groups in the biopolymer (Liu et al., 2014).

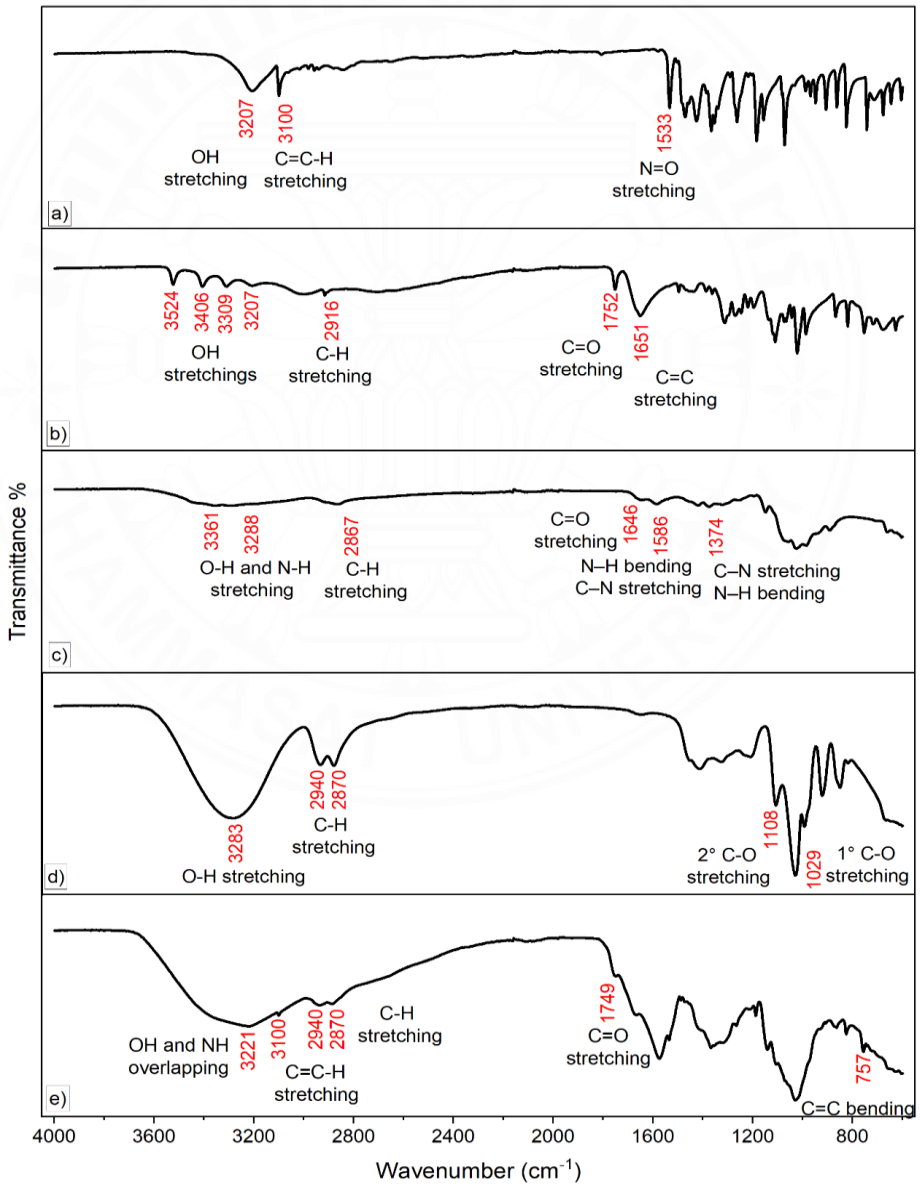
Glycerol (Figure 4.5d) exhibits a broad O–H stretching band centered at 3283 cm^{-1}, a result of the high hydroxyl content and extensive hydrogen bonding. Symmetric and asymmetric C–H stretching vibrations are visible at 2940 and 2870 cm^{-1}, respectively. Moreover, peaks at 1108 and 1029 cm^{-1} correspond to secondary and primary C–O stretching vibrations, respectively, confirming the polyol structure (Obeidat et al., 2019).

The FTIR spectrum of the optimized composite film (F6) (Figure 4.5e) reveals several important modifications reflecting component interactions. A broad absorption centered around 3221 cm^{-1} corresponds to overlapping O–H and N–H_2 stretching bands primarily from chitosan, glycerol, and residual ascorbic acid

(Liping et al., 2020). This band exhibits a downward shift relative to pure chitosan, suggesting reduced availability of free amino groups, likely due to complexation between chitosan and ascorbic acid, resulting in the formation of a chitosan ascorbate (Guo et al., 2022; Liping et al., 2020).

Figure 4.5

FTIR spectra of (a) metronidazole, (b) ascorbic acid, (c) chitosan, (d) glycerol, and (e) the optimized film formulation (F6), recorded in the range of 4000–600 cm⁻¹, highlighting characteristic functional group vibrations for each component and confirming their presence in the final composite film.



The C–H stretching peaks are retained in the 2940–2870 cm^{-1} region, confirming the incorporation of glycerol. The distinct C–H stretching band at 3100 cm^{-1} also persists, verifying the presence of metronidazole within the film matrix. Although some metronidazole-specific bands are obscured due to spectral overlap, their integration is evident from this diagnostic region. The intense 1752 cm^{-1} C=O stretching band of pure ascorbic acid is absent in the film, replaced by a weaker, slightly shifted peak at 1749 cm^{-1}. This shift provides further evidence of interaction with chitosan, affirming the formation of a complex (A. M. Elbarbary, 2014; Halim & Kamari, 2017; Tan et al., 2019). Moreover, the C=C bending vibration at 757 cm^{-1} supports the retention of the ascorbate moiety within the matrix (Kristó et al., 2022; Tan et al., 2019).

In the lower wavenumber region, the distinct C–O stretching peaks of glycerol at 1108 and 1029 cm^{-1} appear as a single broadened band in the film, suggesting the establishment of hydrogen bonding between glycerol and chitosan hydroxyl groups (Leceta et al., 2013).

4.3.2 Differential Scanning Calorimetry (DSC) Analysis

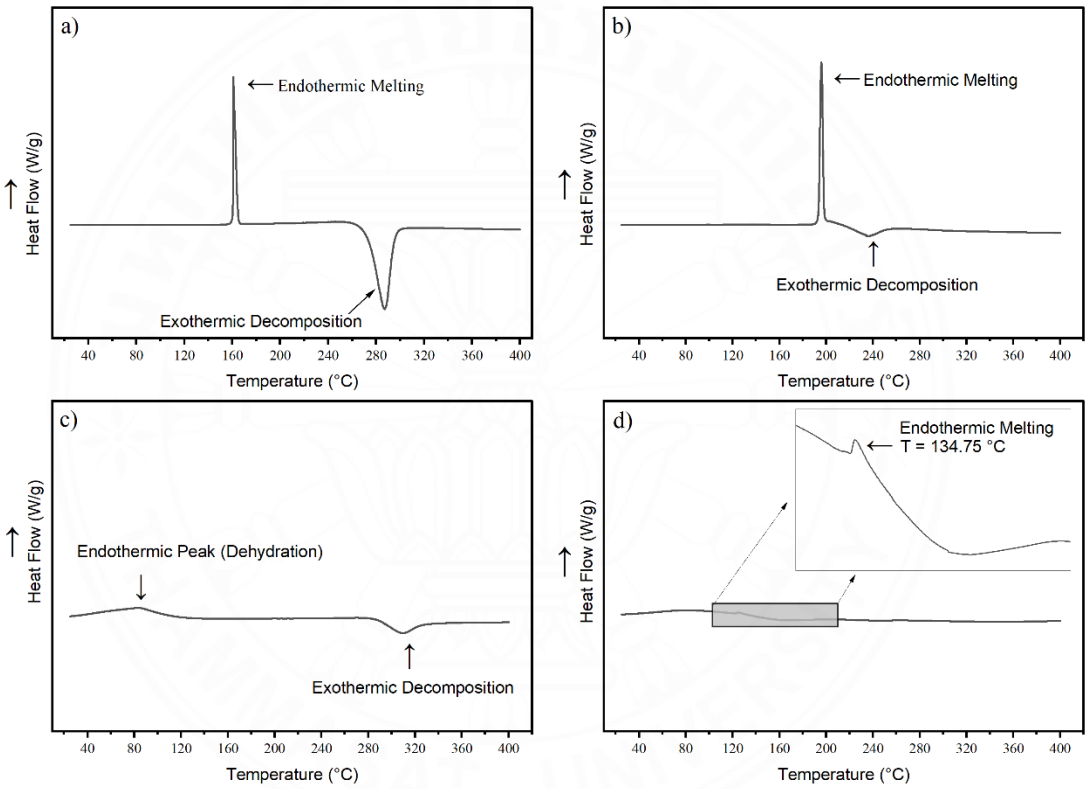
Differential Scanning Calorimetry (DSC) was employed to investigate the thermal behavior of individual solid components—metronidazole, ascorbic acid, and chitosan—and the optimized film formulation (F6). The thermograms of all samples are presented collectively in Figure 4.6. The DSC thermogram of chitosan (Figure 4.6c) displayed two prominent thermal transitions. An endothermic peak near 84 °C was observed, which is attributed to the evaporation of physically bound moisture. A second broad exothermic transition at approximately 310 °C is associated with the thermal degradation of the chitosan polymer chain. These transitions are consistent with previous reports on the thermal characteristics of chitosan (Ta et al., 2021).

As shown in Figure 4.6b, the DSC thermogram of ascorbic acid displayed a pronounced endothermic peak at approximately 194 °C, indicating its melting point. This was followed by an exothermic event centered near 237 °C, which signifies the onset of thermal decomposition (Ioan et al., 2009; Song et al., 2024). In the case of metronidazole (Figure 4.6a), its DSC curve exhibited a clear endothermic peak at 161 °C, representing its melting, and an exothermic peak at approximately

287 °C, which is associated with its thermal degradation process (Szentmihályi et al., 2022).

Figure 4.6

DSC thermograms of (a) metronidazole, (b) ascorbic acid, (c) chitosan, and (d) the optimized film formulation (F6), illustrating characteristic endothermic melting transitions and exothermic decomposition events for each component, with changes in thermal behavior in F6 indicating possible intermolecular interactions or component compatibility within the film matrix.



The DSC thermogram of the optimized film (F6), also illustrated in Figure 4.6d, demonstrated significant deviations from those of the individual components. Notably, several characteristic thermal events observed in the pure substances were absent in the thermogram of the film formulation. The disappearance of the ascorbic acid melting peak suggests that this compound exists predominantly in an amorphous form within the film matrix. This transformation is likely due to molecular dispersion and the formation of intermolecular ionic interactions between ascorbic acid and chitosan, resulting in a stable complex (Aresta et al., 2013; Tian et al., 2009).

Furthermore, a minor endothermic peak was detected at approximately 135 °C in the film, which is lower than the melting point of pure metronidazole. This downward shift in melting temperature implies a reduction in the crystalline nature of metronidazole when incorporated into the polymeric film. Such a decrease in crystallinity can be attributed to the molecular encapsulation of metronidazole within the polymeric matrix, leading to its partial amorphization and altered thermal behavior (Farshforoush et al., 2017).

4.3.3 Powder X-ray Diffraction (PXRD) Analysis

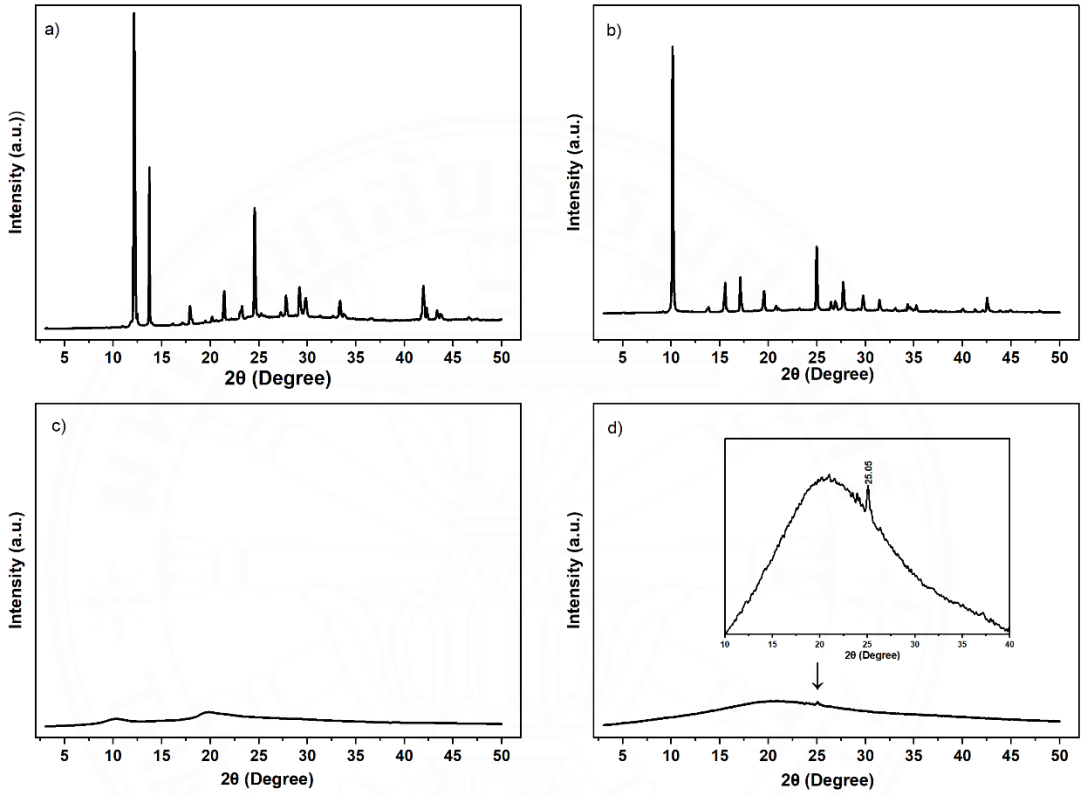
Figure 4.7 illustrates the powder X-ray diffraction (PXRD) profiles of each individual solid component alongside that of the optimized film formulation. As shown in Figure 4.7a, the PXRD spectrum of metronidazole similarly demonstrates a crystalline profile, indicated by multiple sharp and intense reflections. The PXRD pattern of ascorbic acid (Figure 4.7b) displays a series of well-defined, sharp diffraction peaks, confirming its highly crystalline nature. These peaks are consistent with those reported in prior literature for crystalline ascorbic acid (Khan et al., 2023). The diffraction pattern (Figure 4.7c) of chitosan powder reveals its semicrystalline nature, as evidenced by its broad, halo-like profile, which is characteristic of partially ordered polymeric structures (Gómez-Burgaz et al., 2009).

However, upon incorporation of the active pharmaceutical ingredients into the film matrix, significant changes in the diffraction pattern (Figure 4.7d) were observed. Specifically, the optimized film exhibited a single broad, low-intensity diffraction signal at $2\theta = 25.05°$, as denoted by an asterisk in Figure 4.7d. Notably, the characteristic sharp peaks associated with both metronidazole and ascorbic acid were largely absent in the film's diffractogram.

The disappearance of ascorbic acid's distinct crystalline peaks within the polymeric film matrix suggests a molecular dispersion of the compound and implies potential interactions between the polymer constituents and the active molecule. Such interactions may disrupt the regular crystalline lattice of ascorbic acid, thereby preventing its recrystallization within the film (Scolari et al., 2019). Moreover, the presence of weak, diffuse diffraction peaks in the film's PXRD profile indicates that the incorporated drug (metronidazole) exists predominantly in a poorly crystalline or amorphous state.

Figure 4.7

PXRD patterns of (a) metronidazole, (b) ascorbic acid, (c) chitosan, and (d) the optimized film formulation (F6), demonstrating the crystalline nature of the pure compounds and the reduced crystallinity or amorphous characteristics of the optimized film, indicative of molecular dispersion and successful component integration.



The suppressed crystallinity observed in PXRD analysis strongly correlates with the DSC findings, which also revealed the absence or depression of characteristic melting peaks of the drugs in the film. Together, these thermal and structural data support the hypothesis that the optimized film effectively converts the active compounds into an amorphous (or reduced crystalline form).

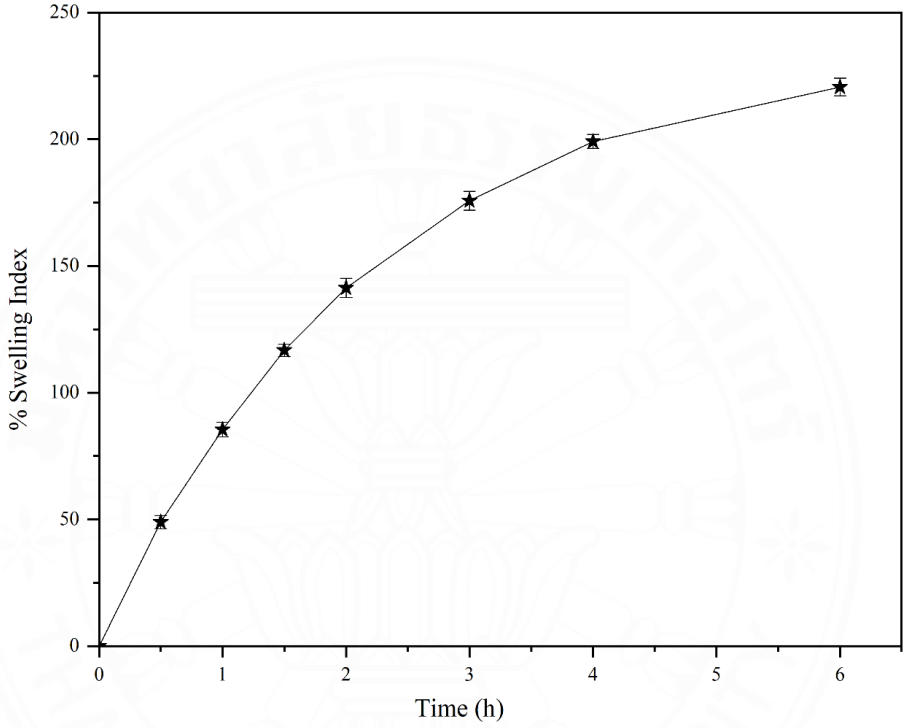
4.3.4 Swelling Index Analysis

The swelling capacity of topical films is a critical parameter in evaluating their suitability for wound management applications, as it directly influences their ability to absorb wound exudates and maintain a moist environment conducive to tissue regeneration (Ghorbani et al., 2022; Silva et al., 2023). In this study, the swelling behavior of the optimized film formulation (F6) (Figure 4.8) was systematically

assessed in simulated wound fluid at 37 °C over predefined time intervals (0.5, 1, 1.5, 2, 3, 4, and 6 h) (Ahmad et al., 2020).

Figure 4.8

Swelling profile of the optimized film formulation (F6) in simulated wound fluid at 37 °C over the indicated time intervals, illustrating its fluid absorption capacity and potential suitability for wound exudate management.



The results demonstrated a time-dependent increase in the swelling ratio, characterized by a pronounced initial surge, indicative of rapid water uptake, followed by a plateau phase where the swelling levels stabilized. This biphasic swelling profile suggests an initial phase dominated by rapid hydration and polymer chain relaxation, succeeded by a diffusion-limited equilibrium state wherein the polymer matrix becomes saturated with fluid.

The observed swelling behavior can be primarily attributed to the presence of hydrophilic functional groups embedded within the polymeric network. These polar moieties exhibit a high affinity for water molecules, thereby facilitating efficient fluid absorption through hydrogen bonding (Olewnik-Kruszkowska et al., 2021; Silva et al., 2023). Such physicochemical characteristics significantly enhance the film's fluid-handling capacity, underscoring its potential effectiveness as a wound

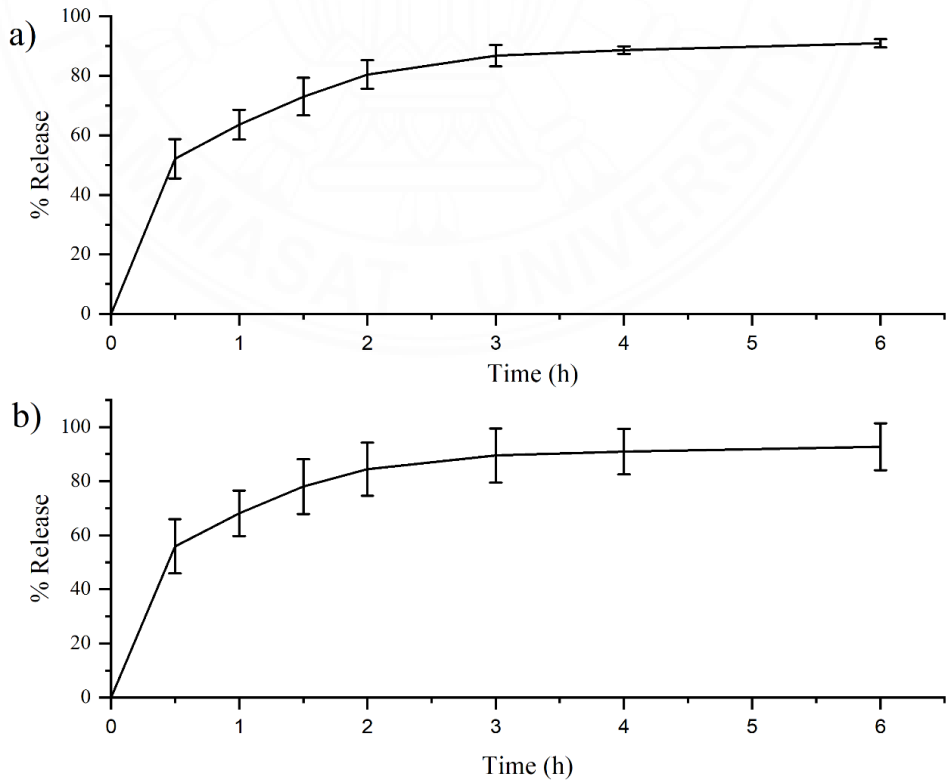
dressing material. By efficiently managing wound exudate and maintaining a hydrated microenvironment, the film supports key aspects of the wound healing process, including autolytic debridement and epithelial cell migration (Ghorbani et al., 2022; Silva et al., 2023). Therefore, the favorable swelling profile of the optimized formulation further corroborates its suitability for advanced wound care applications.

4.3.5 In Vitro Drug Release Studies

The release of APIs from a drug delivery system is crucial in achieving optimal therapeutic outcomes at the intended site of action (Khan et al., 2020). Figure 4.9 illustrates the in vitro release profiles of ascorbic acid and metronidazole from the optimized film formulation. Both compounds exhibited a biphasic release pattern, characterized by an initial burst release followed by a prolonged, sustained release phase. Within a 6-hour period, the cumulative release of ascorbic acid and metronidazole reached 92.7% and 90.9%, respectively.

Figure 4.9

Biphasic release profiles of (a) metronidazole and (b) ascorbic acid from the optimized film (F6), showing an initial burst followed by sustained release, with cumulative releases of 90.9% and 92.7% respectively over 6 hours—ideal for wound healing applications.



The observed rapid initial release is likely attributed to the diffusion of the APIs, which is facilitated by the rapid hydration and subsequent swelling of the polymeric film matrix (Khan et al., 2020). Furthermore, the release behavior may also be influenced by the physicochemical nature of the active compounds, specifically their low crystallinity or amorphous state, which enhances their solubility and promotes faster dissolution upon exposure to the dissolution medium.

The release rate transitions into a sustained pattern following the initial burst phase. This phase is primarily governed by the diffusion of the remaining drug molecules through the now-swollen polymeric network, which acts as a barrier and gradually slows their mobility and release (Khan et al., 2020; Savencu et al., 2024). For wound healing applications, such a release profile is particularly advantageous. The initial burst ensures a rapid onset of therapeutic action at the wound site, which is essential for early-stage microbial control and inflammation management (Savencu et al., 2024). Meanwhile, the subsequent sustained release phase aids in maintaining adequate local drug concentrations over an extended period, thereby supporting continued therapeutic activity and promoting wound recovery (Ruffo et al., 2022; Savencu et al., 2024).

CHAPTER 5
CONCLUSION

In this study, a chitosan-based polymeric film system plasticized with glycerol was successfully developed and optimized for the simultaneous topical delivery of ascorbic acid and metronidazole. The film-forming solution was prepared via a green, solvent-casting approach utilizing ascorbic acid as both an active ingredient and a chitosan-solubilizing agent, eliminating the requirement for additional mineral or organic acids. This simplified formulation strategy enhances biocompatibility and ease of manufacturing, making it highly relevant for wound care applications.

Distinct film formulations were designed and evaluated using Box-Behnken design (BBD). This approach enabled efficient optimization of formulation parameters and their effects on mechanical characteristics (ultimate tensile strength and elongation at break) and pH, all of which are critical for topical film performance.

The optimized formulation (F6), containing 3.0% w/w chitosan, 4.5% w/w ascorbic acid, and 30% w/w glycerol (relative to chitosan), demonstrated favorable structural and functional characteristics.

Swelling studies revealed that the optimized film exhibited a swelling degree of 220.6% after 6 hours, reflecting its high-water uptake capacity and potential to maintain a moist wound environment, which is critical for enhancing healing, absorbing exudate, and improving drug diffusion.

In vitro drug release studies revealed a desirable biphasic release profile, characterized by an initial burst release followed by a sustained release phase. After 6 hours, the cumulative drug release reached 92.7% for ascorbic acid and 90.9% for metronidazole, effectively providing both immediate and prolonged therapeutic effects—ideal for wound healing applications that require rapid antimicrobial and antioxidant actions followed by sustained activity to support tissue regeneration.

Complementary physicochemical analyses further confirmed the success of the formulation. Fourier-transform infrared spectroscopy (FTIR) evidenced the formation of chitosan ascorbate and hydrogen bonding interactions between chitosan and glycerol. Differential scanning calorimetry (DSC) and powder X-ray diffraction

(PXRD) revealed significant suppression of drug crystallinity, indicating drug amorphization within the polymer matrix, a property that enhances drug solubility.

In conclusion, the chitosan-based film developed in this work exhibited optimal mechanical strength, flexibility, pH compatibility, swelling capacity, and controlled dual-drug release. The findings establish a foundation for future in vivo evaluations, stability studies, and potential clinical translation in the field of advanced wound therapeutics.

REFERENCES

A. M. Elbarbary, N. M. E.-S. A. E. A. H. (2014). Antioxidative properties of irradiated chitosan/vitamin C nanoparticles and their use as food additive for lipid storage. *Egyptian Journal of Radiation Sciences and Applications*, *27*(1), 119-133.

Abla, K. K., Mneimneh, A. T., Allam, A. N., & Mehanna, M. M. (2023). Application of box-behnken design in the preparation, optimization, and in-vivo pharmacokinetic evaluation of oral tadalafil-loaded niosomal film. *Pharmaceutics*, *15*(1), 173.

Adekogbe, I., & Ghanem, A. (2005). Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering. *Biomaterials*, *26*(35), 7241-7250.

Ahmad, N., Tayyeb, D., Ali, I., N, K. A., Ahmad, W., Ur Rehman, A., Khan, A. H., & Iqbal, M. S. (2020). Development and characterization of hemicellulose-based films for antibacterial wound-dressing application. *Polymers*, *12*(3), 548.

Ahmed, A., Getti, G., & Boateng, J. (2021). Medicated multi-targeted alginate-based dressings for potential treatment of mixed bacterial-fungal infections in diabetic foot ulcers. *International Journal of Pharmaceutics*, *606*, 120903.

Al Hanbali, O. A., Khan, H. M. S., Sarfraz, M., Arafat, M., Ijaz, S., & Hameed, A. (2019). Transdermal patches: design and current approaches to painless drug delivery. *Acta Pharmaceutica*, *69*(2), 197-215.

Amorim, J. D. P. D., Cavalcanti, Y. D. F., Medeiros, A. D. L. M. D., Silva Junior, C. J. G. D., Durval, I. J. B., Costa, A. F. D. S., & Sarubbo, L. A. (2024). Synthesis of transparent bacterial cellulose films as a platform for targeted drug delivery in wound care. *Processes*, *12*(7), 1282.

Anbukarasu, P., Sauvageau, D., & Elias, A. (2015). Tuning the properties of polyhydroxybutyrate films using acetic acid via solvent casting. *Scientific Reports*, *5*, 17884.

Aresta, A., Calvano, C. D., Trapani, A., Cellamare, S., Zambonin, C. G., & De Giglio, E. (2013). Development and analytical characterization of vitamin(s)-loaded chitosan nanoparticles for potential food packaging applications. *Journal of Nanoparticle Research*, *15*, 1592.

Asare-Nkansah, S., Kwakye, J., Adosraku, R., & Bekoe, S. (2014). A new dimension in medicines quality monitoring: using surrogate reference standards in RP-HPLC assay of pharmaceuticals. *International Journal of Pure and Applied Chemistry*, *9*, 85-95.

Aydin, H., Tatar, C., Savas, O. A., Karsidag, T., Ozer, B., Dursun, N., Bekem, A., Unal, A., & Tuzun, I. S. (2019). The effects of local and systemic administration of proline on wound healing in rats. *Journal of Investigative Surgery*, *32*(6), 523-529.

Basoulis, D., Fragiadaki, K., Tentolouris, N., Sfikakis, P. P., & Kokkinos, A. (2016). Anti-tnfα treatment for recalcitrant ulcerative necrobiosis lipoidica diabeticorum: A case report and review of the literature. *Metabolism*, *65*(4), 569-573.

Bechara, N., Flood, V. M., & Gunton, J. E. (2022). A systematic review on the role of vitamin C in tissue healing. *Antioxidants*, *11*(8), 1605.

Beg, S., & Akhter, S. (2021). Box–behnken designs and their applications in pharmaceutical product development. In S. Beg & S. Akhter (Eds.), *Design of experiments for pharmaceutical product development: Volume I – Basics and fundamental principles* (pp. 77–85). Springer.

Boateng, J. S., Sarheed, O., Ahmed, A., & Shouqair, D. (2016). Antimicrobial dressings for improving wound healing. In V. A. Alexandrescu (Ed.), *Wound healing – New insights into ancient challenges* (pp. 377–402). IntechOpen.

Boateng, J. S., Hafezi, F., Tabriz, A. G., & Douroumis, D. (2023). 3d printed composite dressings loaded with human epidermal growth factor for potential chronic wound healing applications. *Journal of Drug Delivery Science and Technology*, *86*, 104684.

Boateng, J. S., Matthews, K. H., Stevens, H. N. E., & Eccleston, G. M. (2008). Wound healing dressings and drug delivery systems: A review. *Journal of Pharmaceutical Sciences*, *97*(8), 2892-2923.

Borges-Vilches, J., Poblete, J., Gajardo, F., Aguayo, C., & Fernández, K. (2021). Graphene oxide/polyethylene glycol aerogel reinforced with grape seed extracts as wound dressing. *Journal of Materials Science*, *56*, 16082 - 16096.

Brereton, R. G. (2019). ANOVA tables and statistical significance of models. *Journal of Chemometrics*, *33*(3), E3019.

Broussard, K. C., & Powers, J. G. (2013). Wound dressings: selecting the most appropriate type. *American Journal of Clinical Dermatology*, *14*(6), 449-459.

Brown, C. D., Kreilgaard, L., Nakakura, M., Caram-Lelham, N., Pettit, D. K., Gombotz, W. R., & Hoffman, A. S. (2001). Release of pegylated granulocyte-macrophage colony-stimulating factor from chitosan/glycerol films. *Journal of Controlled Release*, *72*(1-3), 35-46.

Bui, V. K. H., Park, D., & Lee, Y. C. (2017). Chitosan combined with ZnO, TiO_2, and Ag nanoparticles for antimicrobial wound healing applications: A mini review of the research trends. *Polymers*, *9*(1), 21.

Carra, J. B., Wessel, K. B. B., Pereira, G. N., Oliveira, M. C., Pattini, P. M. T., Masquetti, B. L., Amador, I. R., Bruschi, M. L., Casagrande, R., Georgetti, S. R., Verri, W. A., Jr., Nakazato, G., Vignoli, J. A., Camilios-Neto, D., & Baracat, M. M. (2024). Bioadhesive polymeric films containing rhamnolipids, an innovative antimicrobial topical formulation. *American Association of Pharmaceutical Scientists*, *25*(6), 177.

Castro, D. L., & Santos, V. L. (2015). Controlling wound odor with metronidazole: A systematic review. *Journal of School of Nursing – University of São Paulo*, *49*(5), 858-863.

Catanzano, O., Docking, R., Schofield, P., & Boateng, J. (2017). Advanced multi-targeted composite biomaterial dressing for pain and infection control in chronic leg ulcers. *Carbohydrate Polymers*, *172*, 40-48.

Chereddy, K. K., Her, C. H., Comune, M., Moia, C., Lopes, A., Porporato, P. E., Vanacker, J., Lam, M. C., Steinstraesser, L., Sonveaux, P., Zhu, H., Ferreira, L. S., Vandermeulen, G., & Préat, V. (2014). PLGA nanoparticles loaded with host defense peptide IL-37 promote wound healing. *Journal of Controlled Release*, *194*, 138-147.

Choi, J. U., Lee, S. W., Pangeni, R., Byun, Y., Yoon, I. S., & Park, J. W. (2017). Preparation and in vivo evaluation of cationic elastic liposomes comprising highly skin-permeable growth factors combined with hyaluronic acid for enhanced diabetic wound-healing therapy. *Acta Biomaterialia*, *57*, 197-215.

Cirri, M., Maestrelli, F., Scuota, S., Bazzucchi, V., & Mura, P. (2021). Development and microbiological evaluation of chitosan and chitosan-alginate microspheres for vaginal administration of metronidazole. *International Journal of Pharmaceutics, 598*, 120375.

Cooper, B., & Bachoo, P. (2018). Extracorporeal shock wave therapy for the healing and management of venous leg ulcers. *Cochrane Database of Systematic Reviews, 6*(6), Cd011842.

Daeschlein, G. (2013). Antimicrobial and antiseptic strategies in wound management. *International Wound Journal, 10*(1), 9-14.

Dave, V., Kushwaha, K., Yadav, R. B., & Agrawal, U. (2017). Hybrid nanoparticles for the topical delivery of norfloxacin for the effective treatment of bacterial infection produced after burn. *Journal of Microencapsulation, 34*(4), 351-365.

De Souza, K. S., Cantaruti, T. A., Azevedo, G. M., Jr., Galdino, D. A., Rodrigues, C. M., Costa, R. A., Vaz, N. M., & Carvalho, C. R. (2015). Improved cutaneous wound healing after intraperitoneal injection of alpha-melanocyte-stimulating hormone. *Experimental Dermatology*, *24*(3), 198-203.

Demir, A., Arık, B., Ozdogan, E., & Seventekin, N. (2010). A new application method of chitosan for improved antimicrobial activity on wool fabrics pretreated by different ways. *Fibers and Polymers*, *11*(3), 351-356.

Dingsdag, S. A., & Hunter, N. (2018). Metronidazole: An update on metabolism, structure-cytotoxicity and resistance mechanisms. *Journal of Antimicrobiology and Chemotherapy*, *73*(2), 265-279.

Do Amaral Sobral, P. J., Gebremariam, G., Drudi, F., De Aguiar Saldanha Pinheiro, A. C., Romani, S., Rocculi, P., & Dalla Rosa, M. (2022). Rheological and viscoelastic properties of chitosan solutions prepared with different chitosan or acetic acid concentrations. *Foods*, *11*(17), 2692.

Dobaria, N. B., Badhan, A. C., & Mashru, R. (2009). A novel itraconazole bioadhesive film for vaginal delivery: Design, optimization, and physico-dynamic characterization. *American Association of Pharmaceutical Scientists, 10*, 951-959.

Edikresnha, D., Suciati, T., Munir, M. M., & Khairurrijal, K. (2019). Polyvinylpyrrolidone/cellulose acetate electrospun composite nanofibres

loaded by glycerine and garlic extract with in vitro antibacterial activity and release behaviour test. *RSC Advances*, *9*(45), 26351-26363.

Edwards, D. I. (1979). Mechanism of antimicrobial action of metronidazole. *Journal of Antimicrobiology and Chemotherapy*, *5*(5), 499-502.

El-Shanshory, A. A., Agwa, M. M., Abd-Elhamid, A. I., Soliman, H. M. A., Mo, X., & Kenawy, E.-R. (2022). Metronidazole topically immobilized electrospun nanofibrous scaffold: Novel secondary intention wound healing accelerator. *Polymers*, *14*(3), 454.

Elshamy, S., Khadizatul, K., Uemura, K., Nakajima, M., & Neves, M. A. (2021). Chitosan-based film incorporated with essential oil nanoemulsion foreseeing enhanced antimicrobial effect. *Journal of Food Science and Technology*, *58*(9), 3314-3327.

Escárcega-Galaz, A. A., Sánchez-Machado, D. I., López-Cervantes, J., Sanches-Silva, A., Madera-Santana, T. J., & Paseiro-Losada, P. (2018). Mechanical, structural, and physical aspects of chitosan-based films as antimicrobial dressings. *International Journal of Biological Macromolecules*, *116*, 472-481.

Falanga, V., Brem, H., Ennis, W. J., Wolcott, R., Gould, L. J., & Ayello, E. A. (2008). Maintenance debridement in the treatment of difficult-to-heal chronic wounds. recommendations of an expert panel. *Ostomy Wound Management, Supplement*, 2-13.

Farshforoush, P., Ghanbarzadeh, S., Goganian, A. M., & Hamishehkar, H. (2017). Novel metronidazole-loaded hydrogel as a gastroretentive drug delivery system. *Iranian Polymer Journal*, *26*(12), 895-901.

Feng, P., Luo, Y., Ke, C., Qiu, H., Wang, W., Zhu, Y., Hou, R., Xu, L., & Wu, S. (2021). Chitosan-based functional materials for skin wound repair: mechanisms and applications. *Frontiers in Bioengineering and Biotechnology*, *9*, 650598.

Fernandes, A. S., De Souza Ferreira, S. B., & Bruschi, M. L. (2023). Design as a strategy for evaluation of the mechanical properties of binary mixtures composed of poly(methyl vinyl ether-alt-maleic anhydride) and Pluronic F127 for biomedical applications. *Journal of the Mechanical Behavior of Biomedical Materials*, *138*, 105608.

Firlar, I., Altunbek, M., Mccarthy, C., Ramalingam, M., & Camci-Unal, G. (2022). Functional hydrogels for treatment of chronic wounds. *Gels*, *8*(2), 127.

Freeman, C. D., Klutman, N. E., & Lamp, K. C. (1997). Metronidazole: A therapeutic review and update. *Drugs*, *54*(5), 679-708.

Gainza, G., Chu, W. S., Guy, R. H., Pedraz, J. L., Hernandez, R. M., Delgado-Charro, B., & Igartua, M. (2015). Development and in vitro evaluation of lipid nanoparticle-based dressings for topical treatment of chronic wounds. *International Journal of Pharmaceutics*, *490*(1-2), 404-411.

Gainza, G., Pastor, M., Aguirre, J. J., Villullas, S., Pedraz, J. L., Hernandez, R. M., & Igartua, M. (2014). A novel strategy for the treatment of chronic wounds based on the topical administration of rhEGF-loaded lipid nanoparticles: In vitro bioactivity and in vivo effectiveness in healing-impaired db/db mice. *Journal of Controlled Release*, *185*, 51-61.

Gao, J. (2024). R-squared (R^2) – How much variation is explained? *Research Methods In Medicine & Health Sciences*, *5*(4), 104-109.

Genesi, B. P., De Melo Barbosa, R., Severino, P., Rodas, A. C. D., Yoshida, C. M. P., Mathor, M. B., Lopes, P. S., Viseras, C., Souto, E. B., & Ferreira Da Silva, C. (2023). Aloe vera and copaiba oleoresin-loaded chitosan films for wound dressings: microbial permeation, cytotoxicity, and in vivo proof of concept. *International Journal of Pharmaceutics*, *634*, 122648.

Ghahremani-Nasab, M., Akbari-Gharalari, N., Rahmani Del Bakhshayesh, A., Ghotaslou, A., Ebrahimi-Kalan, A., Mahdipour, M., & Mehdipour, A. (2023). Synergistic effect of chitosan-alginate composite hydrogel enriched with ascorbic acid and alpha-tocopherol under hypoxic conditions on the behavior of mesenchymal stem cells for wound healing. *Stem Cell Research and Therapy*, *14*(1), 326.

Ghorbani, M., Hassani, N., & Raeisi, M. (2022). Development of gelatin thin film reinforced by modified gellan gum and naringenin-loaded zein nanoparticle as a wound dressing. *Macromolecular Research*, *30*, 397- 405.

Gómez-Burgaz, M., Torrado, G., & Torrado, S. (2009). Characterization and superficial transformations on mini-matrices made of interpolymer complexes of chitosan

and carboxymethylcellulose during in vitro clarithromycin release. *European Journal of Pharmaceutics and Biopharmaceutics*, *73(1)*, 130-139.

Guo, Y., Qu, Y., Yu, J., Song, L., Chen, S., Qin, Z., Gong, J., Zhan, H., Gao, Y., & Zhang, J. (2022). A chitosan-vitamin C-based injectable hydrogel improves cell survival under oxidative stress. *International Journal of Biological Macromolecules*, *202*, 102-111.

Halim, A. L. A., & Kamari, A. (2017, May 01). Active biopolymer film based on carboxymethyl cellulose and ascorbic acid for food preservation. *Proceedings of the International Conference on Education, Mathematics and Science 2016*, 1847(1).

Hanna, J. R., & Giacopelli, J. A. (1997). A review of wound healing and wound dressing products. *Journal of Foot and Ankle Surgery*, *36*(1), 2-14.

Harding, K. G., Morris, H. L., & Patel, G. K. (2002). Science, medicine and the future: Healing chronic wounds. *British Medical Journal*, *324*(7330), 160-163.

Hernández Ceruelos, A., Romero-Quezada, L. C., Ruvalcaba Ledezma, J. C., & López Contreras, L. (2019). Therapeutic uses of metronidazole and its side effects: An update. *European Review for Medical and Pharmacological Sciences*, *23*(1), 397-401.

Hissae Yassue-Cordeiro, P., Zandonai, C. H., Pereira Genesi, B., Santos Lopes, P., Sanchez-Lopez, E., Garcia, M. L., Camargo Fernandes-Machado, N. R., Severino, P., E, B. S., & Ferreira Da Silva, C. (2019). Development of chitosan/silver sulfadiazine/zeolite composite films for wound dressing. *Pharmaceutics*, *11*(10), 535.

Ioan, B., Marieta, M.-P., Irina, K., & Sorin, I. F. (2009). Spectroscopic investigation of the interaction between β-cyclodextrin and ascorbic acid. *Journal of Physics: Conference Series*, *182*(1), 012004.

Irfan, M., Rabel, S., Bukhtar, Q., Qadir, M. I., Jabeen, F., & Khan, A. (2016). Orally disintegrating films: a modern expansion in drug delivery system. *Saudi Pharmaceutical Journal*, *24*(5), 537-546.

Ito, N., Itoh, N., & Kameshima, S. (2023). Volatile sulfur compounds produced by the anaerobic bacteria *Porphyromonas spp*. Isolated from the oral cavities of dogs. *Veterinary Sciences*, *10*(8), 503.

Janjarasskul, T., Tananuwong, K., & Krochta, J. M. (2011). Whey protein film with oxygen scavenging function by incorporation of ascorbic acid. *Journal of Food Science*, *76*(9), 561-568.

Jillani, U., Mudassir, J., Ijaz, Q. A., Latif, S., Qamar, N., Aleem, A., Ali, E., Abbas, K., Wazir, M. A., Hussain, A., Abbas, N., & Arshad, M. S. (2022). Design and characterization of agarose/HPMC buccal films bearing ondansetron HCl in vitro and in vivo: Enhancement using iontophoretic and chemical approaches. *Biomed Research International*, *2022*, 1662194.

Khan, B. A., Ullah, S., Khan, M. K., Uzair, B., Menaa, F., & Braga, V. A. (2020). Fabrication, physical characterizations, and in vitro, in vivo evaluation of ginger extract-loaded gelatin/poly (vinyl alcohol) hydrogel films against burn wound healing in animal model. *American Association of Pharmaceutical Scientists*, *21*(8), 323.

Khan, N., Singh, A. K., & Saneja, A. (2023). Preparation, characterization, and antioxidant activity of L-ascorbic acid/HP-*β*-cyclodextrin inclusion complex-incorporated electrospun nanofibers. *Foods*, *12*(7), 1363.

Khodabakhshi, D., Eskandarinia, A., Kefayat, A., Rafienia, M., Navid, S., Karbasi, S., & Moshtaghian, J. (2019). In vitro and in vivo performance of a propolis-coated polyurethane wound dressing with high porosity and antibacterial efficacy. *Colloids and Surfaces B: Biointerfaces*, *178*, 177-184.

Kianfar, F., Chowdhry, B. Z., Antonijevic, M. D., & Boateng, J. S. (2012). Novel films for drug delivery via the buccal mucosa using model soluble and insoluble drugs. *Drug Development and Industrial Pharmacy*, *38*(10), 1207-1220.

Kim, H., Lee, J. A., Sim, H. J., Lima, M. D., Baughman, R. H., & Kim, S. J. (2016). Temperature-responsive tensile actuator based on multi-walled carbon nanotube yarn. *Nano-micro Letters*, *8*(3), 254-259.

Kim, H. S., Sun, X., Lee, J.-H., Kim, H.-W., Fu, X., & Leong, K. W. (2019). Advanced drug delivery systems and artificial skin grafts for skin wound healing. *Advanced Drug Delivery Reviews*, *146*, 209-239.

Kloth, L. C. (2014). Electrical stimulation technologies for wound healing. *Advance Wound Care (New Rochelle)*, *3*(2), 81-90.

Kowalczyk, D. (2016). Biopolymer/Candelilla wax emulsion films as carriers of ascorbic acid – a comparative study. *Food Hydrocolloids*, *52*, 543-553.

Kristó, K., Módra, S., Hornok, V., Süvegh, K., Ludasi, K., Aigner, Z., Kelemen, A., Sovány, T., Pintye-Hódi, K., & Regdon, G., Jr. (2022). Investigation of surface properties and free volumes of chitosan-based buccal mucoadhesive drug delivery films containing ascorbic acid. *Pharmaceutics*, *14*(2), 345.

Kumar, R. (2020). Modified mix design and statistical modelling of lightweight concrete with high volume micro-fine waste additive via the box-behnken design approach. *Cement and Concrete Composites*, *113*, 103706.

Lalezari, S., Lee, C. J., Borovikova, A. A., Banyard, D. A., Paydar, K. Z., Wirth, G. A., & Widgerow, A. D. (2017). Deconstructing negative pressure wound therapy. *International Wound Journal*, *14*(4), 649-657.

Las Heras, K., Igartua, M., Santos-Vizcaino, E., & Hernandez, R. M. (2020). Chronic wounds: current status, available strategies, and emerging therapeutic solutions. *Journal of Controlled Release*, *328*, 532-550.

Layek, B., Rahman Nirzhor, S. S., Rathi, S., Kandimalla, K. K., Wiedmann, T. S., & Prabha, S. (2019). Design, development, and characterization of imiquimod-loaded chitosan films for topical delivery. *American Association of Pharmaceutical Scientists*, *20*(2), 58.

Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Pecoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Archives of Dermatological Research*, *130*(4), 489-493.

Leceta, I., Guerrero, P., & De La Caba, K. (2013). Functional properties of chitosan-based films. *Carbohydrate Polymers*, *93*(1), 339-346.

Leonhardt, E. E., Kang, N., Hamad, M. A., Wooley, K. L., & Elsabahy, M. (2019). Absorbable hemostatic hydrogels comprising composites of sacrificial templates and honeycomb-like nanofibrous mats of chitosan. *Nature Communications*, *10*(1), 2307.

Li, B., Wang, J., Gui, Q., & Yang, H. (2020). Drug-loaded chitosan film prepared via facile solution casting and air-drying of plain water-based chitosan solution for ocular drug delivery. *Bioactive Materials*, *5*(3), 577-583.

Li, X., Bai, H., Yang, Y., Yoon, J., Wang, S., & Zhang, X. (2019). Supramolecular antibacterial materials for combatting antibiotic resistance. *Advanced Materials*, *31*(5), 1805092.

Li, X., Ma, M., Ahn, D. U., & Huang, X. (2019). Preparation and characterization of novel eggshell membrane-chitosan blend films for potential wound-care dressing: from waste to medicinal products. *International Journal of Biological Macromolecules*, *123*, 477-484.

Liping, L., Kexin, L., Huipu, D., Jia, L., & Jie, Z. (2020). Study on preparation of a chitosan/vitamin C complex and its properties in cosmetics. *Natural Product Communications*, *15*(10), 1.

Lipsky, B. A., & Hoey, C. (2009). Topical antimicrobial therapy for treating chronic wounds. *Clinical Infectious Diseases*, *49*(10), 1541-1549.

Liu, K., Lin, X., Chen, L., Huang, L., & Cao, S. (2014). Dual-functional chitosan–methylisothiazolinone/microfibrillated cellulose biocomposites for enhancing antibacterial and mechanical properties of agar films. *Cellulose*, *21*, 519-528.

Liu, T., Zhang, Z., Liu, J., Dong, P., Tian, F., Li, F., & Meng, X. (2022). Electrospun kaolin-loaded chitosan/PEO nanofibers for rapid hemostasis and accelerated wound healing. *International Journal of Biological Macromolecules*, *217*, 998-1011.

Löfmark, S., Edlund, C., & Nord, C. E. (2010). Metronidazole is still the drug of choice for the treatment of anaerobic infections. *Clinical Infectious Diseases*, *50*(1), 16-S23.

Lord, M. S., Cheng, B., Mccarthy, S. J., Jung, M., & Whitelock, J. M. (2011). The modulation of platelet adhesion and activation by chitosan through plasma and extracellular matrix proteins. *Biomaterials*, *32*(28), 6655-6662.

Love, E. (2010). Design, evaluation, parameters and marketed products of transdermal patches: A review. *Journal of Pharmacy Research*, *3*(2), 235–240.

M M Agwa, S. S., Nagwa A. Atwa, Heba A. Dahdooh, Ramadan E El Lithy, , & Elmotasem, H. (2022). Potential of frankincense essential oil-loaded whey protein nanoparticles embedded in frankincense resin as a wound healing film based on green technology. *Journal of Drug Delivery Science and Technology*, *71*, 103291.

Manconi, M., Manca, M. L., Caddeo, C., Valenti, D., Cencetti, C., Diez-Sales, O., Nacher, A., Mir-Palomo, S., Terencio, M. C., Demurtas, D., Gomez-Fernandez, J. C., Aranda, F. J., Fadda, A. M., & Matricardi, P. (2018). Nanodesign of new self-assembling core-shell gellan-transfersomes loading baicalin and in vivo evaluation of repair response in skin. *Nanomedicine*, *14*(2), 569-579.

Matta, M. D. D., Sarmento, S. B. S., Oliveira, L. M. D., & Zocchi, S. S. (2011). Mechanical properties of pea starch films associated with xanthan gum and glycerol. *Starch*, *63*, 274-282.

Meng, H., Yang, D., Tu, Y., & Yan, J. (2017). Turn-on fluorescence detection of ascorbic acid with gold nanoclusters. *Talanta*, *165*, 346-350.

Mi, F. L., Shyu, S. S., Wu, Y. B., Lee, S. T., Shyong, J. Y., & Huang, R. N. (2001). Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials*, *22*(2), 165-173.

Moores, J. (2013). Vitamin C: A wound healing perspective. *British Journal of Community Nurses*, 18(Suppl 12), S6–S11.

Mori, D., Rathod, P., Parmar, R., Dudhat, K., & Chavda, J. (2020). Preparation and optimization of multi-functional directly compressible excipient: An integrated approach of principal component analysis and design of experiments. *Drug Development and Industrial Pharmacy*, *46*(12), 2010-2021.

Nelson, E. A., & Bell-Syer, S. E. (2014). Compression for preventing recurrence of venous ulcers. *Cochrane Database of Systematic Reviews*, *2014*(9), 002303.

Ng, S. F., Chew, Y. M., Chng, P. E., & Ng, K. S. (2018). An insight of linear regression analysis. *Scientific Research Journal*, *15*(2), 1-16.

Notario-Pérez, F., Cazorla-Luna, R., Martín-Illana, A., Galante, J., Ruiz-Caro, R., Das Neves, J., & Veiga, M. D. (2020). Design, fabrication and characterisation of drug-loaded vaginal films: State-of-the-art. *Journal of Controlled Release*, *327*, 477-499.

Nour, S., Baheiraei, N., Imani, R., Khodaei, M., Alizadeh, A., Rabiee, N., & Moazzeni, S. M. (2019). A review of accelerated wound healing approaches: Biomaterial-assisted tissue remodeling. *Journal of Materials Science: Materials in Medicine*, *30*(10), 120.

O'Meara, S., Al-Kurdi, D., Ologun, Y., & Ovington, L. G. (2010). Antibiotics and antiseptics for venous leg ulcers. *Cochrane Database of Systematic Reviews*, 2010(1), Cd003557.

Obeidat, S. M., Y., H. A., & And Mahmoud, A. (2019). Identification and quantification of diethylene glycol contamination in glycerine raw material. *Spectroscopy Letters*, *52*(1), 60-65.

Olewnik-Kruszkowska, E., Gierszewska, M., Grabska-Zielińska, S., Skopińska-Wiśniewska, J., & Jakubowska, E. (2021). Examining the impact of squaric acid as a crosslinking agent on the properties of chitosan-based films. *International Journal of Molecular Sciences*, *22*(7), 3329.

Otero-Viñas, M., & Falanga, V. (2016). Mesenchymal stem cells in chronic wounds: the spectrum from basic to advanced therapy. *Advance Wound Care (New Rochelle)*, *5*(4), 149-163.

Patel, P., Parmar, K., Patel, D., Kumar, S., Trivedi, M., & Das, M. (2018). Inhibition of amyloid fibril formation of lysozyme by ascorbic acid and a probable mechanism of action. *International Journal of Biological Macromolecules*, *114*, 666-678.

Patil, N., & Wairkar, S. (2024). Chitosan and a cellulose-based mupirocin topical film-forming spray: optimization, in vitro characterization, antimicrobial studies, and wound healing activity. *International Journal of Biological Macromolecules*, *254*(1), 127622.

Paul, J. C., & Pieper, B. A. (2008). Topical metronidazole for the treatment of wound odor: a review of the literature. *Ostomy Wound Management*, *54*(3), 18-27.

Peng, L., & Dai, Y. (2020). Effect of metronidazole combined with autolytic debridement for the management of malignant wound malodor. *Journal of International Medical Research*, *48*(4), 300060519889746.

Percival, N. J. (2002). Classification of wounds and their management. *Surgery*, *20*(5), 114-117.

Qiao, C., Ma, X., Wang, X., & Liu, L. (2021). Structure and properties of chitosan films: effect of the type of solvent acid. *Food Science and Technology*, *135*, 109984.

Ruffo, M., Parisi, O. I., Dattilo, M., Patitucci, F., Malivindi, R., Pezzi, V., Tzanov, T., & Puoci, F. (2022). Synthesis and evaluation of wound healing properties of hydro-diab hydrogel loaded with green-synthetized agnps: in vitro and in ex vivo studies. *Drug Delivery and Translational Research*, *12*(8), 1881-1894.

Saghazadeh, S., Rinoldi, C., Schot, M., Kashaf, S. S., Sharifi, F., Jalilian, E., Nuutila, K., Giatsidis, G., Mostafalu, P., Derakhshandeh, H., Yue, K., Swieszkowski, W., Memic, A., Tamayol, A., & Khademhosseini, A. (2018). Drug delivery systems and materials for wound healing applications. *Advanced Drug Delivery Reviews*, *127*, 138-166.

Sampaopan, Y., & Suksaeree, J. (2022). Formulation development and pharmaceutical evaluation of *Lysiphyllum Strychnifolium* topical patches for their anti-inflammatory potential. *American Association of Pharmaceutical Scientists*, *23*(5), 116.

Sanchez, D. A., Schairer, D., Tuckman-Vernon, C., Chouake, J., Kutner, A., Makdisi, J., Friedman, J. M., Nosanchuk, J. D., & Friedman, A. J. (2014). Amphotericin B-releasing nanoparticle topical treatment of *Candida Spp* in the setting of a burn wound. *Nanomedicine*, *10*(1), 269-277.

Sarheed, O., Abdul Rasool, B. K., Abu-Gharbieh, E., & Aziz, U. S. (2015). An investigation and characterization on alginate hydogel dressing loaded with metronidazole prepared by combined inotropic gelation and freeze-thawing cycles for controlled release. *American Association of Pharmaceutical Scientists*, *16*(3), 601-609.

Savencu, I., Iurian, S., Bogdan, C., Spînu, N., Suciu, M., Pop, A., Țoc, A., & Tomuță, I. (2024). Design, optimization and pharmaceutical characterization of wound healing film dressings with chloramphenicol and ibuprofen. *Drug Development and Industrial Pharmacy, 50*(5), 446-459.

Schilrreff, P., & Alexiev, U. (2022). Chronic inflammation in non-healing skin wounds and promising natural bioactive compounds treatment. *International Journal of Molecular Sciences, 23*(9), 4928.

Scolari, I. R., Páez, P. L., Sánchez-Borzone, M. E., & Granero, G. E. (2019). Promising chitosan-coated alginate-tween 80 nanoparticles as rifampicin coadministered

ascorbic acid delivery carrier against *Mycobacterium tuberculosis*. *American Association of Pharmaceutical Scientists*, *20*(2), 67.

Sezer, A. D., Hatipoğlu, F., Cevher, E., Oğurtan, Z., Baş, A. L., & Akbuğa, J. (2007). Chitosan film containing fucoidan as a wound dressing for dermal burn healing: preparation and in vitro/in vivo evaluation. *American Association of Pharmaceutical Scientists, 8*(2), 39.

Shrivastava, S., & Daharwal, S. J. (2022). Box-behnken design avenue for development and validation of high-performance thin-layer chromatography method for estimation of rutin in Hemidesmus indicus. *Biomedical Chromatography*, *36*(1), e5236.

Silva, A., Carreira, P., Santos, C., Silva, M., & Mateus, A. (2019). Film: Using electrospinning to create bioactive films. *Applied Mechanics and Materials*, *890*, 260-267.

Silva, C. L., Pereira, J. C., Ramalho, A., Pais, A. A. C. C., & Sousa, J. J. S. (2008). Films based on chitosan polyelectrolyte complexes for skin drug delivery: Development and characterization. *Journal of Membrane Science*, *320*(1), 268-279.

Silva, C. N. S., Cruz, M. V., Fernandes, K. F., & Batista, K. A. (2023). Production of anti-inflammatory films based on cashew gum polysaccharide and polyvinyl alcohol for wound dressing applications. *3 Biotech*, *13*(9), 299.

Sim, P., Strudwick, X. L., Song, Y., Cowin, A. J., & Garg, S. (2022). Influence of acidic pH on wound healing in vivo: A novel perspective for wound treatment. *International Journal of Molecular Sciences*, *23*(21), 13655.

Sizílio, R. H., Galvão, J. G., Trindade, G. G. G., Pina, L. T. S., Andrade, L. N., Gonsalves, J. K. M. C., Lira, A. A. M., Chaud, M. V., Alves, T. F. R., Arguelho, M. L. P. M., & Nunes, R. S. (2018). Chitosan/PVP-based mucoadhesive membranes as a promising delivery system of betamethasone-17-valerate for aphthous stomatitis. *Carbohydrate Polymers*, *190*, 339-345.

Smith, D. R., Escobar, A. P., Andris, M. N., Boardman, B. M., & Peters, G. M. (2021). Understanding the molecular-level interactions of glucosamine-glycerol assemblies: A model system for chitosan plasticization. *ACS Omega*, *6*(39), 25227-25234.

Son Tae-Won, K. O. K., Lim Hak-Sang, Byung Giu Kim (2006). Effects of mixing ratio on the mechanical and thermal properties of polyelectrolyte complex film. *Macromolecular Research*, *14*, 267-271.

Song, J.-E., Jun, S.-H., Ryoo, J.-Y., & Kang, N.-G. (2024). Formulation of ascorbic acid and betaine-based therapeutic deep eutectic system for enhanced transdermal delivery of ascorbic acid. *Pharmaceutics*, *16*(5), 687.

Sreeja, V., Jayaprabha, K. N., & Joy, P. A. (2015). Water-dispersible ascorbic-acid-coated magnetite nanoparticles for contrast enhancement in MRI. *Applied Nanoscience*, *5*(4), 435-441.

Stanković, M., Frijlink, H. W., & Hinrichs, W. L. (2015). Polymeric formulations for drug release prepared by hot melt extrusion: Application and characterization. *Drug Discovery Today*, *20*(7), 812-823.

Suárez, L. J., Arce, R. M., Gonçalves, C., Furquim, C. P., Santos, N. C. D., Retamal-Valdes, B., & Feres, M. (2024). Metronidazole may display anti-inflammatory features in periodontitis treatment: A scoping review. *Molecular Oral Microbiology*, *39*(4), 240-259.

Szentmihályi, K., Klébert, S., May, Z., Bódis, E., Mohai, M., Trif, L., Feczkó, T., & Károly, Z. (2022). Immobilization of metronidazole on mesoporous silica materials. *Pharmaceutics*, *14*(11), 2332.

Ta, Q., Ting, J., Harwood, S., Browning, N., Simm, A., Ross, K., Olier, I., & Al-Kassas, R. (2021). Chitosan nanoparticles for enhancing drugs and cosmetic components penetration through the skin. *European Journal of Pharmaceutical Sciences*, *160*, 105765.

Tan, W., Dong, F., Zhang, J., Zhao, X., Li, Q., & Guo, Z. (2019). Physical and antioxidant properties of edible chitosan ascorbate films. *Journal of Agricultural and Food Chemistry*, *67*(9), 2530-2539.

Thevi, T., Abas, A. L., & Rajan, M. (2024). The effects of vitamin C on wound healing—systematic review. *Indian Journal of Surgery*, *86*(1), 23-29.

Thom, S. R. (2009). Oxidative stress is fundamental to hyperbaric oxygen therapy. *Journal of Applied Physiology*, *106*(3), 988-995.

Tian, X. L., Tian, D. F., Wang, Z. Y., & Mo, F. K. (2009). Synthesis and evaluation of chitosan-vitamin C complex. *Indian Journal of Pharmaceutical Sciences, 71*(4), 371-376.

Tsegay, F., Elsherif, M., & Butt, H. (2022). Smart 3d printed hydrogel skin wound bandages: A review. *Polymers*, *14*(5), 1012.

Utomo, E., Domínguez-Robles, J., Anjani, Q. K., Picco, C. J., Korelidou, A., Magee, E., Donnelly, R. F., & Larrañeta, E. (2023). Development of 3d-printed vaginal devices containing metronidazole for alternative bacterial vaginosis treatment. *International Journal of Pharmaceutics, 5*, 100142.

Varaprasad, K., Jayaramudu, T., Kanikireddy, V., Toro, C., & Sadiku, E. R. (2020). Alginate-based composite materials for wound dressing application: A mini review. *Carbohydrate Polymers*, *236*, 116025.

Voss, G. T., Gularte, M. S., De Oliveira, R. L., Luchese, C., Fajardo, A. R., & Wilhelm, E. A. (2020). Biopolymeric films as delivery vehicles for controlled release of hydrocortisone: Promising devices to treat chronic skin diseases. *Materials Science and Engineering*, *114*, 111074.

Vuddanda, P. R., Montenegro-Nicolini, M., Morales, J. O., & Velaga, S. (2017). Effect of surfactants and drug load on physico-mechanical and dissolution properties of nanocrystalline tadalafil-loaded oral films. *European Journal of Pharmaceutical Sciences*, *109*, 372-380.

Wang, J., Jansen, J. A., & Yang, F. (2019). Electrospraying: possibilities and challenges of engineering carriers for biomedical applications: A mini review. *Frontiers in Chemistry*, *7*, 258.

Wang, J., Song, S., Gao, S. L., Muchakayala, R., Liu, R., & Ma, Q.-X. (2017). Mg-ion conducting gel polymer electrolyte membranes containing biodegradable chitosan: preparation, structural, electrical and electrochemical properties. *Polymer Testing*, *62*, 278-286.

Wang, X., Zhao, D., Li, Y., Zhou, X., Hui, Z., Lei, X., Qiu, L., Bai, Y., Wang, C., Xia, J., Xuan, Y., Jiang, P., & Wang, J. (2023). collagen hydrogel with multiple antimicrobial mechanisms as anti-bacterial wound dressing. *International Journal of Biological Macromolecules*, *232*, 123413.

Watanabe, K., Shimo, A., Tsugawa, K., Tokuda, Y., Yamauchi, H., Miyai, E., Takemura, K., Ikoma, A., & Nakamura, S. (2016). Safe and effective deodorization of malodorous fungating tumors using topical metronidazole 0.75 % gel (GK567): A multicenter, open-label, phase III study (RDT. 07. sre. 27013). *Support Care Cancer*, *24*(6), 2583-2590.

Wedmore, I., Mcmanus, J. G., Pusateri, A. E., & Holcomb, J. B. (2006). A special report on the chitosan-based hemostatic dressing: experience in current combat operations. *Journal Of Trauma And Acute Care Surgery*, *60*(3), 655-658.

Weller, C. D., Team, V., & Sussman, G. (2020). First-line interactive wound dressing update: A comprehensive review of the evidence. *Frontiers in Pharmacology*, *11*, 155.

Wittaya-Areekul, S., & Prahsarn, C. (2006). Development and in vitro evaluation of chitosan-polysaccharides composite wound dressings. *International Journal of Pharmaceutics*, *313*(1-2), 123-128.

Wolak, M., Staszewska, T., Juszczak, M., Gałdyszyńska, M., & Bojanowska, E. (2019). Anti-inflammatory and pro-healing impacts of exendin-4 treatment in Zucker diabetic rats: Effects on skin wound fibroblasts. *European Journal of Pharmacology*, *842*, 262-269.

Xu, H. L., Chen, P. P., Zhuge, D. L., Zhu, Q. Y., Jin, B. H., Shen, B. X., Xiao, J., & Zhao, Y. Z. (2017). Liposomes with silk fibroin hydrogel core to stabilize bFGF and promote the wound healing of mice with deep second-degree scald. *Advanced Healthcare Materials*, *6*(19), 1700344.

Xue, M., Zhao, R., Lin, H., & Jackson, C. (2018). Delivery systems of current biologicals for the treatment of chronic cutaneous wounds and severe burns. *Advanced Drug Delivery Reviews*, *129*, 219-241.

Yohannan Panicker, C., Tresa Varghese, H., & Philip, D. (2006). FT-IR, FT-Raman and SERS spectra of vitamin C. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *65*(3-4), 802-804.

Zeng, N., Chen, H., Wu, Y., & Liu, Z. (2022). Adipose stem cell-based treatments for wound healing. *Frontiers In Cell and Developmental Biology*, *9*, *821652*.