

T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

**SAFETY AND EFFICACY OF METALLIZED
HERBAL EXTRACT-LOADED WOUND DRESSING
DESIGNED BY NATURAL AND BIODEGRADABLE-
BASED ELECTROSPUN NANOFIBERS**

DOCTOR OF PHILOSOPHY THESIS
HEBAH AYASH

İSTANBUL-2024

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated and numbered

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.



HEBAH AYASH

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DEDICATION

I dedicate this thesis to my family, whose unwavering support and encouragement have been my greatest source of strength. To my parents, for their endless love and belief in me and their patience and sacrifices throughout this journey. To my close friends who kept asking about me and my cats for unconditional emotional support. Finally, I dedicate this work to myself, to whoever is pursuing knowledge, and to all those who strive to make a difference through their research.



TABLE OF CONTENTS

APPROVAL	ii
DECLARATION	iii
ACKNOWLEDGEMENTS	iv
DEDICATION	v
TABLE of CONTENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF SYMBOLS AND ABBREVIATIONS	xii
ABSTRACT	xv
ABSTRACT (Turkish)	xvi
1. INTRODUCTION	1
2. GENERAL INFORMATION	4
2.1 Wound Healing	4
2.1.1 Natural Body Wound Healing Process	4
2.1.2 Wound Healing General Management	5
2.2 Electrospun nanofibers for wound healing	6
2.2.1 Electrospinning method to produce drug-loaded nanofibers for wound healing	8
2.2.1.1 Basic Principles of Electrospinning	8
2.2.1.2 Factors influencing the electrospinning process	9
2.2.1.3 Incorporation of bioactive agent into electrospun nanofibers	12
2.2.2 Characterization of nanofiber mats	12
2.3 Medical plants in wound healing	13
2.4 Metallic and inorganic nanoparticles for wound healing	22
2.4.1 Metalized nanofibers in wound healing	23
2.5 Combination of herbal extract and metallic/inorganic nanoparticles in the fabrication of nanofibrous wound dressing	25
2.6 Safety of herbal extract-loaded multimodal nanofibers	26
2.6.1 Cytotoxicity and genotoxicity evaluation of nanomaterials	26
2.6.2 Irritation and corrosion tests	30
2.6.3 Sensitization test	31

2.6.4 Toxicity profile of used ingredients	31
3. MATERIALS AND METHODS	33
3.1 Material	33
3.1.1 Chemicals	33
3.1.2 Equipment and Instruments	36
3.2 Method	39
3.2.1 Preparation of electrospinning solutions	39
3.2.2 Electrospinning	46
3.2.3 Characterization	46
3.2.3.1 Characterization of mesoporous silica Nanoparticles	46
3.2.3.2 Characterization of HP Extracts	47
3.2.3.3 Characterization of produced nanofiber mat	47
3.2.4 Assay of St. John's Wort Extract	48
3.2.4.1 Assay of hypericin content after sample preparation	48
3.2.5 Encapsulation Efficiency	49
3.2.6 Release studies	49
3.2.7 Antimicrobial activity	49
3.2.8 Evaluation of <i>in vitro</i> anti-inflammatory effect and cell migration	50
3.2.8.1 Sample preparation	50
3.2.8.2 Evaluation of Anti-inflammatory Activity	50
3.2.8.3 Cell Migration Assay	51
3.2.9 Toxicity studies	51
3.2.9.1 Cell Viability Assay on RAW 264.7 cell line	51
3.2.9.2 Cytotoxicity Assay on L929 cell line	52
3.2.9.3 Ames test	53
3.2.9.4 Comet Assay	57
3.2.10 Statistical Analysis	58
4. RESULTS	59
4.1 FT-IR analysis	59
4.2 Zetasizer analysis	62
4.3 SEM and TEM results	63

4.4 Nanoindentation and tensile strength	73
4.5 Spectrophotometric assay of hypericin	74
4.5.1 Identification HP extracts	74
4.5.2 Assay of St. John's Wort Extract	75
4.5.2.1 Assay of hypericin content after sample preparation	76
4.5.3 Encapsulation efficiency and Release studies	76
4.6 Antimicrobial activity	77
4.7 Anti-inflammatory Activity	80
4.8 Invitro wound healing evaluation	83
4.8.1 Cytotoxicity Assay on L929 cell line	83
4.8.2 Cell Migration Assay	85
4.9 Ames Test	86
4.10 Comet Assay	88
5. DISCUSSION AND CONCLUSION	89
6. REFERENCES	97
7. APPENDICES	111

LIST OF TABLES

Table 1. Comparison between electrospun nanofibers and traditional wound dressing	7
Table 2. Most commonly used polymers' types and applications	10
Table 3. The different polymer blends' preparation ratios, methods, active agents added, and electrospinning parameters.	40
Table 4. Antimicrobial Activities of Compounds (Zone of Inhibition/diameter in mm)	79
Table 5. Effects of different forms of fiber films on the viability of RAW264.7 macrophage cells and the effects of the different forms of fiber films on nitrite levels and % nitrite inhibition in RAW 264.7 cells stimulated with 1 µg/mL LPS.	81
Table 6. Cytotoxicity results of the herbal extract loaded nanoparticle fiber extract on L929 cell line	84
Table 7. Mutagenicity of the sample in Salmonella typhimurium TA98 and TA100 strains.	87

LIST OF FIGURES

Figure 1. Normal wound healing stages	5
Figure 2. Electrospinning basic principle	9
Figure 3. Prepared M16 polymer blends ready for electrospinning	46
Figure 4. FTIR spectrum of CS powder	59
Figure 5. FTIR spectrum of PVA powder	60
Figure 6. FTIR spectrum of PVA and CS powder mixture	60
Figure 7. FTIR spectrum of PVA and CS nanofibers	61
Figure 8. FTIR spectrum of MSNs powder	61
Figure 9. Particle size intensity of MSNs	62
Figure 10. Zeta potential of MSNs	63
Figure 11. MSNs SEM and TEM: a and b are SEM images at 1K X and 2.5K. c and d are TEM images at 60K X and 80K X.	63
Figure 12. SEM images of nanofiber M3 and M5 at 500 X and 5K X	64
Figure 13. SEM images of nanofiber M6 and M6a at 500 X and 5K X	65
Figure 14. SEM images of nanofiber M7 at 500 X, 5K X, and 7.5K X	65
Figure 15. SEM images of nanofiber M8a and M8b at 1K X, and 7.5K X	66
Figure 16. SEM images of nanofiber M9 on foil at 250 X	67
Figure 17. SEM images of nanofiber M9 thread-like structure at 7.5K X	67
Figure 18. SEM images of nanofiber M10 at 500K X	68
Figure 19. SEM images of nanofiber M10 at 7.5K X	68
Figure 20. SEM images of M12 nanofiber at 250 X, 2.5K X, and 7.5K X	69
Figure 21. SEM images of M13 nanofiber at 250 X, 2.5K X, and 7.5K X	70
Figure 22. SEM images of M14 nanofiber at 250 X, 2.5K X, and 7.5K X	70
Figure 23. SEM images of M15 nanofiber at 5K X	71

Figure 24. SEM images of M16 nanofiber at 250 X and 2.5K X	72
Figure 25. SEM images of M16 nanofiber at 5K X	72
Figure 26. The physical structure of the produced nanofiber mat	72
Figure 27. The representative images of indentation regions for calculation of Young's modulus	73
Figure 28. AFM-based Young's modulus of electrospun single fibers	73
Figure 29. Strain (in %) against stress (in MPa)	74
Figure 30. Hypericin absorbance spectrum scan of different herbal extracts	74
Figure 31. Hypericin in Methanol calibration curve	75
Figure 32. Hypericin in Buffer calibration curve	75
Figure 33. Hypericin calibration curve after sample preparation	76
Figure 34. Actual calculated hypericin content.	76
Figure 35. Hypericin release profile from nanofibers	77
Figure 36. The average of zone of inhibitions	78
Figure 37. The effects of different concentrations of nanofiber mats extract on cell viability (A), on nitrite production (B) on LPS stimulated RAW 264.7 cell	82
Figure 38. Cell viability of herbal extract loaded nanoparticle fiber extract on L929 cell lines	85
Figure 39. The effect of the herbal extract loaded fiber film sheets on cell migration.	85
Figure 40. Cell migration (%) of the herbal extract loaded with nanoparticles film fiber with respect to concentration on 24 th hour compared to the medium group.	86
Figure 41. The representative images of negative and positive control results for comet assay	88
Figure 42. Quantification of comet extent by image analysis.	88

LIST OF SYMBOLS AND ABBREVIATIONS

2-AF: 2-Aminofluorene

AFM: Atomic Force Microscopy

AG: Acacia Gum

ARE: Antioxidant/Electrophile Response Element

ASTM D 638: American Society for Testing and Materials standard for tensile properties of plastics

ATCC®: American Type Culture Collection

CS: Chitosan

Ctrl: Control group

DLS: Dynamic Light Scattering

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

ELISA: Enzyme-Linked Immunosorbent Assay

F: Raw fiber

F + H: Raw fiber with herbal extract

F + H + Si: Raw fiber with herbal extract and MSNs

F + Si: Raw fiber with MSNs

FTIR: Fourier-transform Infrared Spectroscopy

G: Gelatin

HPE: Hypericum perforatum extract

IND: Indomethacin

KCl: Potassium Chloride

L-NAME : N(gamma)-nitro-L-arginine methyl ester (1+)

LOD: Limit of Detection

LOQ: Limit of Quantification

LPS: Lipopolysaccharide

MGA: Minimal Glucose Agar

MSNs: Mesoporous Silica Nanoparticles

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NTA: Nanoparticle Tracking Analysis

NO: Nitric oxide

NP: 4-Nitro-o-phenylenediamine

PVA: Polyvinyl Alcohol

PBS: Phosphate-buffered saline

Pen-Strep: Penicillin-Streptomycin

RS comet assay: Reconstructed Skin Comet Assay

RSMN: Reconstructed Skin Micronucleus Test

RPM: Revolutions Per Minute

SA: Sodium Azide

SEM: Scanning Electron Microscopy

S9: A rat liver extract containing active liver enzymes

SPM: Scanning Probe Microscopy

SRB: Sulforhodamine B

TEM: Transmission Electron Microscopy

TNF- α : Tumor Necrosis Factor-alpha

TRIS: Tris(hydroxymethyl)aminomethane

UV-Visible: Ultraviolet-visible

VB: Vogel-Bonner

WHO: World Health Organization

%: Percent

°C: Degrees Celsius

cm: Centimeter

g: Gram

hr: Hour

kV: Kilovolt

λ: Wavelength

L: Liter

mA: Milliampere

mL: Milliliter

mM: Millimolar

mm: Millimeter

μg: Microgram

μL: Microliter

μM: Micromolar (concentration)

nm: Nanometer

pH: Potential of Hydrogen (acidity or alkalinity)

rpm: Revolutions Per Minute

V: Voltage

R²: Coefficient of determination, a measure of linear regression

ABSTRACT

Ayash, H. (2024). Safety and Efficacy of Herbal Extract-loaded Wound Dressing Designed by Natural and Biodegradable Based Electrospun Nanofibers. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology PhD thesis, İstanbul.

Wound dressing is essential for healing, providing a protective barrier and supporting recovery. The global demand for wound care is increasing, with millions suffering from burns and chronic skin ulcers. Electrospinning is a promising technique for wound healing applications, creating nanostructures resembling the natural extracellular matrix. Herbal extracts, known for their therapeutic properties like antioxidants and antibacterial agents, enhance wound care. Incorporating metal nanoparticles further boosts infection prevention and wound healing promotion. This study aims to produce a safe and efficient wound dressing of metallized herbal-loaded nanofibers made of natural and biodegradable materials. A formulation made of Polyvinyl alcohol, Chitosan, and Gelatin was used to produce electrospun nanofibers. Mesoporous silica nanoparticles and *Hypericum perforatum* extract were used to be loaded into the nanofibers. The characterization, loading efficacy, releasing profile, *in vitro* efficacy, and safety were tested. The study achieved successful production and optimization of nanofiber mats incorporating herbal extracts and mesoporous silica nanoparticles. Key advancements included refined polymer ratios, solvent choices, and electrospinning parameters, which enhanced the nanofibers' physical properties and structural integrity. Characterization confirmed the effective integration of mesoporous silica nanoparticles and herbal extracts with an encapsulation efficacy 86-96%. The nanofibers demonstrated a rapid and sustained release profile, substantial antimicrobial activity, and significant anti-inflammatory effects, particularly with metallized herbal-loaded variants. Despite some possible cytotoxicity at high concentrations, the nanofibers showed no mutagenic or genotoxic effects, indicating promising safe therapeutic potential for future clinical applications.

Keywords

Nanofibers; Electrospinning; Multimodal nanostructure; Herbal extract; Wound healing.

ÖZET

Ayash, H. (2024). Doğal ve Biyolojik Olarak Parçalanabilir Bazlı Elektroeğrilmiş Nanofiberlerden Tasarlanmış Bitkisel Ekstrakt Yüklü Yara Örtüsünün Güvenliği ve Etkinliği. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD., Doktora Tezi. İstanbul.

Yara pansumanı, iyileşme sürecinin kritik bir bileşeni olup, koruyucu bir bariyer oluşturarak iyileşmeyi optimize eder. Yanıklar ve kronik cilt ülserleri gibi yaygın rahatsızlıklar, yara bakımına yönelik küresel talebin hızla artmasına yol açmaktadır. Bu bağlamda, elektrospinning, doğal hücre dışı matrise benzer nanoyapılar üreterek yara iyileştirme uygulamalarında umut vadeden bir teknoloji olarak öne çıkmaktadır. Antioksidan ve antibakteriyel özelliklere sahip bitkisel özlerin kullanımı, yara bakımını iyileştirirken, metal nanopartiküllerin entegre edilmesi enfeksiyonların önlenmesini ve yara iyileşmesinin hızlandırılmasını daha da etkin hale getirmektedir. Bu çalışmanın amacı, metalize bitkisel yüklü nanofiberlerden oluşan, güvenli ve etkili bir yara pansumanı geliştirmektir. Bu doğrultuda, elektrospun nanofiberlerin üretimi için polivinil alkol, kitosan ve jelatin içeren bir formülasyon kullanılmıştır. Nanofiberler, mezogözenekli silika nanopartikülleri ve *Hypericum perforatum* özütü ile yüklenmiştir. Çalışmada, nanofiberlerin karakterizasyonu, yükleme etkinliği, salım profili, *in vitro* etkinlik ve güvenliği değerlendirilmiştir. Elde edilen bulgular, bitkisel özler ve mezogözenekli silika nanopartiküllerini içeren nanofiberler başarılı bir şekilde üretildiğini ve optimize edildiğini göstermiştir. Bu süreçte, polimer oranlarının rafine edilmesi, çözücü seçimlerinin optimize edilmesi ve elektrospinning parametrelerinin iyileştirilmesi gibi önemli ilerlemeler kaydedilmiştir. Karakterizasyon çalışmaları, mezogözenekli silika nanopartiküllerin ve bitkisel özlerin %86-96 kapsülleme etkinliği ile etkili bir şekilde entegre edildiğini doğrulamıştır. Nanofiberler, özellikle metalize bitkisel yüklü varyantlarda, hızlı ve sürekli bir salım profili, belirgin antimikrobiyal aktivite ve güçlü anti-inflamatuar etkiler sergilemiştir. Yüksek konsantrasyonlarda sitotoksikite belirtilerine rağmen, nanofiberlerin mutajenik veya genotoksik etki göstermemesi, gelecekteki klinik uygulamalar için umut verici bir terapötik potansiyele işaret etmektedir.

Anahtar kelimeler

Nanofiberler; Elektrospinning; Multimodal nanoyapı; Bitkisel ekstrakt; Yara iyileşmesi.

1. INTRODUCTION

The demand for wound care and treatment is increasing worldwide. Records from the United States showed that 1.25 million patients suffered from burns while 6.5 million patients endured chronic skin ulcers due to pressure, venous stasis, or diabetes mellitus ¹ According to the World Health Organization (WHO), it's estimated that burns cause 180,000 deaths every year. Furthermore, the prevalence of diabetes is expected to rise, with more than 640 million people projected to have diabetes by 2030 ^{2,3}.

Given the timing of this study coinciding with the ongoing conflicts in Ukraine and the Gaza Strip, it is essential to consider the impact of war-related injuries. In conflict zones, inadequate wound treatment and poor wound healing contribute significantly to mortality rates. War-related injuries often result in extensive tissue damage, triggering a robust systemic inflammatory response and increasing the risk of complications, particularly infections caused by bacteria or fungi ⁴. It has been reported that one of the leading causes of death, especially within the first 24 hours, is sepsis, accounting for 2-9% of preventable deaths. The infection rate has been estimated as 34% in Afghanistan and 28% in Iraq. Permanent disabilities often follow such injuries. During the 2014 Gaza war, blasts and explosions were among the primary causes of war injuries, resulting mainly in burns and numerous shrapnel wounds ⁵.

The burden of these war-related injuries on individuals and communities is significant, particularly given the economic conditions and the lack of essential medical resources in conflict zones⁶. Given that wounds remain a global health concern, particularly chronic wounds, the development of alternative treatments is imperative. This is especially crucial considering the limitations of current treatments, particularly in terms of antibacterial activity. Although plain gauze is widely used due to its affordability and accessibility, its limitations have spurred the development of alternative materials such as films, foams, sponges, hydrogels, and nanofibers⁷.

An ideal wound dressing should be able to absorb extra fluid discharge and shield the incision from microbial invasion while allowing gas exchange and preserving a damp healing environment at the wound site. It has to be non-toxic, biocompatible, and degradable. It must

also promote angiogenesis and tissue regeneration and be simple to replace and remove as it does not stick to the wound to be ideal ⁷.

Developing an effective wound dressing for skin injuries remains a significant challenge. Nanofiber dressings have garnered considerable interest among scientists in this field due to their numerous advantages over conventional dressings. These dressings have the ability to mimic tissue and establish an optimal environment for wound healing, surpassing the capabilities of traditional dressings. Additionally, nanofiber dressings offer the potential for incorporating therapeutic or antimicrobial agents, thereby creating functionalized dressings with enhanced healing properties ¹.

Among the techniques employed in creating nanofibers, electrospinning stands out as the most widely used method for producing nanofibers ideal for wound dressings. This method proves highly effective in obtaining membranes with fiber structures of varying diameters, ranging from nanometers to micrometers. Electrospinning is characterized by its cost-effectiveness, simplicity, and unique approach, utilizing electrostatic forces to generate uniform nanofibers from polymer solutions ⁸.

Nanofibers produced through this technique possess several notable characteristics, including their ability to mimic the structure of the extracellular matrix, provide protection against microbial infection, exhibit biocompatibility and biodegradability, offer favorable water vapor transmission rates, and facilitate sufficient gas exchange. These attributes make them an excellent choice for applications in wound healing. Furthermore, owing to their extensive surface area and porous nature, nanofibers can accommodate various substances such as medicines, vitamins, minerals, and herbal extracts. Moreover, the release of drugs can be controlled by adjusting the fiber structure and morphology⁹.

Applications of electrospinning technology in medicine include tissue engineering scaffolds, drug delivery, and wound dressings. The simultaneous production of polymers containing natural and synthetic materials can enhance the biocompatibility and biodegradability of nanomaterials, along with optimizing their chemical, physical characteristics, and functionalities¹⁰.

Herbal treatments have a long history of use in healing illnesses throughout human history. Folklore cultures employ a variety of plants to treat cuts, burns, and wounds. The unique properties of nanofibers, including their high surface area, porosity, and resemblance to the extracellular matrix make them a distinctive method for delivering herbal medicines and natural bioactive components¹¹.

It is conceivable to produce electrospun nanofibers by combining polymers with herbal medication. Hence, there should be more studies concentrating on their efficacy and safety, co-encapsulation with inorganic materials like metallic nanoparticles, the synergistic activity of herbal extracts with other components, and the control of release, alongside exploring the feasibility of large-scale production¹².

This study aims to prepare a safe nontoxic nanofibers mat containing plant extract with wound-healing properties. Scientific literature supports the idea that medicinal plants, which promote coagulation, enhance collagen production, epithelial formation, and possess anti-inflammatory, antifungal, antibacterial, and antioxidant properties are beneficial for wound healing¹³. St. John's Wort, *Hypericum perforatum* extract (HPE), traditionally used in wound and burn treatments in Turkey, has been subject to both *in vitro* and *in vivo* studies regarding wound healing¹⁴. Apart from various flavonoids, phenolic compounds like hyperforin and hypericin in HPE provide anti-inflammatory and antibacterial properties. In this project, various platforms based on modern nanofibers of biocompatible and biodegradable polymers will be produced using the electro-spinning method, incorporating HPE and nonorganic metallic nanoparticles. The safety and efficacy of the produced nanofibers for wound healing will be thoroughly examined.

2. GENERAL INFORMATION

2.1 Wound Healing

2.1.1 Natural body wound healing process

A wound is defined as a disruption of the normal structure and function of the skin, along with its associated underlying soft tissue structures. There are two types of wounds: acute wounds, brought on by trauma; and chronic wounds, which are linked to physiological deficits that hinder or impede wound healing and are typically accompanied by an underlying illness ^{1,15}.

The ideal wound healing process involves organized cell migration and recruitment of endothelial cells for angiogenesis. It can be described in phases including hemostasis, inflammation, proliferation, and maturation ¹⁶.

Immediately after injury, the hemostasis phase begins with vasoconstriction, thrombocyte migration, and platelet aggregation, followed by clot formation within 15min. The Inflammation phase, also known as the lag phase, takes about 3 days to complete, but may last up to 6 days. During this phase, capillaries vasodilate and permeability increases, allowing the migration of inflammatory cells which mature and release signals (Chemotaxis). This leads to increased portability, responsible for edema, leukocyte migration, and phagocytosis activity.

The proliferation phase can be divided into two stages: epithelialization and fibroplasia, beginning 24-48 hours after the wound occurs. Epithelial cells from surrounding tissue migrate into the wound boundaries to create a barrier against germs and foreign objects. This process is more efficient in clean, closed surgical wounds. Fibroplasia involves increased fibroblasts proliferation, responsible for producing ground substance and collagen synthesis. In pathological conditions, increased matrix synthesis leads to fibrosis, forming scar tissue known as keloid.

The last phase is maturation, where collagen cross-linking, remodeling, wound contraction, and repigmentation occur. This phase lasts for several weeks to months following the wound occurrence, marked by the replacement of granulation tissue with scar

tissue. Over time, type I collagen increases while type III collagen, water, and proteoglycans decrease. The degree of tissue damage, along with variables such as infections and physical activity, can delay healing or weaken the tensile strength of the scar ¹⁵. The wound healing stages are illustrated in Figure 1.

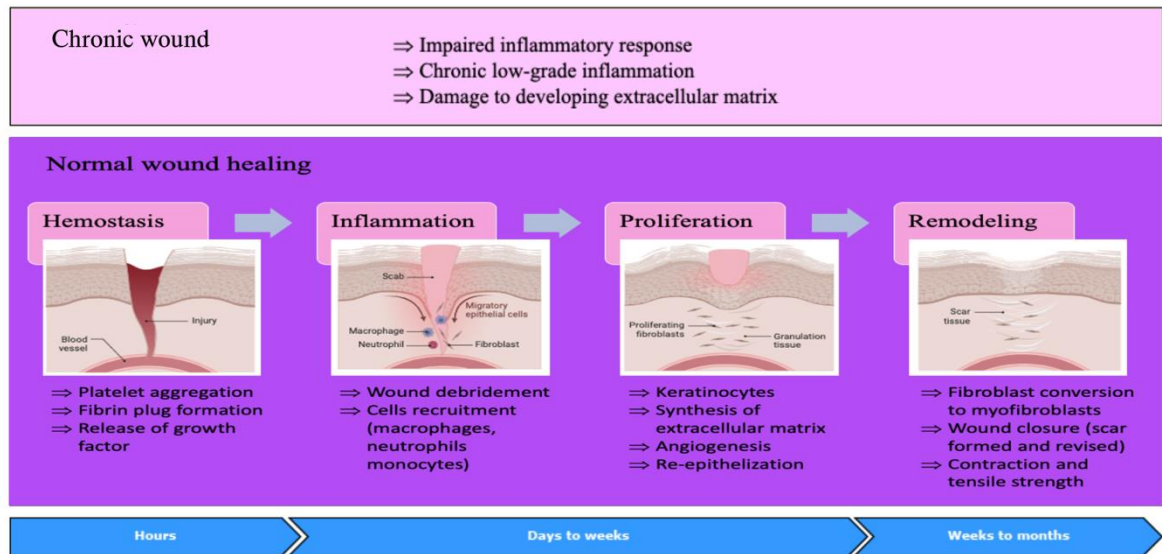


Figure 1. Normal wound healing stages

2.1.2 Wound healing general management

The local treatment of wounds aims to reduce pain and itching, minimizing infection, and control bleeding. In cases of chronic wounds, addressing the most troublesome issues affecting the patient physically and emotionally is crucial, such as excess exudate that can lead to unpleasant odors. Local wound care typically involves debridement and the application of appropriate wound dressings. Ensuring proper local care is vital in preparing the wound bed for procedures like skin graft or flap, or for closure when necessary. Therapeutic options such as coagulants, local anesthetics, anti-inflammatories, and antimicrobial agents can be employed to promote wound healing. However, it's important to note that no single treatment modality can address all aspects of the healing process ¹⁷.

The speed at which a wound heals, the strength and function of the healed skin, and the aesthetic appearance of the resulting scar can all be significantly influenced by the proper

application and replacement of a dressing. There isn't a single dressing that universally suits every condition; instead, clinicians should evaluate each wound individually and choose the appropriate dressing accordingly. While a moderately moist wound bed can facilitate healing, excessive moisture can lead to maceration. Ideally, a dressing for a specific wound should absorb excess drainage while maintaining an optimal level of moisture. In addition to these primary functions, certain dressings may also offer secondary benefits such as reduced pain during changes, odor control, anti-inflammatory or mild debridement properties, and local antibacterial actions ^{17,18}.

The objective of this experiment is to develop a wound dressing with optimal properties, enhanced with antimicrobial and anti-inflammatory activity, to promote effective healing.

2.2 Electrospun nanofibers for wound healing

Developing an effective wound dressing for skin injuries remains challenging, particularly in preventing delayed wound healing process caused by bacterial infections, underlying medical conditions, aging, and other factors. Nanofiber dressings have garnered interest among scientists in this field due to their advantages over conventional dressings, including enhanced anti-inflammatory and antimicrobial activity, improved absorption of exudates, reduced need for frequent dressing replacements, and better mechanical properties ¹⁶.

The advancement of nanotechnology is essential to address the limitations of commonly used dressings and to enhance wound healing outcomes. Nanofiber mats offer distinct advantages, including a highly porous structure that provides a large surface area, enabling efficient gas permeation and exudate absorption. These characteristics support crucial aspects of wound healing such as hemostasis, exudate management, moisture retention, skin regeneration, and cellular respiration. Moreover, the morphological similarity of nanofiber mats to the extracellular matrix promotes cell adhesion, proliferation, and differentiation ¹.

By using natural, synthetic, or a combination of polymers as precursor solutions for electrospinning, nanofiber dressings can be tailored to possess desirable qualities such as biodegradability, biocompatibility, non-toxicity, low antigenicity, and ready availability.

The future of wound healing lies in the integration of active agents, sourced from natural (such as herbal extracts) or synthetic (such as antimicrobial or anti-inflammatory drugs) origins, into nanofibers. This approach accelerates wound healing and tissue regeneration significantly. Electrospun mats augmented with such functionalities hold promise as personalized bandages, offering tailored treatment solutions. Moreover, electrospun mats can prolong and enhance the release profile of antibacterial agents, distinguishing them apart from conventional antibacterial ointments. By reducing the frequency of dressing changes, patient comfort is increased, and the overall cost of treatment is lowered ⁸. Table 1 compares the characteristics of electrospun nanofibers and traditional wound dressings.

Table 1. Comparison between electrospun nanofibers and traditional wound dressing

Feature	Electrospun Nanofibers	Traditional Wound Dressings
Structure	Mimics the extracellular matrix of natural skin, with high surface area and porosity	Varied structure depending on the type (gauze, hydrogels, alginates)
Advantages	• Enhanced wound healing: Promotes cell proliferation, migration, and differentiation. • Controlled drug delivery: Can be loaded with drugs for sustained release and targeted therapy. • Antibacterial properties: Can be incorporated with antibacterial agents to reduce infection. • Conformability: Adapts well to uneven wound surfaces.	• Simple and readily available. • Relatively low cost. • May provide initial protection and absorption.

	• Biocompatibility: Generally well-tolerated by the body.	
Disadvantages	• Complex and expensive to manufacture. • Requires specialized techniques for evaluation. • Long-term stability and efficacy may require further research.	• Limited ability to promote wound healing. • May not effectively manage exudate. • Risk of sticking to the wound bed, causing damage during removal. • May not conform well to complex wounds.
Applications	Chronic wounds, diabetic ulcers, burns, surgical wounds	Superficial wounds, minor cuts and scrapes, initial wound management

2.2.1 Electrospinning method to produce drug-loaded nanofibers for wound healing

2.2.1.1 Basic Principles of Electrospinning

Electrospinning is an electrohydrodynamic process in which electrically charged fluids are set in motion under the influence of applied electric fields. During electrospinning, a liquid droplet is electrified to create a jet, which is then stretched and elongated to form fibers.

The electrospinning apparatus primarily consists of a high-voltage power supply, a collector, and a spinneret. When voltage is applied, the electrospinning fluid is injected into the spinneret at a low feed rate. As the process continues, the morphology of the fluid gradually changes until it reaches a critical voltage. At this point, the drop at the tip, supported by surface tension, forms a structure known as a Taylor cone. Before the fibers are collected on the collector, the liquid jet must evaporate once it reaches a certain distance. This stage marks the onset of bending and whiplash, as illustrated in Figure 2^{19–23}.

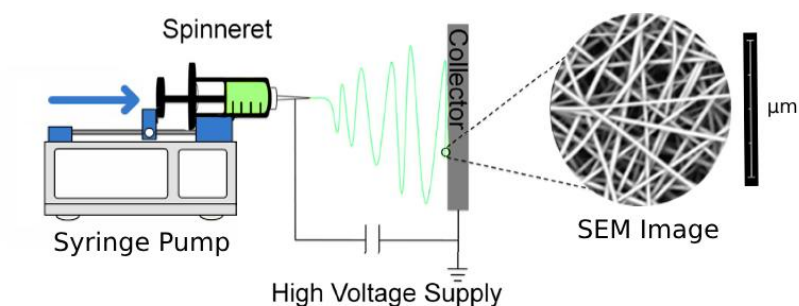


Figure 2. Electrospinning basic principle

2.2.1.2 Factors influencing the electrospinning process

Numerous factors can influence electrospinning, which can be categorized into three main groups related to the apparatus, precursor solution, and environmental conditions.

(a) Apparatus

Variables concerning the apparatus can result from the apparatus setup or parameter adjustments. Electrospinning apparatus can vary from conventional to coaxial or multiaxial setups, offering the advantage of producing core/shell fibers using multiple solutions. Another variable is the type of collector employed, which can be stationary or more advanced, such as rotating drums aiding in fiber alignment and arrangement control. Key apparatus parameters include the applied voltage, spinneret-to-collector distance, and flow rate.

Increasing the flow rate can enlarge pore size and fiber diameter, but it may also lead to bead formation due to insufficient stretching and solvent volatilization. Smaller diameter nanofibers can be achieved by raising the applied voltage and enhancing polymer stretching, but surpassing a critical value may result in increased fiber diameter and bead formation. While nanofiber diameter decreases with increasing distance, maintaining an appropriate distance between the needle tip and collector is crucial to prevent large-diameter and defective nanofibers^{19–22}.

(b) Precursor solution

To optimize the properties of a precursor solution, such as conductivity, surface tension, viscosity, and concentration, the selection of the appropriate polymer and solvent is crucial. The concentration must be adjusted to reach the critical value where uniform nanofibers are formed without bead formation.

The precursor solution typically consists of polymer solutions or pure polymer. The polymer matrix can be synthetic, natural, or a combination of both to achieve the desired properties of the nanofiber. Natural polymers derived from renewable resources, are inherently biocompatible and biodegradable. Proteins, collagen, silk, and polysaccharides (such as chitosan, cellulose, and hyaluronic acid) have all been utilized in electrospinning for localized medication delivery. In wound healing applications, synthetic polymers like polyvinyl alcohol, polylactide, polyethylene oxide, and polycaprolactone are commonly employed. These synthetic polymers offer superior mechanical properties compared to natural polymers and can be electrospun into nanofiber mats more easily due to their compatibility with a wider range of solvents. In the creation of wound healing scaffolds, biopolymers are often combined with synthetic polymers to modify the degradative, mechanical, and/or morphological properties of the porous fiber mat, tailored to the individual needs of each patient ^{24,25}. Table 2 summarizes commonly used polymers in electrospinning and their applications.

Table 2: Most commonly used polymers' types and applications

Category		Polymer Examples	Applications
Synthetic	Non-biodegradable	• Polystyrene (PS) • Poly(vinyl chloride) (PVC) • Polyacrylonitrile (PAN) • Polyurethane (PU)	• Air filtration • Water filtration • Oil-water separation • Battery separators • Sensors
	Biocompatible & Biodegradable	• Poly(vinyl alcohol) (PVA) • Poly(lactic acid) (PLA) • Poly(glycolic acid) (PGA) • Poly(lactic-co-glycolic acid) (PLGA) • Poly(ethylene oxide) (PEO) • Polycaprolactone (PCL)	• Tissue engineering • Drug delivery • Wound healing • Scaffolds for cell growth • Filtration

		● Poly(hydroxybutyrate) (PHB)		
Natural	Polysaccharide	Plant	● Alginate ● Dextran ● Cellulose	● Wound healing ● Drug delivery ● Tissue engineering ● Enzyme immobilization ● Scaffolds for cell growth ● Cosmetics
		Animal	● Chitosan (CS) ● Hyaluronic acid	
	Protein	● Gelatine ● Collagen ● Silk fibroin		

Gelatin, a naturally occurring protein biopolymer that is biocompatible and biodegradable, is commonly produced as translucent, pale-yellow particles or flakes through the hydrolysis of collagen. It's inexpensive, widely available, and soluble in acetic acid, glycerin, and hot water. Typically, to enhance its performance, gelatin is used in combination with a synthetic polymer.

Chitosan exemplifies natural polymers, undergoing deacetylation to transform from chitin into a polysaccharide with a versatile structure and wide-ranging applications. Its potential in pharmacy and wound treatment is augmented by the flexibility of the hydroxyl group and the amino activity along the backbone. Studies have highlighted its antibacterial effectiveness against *Escherichia coli* (*E. coli*), and *Staphylococcus aureus* (*S. aureus*), as along with its capacity to enhance wound healing, as evidenced by mouse wound models.

Polyvinyl alcohol (PVA), a colorless synthetic polymer, dissolves in water above 95°C. It has garnered significant attention in the medical realm, particularly in prosthetic joints and bandages. PVA loaded with *Nigella sativa* nanofibrous mat demonstrates promising wound healing characteristics and accelerated recovery periods, as indicated by wound healing analyses^{16,24}.

(c) Environmental conditions

Ambient parameters, particularly temperature and humidity, play a significant role in fiber synthesis and should be carefully monitored and controlled during the process. Higher temperatures typically result in a decrease in solution viscosity, leading to a reduction in

average fiber diameter. Conversely, increased humidity can cause polymer swelling, increasing average fiber diameter ²⁵.

2.2.1.3 Incorporation of bioactive agent into electrospun nanofibers

Incorporating bioactive agents into electrospun nanofibers can be achieved through various methods, depending on the desired qualities and properties of the final product. When a burst release is desired, an active agent "drug" is suspended in the electrospinning blend to distribute it throughout the fibers or concentrate it at the fiber's surface. Coaxial electrospinning is employed when abrupt release is not desirable or when the active agent is sensitive to solvents. This technique produces core/shell fibers using a concentric or coaxial needle configuration, creating separate layered channels for two or more solutions. Emulsion electrospinning is another method for achieving a core/shell structure, utilizing an emulsion as the feeding solution and a surfactant to separate the different phases. This technique is beneficial for shielding proteins, DNA, and peptides from harsh organic solvents or hydrophilic medications, allowing their inclusion. Finally, dip coating involves immersing electrospun mats in a solution to impart desired qualities ^{1,23,24,26,27}.

2.2.2 Characterization of nanofiber mats

Characterizing the nanofiber mats produced by electrospinning involves evaluating various aspects, primarily their structure and mechanical properties, depending on the intended application.

Morphological characterization is typically conducted using Scanning Electron Microscopy (SEM), which enables the identification of the shape, size, and distribution of electrospun nanofibers. SEM provides high-resolution images that facilitate the measurement of fiber diameter, distribution, and detection of defects such as beads or cracks. Transmission Electron Microscopy (TEM) offers even higher resolution than SEM and can reveal finer details such as internal structure, crystallinity, and the presence of embedded materials within the nanofibers. Atomic Force Microscopy (AFM) is another method for more in-depth morphological characterization, allowing precise measurement of the diameter of individual nanofibers along their length and providing insights into surface texture and 3D profile ^{28,29}.

Structural characterization can be achieved using X-ray diffraction (XRD), which helps determine the crystal structure and orientation of polymer chains within the nanofibers, providing information about crystallinity and potential mechanical properties. Fourier-transform infrared spectroscopy (FTIR) analysis reveals the chemical composition and functional groups present in the nanofibers, which is crucial for understanding their interaction with other materials or biological systems³⁰.

Tensile testing is employed to measure the mechanical properties of nanofibers, such as Young's modulus, tensile strength, and elongation at break, essential for understanding their potential applications in areas like filtration or tissue engineering. These data can be obtained using AFM and tensile tester devices^{28,30}.

2.3 Medical plants in wound healing

Throughout human history, herbal remedies have played a significant role in treating various ailments. Many plants have been utilized in folklore cultures for the treatment of burns, wounds, and cuts. The efficacy of these herbal remedies in wound treatment may stem from their antioxidants, antibacterial, and anti-inflammatory properties (Ex: *Sphagneticola trilobata*, *Aloe vera*, *Panax ginseng*, *Hypericum perforatum*). Some herbal extracts have also been shown to support processes such as angiogenesis, collagen formation, and epithelialization (Ex: *Centella asiatica*, *Curcuma longa*)¹³.

Researchers are actively exploring new formulations, dressings, and composition of medicinal plants to develop stable, sustainable, economical, and effective wound care and treatment methods. The availability of innovative materials and the emergence of nanotechnology have led to advancements in wound treatment, making it more cost-effective and patient-focused^{13,24,27}.

Sphagneticola trilobata, also known as *Wedelia trilobata*, is a plant native to the tropical Americas but now widespread in the tropics. Extracts from the leaves of *Sphagneticola trilobata* have been traditionally used to treat conditions such as rheumatism, persistent wounds, and sore arthritic joints. The plant contains luteolin, a flavonoid that exhibits neuroprotective, anti-cancer, antioxidant, and immunomodulatory activities.

Traditional healers use *Sphagneticola trilobata* leaves to treat skin wounds. Luteolin in the plant inhibits the expression of proinflammatory cytokines regulated by NF- κ B, which is beneficial for skin infections and conditions like psoriasis. Studies have shown that extracts from *Sphagneticola trilobata* leaves support fibroblast viability, proliferation, and migration, and exhibit antimicrobial activity against certain bacteria like *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Sphagneticola trilobata*, commonly known as Bay Biscayne Creeping-Oxeye or Singapore Daisy, is a flowering plant belonging to the *Asteraceae* family. It is native to Mexico and Central America but has spread to various tropical and subtropical regions worldwide. *Sphagneticola trilobata* is a low-growing perennial herb with trailing stems and bright yellow daisy-like flowers. The leaves are green, fleshy, and triangular in shape, with three distinct lobes hence the species name "*trilobata*." In traditional medicine, *Sphagneticola trilobata* has been used for its medicinal properties, including wound healing, anti-inflammatory effects, and as a remedy for skin conditions. The plant contains various phytochemicals such as flavonoids, terpenoids, alkaloids, and saponins, which contribute to its potential therapeutic effects. *Sphagneticola trilobata* has been traditionally used for wound healing purposes, possibly due to its antimicrobial and anti-inflammatory properties. Studies have shown that extracts from *Sphagneticola trilobata* leaves may promote healing in incision wounds. Recent studies have focused on the pharmacological activities of *Sphagneticola trilobata*, including its wound healing potential, antimicrobial effects, and anti-inflammatory properties. *Sphagneticola trilobata* is a hardy plant that thrives in tropical and subtropical climates. It is often grown as ground cover due to its spreading nature and attractive flowers. While *Sphagneticola trilobata* is generally considered safe when used appropriately, individuals with known allergies to plants in the *Asteraceae* family should exercise caution. As with any herbal remedy, it is advisable to consult a healthcare provider before using *Sphagneticola trilobata*, especially if you are pregnant, nursing, or have underlying health conditions. In conclusion, *Sphagneticola trilobata* is a plant with potential medicinal properties, particularly in wound healing and skin health. Further research is needed to explore its full range of therapeutic benefits and mechanisms of action^{31,32}.

Aloe vera is a succulent plant species known for its healing properties and is used in various traditional and modern medicinal applications. It contains a variety of bioactive compounds such as glycosides, polysaccharides, saponins, pyrocatechol, anthraquinones,

acemannan, phytol, oleic acid, and water-soluble polysaccharides. Acetone extracts from *Aloe vera* leaves exhibit antimicrobial activity, particularly against gram-positive bacterial species. Compounds like saponins, acemannan, and anthraquinone derivatives in *Aloe vera* have proven antimicrobial properties. Acemannan, a large mucopolysaccharide in *Aloe vera*, stimulates macrophages and T cells, induces the transcription of proinflammatory mRNAs, and promotes phagocytosis. *Aloe vera* is commonly used for wound healing due to its antimicrobial and anti-inflammatory properties, making it effective in treating various skin conditions and promoting tissue regeneration at the wound site. *Aloe vera* is a perennial plant with thick, fleshy green leaves that contain a gel-like substance. The plant belongs to the *Liliaceae* family and is native to the Arabian Peninsula but is now cultivated worldwide in tropical and subtropical regions. *Aloe vera* has a long history of traditional use in various cultures for its healing properties, particularly in skincare, wound healing, and digestive health. It is commonly used in topical applications for sunburn, minor burns, cuts, and skin irritations. *Aloe vera* gel contains a variety of bioactive compounds, including polysaccharides, glycoproteins, enzymes, vitamins, minerals, and antioxidants. Key components of *Aloe vera* gel include acemannan, aloin, aloe-emodin, and various polysaccharides. *Aloe vera* is known for its wound healing properties, promoting skin regeneration, reducing inflammation, and accelerating the healing process. The plant exhibits anti-inflammatory effects, which can help alleviate skin inflammation and irritation. *Aloe vera* has antimicrobial properties that can help prevent infections in wounds and promote healing. *Aloe vera* is a popular ingredient in skincare products due to its moisturizing, soothing, and anti-aging properties. It is used to treat various skin conditions such as eczema, psoriasis, acne, and dry skin. *Aloe vera* juice is consumed for its potential benefits in promoting digestive health, relieving constipation, and soothing gastrointestinal issues. It is believed to have a mild laxative effect due to its anthraquinone content. While *Aloe vera* is generally safe for topical use, some individuals may be allergic to the plant. When consuming *Aloe vera* internally, it is essential to use products specifically intended for oral consumption and follow recommended dosages to avoid potential side effects. In conclusion, *Aloe vera* is a versatile plant with a wide range of medicinal and therapeutic properties, particularly in wound healing, skin care, and digestive health. Its natural compounds make it a valuable ingredient in various health and beauty products^{33–35}.

Panax ginseng, commonly known as ginseng, is a popular medicinal plant consumed in regions like China, Japan, Korea, and Eastern Siberia. It is believed to enhance immunity, physical agility, and reduce fatigue. *Panax ginseng* is used to treat conditions like depression, anxiety, and chronic fatigue disorders. It has vasodilatory effects, helps control blood lipids, reduces inflammation, and possesses antioxidant, anti-cancer, antibacterial, anti-allergic, anti-aging, and immunomodulatory properties. The plant contains bioactive compounds, with ginsenosides being the most potent active constituents. *Panax ginseng* extracts have been shown to protect the skin from UVB irradiation, improve wound healing, strengthen keratinocyte migration, induce proliferation, and increase collagen production in human dermal fibroblasts. Studies also indicate that ginsenoside Rb2 from *Panax ginseng* promotes epidermal development and enhances wound healing processes. *Panax ginseng*, commonly known as Asian ginseng or Korean ginseng, is a perennial plant native to the mountainous regions of Eastern Asia, including Korea, China, and Russia. *Panax ginseng* is a slow-growing plant with fleshy roots and distinctive leaves that grow in a circular pattern. The plant belongs to the *Araliaceae* family and is highly valued for its medicinal properties. *Panax ginseng* has a long history of use in traditional Chinese medicine and is considered an adaptogen, a substance that helps the body adapt to stress and promotes overall well-being. It is used to boost energy, improve cognitive function, enhance physical endurance, and support the immune system. *Panax ginseng* contains a variety of bioactive compounds, with ginsenosides being the primary active constituents responsible for its pharmacological effects. Other components include polysaccharides, peptides, polyacetylenes, and fatty acids. *Panax ginseng* is known for its adaptogenic properties, helping the body cope with physical and mental stress. It is used to increase energy levels, reduce fatigue, and improve physical endurance. *Panax ginseng* is believed to enhance cognitive function, memory, and concentration. The plant is used to support the immune system and improve overall health. *Panax ginseng* is popular among athletes and individuals seeking to improve physical performance and stamina. It is used to enhance mental clarity, focus, and alertness. *Panax ginseng* is believed to help reduce stress, anxiety, and fatigue. Numerous studies have investigated the pharmacological effects of *Panax ginseng*, including its impact on energy metabolism, cognitive function, immune response, and overall health. Research suggests that ginsenosides in *Panax ginseng* may have antioxidant, anti-inflammatory, and

neuroprotective properties. While *Panax ginseng* is generally considered safe for most people when used appropriately, it may interact with certain medications and have side effects in some individuals. It is advisable to consult a healthcare provider before using *Panax ginseng*, especially if you have underlying health conditions or are taking medications. In conclusion, *Panax ginseng* is a valuable medicinal plant with a wide range of potential health benefits, particularly in supporting energy, cognitive function, and overall well-being. Its adaptogenic properties make it a popular natural remedy in traditional medicine and modern healthcare practices ^{36,37}.

Centella asiatica, also known as Gotu Kola or Indian Pennywort, is a medicinal plant with significant therapeutic properties. It has a long history of traditional use in various cultures. It is a perennial herb native to Asia, particularly found in countries like India, China, and Indonesia. *Centella asiatica* contains bioactive compounds such as asiaticoside and madecassoside, which contribute to its healing properties. Studies have shown that *Centella asiatica* promotes wound healing by stimulating fibroblast proliferation, collagen synthesis, and angiogenesis. It also exhibits anti-inflammatory and antioxidant effects, making it beneficial for skin health and regeneration. *Centella asiatica* has been researched for its antimicrobial activity and its ability to support fibroblast viability and migration. It has been used for centuries in traditional medicine systems such as Ayurveda, Traditional Chinese Medicine, and African herbal medicine. *Centella asiatica* has been extensively studied for its wound-healing properties. It promotes the synthesis of collagen, which is essential for skin regeneration and wound closure. It also enhances the proliferation of fibroblasts, the cells responsible for producing collagen and other extracellular matrix components. The herb exhibits anti-inflammatory effects, which can help reduce inflammation in the body and promote healing processes. *Centella asiatica* contains antioxidants that help protect cells from damage caused by free radicals, thereby supporting overall health and skin regeneration. Due to its collagen-stimulating properties, *Centella asiatica* is used in skincare products to improve skin elasticity, reduce the appearance of scars, and promote overall skin health. Some studies suggest that *Centella asiatica* may have cognitive-enhancing effects and could potentially benefit memory and brain function. *Centella asiatica* has been used to improve symptoms of venous insufficiency, such as varicose veins and swelling in the legs. It has been reported to have calming effects and may help reduce anxiety and stress levels. *Centella*

asiatica can be consumed in various forms, including teas, extracts, capsules, and topical creams. It is generally considered safe for most people when used in recommended doses. However, individuals with specific health conditions or those taking medications should consult a healthcare provider before use. Overall, *Centella asiatica* is a versatile herb with a wide range of potential health benefits, particularly in wound healing, skin health, cognitive function, and stress management ³⁸.

Turmeric, scientifically known as *Curcuma longa*, is a perennial herb belonging to the ginger family. It is highly valued for its rhizomes (underground stems), which are harvested for both culinary and medicinal purposes. The plant features long leaves and striking spikes of yellow flowers. The primary bioactive compound found in turmeric is curcumin, responsible for its vibrant yellow hue and numerous health benefits. With a rich history in traditional medicine, particularly in Ayurveda and Traditional Chinese Medicine, turmeric is renowned for its anti-inflammatory, antioxidant, and healing properties. Curcumin exhibits potent anti-inflammatory effects, making it beneficial for various conditions, including digestive issues, skin disorders, arthritis, and respiratory problems. Additionally, turmeric is known for its antioxidant properties, helping combat oxidative stress and free radical damage. When used topically, turmeric aids in wound healing due to its antimicrobial and anti-inflammatory properties. Turmeric supports various aspects of health, including digestive health, joint health, and skin care, offering relief from digestive issues, joint pain, and skin conditions. Extensive research has explored the potential therapeutic effects of curcumin in conditions such as cancer, cardiovascular disease, neurodegenerative disorders, and metabolic syndrome. Research suggests that curcumin's diverse molecular targets contribute to its broad range of biological effects. While turmeric is generally safe when consumed in moderation as a spice, high doses of curcumin supplements may cause gastrointestinal issues in some individuals so it is advisable to consult a healthcare provider before using curcumin supplements, especially for individuals with specific health concerns. In conclusion, *Curcuma longa*, or turmeric, is a versatile herb with curcumin as its key compound, offering a wide range of health benefits including anti-inflammatory, antioxidant, and healing properties that have established it as a popular natural remedy in traditional and modern medicine ^{39,40}.

Hypericum perforatum, commonly known as St. John's Wort, is a medicinal plant with a long history of traditional use for various health conditions. It contains bioactive compounds such as hypericin, hyperforin, flavonoids, and phenolic acids. St. John's Wort is primarily known for its antidepressant properties and is used to treat mild to moderate depression. It works by inhibiting the reuptake of neurotransmitters like serotonin, dopamine, and norepinephrine in the brain. Additionally, St. John's Wort has anti-inflammatory, antioxidant, and antimicrobial properties^{41,42}. It is also used to alleviate symptoms of anxiety, sleep disorders, and nerve pain. However, it is essential to consult a healthcare provider before using St. John's Wort, as it can interact with certain medications and may have side effects. *Hypericum perforatum* is a perennial herb with bright yellow flowers that bloom around the time of St. John's Day (June 24th), hence its name. The plant belongs to the *Hypericaceae* family and is native to Europe, but it is also found in other regions of the world. St. John's Wort has a long history of use in traditional medicine for various ailments, including depression, anxiety, wounds, and nerve pain. It is also used topically for its anti-inflammatory and wound-healing properties. *Hypericum perforatum* contains a range of bioactive compounds, including hypericin, hyperforin, flavonoids, and phenolic acids. Hypericin and hyperforin are considered the primary active constituents responsible for the plant's medicinal effects. St. John's Wort is well known for its antidepressant properties and is commonly used as a natural remedy for mild to moderate depression^{43,44}. It may also have anxiolytic effects, helping to reduce anxiety and promote relaxation. St. John's Wort has anti-inflammatory properties that can be beneficial for conditions such as arthritis and skin inflammation. When applied topically, it is believed to promote wound healing and reduce inflammation. St. John's Wort is used to improve mood, alleviate symptoms of depression, and enhance overall emotional well-being. It may help reduce nerve pain and discomfort associated with conditions like sciatica and neuralgia. Some people use St. John's Wort to improve sleep quality and alleviate insomnia. Numerous studies have investigated the efficacy of St. John's Wort in treating depression and other conditions, with mixed results. Research suggests that hypericin and hyperforin may modulate neurotransmitters in the brain, such as serotonin, dopamine, and norepinephrine⁴³. While St. John's Wort is generally well-tolerated, it can interact with certain medications, including antidepressants, birth control pills, and blood thinners. Side effects may include gastrointestinal upset, skin sensitivity to

sunlight, and potential allergic reactions. In conclusion, *Hypericum perforatum*, or St. John's Wort, is a popular herbal remedy with potential benefits for mood support, nerve pain relief, and wound healing ⁴². It is essential to use this herb cautiously, especially when combining it with other medications, and consult a healthcare provider before starting any new herbal regimen.

For this research, St. John's Wort, *Hypericum perforatum* extract (HPE) as were used as the main active ingredient. St. John's Wort is known in several cultures for its use in treating several conditions as depression, ulcers, dyspepsia, abdominal pains, burns, bacterial infections, migraine, headaches, and sciatica. In Turkish traditional medicine, the water extract of the flowering aerial parts of has been used as a remedy against a variety of illnesses, such as peptic ulcers, diabetes mellitus, neuralgia, gastritis, hemorrhoids, and urogenital inflammations. Additionally, it has been noted that a common home cure for the quick healing of cuts and burns is the olive oil macerate of blooming herbs administered externally ¹⁴. Among the many compounds found in the plant, naphthodianthrone (mainly hypericin, pseudohypericin, and their derivatives), phloroglucinols, flavonoids, biflavones, phenylpropanes, and proanthocyanidins which play important roles in the main pharmacological effects besides the presence of lesser amounts of tannins, xanthones, essential oils, and amino acids. The extracts are proven to have photodynamic, antimicrobial, antidepressant, and anti-inflammatory activities whereas methanolic extracts are found to be the best extraction method to obtain a homogenous drug sample, representative of all the major metabolites and with fewer degradation products ⁴⁴⁻⁴⁷.

Another natural herbal product that caught the researchers' interest for its wound-healing properties is Acacia or Arabic gum produced as an exudate by the *Acacia Senegal* tree's stem and branches. It is widely recognized for its unique properties, including water solubility, emulsifying capabilities, and stability enhancement. Acacia gum is a polymer of complex carbohydrates composed of uronic acid, galactose, arabinose, and rhamnose. This composition made it useful applications in the medical field and helpful for treating several health conditions. Acacia gum acts as a prebiotic, promoting the growth of beneficial bacteria in the gut. This can help improve gut health and digestion. Studies have shown that Acacia gum may have anti-inflammatory effects, which can be beneficial in conditions characterized

by inflammation such as arthritis. Acacia gum has been reported to have a hypolipidemic effect, helping to lower lipid levels in the blood. This can be beneficial for individuals with high cholesterol levels. Acacia gum has been studied for its potential anti-diabetic effects, showing improvements in blood glucose levels and glycemic control in diabetic individuals. By helping to lower lipid levels and potentially reducing inflammation, Acacia gum may have positive effects on cardiovascular health and reduce the risk of heart disease. Some studies suggest that Acacia gum may have benefits for renal health, although more research is needed in this area. It is important to note that while Acacia gum shows promise in these medical applications, further research is needed to fully understand its mechanisms of action and potential benefits for various health conditions. Overall, Acacia gum is a versatile ingredient with a wide range of applications in the food industry, health sector, and beyond, making it a valuable natural resource with functional and nutritional benefits as well as its antioxidant, hemostatic, non-hemolytic, and antibacterial properties make it an important candidate for healing promotion.^{48,49}

2.3.1 Herbal-loaded electrospun nanofibers

In a study from Iran, Semelil, a substance derived from herbs, was incorporated into electrospun nanofibers for wound treatment. Using a blend of Chitosan and Polyethylene oxide in a 90:10 weight ratio, researchers created homogenous, bead-free nanofibrous mats containing 10–50% weight extract, showing promise as wound dressing for persistent ulcers⁵⁰.

In a study from Turkey, they incorporated St. John's Wort infused oil with chitosan cryogel as a wound dressing material. They investigated antimicrobial activity, antioxidant, and DNA cleavage. The results showed antimicrobial activity, especially against *E. coli* and *L. pneumophila*, and the presence of scavenging activity proves it is a promising candidate for wound healing⁵¹.

In another study, a silk fibroin-curcumin-based nanofiber, in combination with polycaprolactone and polyvinyl alcohol, achieved sustained drug release and promoted oxygen delivery to diabetic wound. *In vivo* studies for wound healing efficacy in diabetes-

induced mice showed a higher wound healing rate compared to the control, higher potential of the nanofibrous tissue formulation, and no dermal irritation ⁵².

In another study from Turkey, they were able to produce electrospun polycaprolactone /Gelatine nanofiber dressing loaded with Aloe vera or St. John's Wort. The aim was to evaluate their efficacy in diabetic wound treatment. Using FTIR analysis, they compared the changes in the quantity and molecular makeup of phospholipids, proteins, and lipids in diabetic rats' blood and serum samples. The results showed that treating diabetic rats with Aloe vera and St. John's Wort caused the blood's and serum's chemical structure to return to what was seen in the control group. By measuring the levels of tumor necrosis factor- α (TNF- α), total antioxidant status (TAS), and total oxidant level (TOS), they were also able to assess the inflammatory response. They observed that while TNF- α levels rose in the St. John's Wort group, TOS decreased in the both Aloe vera and St. John's Wort groups. They concluded that St. John's Wort loaded nanofibers were better in treating diabetic wounds compared to the Aloe vera loaded ones ⁵³.

2.4 Metallic and inorganic nanoparticles for wound healing

Metals such as gold, silver, iron, and platinum, as well as semiconductors like silicon, germanium, and cadmium selenide nanoparticles, play a significant role in wound healing due to their exceptional qualities, including antibacterial properties and the ability to overcome antibiotic resistance ⁵⁴. Inorganic and metallic nanoparticles are increasingly utilized in the medical field for drug delivery, imaging, diagnostic purposes, and tissue engineering. To regulate release kinetics from biomaterials, inorganic nanoparticles have begun to be incorporated into nanofibers ⁵⁵.

Topical application of gold nanoparticles has been shown to significantly enhance the expression of growth factors, cytokines (IL10 and IL4), and collagen, thereby accelerating wound healing processes ⁵⁶.

Silver nanoparticles (AgNPs) play a significant role in wound healing ⁵⁷. Silver ions are highly effective antibacterial agents, particularly against biofilm-forming bacteria and

multidrug-resistant organisms commonly found in chronic wounds. AgNPs are excellent candidates for tissue engineering and wound treatments due to their unique properties.

Zinc oxide nanoparticles (ZnONPs) are another type of inorganic nanoparticle that exhibits promising properties for wound healing. The benefits of ZnONPs include their skin permeability, biocompatibility, and exceptional regeneration capabilities *in vivo*, achieved through tissue granulation, keratinocyte migration, collagen fiber deposition, and re-epithelialization ⁵⁸.

Iron oxide nanoparticles aid in wound healing by releasing iron metal ions with antibacterial properties, which can overcome bacterial resistance. Clinically, iron nanoparticles can be used to alleviate anemia and glucose abnormalities and promote wound healing through these mechanisms. Chronic wounds benefit from the use of iron nanoparticles, which may have applications beyond wound care ⁵⁴.

Mesoporous silica nanoparticles (MSNs) possess unique properties such as large surface area, porous structure, and surface-active groups attracting biomedical researchers for their potential in drug delivery, photodynamic therapy, biosensors, and tissue engineering ⁵⁹. Silica nanoparticles have been shown to have beneficial effects on wound healing due to their hemostatic properties, helping to stop bleeding ⁶⁰.

2.4.1 Metalized nanofibers in wound healing

Metallized nanofibers represent a novel class of nanomaterials with diverse applications in biomedicine. Their unique physicochemical properties, including high specific surface area and superior surface, magnetic, optical, and electronic characteristics, make metal ions highly valuable in contemporary medicine ^{55,61}.

Metallic nanofiber-based composites are multimodal nanostructures formed by incorporating metal nanoparticles into polymer nanofibers during electrospinning. The combination of metallization with electrospinning facilitates the production of metallized nanofibers. These hybrid nanocomposites can be created through in situ reactions of metallic

precursors within electrospun polymer nanofibers ⁶². Another fabrication method involves adding precursor metal nanoparticles to the polymer solution, followed by electrospinning to form nanofibers.

The integration of nanofibers' multiple qualities with the various properties of metallic nanoparticles, such as high formability, stability, and electrical and thermal conductivity, results in versatile materials. Metallized nanofibers are particularly suitable as scaffolds in tissue engineering due to their exceptional antimicrobial and plasticity. Additionally, their capacity for local therapy and targeted drug delivery can be enhanced by loading various medications onto the nanofibers. The excellent absorption capabilities and porous structure of metallized nanofibers make them ideal for drug and cell delivery ⁶³.

Encapsulated metallic ions in electrospun nanofibers have been evaluated for their antibacterial and angiogenesis-promoting function, aiding in wound repair ⁶¹. Nanofibrous materials loaded with metallic nanoparticles, such as AgNPs, have demonstrated enhanced therapeutic outcomes in wound healing applications ⁶⁴. For example, the dispersion of AgNPs in a chitosan matrix is electrospun to create immobilized cubic structure silver nanofibers ⁶⁵.

Other metal ions have also been incorporated into polymer nanofibers, with their wound-healing effects being thoroughly investigated. Zinc ions, for instance, can support tissue regeneration, repair, cell proliferation, and differentiation. Studies indicate that zinc ions can aid in wound treatment, prevent infection, and possess antibacterial properties. Additionally, magnesium ions have been found to renew and repair soft tissues ⁶⁶.

The advantages of metallized nanofiber composites include strong mechanical properties, providing a non-toxic and antibacterial foundation for the development of drug-loaded wound healing platforms.

Scientists have successfully incorporated MSNs into chitosan to form porous chitosan-silica composite microspheres. These microspheres leverage the hemostatic properties of both components for controlling traumatic hemorrhaging. In a rat liver laceration model, this composite demonstrated positive results with no adverse effects on cytotoxicity or histological analysis ⁶⁷.

Researchers have also produced gentamicin-loaded MSNs-PCL nanofibers that provide sustained release of the antibiotic, which is crucial for preventing post-surgery wound infections. MSNs functioned as drug delivery vehicles, acting as release modifiers and protectors of bioactivity. After 40 hours, only 50% of the drug was released, with complete release taking 136 hours. The antimicrobial activity of the produced fiber was confirmed ⁶⁸.

2.5 Combination of herbal extract and metallic/inorganic nanoparticles in the fabrication of nanofibrous wound dressing

One of the promising methods for enhancing the biocompatibility and bioavailability of phytochemical compounds is the application of nanotechnology-based therapies. This approach involves incorporating naturally occurring substances from plants into nano-based delivery systems, such as nanoparticles and nanofibers.

Nanomaterials loaded with plant extracts are environmentally friendly and non-toxic, allowing for controlled and synergistic release of the active plant material in the wound area. Given that several plant extracts and their natural constituents used in nanoformulations have demonstrated strong efficacy in wound treatment, they hold potential for development into future pharmaceutical products ⁶⁹.

Nanocarriers, including nanoemulsions, nanohydrogels, nanoliposomes, and nanofibers, significantly increase the effectiveness and solubility of poorly soluble herbal medicines. The unique properties of plant extract-loaded nanofibers enhance their antimicrobial, anti-inflammatory, antioxidant, antiseptic, and antiviral capabilities, thereby increasing their wound-healing potential ⁷⁰.

A study from India incorporated MSNs into PCL/Curcumin nanofibers. The researchers conducted antibacterial studies on both gram-positive and gram-negative bacteria using Swiss 3T6 cell lines, and *in vivo* on female Wister rats. The results demonstrated improvement in the zone of inhibition, re-epithelization of tissue, collagen deposition, and granulation tissue formation. When the fabricated nanofibers were applied to rats, they achieved 99% scar-less wound healing within 21 days ⁷¹.

In our research, we attempted to incorporate HPE, AG, and MSNs to produce a wound-dressing mat of nanofibers.

2.6 Safety of herbal extract-loaded multimodal nanofibers

The safety of nanofibers loaded with various active ingredients, including herbal constituents, chemicals, and metals must be thoroughly investigated through toxicity tests due to the current lack of comprehensive information in this area. Among the toxicity tests, *in vitro* cytotoxicity testing, irritation, and sensitization are considered the primary evaluation. In addition to these tests, assessing the genotoxicity of new materials is crucial, as it represents a significant toxicity endpoint. Therefore, genotoxicity testing should be conducted alongside other standard toxicity assessments to ensure the safety of new nanomaterial-based products.

2.6.1 Cytotoxicity and genotoxicity evaluation of nanomaterials

Numerous techniques can be used to test for cytotoxicity. The most popular method is the viability test, which measures a cell's capacity to multiply, endure, and form colonies according to OECD Test Guidelines. Applying membrane-impermeable dyes to measure membrane integrity allows staining only the cells whose membranes have been damaged. Examples of staining techniques include the Trypan blue exclusion test and dual staining with fluorescent dyes, such as fluorescein diacetate for live cells and propidium iodide for dead cells, followed by microscopy or flow cytometry analysis. Another method to identify dead cells is by detecting intracellular material leakage, such as lactate dehydrogenase (LDH). For detection, colorimetric or fluorometric techniques are employed, such as the alamarBlue™ assay and the MTT test, which involve dyes that need to be metabolically activated.

Genotoxicity caused by nanomaterial exposure can lead to transient or permanent genetic changes. There are two primary mechanisms for nanomaterial induced genotoxicity: direct and indirect. Direct genotoxicity occurs when there is physical contact between nanomaterial and the nucleus, resulting in DNA damage such as chromosomal damage, significant DNA malformations, or breaks and other lesions in the DNA. In this case

nanomaterial can interact directly with the genome Indirect genotoxicity involves the release of harmful ions or reactive oxygen species (ROS) from the dissolution of nanomaterial, creating intracellular ROS through a Fenton-type reaction, which causes oxidative stress. While the damage can be repaired, free radicals can cause strand breaks, purine or pyrimidine oxidation lesions, gene mutations, and more extensive chromosomal damage. Additionally, DNA damage may be induced indirectly by molecules that interact with DNA or obstruct DNA replication and cell division such as protein kinases, which control cell cycle activities like DNA replication and cell division.

The genotoxicity of nanomaterials varies with dose, length of exposure, size, surface characteristics, chemical composition, and shape. The primary determinant of the genotoxicity mechanism is the uptake method. For direct damage, smaller nanomaterials have a higher chance of entering the nucleus through nuclear pores. Larger nanomaterials can enter the nucleus either by endocytosis or when the cell undergoes mitosis. Indirect damage is more common, as nanomaterial induces inflammation due to an oxidative burst and may result in chronic immune cell response ^{72,73}

Two *in vitro* test methodologies are recommended for nanotoxicology risk assessment due to the widespread application of genotoxicity testing.

a) Tests for mammalian gene mutation.

Thymidine kinase (Tk) and hypoxanthine phosphoribosyltransferase (Hprt) genes are the primary assays used as a basis for gene mutation testing of non-metallic nanomaterials. These assays are described in the OECD Test Guidelines (TGs) (OECD TG 476 and OECD 490), with minor modifications for nanomaterials exposure and characterization. For example, the murine lymphoma (MLA) assay can identify a broad range of genetic damage, such as promoter hypermethylation-induced epigenetic silencing of the functional Tk allele and gene deletion.

b) DNA and chromosomal damage tests.

DNA damage detection is essential when evaluating the hazard of nanomaterials. The preferred technique for assessing DNA strand breakage and quantifying damage to cellular DNA is the comet assay. Due to its high sensitivity, consistent experimental design and standardized conditions are crucial to prevent variability in results. In addition to measuring strand breakage, the basic comet assay can identify damaged bases and the effects of oxidative stress on DNA through the use of a lesion-specific endonuclease, such as formamido pyrimidine DNA glycosylase (Fpg), after the lysis step.

DNA damage response biomarkers include DNA double-strand breaks (DSBs) and DNA damage foci. Localized accumulations or modifications of DNA damage response proteins, such as the microscopic imaging observation of γ H2AX after immunocytochemical detection or fluorescence protein tagging, are also utilized. γ H2AX, a phosphorylated histone protein, has been suggested in several studies as a potential biomarker of DNA double-strand breaks induced by genotoxicants. Numerous studies have employed the γ H2AX phosphorylation technique to quantify DNA damage produced by various nanomaterials, including titanium dioxide, gold, silica, polystyrene, carbon nanotubes, zinc oxide, and silica oxide. These studies demonstrate that this technique can be a valuable instrument for evaluating the genotoxic potential of nanomaterials.

Clastogenicity (structural chromosome damage) and aneugenicity (numerical chromosome alterations) are the two key genotoxicity endpoints. Agents causing structural chromosome or chromatid breaks, dicentrics, and other abnormal chromosomes, including the loss of chromosomes, can be assayed by chromosomal aberration tests. However, because this test is time-consuming, an *in vitro* micronucleus assay, which detects micronuclei in the cytoplasm of interphase cells, is often used to identify chromosomal damage. Micronuclei can arise in the cytoplasm from chromosomes, chromatid fragments, or whole chromosomes that lag behind in cell division. The micronucleus assay is capable of identifying both aneugenic and clastogenic effects. This assay described in the OECD TG 487 (2016), is performed in several cell models, such as human or other mammalian cell lines. Cytochalasin B is used to inhibit cytokinesis, preventing daughter cells from dividing after mitosis and forming binucleated cells instead. This assay is frequently used to test for nanomaterials;

however, to allow for nanomaterial uptake by cells, exposure to nanomaterials must occur before the addition of cytochalasin B.

In *in vitro* investigations, before adding nanomaterials stock solutions to cultured cells, they are usually diluted in a cell culture medium. The particle surface interactions of nanomaterials whether they involve particle-particle or particle-cell interactions, are altered when nanomaterials are suspended in cell culture medium or other biological fluids. These alterations can change the size, shape, composition, and surface chemistry of the nanomaterial as well as the temperature, pH, ionic strength, and protein content of the surrounding fluid. Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) are used to determine the size and size distribution of nanomaterials, while microscopic methods including AFM, SEM, and TEM are used to directly observe nanomaterials. The Zeta potential, a technique for measuring the electrostatic potential of the electrical double layer at the location where NMs are suspended, can be used to describe the surface charge of nanomaterials.

As a new advanced *in vitro* models for the assessment of genotoxicity on human skin, two models have been developed: the reconstructed skin comet assay (RS comet assay) and the reconstructed skin micronucleus test (RSMN). It has been demonstrated that commercially available reconstructed skin tissues (EpiDermTM, Phenion^{®FT}, and EpiSkinTM) exhibit the same metabolic characteristics as natural skin. For example, in an RSMN study using silica nanoparticles and the EpiDermTM model, it was determined that thorough exposure characterization and uptake evaluation techniques are essential for effectively interpreting nano(geno)toxicity research.

Based on the above, the safety of using nanofibers has been evaluated in some studies. The cytotoxicity of composite chitosan microspheres loaded with mesoporous silica was tested using the MTT method, evaluating the cytotoxicity on mouse embryonic fibroblast cells. The results showed good biocompatibility and non-cytotoxicity, indicating that they are safe for use in medical applications⁷⁴.

The Sulforodamine B (SRB) assay was used to assess the cytotoxicity of acacia chitosan/nanocellulose-based nanofibers on non-tumor primary cells (PLP2). The GI50

values were calculated after the nanofibers were evaluated at concentrations ranging from 8 to 0.125 mg/mL diluted in water. Non-tumor cells did not exhibit cytotoxicity, indicating biocompatibility in the results ⁷⁵.

Studies have shown that mesoporous silica nanoparticles (MSNs) in the size range of 25–100 nm exhibited genotoxicity when HT-29 cells were exposed for 24 hours period. However, the cytotoxicity study using the MTT assay indicated no deterioration for average sizes of 30–300 nm. Nonetheless, dosing exposure higher than 200 µg/mL showed significant cytotoxicity ⁵⁹.

2.6.2 Irritation and corrosion tests

Experiments assessing skin irritation and corrosion have historically relied on animal testing, often involving rabbits, in accordance with the guidelines outlined in OECD Test Guideline (TG) 404. However, increasing ethical awareness regarding animal welfare has driven the exploration of alternative methods to conduct *in vitro* testing for skin irritation and corrosion, aiming to replace the reliance on animal-based approaches ⁷⁶

Several three-dimensional (3D) human skin models available for purchase have emerged as substitutes for *in vivo* skin irritation testing methods. Notably, for regulatory purposes, SkinEthic™ (EpiSkin Research Institute), EpiDerm™ (MatTech Co., Ashland, MA, USA), and EpiSkin™ (EpiSkin Research Institute) are frequently used. These models utilize a human epidermis reconstructed from non-transformed epidermal keratinocytes. They closely mimic the histological, morphological, biochemical, and physiological characteristics of the human skin's epidermal layer ^{77,78}.

In vitro testing using cultivated skin-derived cell lines has been employed in several studies to evaluate the degree of skin irritation induced by nanomaterials. Titanium nanoparticles (TNPs) and quantum dot nanoparticles exhibited reduced viability in the cultured HaCaT human keratinocytes cell line, but their effect did not extend to a human skin equivalent model ^{79,80}. Conversely, nano silica nanoparticles induced cell death in cultured human keratinocytes, yet they demonstrated no cytotoxicity in a human skin equivalent model ⁸¹. In another study, exposing EpiDerm™ to TiO₂ nanoparticles did not cause any

skin irritation symptoms ⁸². Additionally, the application of TiO₂ nanoparticles and ZnO nanoparticles, or a combination of both did not cause irritation or corrosion when applied to undamaged rabbit skin or KeraSkin, a 3D human skin model ⁸³.

2.6.3 Sensitization test

The covalent interaction of electrophilic substances with primary amines in skin proteins and nucleophilic thiols is the initial critical step in the skin sensitization process. The second major event, occurring in keratinocytes, involves inflammatory reactions and modifications in gene expression linked to specific cell signaling pathways, such as those dependent on the antioxidant/electrophile response element (ARE) ⁸⁴. The ARE-Nrf2 Luciferase KeratinoSens™ test is a tool that is representative of this occurrence and may be used to differentiate between skin sensitizers and non-sensitizers in a way that is consistent with the standards established by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals ⁷⁹. In research conducted by Kim et al. (2021), the ARE-Nrf2 Luciferase KeratinoSens™ test was used to measure skin sensitization induced by seven distinct metal oxide nanoparticles ⁸⁵. The study examined the effects of nickel oxide, titanium oxide, copper oxide, cobalt oxide, zinc oxide, iron oxide, and cerium oxide on KeratinoSens™ cells. Notably, copper oxide nanoparticles elicited a positive response, while the other nanoparticles showed no response.

2.6.4 Toxicity profile of used ingredients

Using ingredients with a low toxicity profile is essential for producing a safe wound-healing dressing. PVA is known for its low toxicity, making it safe for oral use in various pharmaceutical preparations as a coating agent and in several medical applications ^{86,87}. However, the increased use of PVA in electrospinning applications, including wound healing dressing, has raised concerns about its safety. A study investigated the cytotoxicity of electrospun PVA with different molecular weights on human coronary artery smooth muscle cells and human coronary artery endothelial cell cultures ⁸⁸. The study found while the different PVA polymers were non-cytotoxic before electrospinning, the electrospinning process generated small molecular weight fractions that were cytotoxic to cells. Thus,

assuming PVA is safe based solely on its known biocompatibility and biodegradability is insufficient, and more investigation is needed.

Chitosan, approved by the FDA for use in wound dressings and as a dietary component in various countries, is widely regarded as a non-toxic, biologically friendly polymer. However, its toxicity can be influenced by processing and alteration, as it seems to correlate with its charge density. Therefore, toxicity testing is required ⁸⁹. Similarly, while gelatin is a natural protein polymer, its cytotoxicity can vary depending on the conditions and solvents used during the electrospinning process.

Relatively little toxicological information is available from laboratory experiments about St. John's wort. Chronic and subchronic toxicity tests have shown no risk to patients, but there are reports of adverse effects and interactions when consumed orally daily ⁹⁰. Dermatological adverse effects after oral consumption include allergic reactions and photosensitivity ⁹¹. Although there is insufficient toxicity data on the dermatological application of HPE, it may cause skin reactions after sun exposure. The AMES test revealed a slight mutagenic potential for ethanolic extracts ⁹⁰.

Toxicological studies indicate that animals are not negatively impacted by acute or subchronic exposure to modified gum acacia, nor does it have the potential to cause mutations. However, a dermal irritation study identified it as slightly irritating. All abraded locations on rabbit skin showed very modest to well-defined erythema and slight edema one hour after the patch was removed ⁹².

Even though all the raw materials are relatively non-toxic and safe to use, it is still important to verify the safety of the final product and ensure that no toxic properties arise from the components' interaction or the production process.

3. MATERIALS AND METHODS

3.1 Material:

3.1.1 Chemicals:

Chitosan (CS)	Merck chemical company
low or moderate molecular weight (50-190KDa), 75-85% deacetylation	
Polyvinyl alcohol (PVA)	Merck chemical company
High molecular weight (89-98KDa) degree of hydrolysis = 99%	
Gelatin (G) powder food grade	Merck chemical company
St. John's Wort standardized herb extract food supplement capsules	Solgar; USA
St. John's Wort olive oil extract	Hünnap; Türkiye
Acacia gum (AG)	Local herbal shop
Herbal-based Mesoporous Silica Nanoparticles (MSNs)	Laboratory; Tabriz University
Glutardialdehyde	Sigma Aldrich; USA
Doxorubicin hydrochloride	Sigma Aldrich; USA
Rosmarinic acid	Sigma Aldrich; USA
(-)-Epigallocatechin gallate	Sigma Aldrich; USA
Fluorescein diacetate (FDA)	SERVA Electrophoresis GmbH; Germany
Gel Red nucleic acid gel stain	Biotium ;USA

Gel Green	Biotrend; Germany
Agarose MEE0 ultra quality	Carl Roth; Germany
2-Hydroxyethylagarose	Sigma Aldrich; USA
Triton X-100	Sigma Aldrich; USA
Dimethylsulfoxid	Carl Roth; Germany
Ethylenediaminetetraacetic acid	Sigma Aldrich; USA
Disodium salt dihydrate (EDTA)	
Sodium chloride	Carl Roth; Germany
Potassium chloride	Fluka; Switzerland
Potassium dihydrogen phosphate	Merck; Germany
di-Sodium hydrogen phosphate anhydrous	Merck; Germany
Sodium dihydrogen phosphate monohydrate	Merck; Germany
Trizma base	Sigma Aldrich; USA
Methanol	Sigma Aldrich; USA
Ethanol	Sigma Aldrich; USA
1,4-Diazabicyclo [2.2.2] octane	Sigma Aldrich; USA
Dihydroethidium	Calbiochem; Germany
Agar	Sigma Aldrich; USA
L-Histidine	Fluka; Switzerland
Biotin	Sigma Aldrich; USA
β -Nicotinamide adenine	Sigma Aldrich; USA
inucleotide phosphate (NADP)	

D-Glucose 6-phosphate disodium salt hydrate	Sigma Aldrich; USA
Potassium phosphate dibasic	Sigma Aldrich; USA
D-(+)-Glucose	Sigma Aldrich; USA
Sodium azide	Sigma Aldrich; USA
2-Aminofluorene	Sigma Aldrich; USA
2-Aminoanthracene	Sigma Aldrich; USA
4-nitro-o-phenylenediamine	Sigma Aldrich; USA
Mitomycin C	Sigma Aldrich; USA
Lyophilized Standard disc TA98	Moltox; USA
Lyophilized Standard disc TA100	Moltox; USA
Nutrient Broth Oxoid	Moltox; USA
Lyophilized S9	Moltox; USA
Magnesium sulfate (MgSO ₄ .H ₂ O)	Sigma Aldrich; USA
Citric acid monohydrate	Sigma Aldrich; USA
Potassium phosphate (K ₂ HPO ₄)	Sigma Aldrich; USA
Sodium Ammonium	Sigma Aldrich; USA
Phosphate (NaH ₂ NH ₄ (PO ₄ .4H ₂ O))	
Ampicillin trihydrate	Sigma Aldrich; USA
Sodium hydroxide	Sigma Aldrich; USA
Bovine Serum Albumin (BSA)	Sigma Aldrich; USA
Dulbecco's Modified	Sigma Aldrich; USA

Eagle's Medium/Ham's Nutrient Mixture F12	
RPMI-1640 Medium	Sigma Aldrich; USA
Sodium pyruvate	Sigma Aldrich; USA
DMEM (Dulbecco's Modified Eagle Medium)	Sigma Aldrich; USA
Fetal Bovine Serum FBS	Gibco; USA
Penicillin-Streptomycin	Sigma Aldrich; USA
Acetic acid	Sigma Aldrich; USA
Giemsa solution	Sigma Aldrich; USA
Dulbecco's Phosphate-Buffered Saline	Gibco; USA
Cytochalasin B	Sigma Aldrich; USA
Trypsin/EDTA	Sigma Aldrich; USA
Phosphate buffered saline (PBS)	Gibco; USA

3.1.2 Equipment and Instruments

Inovenso's electrospinning device	Istanbul; Türkiye
Scanning electron microscopy (SEM)	ZEISS EVO40; Germany
Sputtering device	LEICA EM ACE200; Germany
Fourier transform infrared (FTIR)	Perkin-Elmer Spectrum One series FT-IR apparatus (Version 5.0.1.); USA
Dynamic light scattering (DLS)	Malvern Zetasizer (ZEN3600); UK

Atomic force microscopy (AFM)	Park Systems XE 100; KOR
UV-Visible Spectrophotometer	Thermo Scientific Evolution 300; USA
Fluorescence microscope	Leitz; Germany
Eclipse 55i microscope	Nikon GmbH; Germany
Fluoro Pro MP 5000 camera	Intas Science Imaging Instruments GmbH, Germany
Comet Assay Tank	Cleaver Scientific; UK
Electrophoresis power supply	Biorad; USA
Centrifuge	Sigma 3-16 PK, Germany
Incubator	Heidolph Unimax 1010, Germany
Micropipette (10-100 μ L)	Eppendorf, Germany
Micropipette (20-200 μ L)	Eppendorf, Germany
Micropipette (100-1000 μ L)	Eppendorf, Germany
Micropipette (1-10 μ L)	Capp, Denmark
Vortex	Heidolph Reax Top, Germany
Analytical Balance	Ohaus Explorer 214C, USA
8-Channel Multichannel Pipett	Capp, Denmark
Laminar flow cabin	Esco, Singapore
Refrigerator	Arçelik, Turkey
Deep Freezer (-20°C)	Siemens, Germany
Deep Freezer (-80°C)	New Brunswick, Canada
Phase-contrast invert microscope	Zeiss, Germany
Automatic pipettor	Hirschmann Laborgeräte, Germany

pH meter	Mettler-Toledo MP220, Germany
Waterbath	Gesellschaft Fuer Labortec, Germany
Incubator (37 °C with 5% CO ₂)	Benchmark Scientific, USA
Incubator	Heal Force, China
Autoclave	Systec GmbH, Germany
Microplate reader	Thermo Multiskan Ascent, USA
Multimode microplate reader fluorescence plate reader	Thermo Scintific, Varioskan Lux, USA
Milli Q water device	Merck, Millipore, Germany
Light microscope	Zeiss, Primo Star, Germany
Inverted microscope	Zeiss, Germany
Falcon tube (15-50 mL)	Capp, Denmark
Cell Culture Flask (25 cm ² ; 75 cm ²)	Corning flask, USA
Petri dishes, polystyrene	Sigma Aldrich, Germany
Cryo Vial	Isolab Laborgeräte GmbH, Germany
Eppendorf tubes	Isolab Laborgeräte GmbH, Germany
96 well plate	SPL Life science, Korea
6 well plate	TPP, Switzerland
Autoclavable schott bottle (250 mL; 500 mL; 1 L)	Isolab Laborgeräte GmbH, Germany
Neubauer counting chambers	Isolab Laborgeräte GmbH, Germany
Pipette tip (100-1000 µL)	Mettler Toledo, Germany
Pipette tip (10-100 µL)	Capp, Denmark
Microscope slides	Karl Hecht Assistant, England

Plain and frosted microscope slides	Thermofisher, USA
Cover Glass	Thorlabs, USA
Volumetric flasks (10, 25, 50, 100, 200, 250, 500, 1000 mL)	S&H Labware, Turkey
Balance	Daihan Biomedical, China
Beaker (50, 100, 250, 1000 mL)	Isolab Laborgeräte GmbH, Germany
Hotplate stirrer	Ohaus Europe GmbH, Switzerland
Test tube heater	Hanna Instrument, USA
Staining jar	Isolab Laborgeräte GmbH, Germany
Spatulas	Isolab Laborgeräte GmbH, Germany
Magnetic stir bar	Fisher Scientific, Germany
Graduated cylinder (25, 100, 250 mL)	Isolab Laborgeräte GmbH, Germany
Test tube racks	Isolab Laborgeräte GmbH, Germany
Slide box	Isolab Laborgeräte GmbH, Germany
Parafilm	Fisher Scientific, Germany
Autoclave tape	Isolab Laborgeräte GmbH, Germany

3.2 Method

3.2.1 Preparation of electrospinning solutions:

Biocompatible and biodegradable polymers were selected to produce nanofibers. Initially, a mixture of CS and PVA was used. Subsequently, Gelatin (G) was added to the mixture to enhance the properties of the nanofibers. Various preparation methods and mixture ratios (M) were tested to optimize nanofiber formation. *Hypericum perforatum* (HP) extracts, MSNs, and gum acacia (GA) were incorporated directly into the electrospinning blend to achieve both immediate and sustained release from the nanofiber. Different

concentrations and mixing methods were experimented with until optimal results were obtained. Table 3 details the preparation ratios, methods, and active agents used in the different polymer blends. Figure 3 illustrates M16 polymer blends prepared for electrospinning.

Table 3. The different polymer blends' preparation ratios, methods, active agents added and electrospinning parameters.

Mixture (M)	Composition	Preparation method	Active agents addition	Electrospinning parameters
M1	5% total solids CS: PVA (20:80)	• Dissolved in 70 mL of 2% Acetic acid solvent using a magnetic stirrer at 80 °C for 1.5hr	—	• Distance: 10cm • Flow rate: 1.5mL/hr • Voltage: 32kV
M2	3% total solids CS: PVA (50:50)	• Dissolved in 100mL of 3% Acetic acid solvent using a magnetic stirrer at 80 °C for 1.5hr	—	• Distance: 10cm • Flow rate: 2mL/hr • Voltage: 24kV
M3	1% CS and 4% PVA	• Dissolved in 1% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr	—	• Distance: 12cm • Flow rate: 1mL/hr • Voltage: 24kV
M4	1% CS and 4% PVA	• Dissolved 90% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr	—	• Distance: 12cm • Flow rate: 1mL/hr • Voltage: 24kV
M5	3% CS: 5%PVA (50:50)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr	—	• Distance: 12cm • Flow rate: 1mL/hr • Voltage: 24kV

		• Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr		
M6	3% CS: 8%PVA (50:50)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 20% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M6a: 2mL HP ethanolic extract was added to 30 mL of the mixture	• Distance: 12cm • Flow rate: 0.5mL/hr • Voltage: 26kV
M7	3% CS: 8%PVA (50:50)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M7a: 5% HP ethanolic extract of total polymer content was added to the mixture.	• Distance: 12cm • Flow rate: 0.5mL/hr • Voltage: 26kV
M8	1% CS: 6%PVA (50:50)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr	• M8a: 1% of MSNs of the total polymer content was added to the mixture. • M8b: 0.5% of MSNs of the total polymer content was added to the mixture.	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV

		• Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr		
M9	2% CS: 7%PVA (50:50)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M9a: 2mL Distilled water filtered by 0.45 µm injection filter then was added to 15mL of the mixture. • M9b: 1% of MSNs of the total polymer content was dissolved in 2mL Distilled water and filtered by 0.45 µm injection filter then was added to 15mL of the mixture. • M9c: 5% of HP extract powder of the total polymer content was dissolved in 2mL of Distilled water and filtered by a 0.45 µm injection filter, then added to 15ml of the mixture. • M9d: 1% of MSNs and 5% HP extract powder of the total polymer content was dissolved in 2mL Distilled water and filtered by 0.45 µm injection filter then was added to 15mL of the mixture. • M9e: 5% of HP oil extract of the total polymer content was dissolved in 2mL of Acetone as a co-solvent for the oil before being mixed with the polymer solution.	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV
M10	2% CS: 8%PVA (30:70)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr	• M10a: 2mL Methanol filtered by 0.45 µm injection filter then was added to 20mL of the mixture.	• Distance: 15cm • Flow rate: 1mL/hr

		• Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M10b: 1% of MSNs of the total polymer content was dissolved in 2mL Methanol and filtered by a 0.45 µm injection filter, then added to 20mL of the mixture. • M10c: 5% of HP extract powder of the total polymer content was dissolved in 2mL of Methanol and filtered by a 0.45 µm injection filter, then added to 20mL of the mixture. • M10d: 1% of MSNs and 5% of HP extract powder of the total polymer content was dissolved in 2mL Methanol and filtered by 0.45 µm injection filter then was added to 20mL of the mixture.	• Voltage: 21.5kV
M11	2% CS: 8%PVA (30:70)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M11a: 25% of HP extract powder of the total polymer content was dissolved in 5mL of Methanol and centrifuged, then the supernatant added to 10 mL of the mixture. • M11b: 1% of MSNs and 25% of HP extract powder of the total polymer content was dissolved in 5mL of Methanol and centrifuged, then the supernatant added to 10mL of the mixture.	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV
M12	3% CS: 12%PVA (30:70)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS in 80% Acetic acid	• M12a: HP extract powder containing 5% hypericin of the total polymer content was dissolved in 2mL of Methanol and centrifuged, then the	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV

		using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	supernatant was added to 10mL of the mixture. • M12b: HP extract powder contains 5% hypericin and 1% of MSNs of the total polymer content was dissolved in 2mL of Methanol and centrifuged, then the supernatant was added to 10mL of the mixture. • M12c: 15% of HP oil extract of the total polymer content was added to 10mL of the mixture drop by drop while stirring.	
M13	12%PVA: 3%CS: 10%G (70:15:15)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS and G in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M13a: 100mg of HP extract powder in Methanol then centrifuged, the supernatant was added to 10mL of the mixture. • M13b: similar to M13a with the addition of 1% MSNs of the total polymer content. • M13c: 5% herbal loaded silica 10mL of the mixture.	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV
M14	12%PVA: 3%CS: 10%G (60:15:25)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS and G in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a	—	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV

		magnetic stirrer at 80 °C for 1.5hr		
M15	12%PVA: 3%CS: 10%G (70:15:15)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS and G in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M15a: 100mg of HP extract powder was dissolved in 10 mL Methanol, sonicated for 30min and left to settle for 24hr. After centrifuge the supernatant was transferred into a beaker where the methanol was evaporated at 50 °C. 10mL of the mixture is added after evaporation and mixed with a magnetic stirrer. • M15b: similar to M1a with the addition of 1% MSNs of the total polymer content.	• Distance: 15cm • Flow rate: 1mL/hr • Voltage: 21.5kV
M16	12%PVA: 3%CS: 10%G (70:15:15)	• Dissolve PVA in distilled water using a magnetic stirrer at 80 °C for 1.5hr • Dissolve CS, G and 1% of total solid GA in 80% Acetic acid using a magnetic stirrer at 80 °C for 1.5hr • Mix the two solutions using a magnetic stirrer at 80 °C for 1.5hr	• M16a: 1% of MSNs of the total polymer content was added to the mixture. • M16b: 100mg of HP extract powder was dissolved in 10 mL Methanol, sonicated for 30min and left to settle for 24hr. After centrifuge the supernatant was transferred into a beaker where the methanol was evaporated at 50 °C. 10mL of the mixture is added after evaporation and mixed with a magnetic stirrer. • M16c: similar to M16b with the addition of 1% MSNs of the total polymer content.	• Distance: 15cm • Flow rate: 0.7mL/hr • Voltage: 21.5kV

* All mixtures were left to settle for 24hr before electrospinning

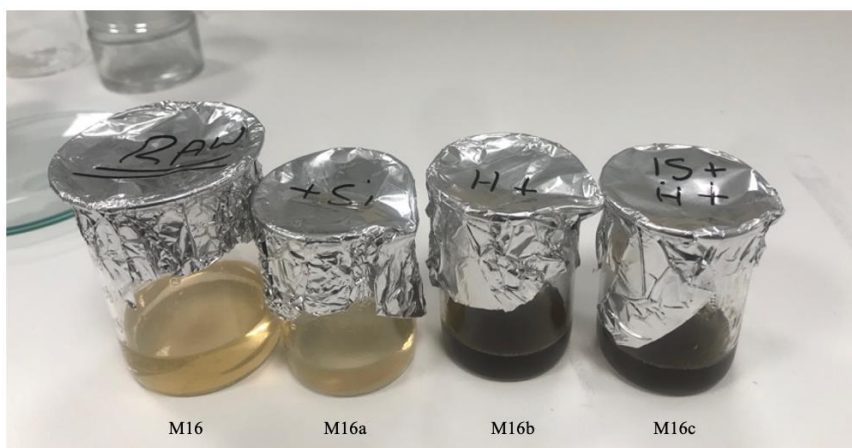


Figure 3. Prepared M16 polymer blends ready for electrospinning

3.2.2 Electrospinning:

The electrospinning processes were conducted using Inovenso's conventional electrospinning device equipped with a stationary collector. During the experiments, adjustments were made to the flow rate, applied voltage, and distance parameters as shown in Table 3. Our facilities' air conditioning system allowed for precise control of the environmental conditions during the electrospinning process.

The polymer solution was loaded into a 10 mL plastic syringe with a blunt-ended needle to produce a nanofiber mat. An aluminum foil was wrapped around a stationary collector positioned at a fixed, predetermined distance from the needle. A syringe pump fed the solution to the needle tip at a consistent, predetermined rate. A direct current positive high voltage wire was connected to both the metallic needle and the collector. The applied voltage, was tested between 18-32 kV and fixed after several trials.

3.2.3 Characterization:

3.2.3.1 Characterization of mesoporous silica nanoparticles

Fourier transform infrared (FT-IR) spectrum was used to characterize and identify the used MSNs. The spectrum was recorded using potassium bromide pellets, the frequencies reported in cm^{-1} . The morphology of the MSNs was determined by SEM and TEM. For SEM analysis, the MSN powder was secured on a glass slide using double-sided conductive carbon

tape and sputtered with a thin layer of gold. For TEM imaging, a small amount of MSN powder was dispersed in 1 mL of ethanol, placed on a specialized grid, and loaded into the TEM instrument. Particle size was determined by DLS and particle charge by zeta potential analysis. For these measurements, a small amount of MSN powder was dispersed in 1 mL of distilled water and then placed in a plastic cuvette for DLS analysis or in a capillary cell for zeta potential measurement. A refractive index of 1.42 was used for both DLS and zeta potential analysis.

To prepare herbal-loaded MSNs, 100 mg of HP extract powder was dissolved in 10 mL ethanol, sonicated for 30 minutes in an ultrasonic water bath, and then centrifuged. Subsequently, 500mg MSNs was mixed with the supernatant and heated at 50 °C until dry. TEM, DLS, and zeta potential analyses were repeated for comparison.

3.2.3.2 Characterization of HP extracts

For the identification HP extracts, hypericin was used as a reference. A UV-Visible spectrophotometer was employed to perform a spectrum scan from 400-700 nm, with the maximum absorbance of hypericin expected between 580-600 nm^{46,47,93}. HP oil and powered capsule extracts, purchased from a local pharmacy, were tested. For the oily extract, acetone was used as the solvent for analysis. For the HP powder extract, two different solvents were tested: methanol and PBS. The powders were dissolved, sonicated for 30 minutes, and then filtrated before scanning.

3.2.3.3 Characterization of produced nanofiber mat

FT-IR spectra of the raw material and synthesized nanofibers were recorded using potassium bromide pellets, with frequencies reported in cm⁻¹. The size and morphology of the produced nanofibrous mats were analyzed using SEM. A small section of each nanofibrous mat was sputtered with a thin layer of gold before SEM analysis. The tensile strength of the specimens was measured using a universal testing machine (Sahand University, Iran). The sample size used was 1x3 cm, and the loading rate was 5 mm/min. AFM analysis was performed using a Park Systems XE 100 instrument in contact mode. The AFM images were analyzed with XEI, an image-processing program for scanning probe

microscopy (SPM) data developed by PSIA. A glass slide was fixed on the collector, and samples were collected during the electrospinning process. Ten points from twelve nanofibers were randomly selected from the slide for each sample. Young's modulus values were calculated using the Hertz mathematical model, which is suitable for polymeric composite samples ²⁸.

3.2.4 Assay of St. John's Wort extract

For the St. John's Wort assay, the spectrophotometric method for hypericin assay was used as described previously ^{46,47,93}. St. John's Wort standardized herb extract food supplement capsules, purchased from a local pharmacy in Turkey, served as the source of the plant extract and as a reference for determining hypericin content. The average capsule content weight was calculated to be 490mg. According to the label, each capsule contained 300 mg HP powder, 175mg standardized HP extract, and 0.5 mg hypericin. Ethanolic and oil herbal extracts were used for spectrum comparison in the range of 400-700 nm.

For the calibration curve, the contents of ten capsules were mixed, and a weight of 490 mg (0.5 mg of hypericin) was used to prepare 2 stock solutions: one in 100 mL methanol and the other in 100 mL Phosphate buffer (pH =7.4). These solutions were sonicated for 10 minutes twice and left to settle for 24 hours. The stock solutions were then filtered, and 6 dilutions of each stock were prepared by serial dilution, with the process repeated three times. Samples were analyzed using a Thermo Scientific Evolution 300 UV-visible (Germany) spectrophotometer.

3.2.4.1 Assay of hypericin content after sample preparation

100 mg of HP extract powder was dissolved in 10 ml Methanol, sonicated for 30 minutes, and left to settle for 24 hours. After centrifugation, the supernatant was transferred into a beaker, and the methanol was evaporated at 50°C. The residue was reconstituted by adding 10 mL of 80% acetic acid and mixing with a magnetic stirrer for 10 minutes. The following concentrations were tested: 100%, 75%, 50%, 25%, 10%, and 5%. Samples were analyzed using a UV-visible spectrophotometer, and a calibration curve was plotted. This procedure was repeated three times.

3.2.5 Encapsulation efficiency

For the determination of the weight of the fibers formed from 1 mL of the polymer mixture, the weight of the aluminum foil was measured before and after the electrospinning process of a known volume of the polymer solution. The average weight from six trials was calculated. To determine the encapsulation efficiency (EE%), fibers equivalent to 1 mL of the polymer mixture (120 mg) were added to a test tube containing 4 mL 80% acetic acid. The mixture was heated in a shaker water bath at 80°C until all fibers dissolved. The test tube was then centrifuged, and the supernatant was analyzed using a spectrophotometer. This process was repeated three times. The EE% was calculated using the following equation:

$$EE\% = \left(\frac{\text{Weight of drug in nanoparticles}}{\text{Weight of feeding drugs}} \right) \times 100$$

3.2.6 Release studies

Fibers equivalent to 1 mL (120 mg) were added to a test tube containing 4 mL Phosphate buffer (pH =7.4) and placed in a shaking water bath set at 100 rpm and 37°C. Samples of 1 mL were pipetted out at 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours. Each 1 mL sample was replaced with 1 mL of fresh PBS solution. A final sample was taken after 12 hours to determine the total amount released.

3.2.7 Antimicrobial activity

The disk diffusion method was employed to evaluate the antimicrobial activity of the compounds against the microorganisms *Enterococcus hirae* (ATCC 10541), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 15442) and *Candida albicans* (ATCC 10231). Standard disks of Ofloxacin (5 µg) served as positive controls and references for the agar-based disk diffusion method. Bacterial and fungal suspensions were adjusted to the turbidity of the 0.5 McFarland standards and inoculated onto Mueller Hinton Agar (for bacteria) and Sabouraud 2 % Dextrose Agar (for fungi) using sterile swabs. Blank paper discs with a diameter of 6.0 mm were impregnated with the test

samples and placed on the agar plates. The zones of growth inhibition around the disks were measured after 18 to 24 hours of incubation at 37°C for bacteria and after 24 to 72 hours of incubation at 25°C for fungi. The antimicrobial activity was determined by measuring the sizes of the inhibitory zones on the agar surface around the discs ⁹⁴.

3.2.8 Evaluation of *in vitro* anti-inflammatory effect and cell migration

3.2.8.1 Sample preparation

The scaffolds were cut into appropriate pieces. Cell culture medium was added to scaffolds at a rate of 6 cm²/mL according to ISO 10993-12:2012, which specifies the biological evaluation of medical devices. The samples were then incubated at 37°C with 5% CO₂ for 24 hours. After the incubation period, the cell culture medium that had been in contact with the scaffolds was diluted with varying amounts of DMEM ⁹⁵.

3.2.8.2 Evaluation of Anti-inflammatory Activity

Nitric oxide (NO) production inhibition was assessed by measuring nitrite levels in the cell culture medium using the Griess reagent (0.1% N-(1-Naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide). RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10⁴ cells per well and incubated for 24 hours at 37°C with 5% CO₂. The cells were pre-treated with various concentrations of nanofiber film extracts (0.0625, 0.125, 0.25, 0.5 mg/mL) for 2 hours, followed by stimulation with 1 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli* 0111(Sigma, USA) for an additional 22 hours. After 24 hours, the cell culture supernatant was collected for analysis. Nitrite concentrations were determined using the Griess reagent. The supernatant was mixed with an equal volume of Griess reagent in a 96-well plate and incubated for 10 minutes at room temperature in the dark. The color development, which correlates with NO levels, was measured at 540 nm using a microplate reader (Thermo Fisher Scientific™, Inc., Waltham, MA, USA). Nitrite concentrations were quantified using a sodium nitrite standard curve. Indomethacin (100 mM) and L-NAME (100 mM) (Sigma Aldrich, St. Louis, Missouri, USA) served as positive controls ^{96–99}.

3.2.8.3 Cell Migration Assay

This experiment was conducted to assess cell migration rates in cultured cells, which is crucial for understanding collective cell migration—a process where a cohesive cell group maintains cell-cell junctions and coordinates cytoskeletal activity among neighboring cells and surrounding tissues. Cell migration is an essential parameter in wound healing, as it is one of the initial steps in the healing process^{100,101}. L929 cells were seeded in a 24-well culture plate at a density of 1.5×10^5 cells/mL in 0.4 mL of DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). The plate was placed in a humidified incubator set at 37°C with 5% CO₂. Before seeding, the culture plate was marked with three evenly spaced parallel lines to serve as reference points for capturing images of created gaps at different time points during the experiment. Following seeding, the cells were incubated for 24 hours until they reached full confluency. An artificial gap was then created in the cell monolayer using a Cell Scratcher (SPL, Life Sciences, South Korea) to simulate tissue injury. The wells were rinsed twice with phosphate-buffered saline (PBS), and 500 µL of fresh media was added to each well. Images of the initial wound (hour 0) were captured using a microscope at 10x magnification (Zeiss AxioCam, Germany). Various concentrations of extracts (0.0625, 0.125, 0.25, 0.5 mg/mL) were applied to the cultures, with DMEM serving as the control group. Images of the wounds were captured again at hour 0 and 24 post-treatment. ImageJ analysis software (NCBI, USA) was used to measure the width of the wounds at 0- and 24-hours post-treatment, enabling the determination of wound closure and calculation of cell healing capacity, expressed as the migration area (%). The healing rate of the cell cultures was calculated using the following formula⁹⁶:

$$\text{Gap closure\%} = [((\text{Gap area at } t_0) - (\text{Gap area at } t_{\text{final}})) / \text{Gap area at } t_{\text{final}}] \times 100$$

3.2.9 Toxicity studies

3.2.9.1 Cell Viability Assay on RAW 264.7 cell line

The RAW 264.7 mouse macrophages (ATCC® TIB-71™) used in this study were sourced from ATCC (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)

supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin-streptomycin (10,000 units/mL and 10,000 mg/mL, respectively) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The culture conditions were maintained at 37°C in a humidified atmosphere with 5% CO₂. Subculturing was performed when confluence reached 80-90%.

To evaluate the impact of herbal extract-incorporated fiber films on cell viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was utilized. RAW264.7 cells were seeded in 96-well plates at a density of 5×10^4 cells per well and incubated for 24 hours in the complete medium. Subsequently, the cells were treated with varying concentrations of nanofiber extracts (0.0625, 0.125, 0.25, 0.5 mg/mL) for another 24 hours. After treatment, MTT reagent (0.5 mg/mL in PBS) (Sigma Aldrich, St. Louis, Missouri, USA) was added and incubated for 2 hours at 37°C with 5% CO₂. Following the removal of the supernatant, isopropanol (100 µL) was added to each well, and the absorbance at 570 nm was measured using a microplate reader (Thermo Fisher Scientific™, Inc., Waltham, MA, USA). The cell viability assay was conducted in triplicate and repeated three times. Cytotoxicity was defined as a cell viability of less than 70% compared to untreated control cultures. The percentage of cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \text{OD}_{570} (\text{sample}) \times 100 / \text{OD}_{570} (\text{medium control})$$

(OD: Optical Density)

3.2.9.2 Cytotoxicity Assay on L929 cell line

The cytotoxicity of herbal extract-loaded nanofibers was assessed using the healthy mouse fibroblast cell line L929, obtained from the American Type Culture Collection (ATCC, USA). L929 cells were cultured in DMEM supplemented with 10% FBS (Gibco, NY, USA), and 1% penicillin-streptomycin (10,000 units/mL and 10,000 mg/mL, respectively), in an incubator with 5% CO₂ at 37°C. L929 cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/mL and incubated for 24 hours. Following incubation, varying concentrations of the herbal extract-loaded nanofiber were administered to the cells. After 24 hours of treatment, 100 µL of 0.5 mg/mL MTT solution was added to each well.

The cells were then cultured for an additional 2 hours. Subsequently, the MTT solution was discarded, and 100 μ L of isopropanol was used to dissolve the formazan crystals⁹⁶. Cytotoxic was defined as cell viability of less than 70% compared to the medium control group. The optical density was measured at 570 nm using an ELISA microplate reader (Thermo Multiskan Spectrum, Finland), and cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \text{OD}_{570} (\text{sample}) \times 100 / \text{OD}_{570} (\text{medium control})$$

(OD: Optical Density)

3.2.9.3 Ames test

The plate incorporation method was used to conduct a mutagenicity assay. Briefly, test tubes were maintained in a 45°C water bath. Then each test tube was prepared with 2 mL of top agar containing a histidine/biotin solution. Subsequently, 0.05 mL of the test substance and 0.1 mL of a 12-14-hour bacterial culture were added to the test tubes. The mixture was vortexed and poured into minimal glucose agar (MGA) plates. After allowing 15 minutes for the top agar to solidify, the petri dishes were inverted and incubated in a 37°C for 48-72 hours. Post incubation, His⁺ bacterial colonies were counted. Solvent control and positive control experiments were performed in parallel to evaluate the results. Some chemicals exhibit mutagenic or antimutagenic activities only after metabolic activation. The S9 fraction, a rat liver extract containing active liver enzymes (P450 activity), was used to metabolize test substances. The Ames test in the presence of S9 was performed by freshly preparing the S9 mixture just before the experiment. The S9-free assay included 2 mL of top agar, histidine-biotin solution, bacterial culture, and the tested substances, followed by the addition of 0.5 mL of the S9 mixture. This was mixed and spread homogeneously on MGA petri dishes. After solidification, plates were incubated at 37°C for 48 hours. Colonies were manually counted post-incubation, and the findings were evaluated. The indirect-acting mutagen 2-aminofluorene (2-AF) was used as a positive mutagen for both TA98 and TA100 strains with the S9 experiment. In the absence of the S9 mix, direct-acting mutagens 4-nitro-o-phenylenediamine (NPD) served as a positive control for the TA98 strain, sodium azide (SA) for TA100 strain^{102,103}.

The mutagenic index (MI) is calculated using the formula: $MI = a/b$ where a is the average number of reverse mutation colonies observed on compound-treated plates, and b is the average number of reverse mutation colonies per plate in the negative control. A sample is considered to exhibit mutagenicity if a dose-response relationship is detected and at least one concentration shows a two-fold increase in the number of mutants ($MI \geq 2$)¹⁰³.

Preparation of *Salmonella* culture and solutions:

Salmonella Typhimurium was streaked onto nutrient agar (containing histidine/biotin/ampicillin) plates from the stock culture. The plates were inoculated for 48 hours at 37°C. A small portion of the subculture was transferred into a test tube containing nutrient broth (oxoid) using a sterile loop and cultured at 37°C for 12-16 hours until reaching the log phase. The prepared liquid bacterial culture was used in the experiment.

Preparation of minimal glucose agar plates for TA98 and TA100 strains:

Agar	15 g
Distilled water	930 ml
50 X VB salt	20 ml
40% glucose	20 g in 50 ml

Preparation of Vogel-Bonner (50xVB salts) solution:

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaH_2NH_4PO_4 \cdot 4H_2O$)	175 g
Distilled water (45 ° C)	670 ml

The total volume is completed to 1000 ml and sterilized in an autoclave at 121 ° C for 15 minutes. Store in a dark environment at room temperature.

Preparation of top agar:

Agar	6 gr
NaCl	5 gr
Distilled water	1000 ml

The total volume is completed to 1000 ml and sterilized in an autoclave at 121 ° C for 15 minutes. Store in a dark environment at room temperature.

Top agar:

Agar	6 gr
NaCl	5 gr
Distilled water	1000 ml

Agar, salt and water were mixed and sterilized for 15 minutes at 121 ° C in an autoclave.

Preparation of 0.5 mM Histidine-Biotin Solution:

D-biotin	12.36 mg
L-histidin.HCl	9.6 mg
Distilled water	100 ml

The final volume was made up to 100 ml with distilled water. Then it was sterilized in an autoclave. This solution was added at 10 ml per 100 ml of top agar during the experiment.

Preparation of minimal glucose agar (MGA) plates:

Agar	15 g
Distilled water	880 ml
50xVB salt	20 ml
40% glucose	50 ml

Agar and water were mixed and dissolved and sterilized for 15 minutes at 121 °C. Then, the solution was cooled to 45 °C and 40% glucose and 50xVB salts were added slowly with stirring, the prepared medium was divided into each petri dish as 30 ml.

Preparation of nutrient broth liquid culture media:

Nutrient Broth	5 g
Distilled water	200 ml

Nutrient broth and water are mixed and sterilized in an autoclave at 121 °C for 15 minutes and stored at 4 °C.

Preparation of S9 microsomal enzyme (10%) S9 mixture:

S9 Microsomal enzyme	1 ml
MgCl ₂ -KCl salts	0,5 ml
Glucose-6-phosphate	0,25 ml
0.1 M NADP	1 ml
0.2 M phosphate buffer (PH 7.4)	12,5 ml
Sterile distilled water	9.875 ml

All chemicals were freshly prepared. All materials were mixed in a sterile falcon tube. The tube in which the solution was prepared was placed in ice and kept cold throughout the experiment.

Preparation of salt solution (1,65 M KCl + 0,4 M MgCl₂):

Potassium chloride (KCl)	61,5 g
Magnesium chloride (MgCl ₂)	40,7 g
Distilled water	500 ml

Preparation of 0.2 M Sodium-phosphate buffer solution (pH = 7.4):

0.2 M Sodium dihydrogen phosphate (NaH ₂ PO ₄ ·H ₂ O)	60 ml (2.76 g/100 ml)
0.2M Disodium hydrogen phosphate (Na ₂ HPO ₄)	440 ml (14.2g/500 ml)

After the mixture is adjusted to pH 7.4, it was sterilized in an autoclave. It was stored in a glass bottle at room temperature.

Preparation of 1 M Glucose-6-Phosphate solution:

Glucose-6-phosphate	2,82 g
Distilled water	10 ml

Sterilization was done with a 0.22 μ m pore diameter filter. It was stored at -20 ° C.

Preparation of 0.1M β -NADP solution:

β -NADP	383 mg
Distilled water	10 ml

Sterilization was done with a 0.22 μ m pore diameter filter. It was stored at -20 ° C.

3.2.9.4 Comet Assay

One day prior to the experiment, L929 cells were seeded in 6-well plates at a density of 3×10^5 cells/well, allowing them to adhere overnight. The cells were then exposed to herbal-loaded metallized nanofibers extract at different concentrations (100%, 50%, and 25%) with continuous exposure for 4 hours. After the incubation period, the cells cultured were harvested from the 6-well plates, centrifuged for 5 minutes at 1000 rpm, and resuspended in the remaining media after discarding the supernatant.

Twenty microliters of this cell solution were mixed with 180 μ l of pre-warmed low melting point agarose. Forty-five microliters of the cell-agarose mixture were added onto a high melting point agarose pre-coated slide and covered with a cover slip. The slides were dried at room temperature for 3 minutes, and the cover slip was carefully removed. The cells on the slides were placed in pre-cooled glass cuvettes containing the lysis solution and allow to undergo lysis at 4°C in the dark.

After lysis, the slides were placed in a cold electrophoresis solution for 20 minutes. Electrophoresis was at a fixed voltage of 25V and a current adjusted to 0.3A (300 mA) by adding or removing the electrophoresis buffer and running for 20 minutes. After

electrophoresis, the slides were washed with distilled water and then rinsed in tris buffer for 5 minutes. The slides were placed in ice-cold methanol (-20°C) for 5 minutes and dried. They were stained with Gel Red solution (20 µl per slide) and covered with a cover slip. The slides were evaluated using BAB Bs200ProP Image System Software under a fluorescence microscope with results expressed as the percentage of DNA in the tail region for a total of 100 cells (50 per slide) ^{102,104}.

Preparation of lysis buffer (pH-10) in 1 L:

2.5 M NaCl	146.10 g/L
100 mM Na ₂ EDTA	37.22 g/L
10 mM TRIS	1.21/L

Preparation of lysis solution: Freshly made and stored at 4°C under darkness

Lysis buffer	66.75 mL
DMSO (10%)	7.5 mL
Triton X (1%)	750 µL

Preparation of electrophoresis buffer (Make up to 2L volume):

5M NaOH	200 g/L
0.2 M Na ₂ EDTA (pH10)	14.89 g/200mL
-Autoclaved	

3.2.10 Statistical Analysis

Each experiment was carried out in triplicate. The GraphPad Prism 9 was used for the statistical analyses (GraphPad Software, Inc., San Diego, CA; The 8.4.3 version). Following the Tukey post hoc tests, one-way ANOVA was used to determine group differences.

4. RESULTS

4.1 FT-IR analysis

FT-IR spectra were acquired for CS and PVA powders individually, followed by their mixture. FT-IR spectrum was performed before and after electrospinning. Figure 4 shows CS's powder spectrum where we can see -OH and -NH bands between $3000\text{--}3500\text{ cm}^{-1}$, -CH stretching around 2800 cm^{-1} , Amide I and II around 1650 cm^{-1} , and 1540 cm^{-1} , -C-O between $1000\text{--}1100\text{ cm}^{-1}$, and saccharide stretch around $800\text{--}900\text{ cm}^{-1}$.

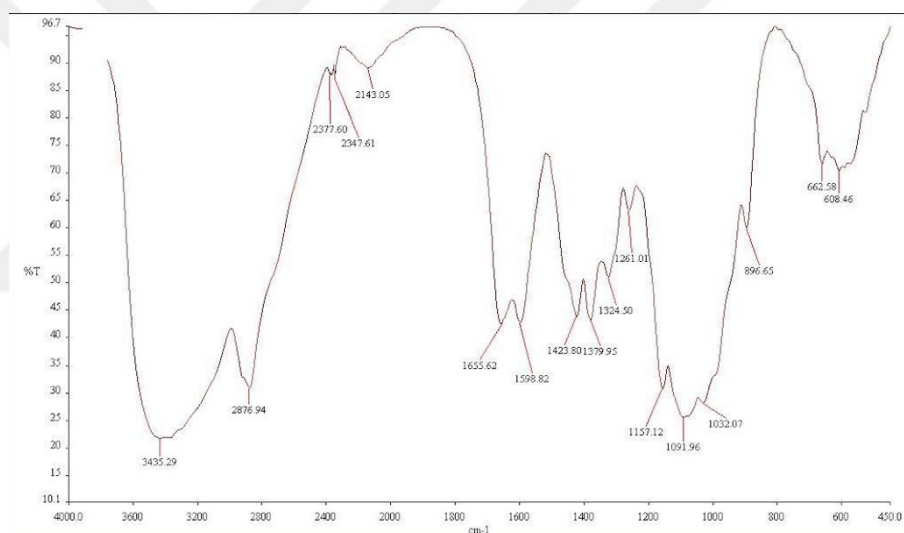


Figure 4. FTIR spectrum of CS powder

PVA powder spectrum is shown in Figure 5. -OH bond band is seen between $3200\text{--}3500\text{ cm}^{-1}$, -CH stretching around 2800 cm^{-1} , -CH bending around 1750 cm^{-1} , and -C-O stretching between $1000\text{--}1100\text{ cm}^{-1}$.

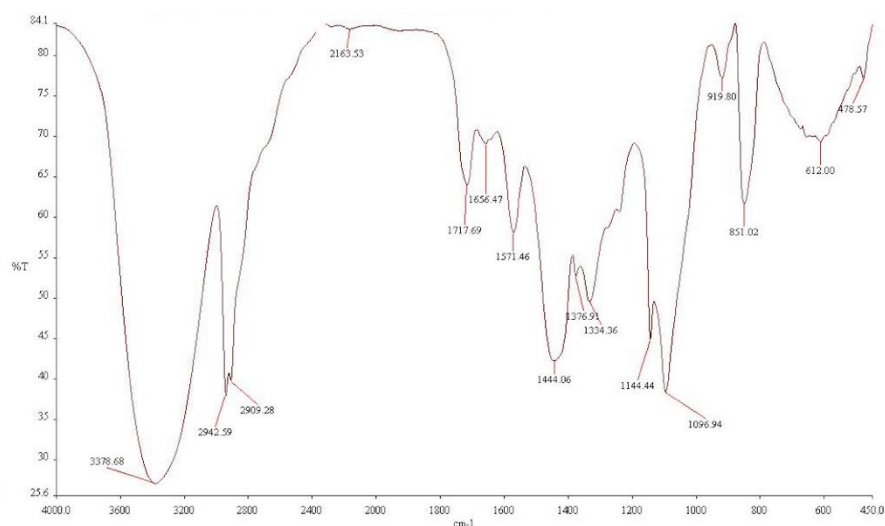


Figure 5. FTIR spectrum of PVA powder

When the two polymers' powders are mixed, the major bands in the spectrum confirm the presence of both compounds (Figure 6).

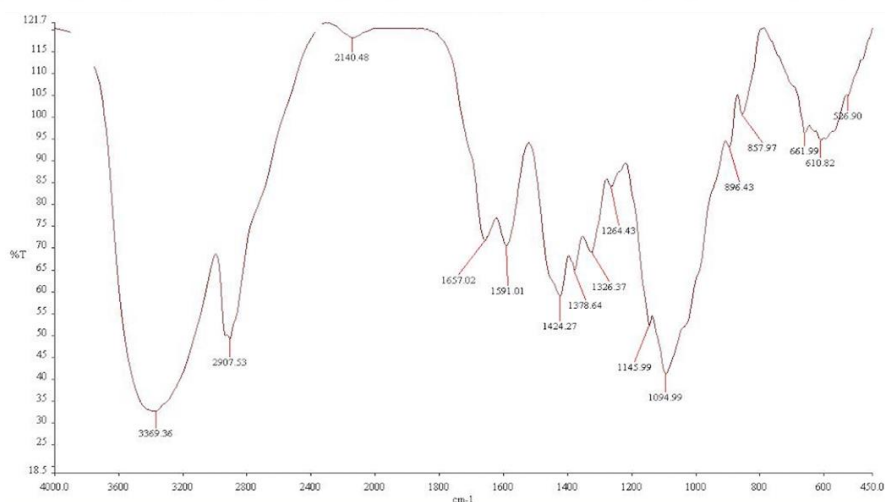


Figure 6. FTIR spectrum of PVA and CS powder mixture

After electrospinning, another FTIR spectrum was analyzed for the formed fiber Figure 7. A sharp peak at around 3400 cm⁻¹ represents the overlap between the -OH and -NH of CS and the -OH of PVA. Peaks around 1560 cm⁻¹ are related to the symmetric deformation of -NH⁺₃ resulting from the protonation of amide groups of CS in the acidic media.

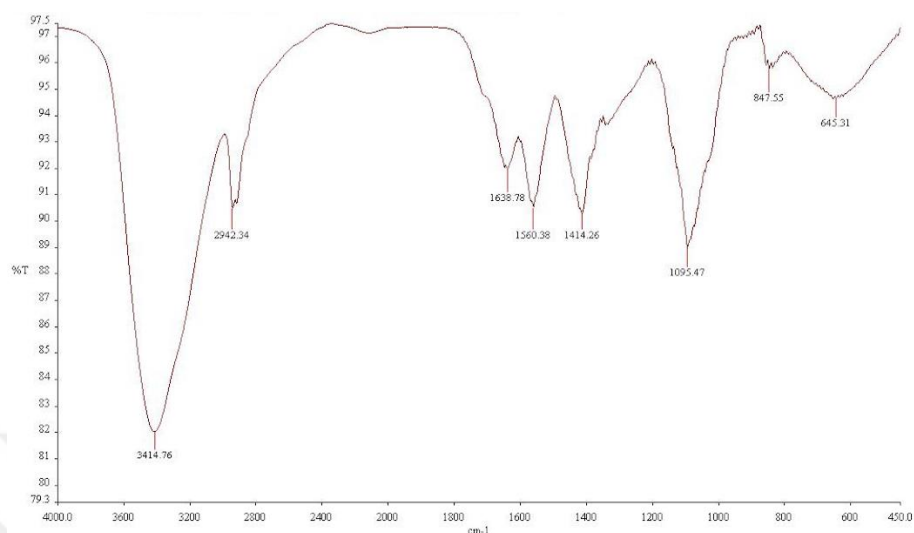


Figure 7. FTIR spectrum of PVA and CS nanofibers

To confirm the chemical structure of MSNs, FT-IR analysis was performed, and the resulting spectrum is presented in Figure 8. The Si-O rocking vibration is shown at 470 cm^{-1} . A broad (hydrogen bonded) hydroxyl stretching band for both silanol Si-O-H and the water hydroxyls was clear around 3500 cm^{-1} in addition to the characteristic H-OH water twisting band around 1640 cm^{-1} . The internal Si-O-Si stretching vibration of the SiO_4 asymmetric band appeared so clear at 1100 cm^{-1} , while the symmetric one is around 800 cm^{-1} . These bands are very intense and correspond to the formation of the SiO_2 network.

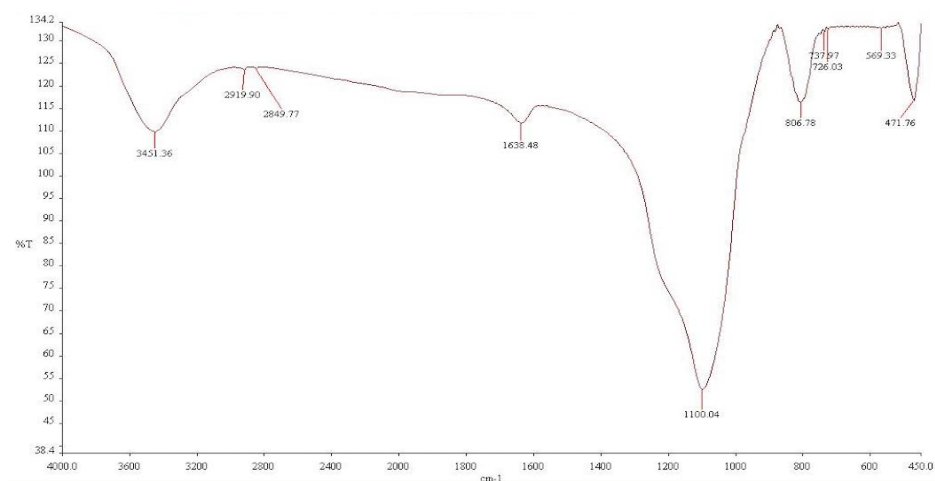


Figure 8. FTIR spectrum of MSNs powder

4.2 Zetasizer analysis

The particle size analyzer revealed two distinct ranges for the MSNs: 150-190 nm and 500-530 nm (Figure 9). Additionally, the Zeta potential analysis indicated an average Zeta potential of -24.1 mV for the particles (Figure 10). The characterization of the lab-prepared herbal loaded MSNs wasn't successful as couldn't determine the size nor the Zeta potentials of the new particles because they had a high sedimentation rate.

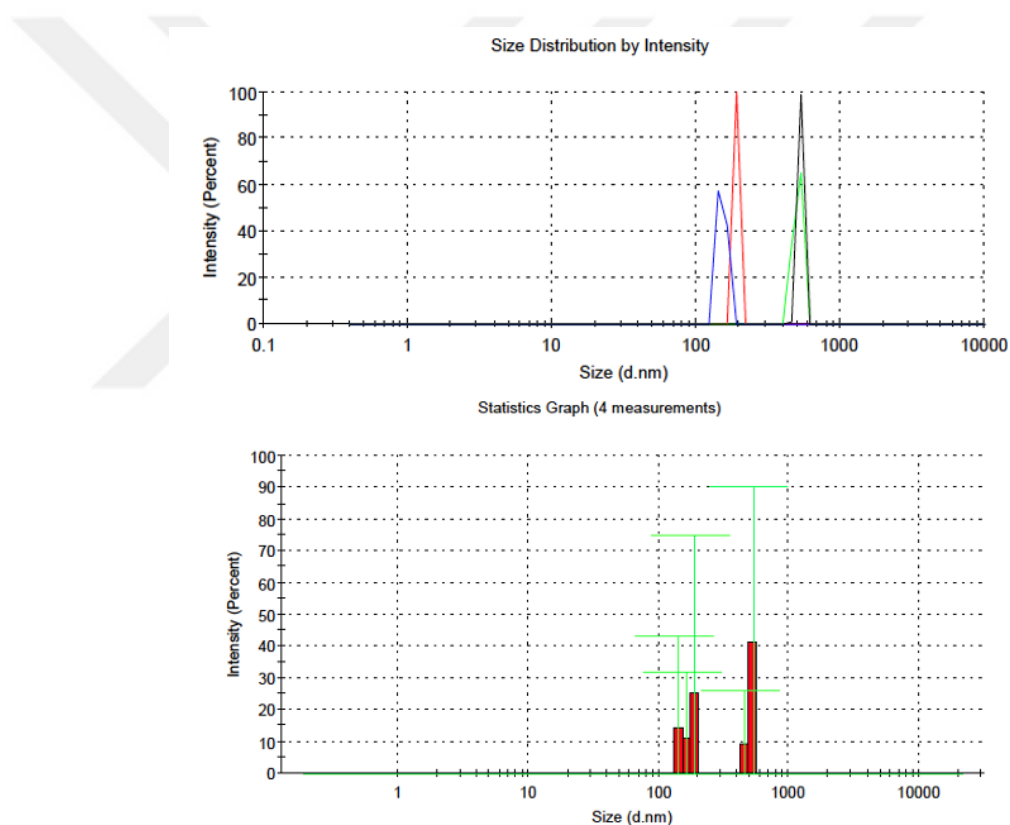


Figure 9. Particle size intensity of MSNs

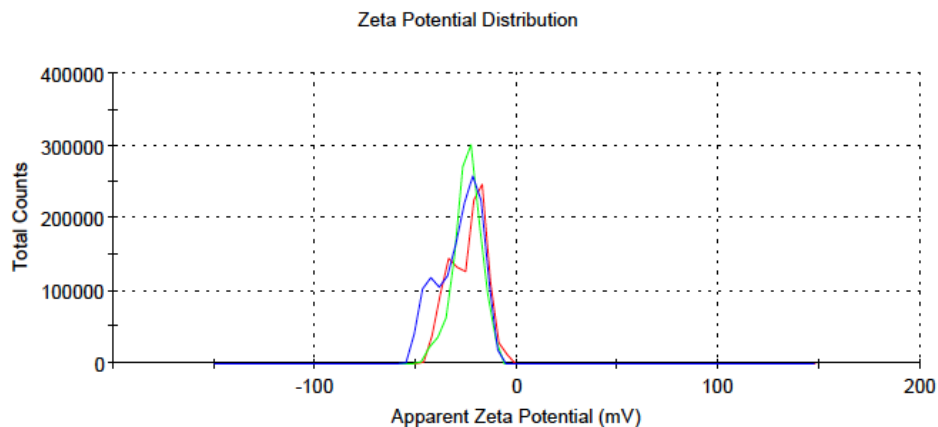


Figure 10. Zeta potential of MSNs

4.3 SEM and TEM results

SEM and TEM images were taken for the MSNs powder to identify the mesoporous structure (Fig. 11).

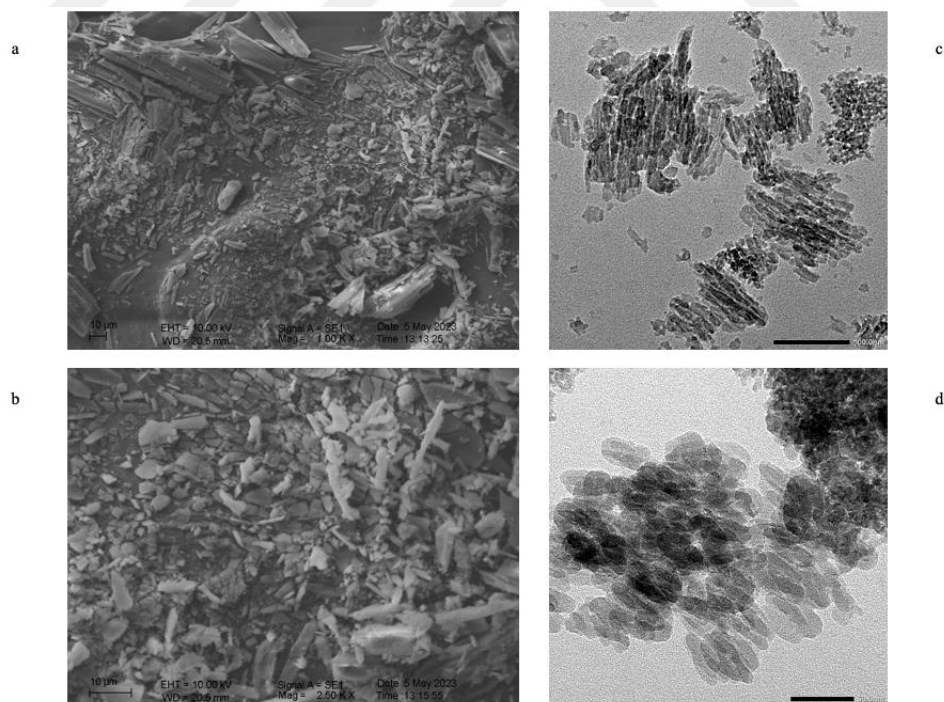


Figure 11. MSNs SEM and TEM: a and b are SEM images at 1K X and 2.5K. c and d are TEM images at 60K X and 80K X.

After electrospinning, the produced nanofiber mats were analyzed by SEM to assess nanofiber formation. Based on the results, modifications were made to the polymer composition and electrospinning parameters until optimal nanofiber formation was achieved.

M1 and M2 failed to electrospun due to the precursor's high viscosity of the precursor solution. M3 and M5 formed a gelatin-like mat and were able to see the nanofiber formation in SEM (Figure 12).

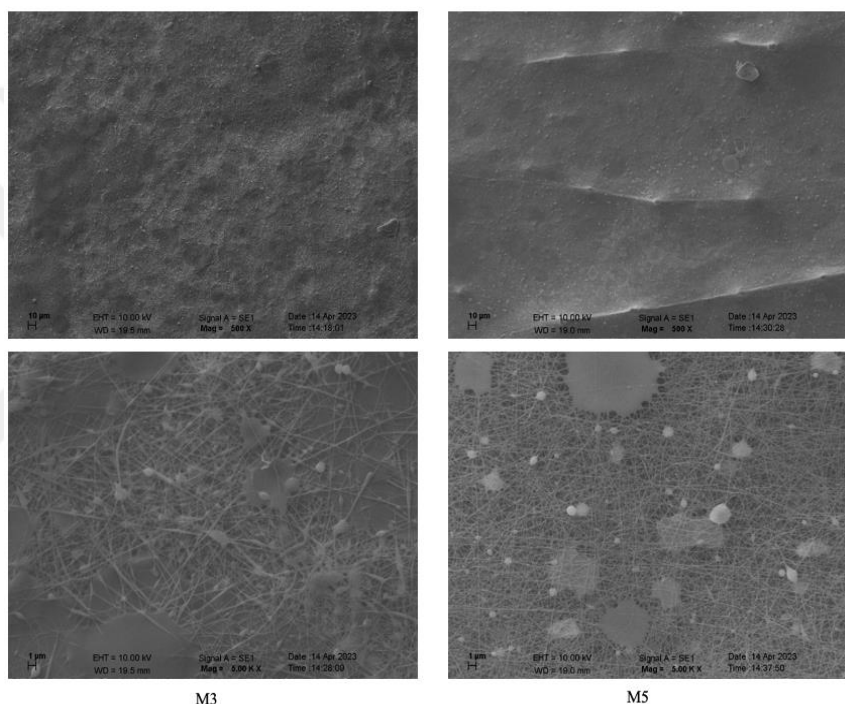


Figure 12. SEM images of nanofiber M3 and M5 at 500 X and 5K X

M4 was unsuccessful due to the low solubility of PVA in a 90% acetic acid solvent. Beginning with M5, we adopted a new approach of dissolving chitosan (CS) and PVA separately in different solvents before mixing them in the desired ratio. This method improved solubility and resulted in a clearer polymer mixture. M6 produced a paper-like mat, similar to M6a, but SEM analysis revealed a smoother surface for M6a. This improvement is likely attributed to the dilution effect from the addition of 2 mL of ethanolic herbal extract (Figure 13). M7, however, resulted in a fluffy fibrous mat with exceptionally thin nanofibers (Figure 14).

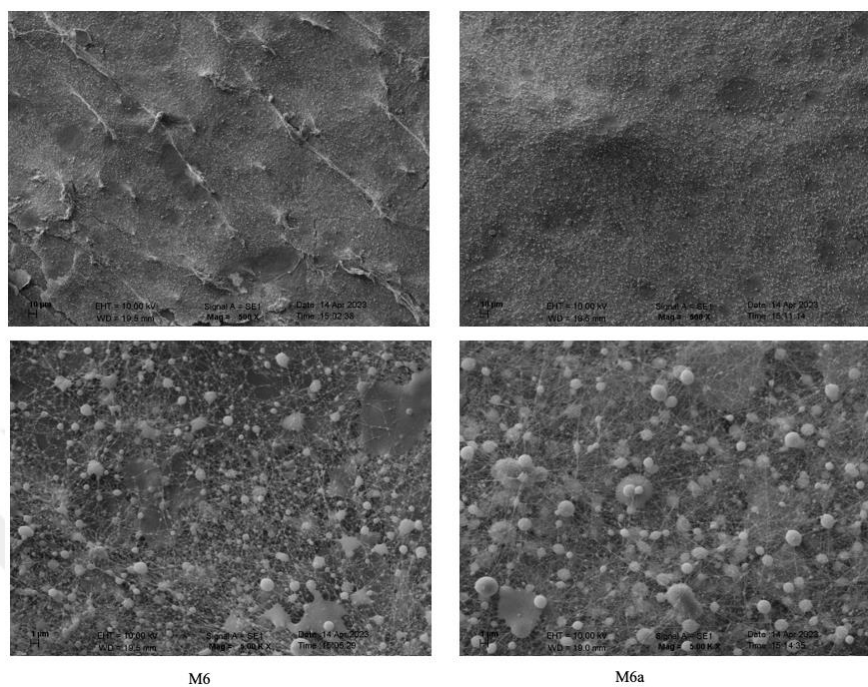


Figure 13. SEM images of nanofiber M6 and M6a at 500 X and 5K X

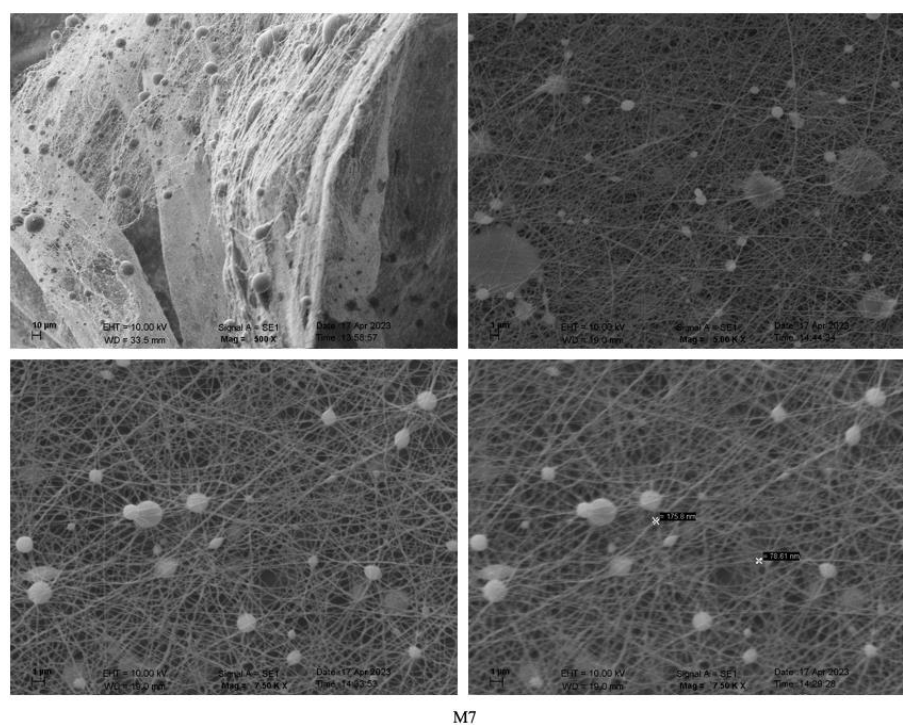


Figure 14. SEM images of nanofiber M7 at 500 X, 5K X, and 7.5K X

A new precursor solution was tested but with the addition of MSNs in 1% and 0.5%. The mat formed was soft paper-like and we can notice by SEM that M8a and M8b have a rough surface and fragile fibers were formed with a lot of beads (Figure 15).

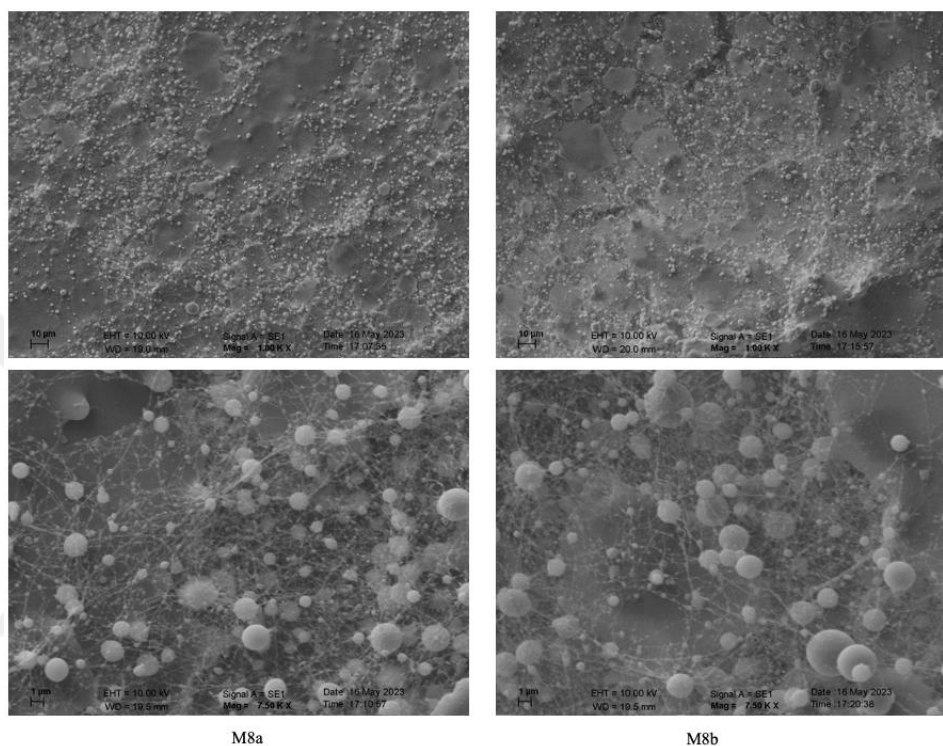


Figure 15. SEM images of nanofiber M8a and M8b at 1K X, and 7.5K X

Given the excessive thinness of the nanofibers produced from M8, we aimed to optimize the polymer concentration by selecting a value between those used in M7 and M8. To ensure consistency, we prepared five different formulations using the same methodology. Solid additives were separately dissolved in distilled water and microfiltered to eliminate large undissolved particles. Despite these efforts, most of the nanofiber mats produced were light and formed fibrous structures that did not adhere well to the collector. SEM analysis of two samples from each preparation—both fibrous structures and nanofiber mats formed on the foil—revealed that the fibrous structures on the foil had rough, uneven surfaces (Figure 16) and exhibited increased bead formation (Figure 17).

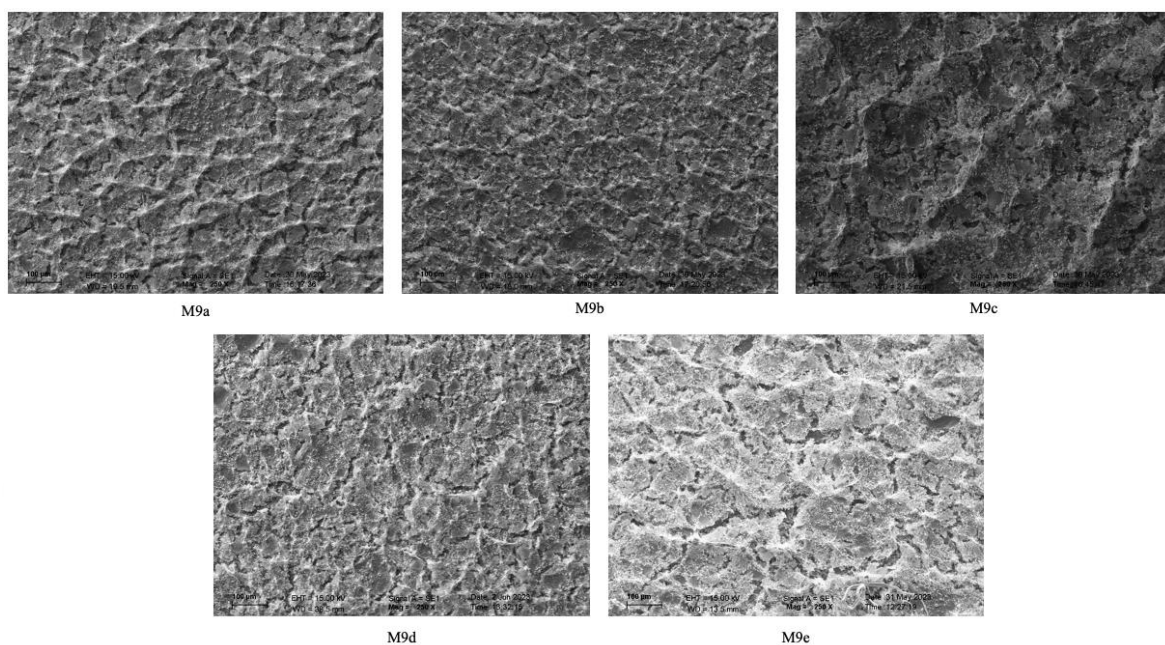


Figure 16. SEM images of nanofiber M9 on foil at 250 X

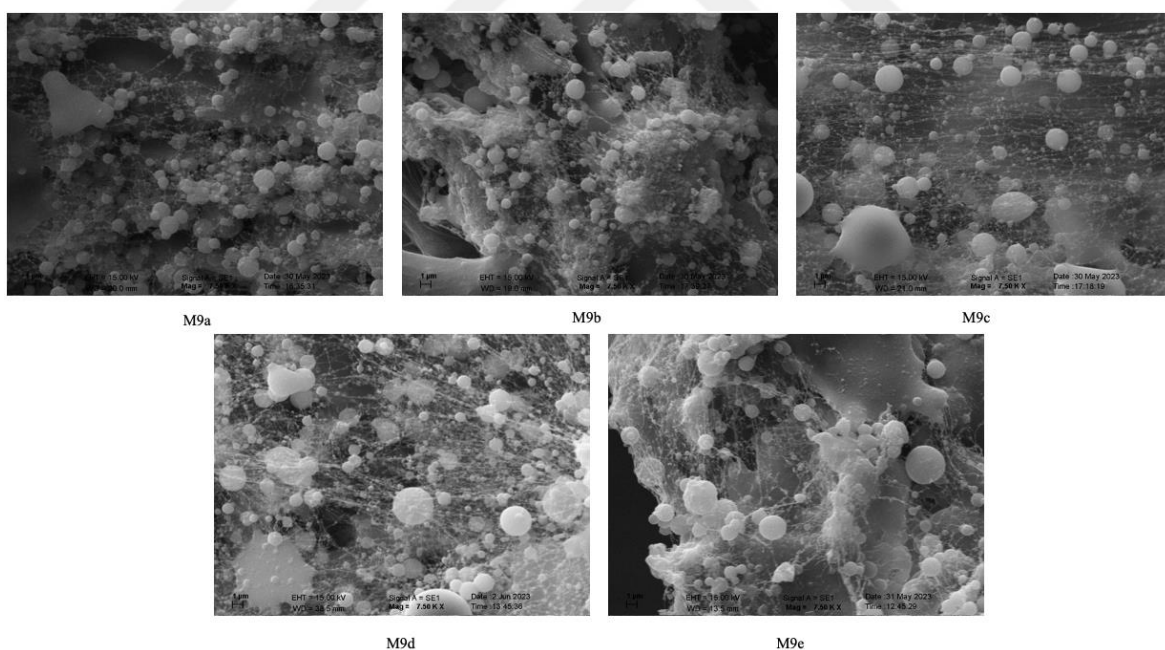


Figure 17. SEM images of nanofiber M9 thread-like structure at 7.5K X

To increase the thickness of the nanofibers and reduce bead formation observed in M9, the preparation of M10 involved decreasing the CS ratio from 50% to 30%, following the recommendation by Xu et al. (2013), who found that lower CS concentrations improved nanofiber quality. Additionally, methanol was used instead of distilled water to dissolve the

herbal extract and MSNs, aiming to enhance the solubility of the active constituents. Despite these modifications, bead formation did not decrease, and M10d, which contained both herbal extract and MSNs, exhibited the highest level of bead formation (Figure 18 and Figure19).

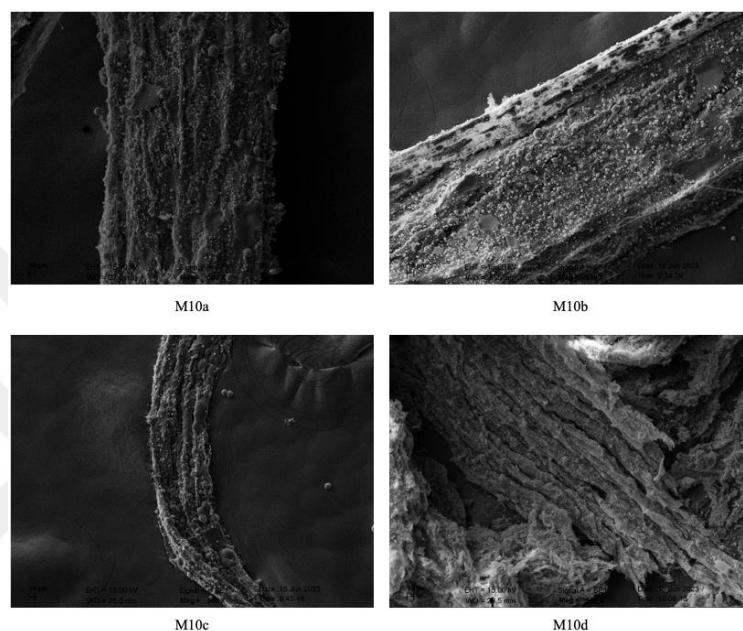


Figure 18. SEM images of nanofiber M10 at 500K X

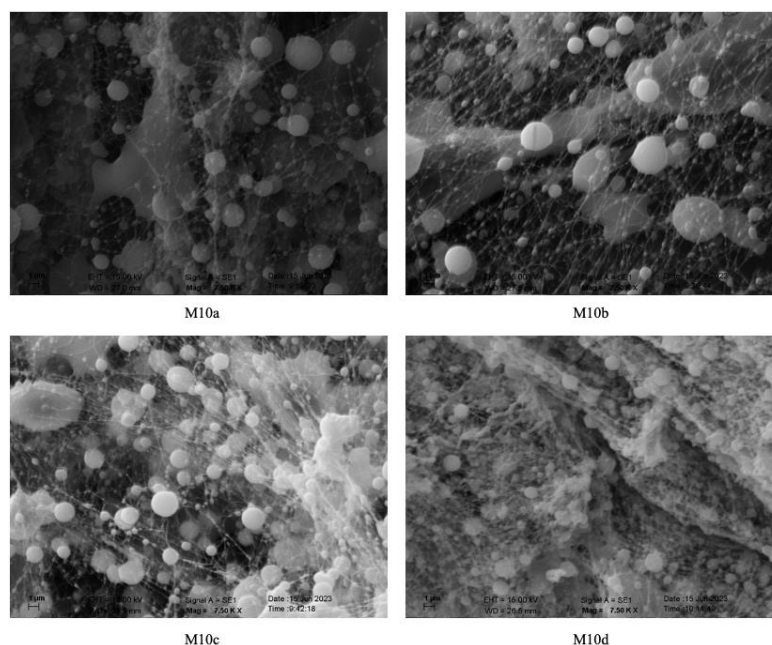


Figure 19. SEM images of nanofiber M10 at 7.5K X

Since there was minimal difference in nanofiber structure between M9 and M10, it indicated the need for further modifications to the precursor solution and electrospinning parameters. M11 was prepared with the same polymer ratios as M10 but with an increased herbal extract concentration of 25% instead of 5%, to facilitate comparison with other studies in terms of antimicrobial activity and encapsulation efficacy. However, the results were not satisfactory, leading to the preparation of M12. In M12, the concentrations of PVA and CS were increased to 12% and 3%, respectively, and 5% hypericin was used instead of 5% powder extract. SEM analysis revealed improved nanofiber formation with reduced bead formation in M12 (Figure 20).

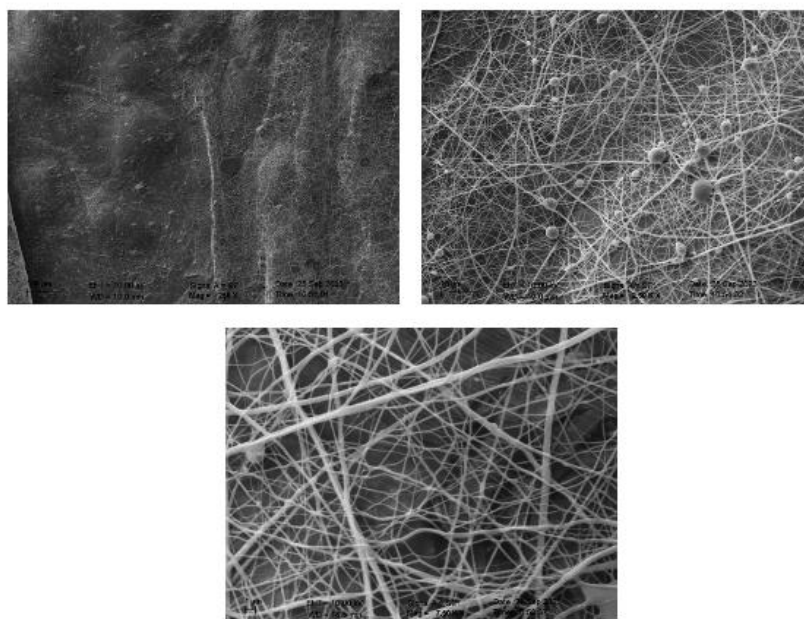


Figure 20. SEM images of M12 nanofiber at 250 X, 2.5K X, and 7.5K X

To enhance the elasticity of the fibrous mat, Gelatin was incorporated into the polymer mixture. Two formulations, M13 and M14, were prepared with PVA:CS ratios of 70:15:15 and 60:15:25, respectively. Both formulations exhibited improved visible appearance and structural integrity. SEM analysis revealed smoother nanofiber formation with reduced bead formation in M13, making it the preferred choice for further studies (Figure 21-22).

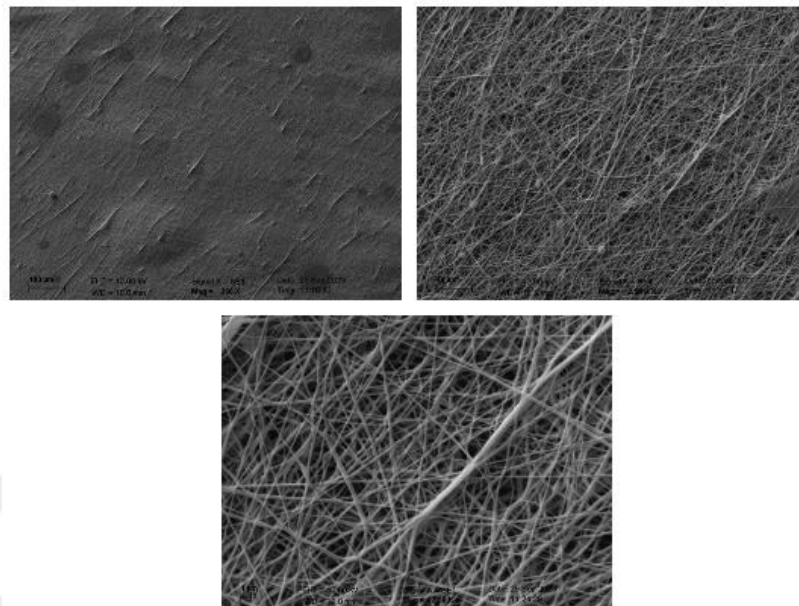


Figure 21. SEM images of M13 nanofiber at 250 X, 2.5K X, and 7.5K X

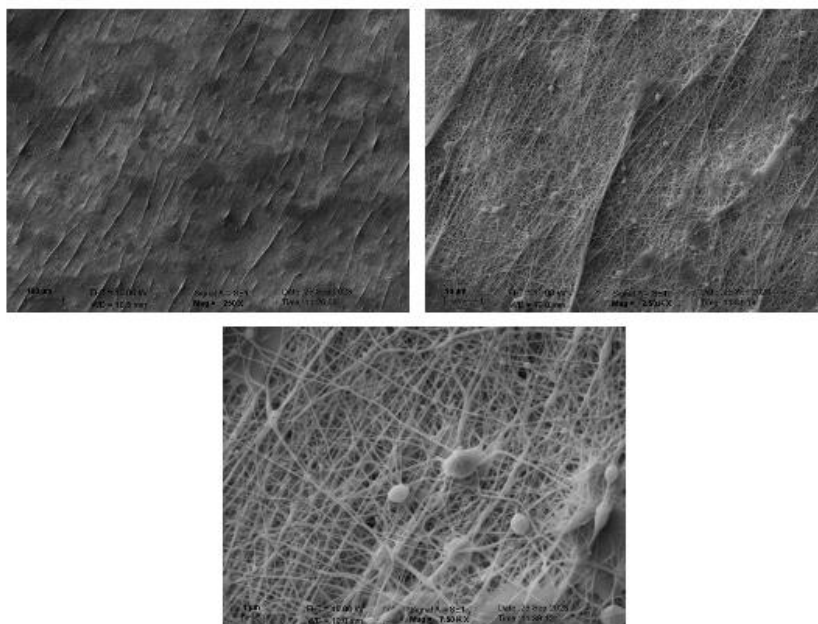


Figure 22. SEM images of M14 nanofiber at 250 X, 2.5K X, and 7.5K X

M15 is similar to M3, with the primary modification being the method of extract addition. In this formulation, the dissolved extract powder in methanol was heated to dryness before being incorporated into the polymer solution. However, in the subvariants M15a and

M15b, the resulting nanofibers displayed less regularity and exhibited more bead formation compared to M15 (Figure 23). These observations suggest that further adjustments to the formulation or processing conditions are necessary to improve the uniformity and quality of the nanofibers.

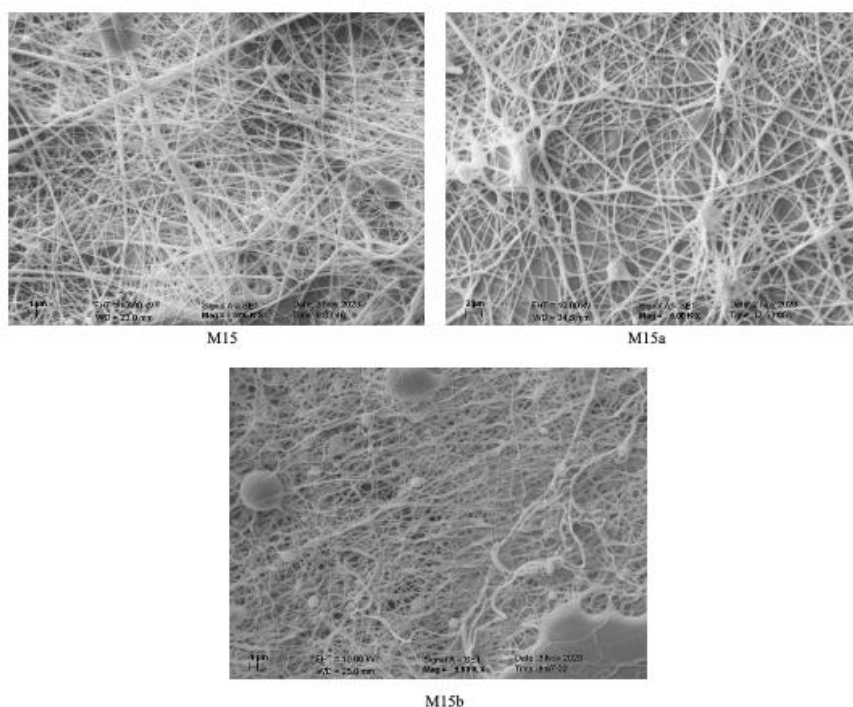


Figure 23. SEM images of M15 nanofiber at 5K X

For the M16 formulation, the addition of acacia gum was implemented to enhance both the elasticity and wound healing properties of the nanofibers. During the experiment, adjustments to the electrospinning parameters further optimized the process. All M16 mixtures resulted in the formation of smooth nanofibrous mats, characterized by well-defined nanofibers without noticeable bead formation (Figures 24 and Figure 25). SEM images revealed significant improvements in nanofiber formation, particularly with the reduction of CS percentage and the inclusion of gelatin and acacia gum. The adjustments in electrospinning parameters also contributed to the improved quality of the M16 fibrous mats, which exhibited nanofiber diameters ranging from 100 to 900 nm. These advancements are evident in the physical structure (Figure 26), where the transition from the thread-like structure observed in M12 to the continuous mat in M15 was further refined in M16, resulting in increased thickness and enhanced strength of the nanofibrous mats.

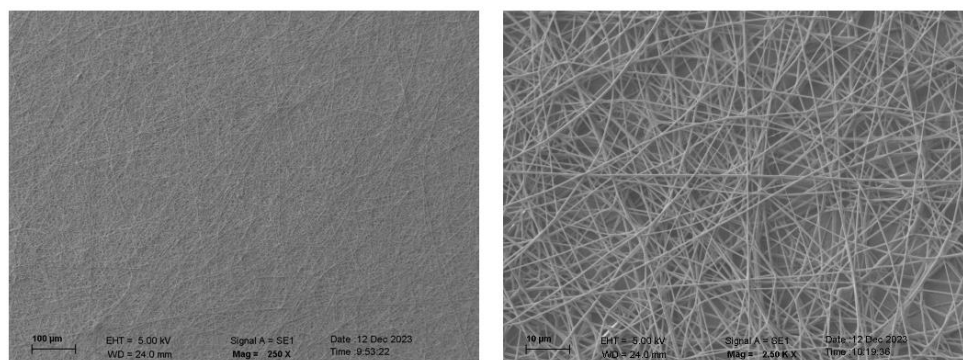


Figure 24. SEM images of M16 nanofiber at 250 X and 2.5K X

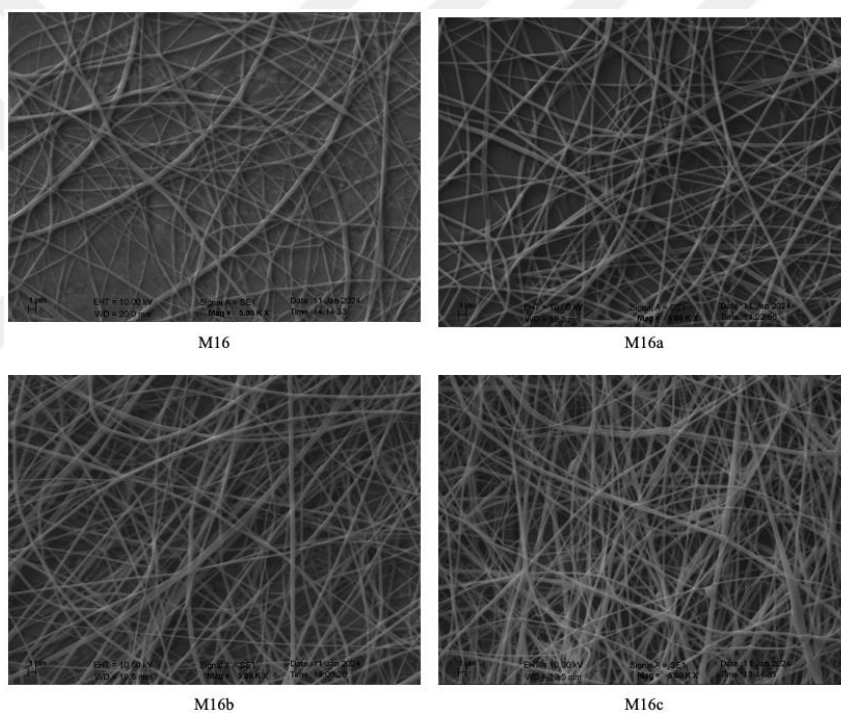


Figure 25. SEM images of M16 nanofiber at 5K X

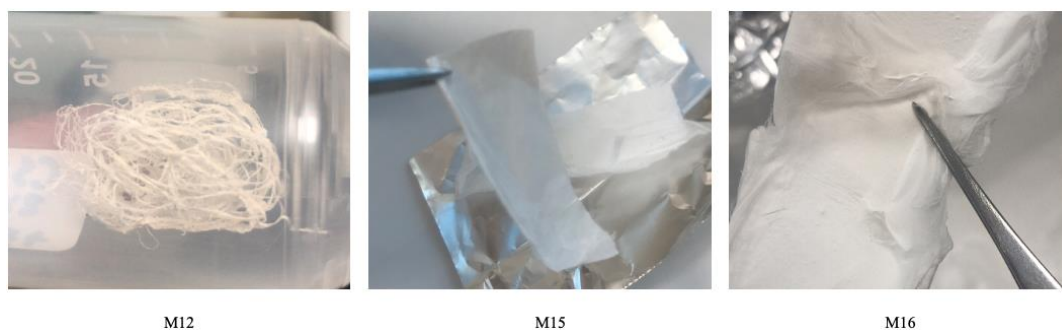


Figure 26. The physical structure of the produced nanofiber mat

4.4 Nanoindentation and tensile strength

Nanoindentation tests to calculate Young's modulus were performed using AFM for samples M16 and M16a (Figure. 27). The average for Young's modulus for M16 was found to be 2.1 MPa, while for M16c, which contains the herbal extract and MSNs added to the polymers, the average was 2.5 MPa (Figure. 28).

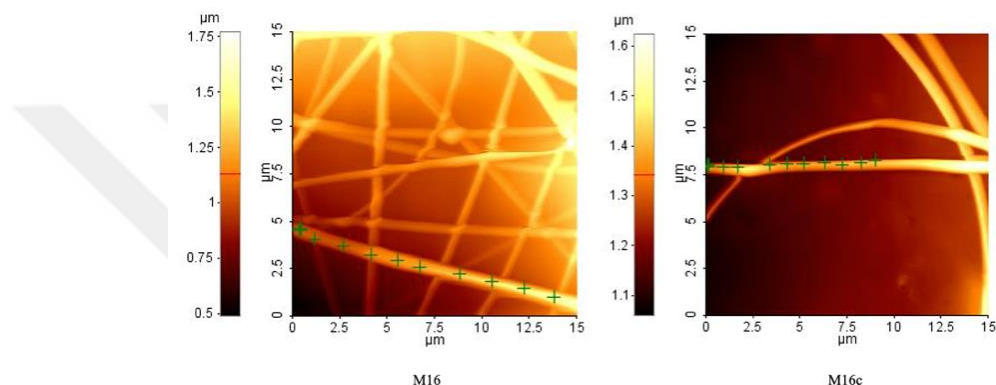


Figure 27. The representative images of indentation regions for calculation of Young's modulus

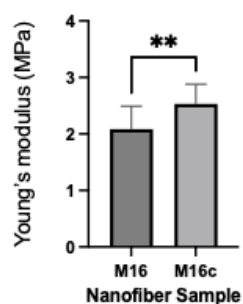


Figure 28. AFM-based Young's modulus of electrospun single fibers
Statistically significant difference was indicated ($p < 0.05$)

The results of M15 nanofibers mat tensile strength from tensile tests conducted on a plastic material according to ASTM D 638. The initial thickness was 0.1 mm. At a speed 5mm/min the Ultimate Tensile Strength is 4.07 MPa, this is the maximum stress that the material can withstand while being stretched before failing as shown in Figure 29.

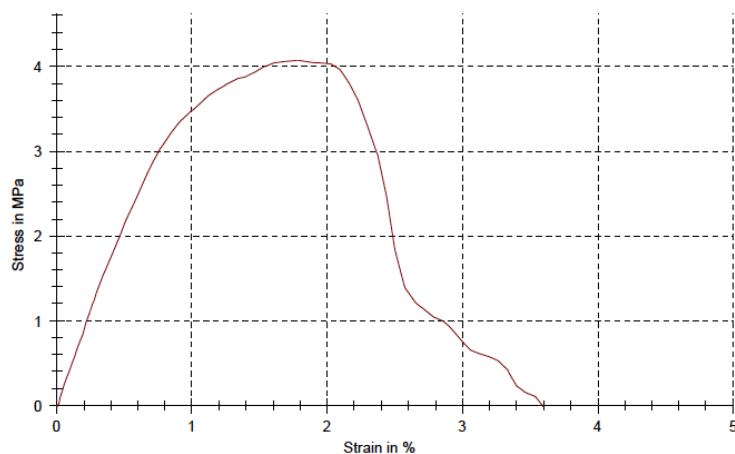


Figure 29. Strain (in %) against stress (in MPa).

The curve shows an initial linear region (elastic behavior) followed by a non-linear region (plastic behavior) until failure.

4.5 Spectrophotometric assay of hypericin

4.5.1 Identification HP extracts

Hypericin was identified in various herbal extracts by performing a spectrum scan from 400-700 nm, with maximum absorbance observed around 590 nm. The powdered herbal extract dissolved in methanol produced a distinct peak, whereas the powdered herbal extract dissolved in phosphate buffer (pH 7.4) and the oil extract dissolved in acetone showed mild peaks (Figure 30).

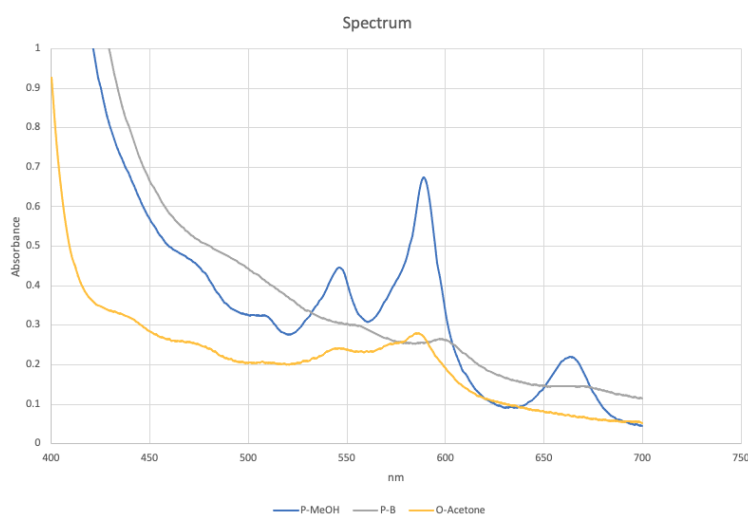


Figure 30. Hypericin absorbance spectrum scan of different herbal extracts

4.5.2 Assay of St. John's Wort extract

For the calibration curve, absorbance at 588 nm was used. Spectrophotometric analysis was conducted using a seven-point calibration curve generated with standardized St. John's Wort herb extract food supplement capsules. Experiments were repeated three times. All calibration curves demonstrated good linear regression, with an average R^2 value of 0.9998 for samples dissolved in methanol, and 0.9975 for samples dissolved in phosphate buffer (Figure 31–32). The limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as follows: for methanol as the solvent $\text{LOD} = 0.098 \times 10^{-3} \text{ mg/ml}$ and $\text{LOQ} = 0.327 \times 10^{-3} \text{ mg/ml}$; for phosphate buffer $\text{LOD} = 0.111 \times 10^{-3} \text{ mg/ml}$ and $\text{LOQ} = 0.371 \times 10^{-3} \text{ mg/ml}$.

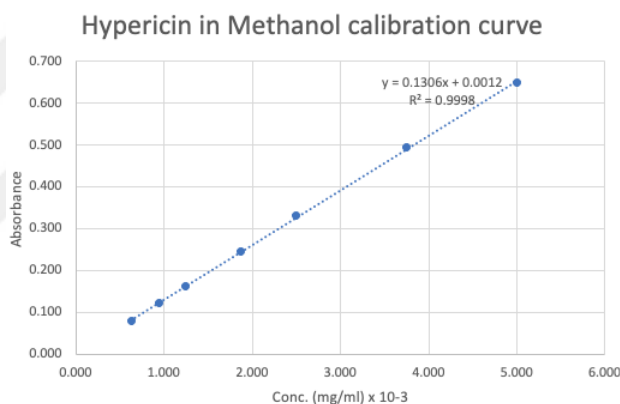


Figure 31. Hypericin in Methanol calibration curve

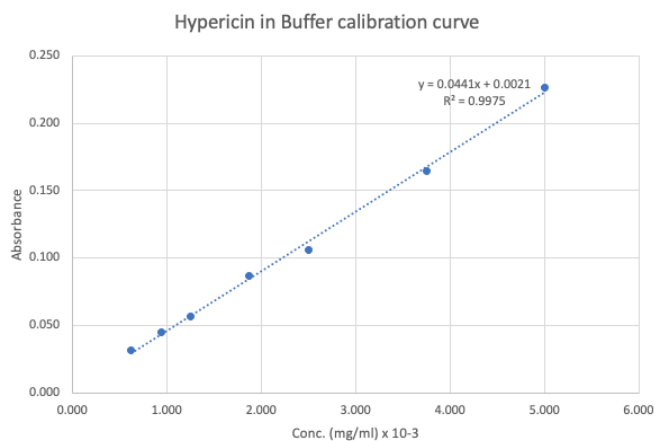


Figure 32. Hypericin in Buffer calibration curve

4.5.2.1 Assay of hypericin content after sample preparation

A new calibration curve was established to determine the hypericin content after sample preparation, ensuring it accurately reflects the actual content added to the electrospinning solution (Figure 33). LOQ and LOD were found to be 4.4% and 1.3%, respectively.

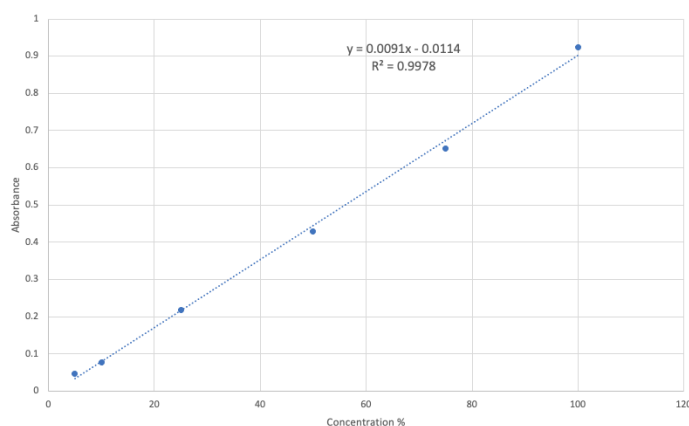


Figure 33. Hypericin calibration curve after sample preparation

4.5.3 Encapsulation efficiency and Release studies

The EE% was calculated to be between 86% and 96%, excluding the absorbance contribution from the raw fiber. There was no significant difference in EE% between the herbal-loaded mats with or without MSNs (Figure 34).

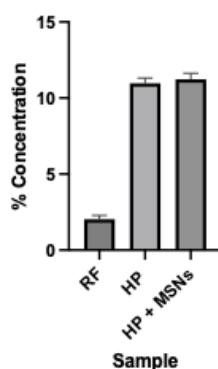


Figure 34. Actual calculated hypericin content.

Statistically insignificant differences were indicated between herbal-loaded nanofibers with or without MSNs ($p = 0.628$) but it was significant compared to raw nanofiber ($p < 0.0001$)

Hypericin release from the nanofibers exhibited an initial rapid release during the first two hours, followed by a slower, more sustained release over the subsequent six hours. The samples containing MSNs demonstrated a slower release rate, with approximately 37% of hypericin released by the 6th hour, compared to 58% for the samples without MSNs (Figure 35). After 12 hours, hypericin release was approximately 43% for the samples with MSNs and 62% for those without MSNs.

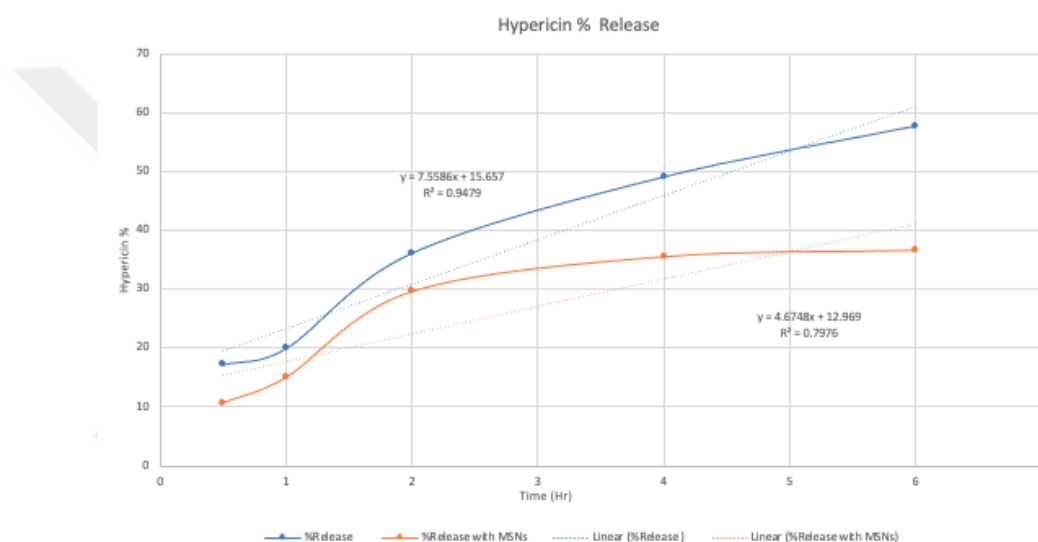


Figure 35. Hypericin release profile from nanofibers

4.6 Antimicrobial activity

Various components of *H. perforatum*, including polysaccharides, contribute to its antimicrobial activity. However, due to the inability to precisely evaluate the content of the powder extract, hypericin was used as a reference for predicted concentration. The results confirm that we were able to produce herbal-loaded nanofibers with antimicrobial activity (Table 4). The different composition and loading methods showed different results. For a better comparison of the antimicrobial activity, methanolic extracts were used which were efficient at around 70% of the control drug activity. All different M10 showed antimicrobial activity with the best activity for M10d but only covering about 39% of control activity

indicating insufficient loading. In M11 the concentration of herbal extract was increased 2 times but the activity only improved to 59% of control activity. Changing the precursor solution to improve loading efficacy followed. The nanofibers without any addition in M10 showed antimicrobial activity but this activity diminished later. This can be explained by the decrease in the percentage of CS from 30 to 15% as CS in the precursor solution as it's known to have antimicrobial activity. M12 didn't show much improvement but since the oil extract addition in M12c didn't show any activity, we stopped using it in the following trials. The improvement of the produced nanofiber mat reflected the improvement of herbal-loading efficacy in M13 as the activity it reached 58% in Mb. M13c where we tried to herbal-loaded MSNs was less effective than adding them separately so decided not to use it in the following trials. The change of loading method from adding methanolic extract in M13 to evaporating it first in M15 showed better results. This method helped in increasing concentration while avoiding adding more solutions and diluting the polymer solution keeping in mind that heating at 80 °C till evaporation may cause a breakdown of the extract components. M15b reached about 70% of control drug activity and is equivalent to methanolic herbal extract. In general, the addition of 1% MSNs showed better activity which can be explained by the modification of the release rate of the loaded herbal extract increasing the duration of the effect. Figure 36 shows the average of inhibition zones obtained for all samples during the experiment.

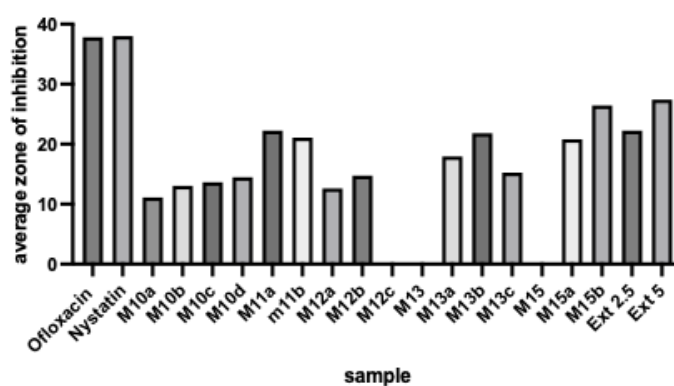


Figure 36. The average of zone of inhibitions

Table 4. Antimicrobial Activities of Compounds (Zone of Inhibition/diameter in mm)

No	Compound	Conc. of test Compound	<i>E. hirae</i> ATCC 10541	<i>E. faecalis</i> ATCC 29212	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 10536	<i>A. baumannii</i> ATCC 19606	<i>P. aeruginosa</i> ATCC 15442	<i>C. albicans</i> ATCC 10231	%*
1	M10a	-	12± 0.75	11± 0.71	11± 1.23	12± 1.12	10± 0.15	10± 0.63	12± 0.45	29
2	M10b	1% MSNs	14± 1.43	15± 1.41	15± 1.25	13± 1.13	11± 0.71	12± 0.52	12± 0.26	35
3	M10c	5 µg/ml	14± 0.75	16± 0.71	15± 1.23	13± 1.12	12± 0.15	13± 0.63	13± 0.45	36
4	M10d	5 µg/ml +1% MSNs	16± 1.43	17± 1.41	16± 1.25	14± 1.13	12± 0.71	13± 0.52	14± 0.26	39
5	M11a	15 µg/ml	23± 0.25	24± 0.31	21± 1.22	22± 0.52	22± 0.14	21± 0.42	23± 0.45	59
6	M11b	15 µg/ml +1% MSNs	22± 1.13	21± 1.23	19± 0.15	20± 1.15	21± 0.75	20± 1.25	25± 0.24	56
7	M12a	5 µg/ml	15± 1.23	12± 1.45	11± 1.23	13± 1.16	11± 0.73	12± 0.45	15± 0.49	34
8	M12b	5 µg/ml +1% MSNs	17± 0.55	18± 0.91	14± 0.57	12± 1.21	14± 1.15	13± 0.28	16± 0.36	39
9	M12c	2 ml oil	-	-	-	-	-	-	-	-
10	M13	-	-	-	-	-	-	-	-	-
11	M13a	5 µg/ml	19± 1.13	18± 1.15	17± 0.16	16± 1.17	17± 0.45	19± 1.35	20± 0.15	48
12	M13b	5 µg/ml +1% MSNs	22± 0.55	23± 0.91	20± 0.57	23± 1.21	20± 1.15	22± 0.28	23± 0.36	58
13	M13c	5 µg/ml**	18± 1.25	16± 1.25	10± 0.16	14± 1.17	14± 0.35	17± 1.59	20± 0.19	41
14	M15	-	-	-	-	-	-	-	-	-
15	M15c	10 µg/ml	20± 1.15	21± 1.18	19± 0.25	21± 1.17	22± 0.75	21± 1.45	22± 0.19	55
16	M15b	10 µg/ml + 1% MSNs	29± 0.53	28± 0.91	24± 0.57	23± 1.21	25± 1.55	27± 0.98	30± 0.52	70
17	Extract***	2.5 µg/ml	21± 0.25	22± 0.25	21± 1.24	23± 0.55	23± 0.25	24± 0.47	22± 0.75	59
18	Extract***	5 µg/ml	27± 0.55	28± 0.91	25± 0.57	27± 1.21	28± 1.15	29± 0.28	28± 0.36	73
19	Ofloxacin	5 µg	35± 0.45	39± 0.15	38± 1.22	37± 1.46	39± 0.25	39± 1.15	-	100
20	Nystatin	100 units	-	-	-	-	-	-	38± 1.25	100
21	Distilled Water	-	-	-	-	-	-	-	-	

* Average efficacy % compared to control

** Herbal-loaded MSNs

*** Methanolic extracts of the herbal powder used as reference

(-) No Activity

(±) Standard Deviation

4.7 Anti-inflammatory Activity

Before the evaluation of anti-inflammatory activity, non-toxic concentrations of samples were determined by cell viability of 70% and above, which both forms and release solutions were observed to be non-cytotoxic for all concentrations. The MTT assay showed that one of the samples had a cytotoxic effect at a concentration 1 mg/mL at 24 h as compared with control cells that received no treatment (Table 5 and Figure 37).



Table 5. Effects of different forms of fiber films on the viability of RAW264.7 macrophage cells and the effects of the different forms of fiber films on nitrite levels and % nitrite inhibition in RAW 264.7 cells stimulated with 1 $\mu\text{g/mL}$ LPS.

Groups	Dose	Cell Viability (%)	Nitrite Level (μM)	Nitrite Inhibition (%)
Ctrl		107.81 $\pm$ 0.63	1.39 $\pm$ 0.39	-
LPS		100.00 $\pm$ 0.29	60.99 $\pm$ 1.62	-
L-NAME	100 μM	92.11 $\pm$ 0.80	30.69 $\pm$ 0.32**	49.65 $\pm$ 1.82
Indomethacin	100 μM	91.85 $\pm$ 0.32	29.62 $\pm$ 0.45**	51.42 $\pm$ 1.34
F (mg/mL)	0.0625	95.46 $\pm$ 1.12	61.03 $\pm$ 1.12	0.00 $\pm$ 2.67
	0.125	92.09 $\pm$ 1.21	59.87 $\pm$ 0.88	1.81 $\pm$ 2.62
	0.25	90.40 $\pm$ 2.53	59.01 $\pm$ 1.55	3.21 $\pm$ 3.54
	0.5	82.07 $\pm$ 2.41	59.23 $\pm$ 1.93	2.87 $\pm$ 3.01
	1	65.14 $\pm$ 3.26		
F+Si (mg/mL)	0.0625	95.11 $\pm$ 0.46	60.39 $\pm$ 2.16	1.01 $\pm$ 0.90
	0.125	90.01 $\pm$ 0.95	59.19 $\pm$ 1.28	2.95 $\pm$ 0.48
	0.25	85.62 $\pm$ 1.73	58.81 $\pm$ 1.48	3.57 $\pm$ 2.18
	0.5	81.45 $\pm$ 1.62	57.97 $\pm$ 1.41	4.95 $\pm$ 0.84
	1	67.46 $\pm$ 2.42		
H+F (mg/mL)	0.0625	97.84 $\pm$ 1.58	50.06 $\pm$ 1.49*	17.86 $\pm$ 4.05
	0.125	94.34 $\pm$ 1.69	45.99 $\pm$ 1.01**	24.59 $\pm$ 1.36
	0.25	89.69 $\pm$ 1.43	38.51 $\pm$ 1.32**	36.83 $\pm$ 2.87
	0.5	81.58 $\pm$ 1.70	34.22 $\pm$ 1.75**	43.82 $\pm$ 4.26
	1	67.58 $\pm$ 2.14		
H+F+Si (mg/mL)	0.0625	93.91 $\pm$ 1.50	46.32 $\pm$ 0.53*	24.03 $\pm$ 2.11
	0.125	91.74 $\pm$ 1.84	43.18 $\pm$ 2.19**	29.23 $\pm$ 2.33
	0.25	89.17 $\pm$ 1.95	36.92 $\pm$ 1.73**	39.43 $\pm$ 3.24
	0.5	80.85 $\pm$ 0.64	29.54 $\pm$ 0.18**	51.54 $\pm$ 1.43
	1	63.25 $\pm$ 0.47		

Values in bold indicate viability below 70% when compared to medium control; Ctrl: Control group treated with DMEM; LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 μM); L-NAME (100 μM): N(gamma)-nitro-L-arginine methyl ester(1+); F: Raw fiber; F + Si: Raw fiber with MSNs; F + H: Raw fiber with herbal extract; F + H + Si: Raw fiber with herbal extract and MSNs. Statistically significant differences were indicated for each compound vs. LPS (* p <0.05, ** p <0.001.)

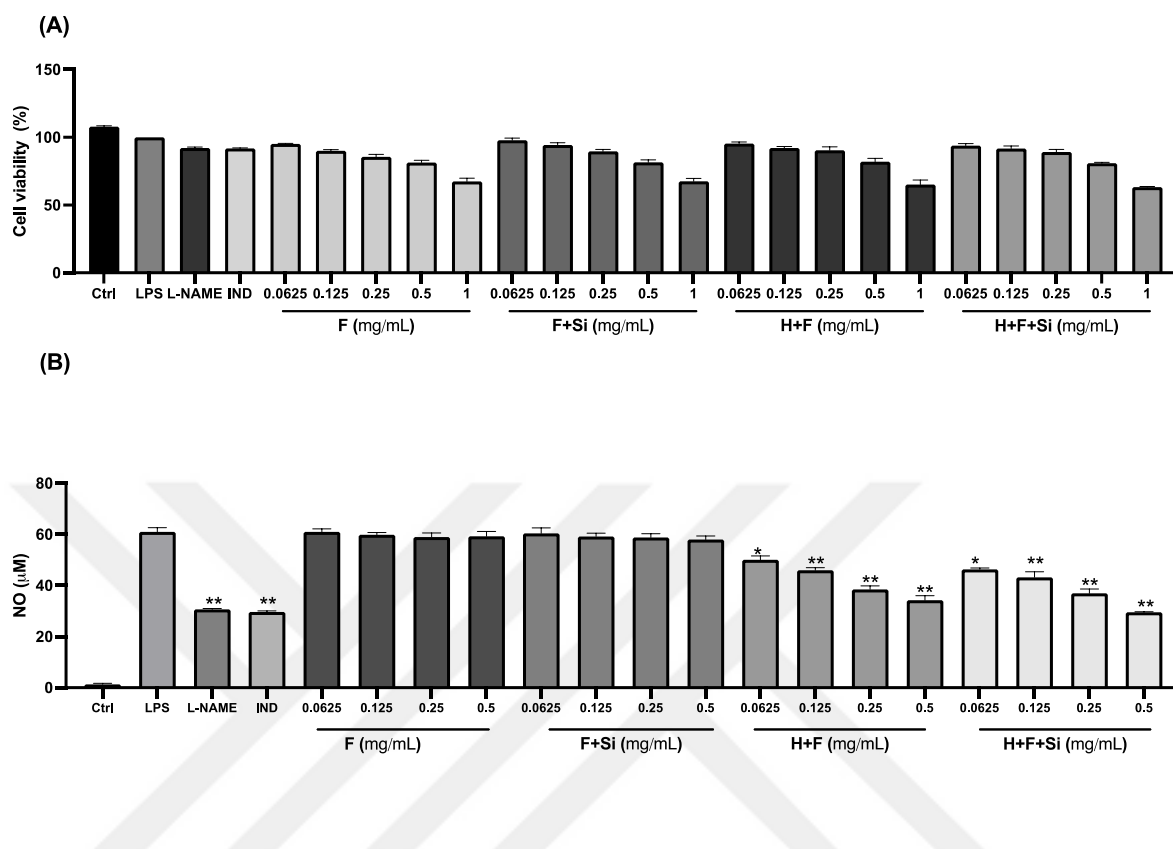


Figure 37. The effects of different concentrations of nanofiber mats extract on cell viability (A), on nitrite production (B) on LPS stimulated RAW 264.7 cell

Ctrl: Control group treated with DMEM; LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; L-NAME (100 μM): N(gamma)-nitro-L-arginine methyl ester(1+); IND: Indomethacin (100 μM); F: Raw fiber; F + Si: Raw fiber with MSNs; F + H: Raw fiber with herbal extract; F + H + Si: Raw fiber with herbal extract and MSNs. Statistically significant differences were indicated for each compound vs. LPS (* $p < 0.05$, ** $p < 0.001$).

The ability of extracts to inhibit LPS-induced nitrite production in RAW 264.7 cells was evaluated in this study, with L-NAME, a direct inhibitory agent of nitrite oxide, and with indomethacin, a potent anti-inflammatory agent, serving as a reference compound. Treatment with LPS (1 μg/mL) led to a significant increase in nitrite levels in the cell culture supernatant (Table 5). NO production after LPS stimulation increased to 60.99 ± 1.62 μM in the untreated cells. However, both metallized herbal-loaded nanofibers and herbal-loaded nanofibers exhibited a concentration-dependent reduction in LPS-induced nitrite production, as shown in Table 5. The treatment of L-NAME and indomethacin significantly inhibited nitrite oxide production by about 49% and 51% respectively ($p < 0.001$).

Moreover, nitrite inhibition of metallized herbal-loaded nanofibers and herbal-loaded nanofibers at their highest studied concentrations was approximately 51% and 43% respectively. The other two nanofiber film sheets do not exert any significant anti-inflammatory effect. This suggests that the incorporated herbal extract within the fiber exerts a strong anti-inflammatory effect. The addition of MSNs nanoparticles potentiates this effect.

4.8 Invitro wound healing evaluation

4.8.1 Cytotoxicity Assay on L929 cell line

The effect of 24 h exposure to different doses of herbal extract loaded nanoparticle fiber extract on L929 cell line were determined, and the detected viability was above 70%. Therefore, different doses of the extract were considered cytotoxic when the cell viability is below 70%. The cytotoxic doses were 0.5 and 1 mg/mL for Raw fiber with herbal extract and MSNs, 0.5 and 1 mg/mL for Raw fiber with MSNs, 1 mg /mL for Raw fiber with herbal extract and 1 mg/mL for Raw fiber extract (Table 6 and Figure 38).

Table 6. Cytotoxicity results of the herbal extract loaded nanoparticle fiber extract on L929 cell line

Groups	Dose (mg/mL)	Cell Viability (%)
Ctrl	-	100.00 ± 0.12
F (mg/mL)	0.0156	99.19 ± 1.71
	0.0312	96.46 ± 3.29
	0.0625	90.26 ± 1.12
	0.125	85.40 ± 2.40
	0.25	82.44 ± 4.02
	0.5	77.03 ± 2.19
	1	61.10 ± 3.35
F+Si (mg/mL)	0.0156	96.88 ± 1.18
	0.0312	95.14 ± 1.49
	0.0625	92.63 ± 0.93
	0.125	89.58 ± 1.57
	0.25	78.86 ± 5.61
	0.5	70.94 ± 2.09
	1	53.30 ± 1.14
H+F (mg/mL)	0.0156	97.36 ± 0.93
	0.0312	95.21 ± 3.16
	0.0625	91.08 ± 1.65
	0.125	88.50 ± 2.79
	0.25	69.78 ± 0.21
	0.5	69.21 ± 0.68
	1	62.61 ± 2.55
H+F+Si (mg/mL)	0.0156	98.54 ± 1.78
	0.0312	93.48 ± 1.36
	0.0625	90.40 ± 2.53
	0.125	85.78 ± 1.42
	0.25	69.65 ± 0.17
	0.5	66.18 ± 4.24
	1	58.83 ± 0.82

*F: Raw fiber, F + Si: Raw fiber with MSNs, F + H: Raw fiber with herbal extract, F + H + Si: Raw fiber with herbal extract and MSNs. Values in bold indicate viability below 70% when compared to medium control.

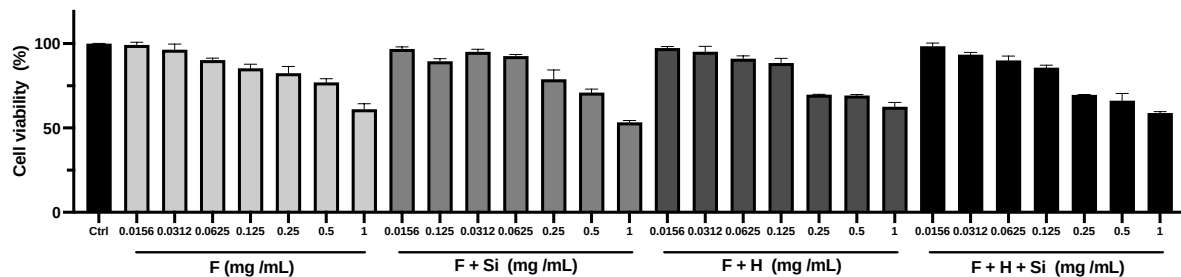


Figure 38. Cell viability of herbal extract loaded nanoparticle fiber extract on L929 cell lines

*F: Raw fiber, F + Si: Raw fiber with MSNs, F + H: Raw fiber with herbal extract, F + H + Si: Raw fiber with herbal extract and MSNs. Values in bold indicate a significant decrease when compared to medium control.

4.8.2 Cell Migration Assay

In this study, different concentrations of samples were used in order to establish healing rate by the mitigation of fibroblast cells compared with the untreated culture medium as control. The chosen concentrations to go through with the cell migration assay are the highest two non-cytotoxic doses for each sample (F: 0.25, 0.5 mg/mL; F+Si: 0.25, 0.5 mg/mL; H+F: 0.0625, 0.25 mg/mL and H+F+Si: 0.0625, 0.25 mg/mL). The cellular proliferation and migration were observed and photographed on 0 and 24 h. The average rates of cellular proliferation of medium control were determined as 74% in the 24th hour (Figure 39-40).

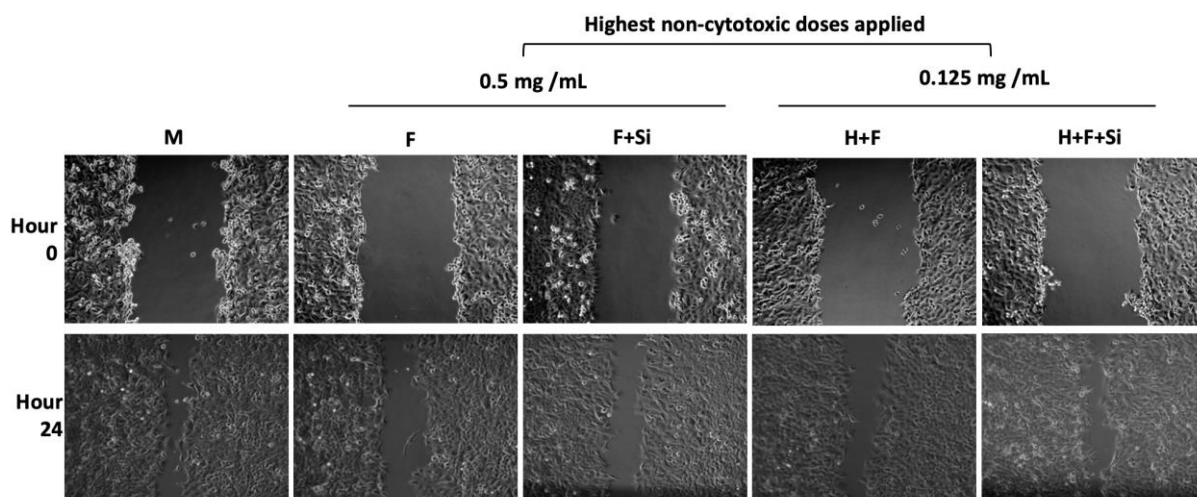


Figure 39. The effect of the herbal extract loaded fiber film sheets on cell migration.

*F: Raw fiber, F + Si: Raw fiber with MSNs, F + H: Raw fiber with herbal extract, F + H + Si: Raw fiber with herbal extract and MSNs.

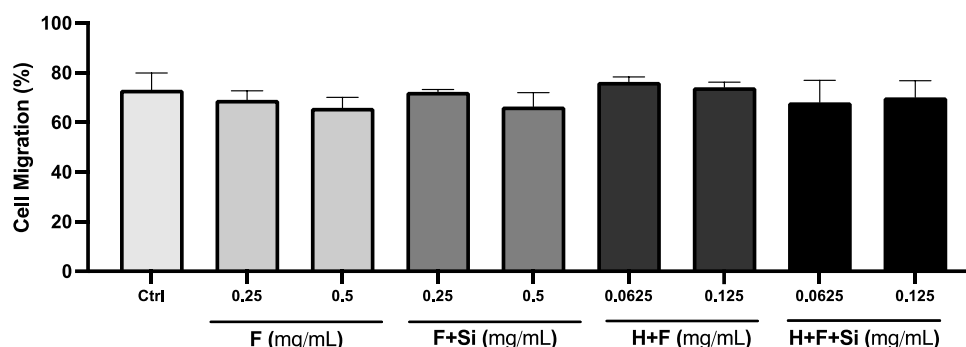


Figure 40. Cell migration (%) of the herbal extract loaded with nanoparticles film fiber with respect to concentration on 24th hour compared to the medium group.

*F: Raw fiber, F + Si: Raw fiber with MSNs, F + H: Raw fiber with herbal extract, F + H + Si: Raw fiber with herbal extract and MSNs.

In the cell migration assay, samples did not indicate a dose-dependent wound healing potential. A control group medium with no treatment is used for comparison. The gap closure of raw fiber with all non-cytotoxic doses and medium was similar. The wound closure for the highest dose of raw fiber was 66% and for the medium was 73%. In light of this knowledge, even when the control group is used as the raw fiber, the gap closure and thus the % cell migration would not reveal a positive potential for metallized herbal-loaded nanofibers. All samples in all concentrations applied show a similar cell migration rate as the control (medium) group.

4.9 Ames Test

Table 7 present the results of the mutagenicity assay for the final product (raw fiber with herbal extract and MSNs) using the TA98 and TA100 strains, both with and without metabolic activation. The positive control exhibited significantly higher frequencies of revertant colonies compared to the negative control ($p < 0.05$) in both strains, with and without S9 activation. None of the tested concentrations induced a significant increase in revertant colonies in either the TA98 or TA100 strains, indicating no mutagenic effect of the tested samples with or without S9 activation.

Table 7. Mutagenicity of the sample in Salmonella typhimurium TA98 and TA100 strains.

Without S9		Number of revertant/plate	
		TA98	TA100
Negative control (dH ₂ O ₂)		25.0 ± 4.2	193.0 ± 18.4
Positive control		822.5 ± 85.6*	1065.5 ± 54.4*
Dose (µg/plate)	100%	22.5 ± 4.8	184.0 ± 11.4
	50%	27.0 ± 1.0	178.0 ± 19.0
	25%	23.0 ± 6.5	190.5 ± 14.5
With S9			
Negative control (dH ₂ O ₂)		35.2 ± 7.5	173.7 ± 19.4
Positive control		721.7 ± 23.4*	696.5 ± 42.4*
Dose (µg/plate)	100%	33.4 ± 1.5	165.3 ± 12.9
	50%	37.3 ± 4.0	177.0 ± 14.0
	25%	36.0 ± 3.6	184.3 ± 10.5

Dunnett's multiple comparison test was carried out for statistical analysis. NPD (20 µg/plate) and SA (1 µg/plate) were used as positive mutagen without S9 activation for TA98 and TA100, respectively. 2-aminofluorene (10 µg/plate) was used as positive mutagen with S9 experiment for both strains. *p < 0.05 versus negative control.

4.10 Comet Assay

Figures 41 and 42 show the results obtained from the experiment. Different concentrations of herbal-loaded metalized nanofiber mat extracts were tested (100%, 50%, 25%). The DNA tail was found to be so clear in the positive control sample while there was no significant difference between the herbal-loaded metallized nanofiber mat and the negative control.

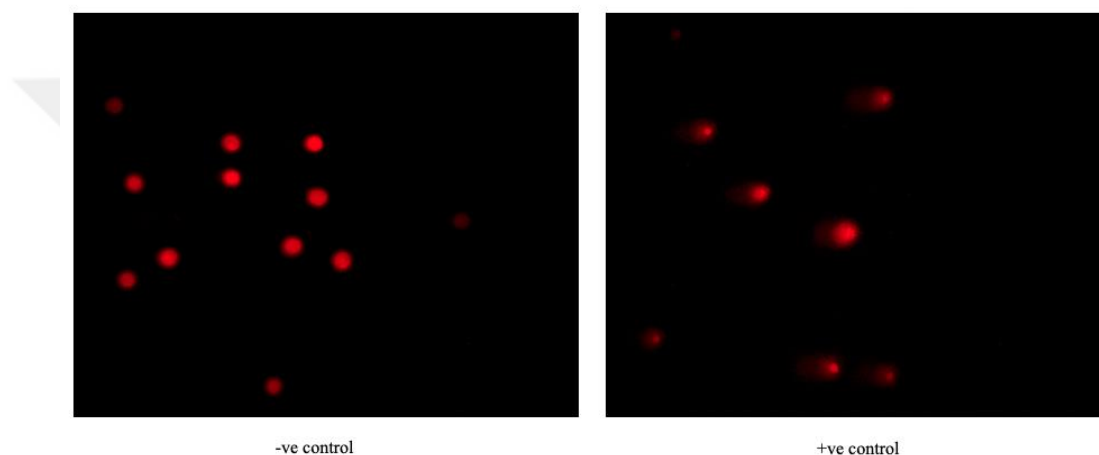


Figure 41. The representative images of negative and positive control results for comet assay

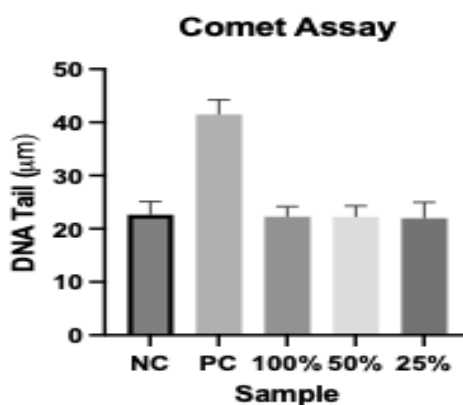


Figure 42. Quantification of comet extent by image analysis.

*NC: negative control, PC: positive control, different concentrations of herbal loaded metalized nanofiber mat (100%, 50%, 25%). Statistically significant differences were indicated between PC and the rest of the samples ($p < 0.0001$).

5. DISCUSSION AND CONCLUSION

This research focuses on the fabrication of metallized herbal-loaded nanofibers via electrospinning, a widely recognized technique for producing nanofibrous materials characterized by high surface area and porosity, making them suitable for wound dressing applications. The process initiates with the preparation of a precursor solution comprising polymers. For this study, CS and PVA, incorporated with herbal extracts such as Acacia gum and St. John's Wort, along with MSNs were used. The electrospinning process involves applying a high voltage to the precursor solution, which causes the polymer solution to be drawn into fine fibers. This method allows for the production of nanofibers with diameters in the nanometer range, which can enhance the surface area for drug loading and improve the release characteristics of the herbal extracts. The modifications that were made to the polymer composition and electrospinning parameters to achieve optimal nanofiber formation indicate a systematic approach to refining the formulation process applied in this study.

The structural analysis of the nanofibers through scanning electron microscopy (SEM) revealed the successful formation of fibrous mats with a desirable morphology. The presence of MSNs within the nanofibers contributes to their mesoporous structure, which is beneficial for drug delivery applications.

Previous studies on nanofiber formulations using combinations of PVA, CS and gelatin have shown that reducing the concentration of CS improves nanofiber formation, resulting in fewer beads, consistent with the findings of this study. In a study, a 3% (w/w) CS solution and 20% (w/w) gelatin solution were prepared by dissolving the components in 90% acetic acid. Mixing these solutions in various ratios and analyzing them with SEM revealed that increasing the gelatin percentage led to more uniform composite nanofibers with significantly fewer beads¹⁰⁵. Similar results were observed when different ratios of CS salt solutions were mixed with PVA solution. Specifically, when the CS/PVA weight ratio was increased to 30/70, SEM micrographs showed no beading¹⁰⁶.

Our results also concluded that increasing the polymer weight percentage enhances nanofiber formation. Consistent with our findings, another study fixed the weight ratio of PVA to CS at 80:20 in 2% acetic acid, varying the PVA/CS precursor concentration from

3% to 5 wt%. The morphology transitioned from a structure with more beads to a uniform fiber structure as the concentration increased from 3% to 5 wt% ¹⁰⁷.

In another study, an 8% (w/v) PVA solution in water and a 2% (w/v) CS and 10% (w/v) gelatin solution in 2% (v/v) acetic acid were prepared. Blends of PVA/CS/G were characterized and compared to PVA alone, PVA/CS, and PVA/G blends. The results indicated that the solution could still produce fibers, although with some bead formation along the fibers, albeit not excessively. Additionally, the fiber diameter increased with the greater volume addition of PVA ¹⁰⁸, which is also consistent with our results.

Acacia gum was utilized alongside PVA to produce electrospinning blends. Specifically, a 10% (w/v) PVA solution and a 1% (w/v) acacia gum solution were separately prepared in double-distilled water and then mixed in a 5:1 ratio for antifungal biofilm formulations ^{12,109}. The addition of 1% (w/v) acacia gum improved nanofiber production in this study, functioning as a thickening agent and offering biological benefits that can promote wound healing ⁴⁸.

The FT-IR results of our study exhibit similarities with previous studies, confirming the reliability and reproducibility of the findings. These results validate the expected performance and characteristics of the material, further supporting its potential application ^{74,107,110,111}.

The nanoindentation test results revealed a significant increase in Young's modulus in the metallized herbal-loaded nanofiber mat compared to the raw nanofiber mat without any additives. However, the tensile strength tests indicated that the M15 nanofiber mat exhibited moderate stiffness, low yield, and ultimate tensile strengths, as well as limited ductility. These mechanical properties are critical in applications where the material is subjected to tensile forces. Given its low tensile strength and limited ductility, the M15 nanofiber mat may not be ideal for use as a wound dressing. For such applications, higher mechanical strength and greater elongation properties are necessary to ensure that the dressing can withstand the stresses of application and movement while providing adequate support for wound healing. Commercial microfibrous dressings typically have a tensile strength of around 10 MPa, which serves as a benchmark for comparison ¹⁰⁶. Due to technical difficulties, tensile testing of the M16 nanofiber mat was not conducted. Further

characterizations of physiochemical properties and stability studies for the produced nanofiber mat were limited in this study since the main focus was safety and efficacy. Follow-up research and more data about the formulation in the biotechnology field are required.

Due to its large surface area, high pore volume, flexible morphology, surface functionalization capabilities, uniform mesoporosity, tunable and narrow pore size distribution, and exceptional biocompatibility and biodegradability, mesoporous silica has garnered significant interest. Mesoporous silica nanoparticles are typically cylindrical, with diameters ranging from 50 to 500 nm and pore sizes between 2 and 20 nm. These pores function as reservoirs and carriers in various applications, including drug delivery, adsorption, and heterogeneous catalysis. Among these, the delivery of therapeutic agents is a particularly prominent application ¹¹². The particle size analysis revealed two distinct particle size ranges: 150-190 nm and 500-530 nm for the MSNs used in the study. These sizes fall within the expected range and are considered safe for nanoparticles. The Zeta potential analysis showed an average Zeta potential of -24.1 mV, indicating that the MSNs were successfully functionalized with carboxylate groups ¹¹³. The TEM and SEM images showed similarity in the structure of some other plant-derived silica nanoparticles such as rice husk and sugarcane bagasse ¹¹².

Several researchers were interested in *Hypericum perforatum* extracts due to their bioactive properties including utilizing it for wound healing. The identification of *Hypericum perforatum* extracts is primarily confirmed by the presence of hypericin, a compound that imparts a characteristic reddish coloration to various prepared solutions. When these solutions are analyzed using spectrophotometry, they exhibit a peak around 590 nm, which corresponds to the reported absorption maximum for hypericin [37,38,84]. The research demonstrated that dissolving the extract powder in methanol yielded the most reliable results. Consistent with previous studies, methanol was selected as the solvent of choice for sample preparation prior to loading into the precursor solution, and an absorption wavelength of 588 nm was used for the hypericin assay in our study. Most studies have primarily focused on using HP oil extract, which has made assays for EE% and release profiles challenging. As a result, these studies often concentrated solely on evaluating the efficacy of the product compared to control and did not investigate EE% or release profiles ^{51,53,114}. In a study,

researchers developed a wound dressing by preparing HP-loaded CS nanoparticles embedded in an agarose film. HP extraction was performed using various ratios of ethanol and methanol. To determine the EE% and release profile, they employed a spectrophotometer to plot a calibration curve based on different concentrations of HP. The loading efficiency was calculated to be $90.1 \pm 2.53\%$, and the release of HP reached a plateau in PBS medium after 4 hours, achieving a release of $82 \pm 5\%$ ¹¹⁵. In this study, different concentrations of HP after sample preparations were used for the calibration curve. In the current study, different concentrations of HP were used for the calibration curve after sample preparation. The EE% ranged from 86% to 96%, and the release profile showed a rapid release during the first two hours, reaching 30% for herbal-loaded nanofibers and 36% for metallized herbal-loaded nanofibers. After 12 hours, the release was 62% and 43%, respectively, indicating a sustained release that allows for prolonged activity of the product.

Our results indicate that the produced metallized herbal-loaded nanofibers enhanced antimicrobial properties, as evidenced by their effective inhibition of bacterial and fungal growth. The variations in loading methods and compositions led to differing levels of antimicrobial activity, with some formulations achieving up to 70% of the antimicrobial efficacy compared to the control drug. Furthermore, the incorporation of MSNs was shown to improve the release rate of the herbal extracts, thereby extending their therapeutic effects. HP extracts exhibit significant antibacterial and antifungal properties, with their effectiveness influenced by the extraction method, solvent used, and seasonal factors. HP extracts are generally more effective against Gram-positive bacteria than Gram-negative ones ⁴⁴. In one study, gelatin nanofibrous scaffolds incorporating HP oil demonstrated antibacterial activity against *E. coli* (7.83 mm) and *S. aureus* (17 mm), indicating sufficient antibacterial properties for potential use in wound dressing materials ¹¹⁴. Another study found that HP oil-loaded CS cryogel scaffolds were effective as antimicrobial agents. The antimicrobial efficacy followed the order: *E. coli* (14 mm) > *L. pneumophila* (13 mm) > *P. aeruginosa* (12 mm) > *S. aureus* (11 mm) > *E. hirae* = *B. cereus* = *C. albicans* (10 mm), highlighting its potential as a candidate for tissue engineering applications ⁵¹. Conversely, an HP ethanolic extract was ineffective against *Candida albicans* and the Gram-negative bacterium *E. coli*, but it significantly impacted the Gram-positive bacterium *S. aureus* ¹¹⁶. Loaded-CS nanoparticle/agarose film containing 160 mg of HP per film demonstrated antibacterial

activity against *E. coli* (16 mm), *S. aureus* (34 mm), and *C. albicans* (15 mm)¹¹⁵. In contrast, our results confirmed the antimicrobial activity of HP methanolic extract against both Gram-positive and Gram-negative bacteria, as well as fungi. The metallized herbal-loaded nanofibers showed the strongest activity, with inhibition zones ranging from 20 to 30 mm, covering approximately 70% of the activity observed in control antibiotics.

In addition to antimicrobial activity, anti-inflammatory activity is a crucial factor in promoting wound healing. Previous studies on HP extract loaded polymers have evaluated anti-inflammatory effects using different parameters, such as *in vivo* studies and measuring oxidative stress indices and TNF- α levels⁵³. In one study, the anti-inflammatory activity of ethanolic extracts from *Hypericum* species was assessed using RAW 264.7 mouse macrophages, revealing that HP inhibited both PGE2 and NO production by more than 30% compared to the vehicle control⁴¹. In the current study, metallized herbal-loaded nanofibers at the highest tested concentration inhibited nitrate production by approximately 51%, which is comparable to the effects of L-NAME and indomethacin treatments. This result indicates that the nanofibers exhibit significant anti-inflammatory activity.

The cell migration assay is a valuable tool for understanding how cells move and migrate in response to various stimuli or conditions, which is critical for wound healing. A study, explored the use of CS/collagen composite films encapsulating allantoin and lidocaine hydrochloride as wound dressings. To simulate wound healing, an *in vitro* scratch assay was performed on Mesenchymal Stem Cells (MSCs), where a gap was created in a cell monolayer, and cell migration was observed over time. The results showed that scaffolds with higher collagen ratios significantly improved closure rates at 6 and 24 hours, suggesting that the formula enhances the early stages of wound healing¹¹⁷. The cell migration assay in this study aimed to evaluate the healing potential of fibroblast cells in response to different sample concentrations, offering insights into their potential use in regenerative medicine. L929 cell lines were utilized, and images of the wounds were captured at 0 and 24 hours post-treatment to assess gap closure and, consequently, the percentage of cell migration. However, the results did not indicate a significant positive effect for metallized herbal-loaded nanofibers, as all samples across all concentrations showed a similar cell migration rate to the control (medium) group. This suggests that any potential enhancement in the early stages

of wound healing would not have been detected in this assay. While the concentrations used in this test demonstrated moderate anti-inflammatory activity, an *in vivo* test might be more effective for evaluating the healing rate of a nanofiber mat ⁵².

The results of this study offer valuable insights into both the biological activity and the safety of the tested samples. The MTT assay on RAW 264.7 cells revealed that while most concentrations were non-cytotoxic, all samples exhibited cytotoxic effects at a concentration of 1 mg/mL after 24 hours. In contrast, the MTT assay on the L929 cell line detected cytotoxicity at lower concentrations: 0.5 mg/mL for raw fiber and fiber with MSNs, and 0.25 mg/mL for both herbal-loaded nanofibers and metallized herbal-loaded nanofibers. These findings underscore the importance of dose management in therapeutic applications. In other studies investigating the cytotoxicity of HP-loaded nanofibers or films, L929 cells were exposed to the tested samples for 24 hours, followed by treatment with MTT solution and incubation until formazan crystals formed. These studies found no cytotoxicity within the 24-hour period, suggesting that the observed cytotoxic effects in the current study may be dose-dependent and emphasizing the need for careful consideration of dosage in future therapeutic applications^{114,115}.

As observed in previous studies, most researchers have focused primarily on providing cytotoxicity results. However, this study extends beyond cytotoxicity by also investigating mutagenicity and genotoxicity, offering a more comprehensive safety assessment of the tested samples. The mutagenicity and genotoxicity results are particularly valuable as they provide critical insights into the safety profile of the samples concerning their potential effects on genetic material. Mutagenicity refers to the ability of a substance to induce mutations in DNA, which can lead to cancer and other genetic disorders. In contrast, genotoxicity encompasses a broader range of effects that can damage DNA and compromise cellular integrity. By examining these aspects, the study contributes to a more thorough understanding of the potential risks associated with the tested materials, which is essential for their safe application in therapeutic contexts. In the mutagenicity testing, the results indicated that the tested concentrations did not induce a significant increase in revertant colonies in either TA98 or TA100 strains, both with and without S9 metabolic activation. This suggests that the samples do not exhibit mutagenic effects, reflecting a favorable safety

profile with respect to genetic toxicity. The absence of mutagenicity is particularly crucial for substances intended for therapeutic use, as it implies a low likelihood of causing harmful genetic alterations in human cells. Furthermore, the comet assay results demonstrated that the herbal-loaded metallized nanofiber mat did not induce significant DNA damage compared to the negative control. This finding further supports the conclusion that the samples are non-genotoxic, as they did not cause detectable DNA strand breaks or other forms of genetic damage. The findings highlight the critical importance of thorough safety evaluations in the development of herbal-loaded nanofibers, particularly for applications in wound healing and tissue regeneration, where biocompatibility and safety are paramount. Further studies may be warranted to explore the long-term effects and underlying mechanisms contributing to the observed safety profile.

In conclusion, the results presented in this study demonstrate the successful formulation and characterization of metallized herbal-loaded nanofibers with promising biological activities and a favorable safety profile.

The research focused on optimizing the formulation and characterization of nanofiber mats incorporating herbal extracts and MSNs. Initial challenges, such as high viscosity and solubility issues with precursor solutions, were effectively overcome through refined preparation techniques. By adjusting polymer ratios, solvent choices, and electrospinning parameters, the study successfully produced nanofiber mats with desirable properties. The incorporation of gelatin and acacia gum significantly enhanced the physical properties and structural integrity of the nanofibers, leading to improved overall performance.

Characterization of the nanofibers confirmed the successful incorporation of herbal extracts and MSNs. Mesoporous silica nanoparticles were accurately characterized, showcasing their structural and functional attributes. Methanol was identified as the optimal solvent for preparing herbal extracts, ensuring high encapsulation efficiency within the nanofibers.

The electrospinning method effectively produced nanofibers with significant antimicrobial properties, with certain formulations achieving up to 70% of the control drug

activity. The inclusion of MSNs enhanced the release characteristics of the herbal extracts, contributing to prolonged therapeutic effects.

Biological testing revealed that the nanofibers exhibited notable anti-inflammatory effects, particularly the metallized herbal-loaded nanofibers, which showed the most significant reduction in nitrite production. However, the cell migration assays did not demonstrate substantial wound healing effects, suggesting that further *in vivo* testing is necessary to fully assess the therapeutic potential of these nanofibers.

Safety and toxicity studies raised some concerns, as the nanofibers exhibited cytotoxicity at certain concentrations. This underscores the need for additional toxicological evaluations and careful consideration of potential interferences with the assays used. Further studies are essential to ensure the safety and efficacy of these nanofibers for potential clinical applications. Importantly, the mutagenicity and genotoxicity assessments revealed that the tested samples did not induce significant mutations or DNA damage, indicating a strong safety profile for potential therapeutic applications. The absence of mutagenic effects, as demonstrated by the results from the Ames and the comet assay, suggests that these metallized herbal-loaded nanofibers could be safely utilized in medical contexts, particularly in wound healing and tissue engineering. These findings support the potential of these nanofibers as safe and effective materials for therapeutic use, especially in applications where maintaining genetic integrity is crucial.

Overall, the findings underscore the potential of these nanofibers as effective and safe biomaterials with promising therapeutic properties for wound healing and tissue engineering applications. These results pave the way for further research and development aimed at optimizing the formulation and exploring their clinical utility. Future research should focus on refining the formulation, enhancing tensile strength, and conducting degradation and stability studies. Additionally, *in vivo* evaluations and investigations into the mechanisms underlying their biological activities are essential to fully establish their therapeutic potential.

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7. APPENDICES

APPENDIX 1. Curriculum Vitae

Personal Informations

Name	Hebah	Surname	Ayash
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Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate	Pharmaceutical toxicology	Yeditepe university	2024
Master	Analytical toxicology	University of Jordan	2014
University	Pharmacy	Applied science university	2008
High school	Science	Al- Safar	2004

All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Languages	Grades (#)
TOFEL iBT	90

Work Experience (Sort from present to past)

Position	Institute	Duration (Year - Year)
Lecturer	Faculty of Pharmacy - Zarqa University	2014 - 2019
Teacher Assistant and Lab Technician	Faculty of pharmacy - Applied Science University	2008 - 2011

Computer Skills

Program	Level
ICDL	good

*Excellent , good, average or basic

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

A review on the application of electrospun herbal extract-loaded metallized nanofiber composites as wound healing promoter: Fabrication, efficacy, and safety – accepted for publication at The Jordan Journal of Pharmaceutical Sciences

Proceedings presented in international scientific meetings and published in proceedings book.

Speaker at HS's 1st International Pharmaceutical Sciences Conference "Exploring New Frontiers in Pharmaceutical Sciences" (2024)
A review on the application of electrospun herbal extract-loaded metallized nanofiber composites as wound healing promoter: Fabrication, efficacy, and safety