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Turmeric/ Oregano extracts as wound healing agents in a diabetic animal model

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School of Science and Engineering

Turmeric/ Oregano Extracts as Wound Healing Agents in a Diabetic Animal Model

A Thesis Submitted to

The Biotechnology Graduate Program

In partial fulfillment of the requirements for
The degree of Master of Science in Biotechnology

By

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Under the Supervision of

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Has been approved by

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DEDICATION

"My family is my life, and everything else comes second as far as what is important to me." Michael Imperioli

To the most important people in my life, my backbone & whole world;

Dad & Mum, I love you so much. I am blessed to be your daughter. You are the light of my eyes. Thank you for your unconditional love, endless support, extreme care, encouragement and bringing me up to what I am now. Thank you for having my back in the hardest situations in my life & never letting me down. None of this work or any other achievement or success would have been done without your support. Wish I make you always proud of me.

My Brother, you are my backbone, supporter and the reason of our happiness. Thank you for being my best faithful friend. Wish you all happiness & success. God bless you for us.

Without all of your love, support & care, I would not have been able to come the person I am now. Your efforts will be never forgotten & no thank you word will give you your rights. For this, with all love I dedicate this work to you.

I will always love you all. I owe you my life.

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ABSTRACT

Diabetic wound infections and pressure ulcers pose a significant challenge to healthcare providers worldwide. With an increased incidence of chronic skin ulcers and a significant financial impact on healthcare systems, reaching $25 billion annually, new methods to treat chronic and diabetic ulcers are in great need. The current study provides new and innovative wound care products that reduce inflammation, clear infection and improve healing time in an animal model of pressure and diabetic ulcers. Animal model with excisional wound & pressure ulcer was done on the dorsal side of the rats in diabetic and non-diabetic groups. Our results showed that pressure ulcer had significantly different pathological features compared to excisional wounds. Diabetes caused skin changes that negatively affects the healing process.

Different turmeric extracts, oregano essential oil and chitosan nanoparticles were tested for their antibacterial & antioxidant activity. Results showed that turmeric ethanolic extract 5%, oregano essential oil 1% & chitosan nanoparticles 1% had the most antibacterial & antioxidant effects. Ointments were synthesized of each herb individually. An i*n vivo* pilot study was conducted on diabetic and non-diabetic rats with pressure ulcer. Results showed that turmeric 5% ointment successfully healed the ulcer in both diabetic and non-diabetic rats by day 15. The oregano 1% ointment achieved complete healing by day 15 in the non-diabetic group while in the diabetic group was achieved by day 21.

The above concentrations were incorporated in different forms (ointment, amorphous hydrogel & nanofibers). Those forms were tested for their antibacterial, cytotoxic effect & *in vivo* using Tegaderm® (commercial wound dressing) as positive control. Our results showed that the designed formulas had significant antibacterial effect as Tegaderm®. On testing the formulations on mouse fibroblast cell line (L929), ointment & hydrogel were non-cytotoxic while nanofibers showed relative cytotoxicity if compared to Tegaderm® that was highly toxic. By testing the formulations *in vivo,* our results showed that by day 15 ointment and nanofibers achieved complete wound closure while hydrogel and Tegaderm® did not.

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List of Abbreviations

Abbreviation	Term
Ab	Absorbance
AGE	Advanced Glycation End-products
AHRQ	Agency for Healthcare Research and Quality
CFU	Colony Forming Unit
ChNP	Chitosan Nanoparticles
CMC	Carboxymethyl Cellulose
COX 2	Cyclooxygenase 2
DI	Deionized water
DL	Demarcation Line
DLS	Dynamic Light Scattering
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl radical
DU	Diabetic ulcers
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GT	Granulation Tissue
H&E	Hematoxylin and Eosin
HBOT	Hyperbaric Oxygen Therapy
HD	Hydrodynamic Diameter
HIF-1α	Hypoxia Inducible Factor-1α
Hr	Hour
I.P	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IGF-I	Insulin Growth Factor
IL-1	Interleukin 1
iNOS	inducible Nitric Oxide Synthase
KV	Kilo Volt
L929	Mouse fibroblast cell line
LOX	Lipoxygenase
MCP-1	Macrophage Chemo attractant Protein-1
MMP	Matrix Metalloproteinase
MTT	Thiazolyl blue tetrazolium bromide
NF-κB	Nuclear Factor Kappa B

NIH	National Institute of Health
NPs	Nanoparticles
NPUAP	National Pressure Ulcer Advisory Panel
OD	Optical Density
PBS	Alkaline Phosphate buffer
PDGF	Platelet Derived Growth Factor
PDI	Polydispersity Index
PG	Prostaglandin
PMNL	Polymorphonuclear Leucocytes
PU	Pressure Ulcers
PVA	Polyvinyl Alcohol
ROS	Reactive Oxygen Species
S. aureus	Staphylococcus aureus
SCT	Subcutaneous Tissue
SEM	Scanning Electron Microscopy
ST	Surrounding Tissue
STZ	Streptozotocin
TEM	Transmission Electron Microscopy
TGF- ß	Transforming Growth Factor Beta
TIMPs	Tissue Inhibitors of MMPs
TNF alpha	Tumor Necrosis Factor alpha
TPP	Tripolyphosphate
UV	Ultraviolet radiation
VAC	Vacuum Assisted Closure
VEGF	Vascular Endothelial Growth Factor
Z- average	Hydrodynamic Size
ZP	Zeta Potential

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Chapter 1 : Literature Review

1.1 Introduction

Chronic wounds such as diabetic foot infections and pressure ulcers, pose a significant challenge to healthcare providers worldwide. Over the past years, the incidence of chronic skin ulcers dramatically increased, leading to a huge financial impact on healthcare systems, reaching $25 billion annually in the United States (Gainza et al., 2015; Han & Ceilley, 2017). Skin ulcers cause high morbidity and mortality rates. A study conducted in 2011 showed the mortality rate in 2 years follow up of skin ulcer patients reached 28% due to chronic skin complications (Escandon et al., 2011).

Diabetes is one of the leading causes for skin infection and chronic ulcers. With the prevalence of diabetes increasing worldwide new wound care products are needed to reduce healing time and improve patients' quality of life (Jhamb et al., 2016).

In the present study, we describe new and innovative wound care product that reduce inflammation, clear infection and reduce healing time in an animal model of diabetic ulcer. The current model improves the understanding of the healing process under different conditions. Using readily available rodents, with low cost and the ability to use a relatively large number of animals, making research more appropriate and reducing statistical errors (Trujillo et al., 2015; Trostrup et al., 2016). The present model avoids the wound contracture characteristic of rodents by compromising the subcutaneous muscle (panniculus carnosus), therefore, making the wound more clinically relevant to human studies (Wong et al., 2011; Seaton et al., 2015).

1.2 Global Impact

Chronic ulcers have negative impact on patients, their families, healthcare systems and economies as well. They negatively affect patients' quality of life, as well as, daily lifestyle and job performance (Herber et al., 2007).

Ulcer management is a costly process reaching £5,000 per patient annually (Kapp et al.,2017 ; Guest et al., 2016). Chronic skin ulcers also affect patients' productivity leading to an economic impact, besides the huge healthcare expenditure, reaching US $25 billion annually (Rahman et al., 2010; Han et al., 2017).

1.3 Healing process in acute and chronic wounds

The normal healing process of the skin is characterized by the following phases: coagulation, acute inflammation, proliferation and remodeling (Khodaeian et al., 2015).

Following acute injury circulating platelets are exposed to collagen leading to the activation of coagulation cascade, where fibrinogen is converted to fibrin leading to thrombus clot formation (Gilbert et al, 2016). This clot is crucial in protecting the wound from infection, as well as preventing further blood loss (Bielefeld et al., 2013). Activated platelets release pro-inflammatory cytokines and growth factors that recruit inflammatory cells to the site of wound to initiate the inflammatory phase (Gilbert et al., 2016; Hameedaldeen et al., 2014).

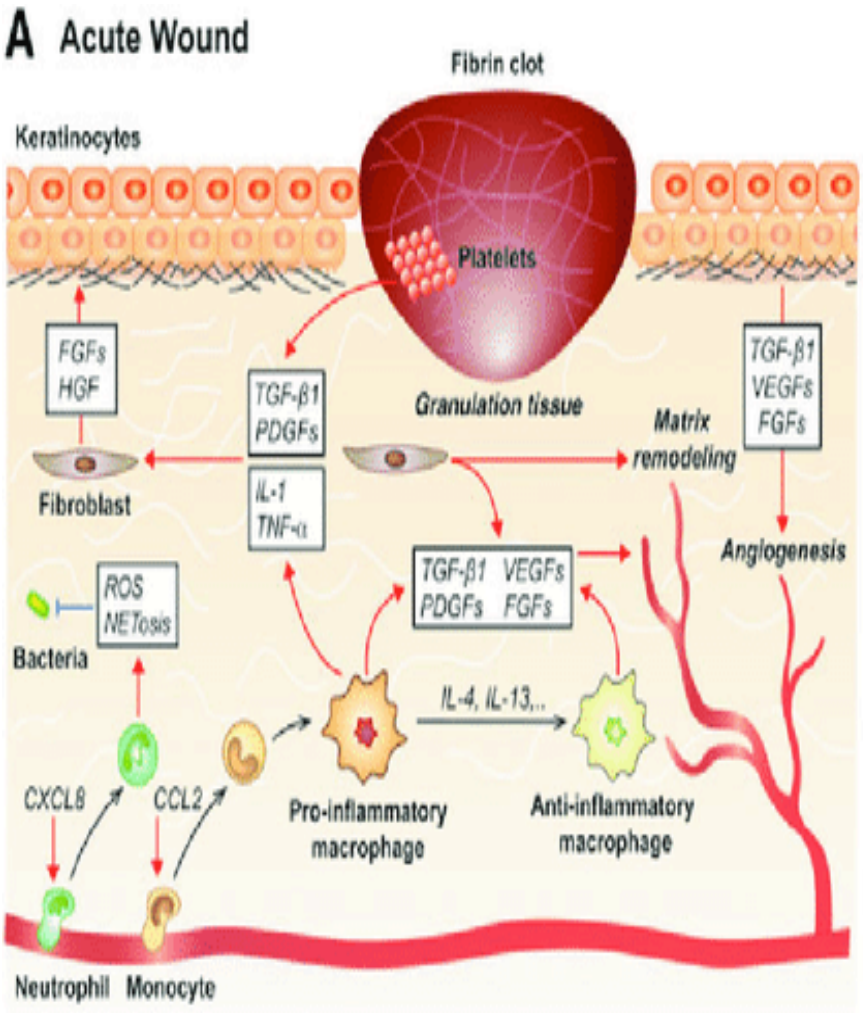
In the inflammatory phase, neutrophils and macrophages are the key players. Neutrophils remove bacteria, foreign objects from the wound, produce proteolytic enzymes such as matrix metalloproteinase (MMP) that break down dead tissue (Hameedaldeen et al., 2014). Monocytes later differentiate to macrophages that phagocytose foreign organisms and dead neutrophils. They also release transforming growth factor beta (TGF-ß) and other cytokines, and thereby enhance fibroblasts and epithelial cells movement into the wound area (Bielefeld et al., 2013; Hameedaldeen et al., 2014).

The proliferation phase overlaps with the inflammatory phase, and is primarily characterized by three major events: angiogenesis; extracellular matrix (ECM) synthesis and re-epithelialization (Gilbert et al., 2016; Emanuelli et al, 2016). In the proliferative phase, macrophages shift to an anti- inflammatory phenotype expressing different anti-inflammatory mediators, proteases and protease inhibitors, and growth factors, such as vascular endothelial growth factor (VEGF) and TGF- ß that encourage cell proliferation and protein synthesis. Endothelial cells and fibroblasts then accumulate in the wound site encouraging occurrence of angiogenesis and fibroplasia, providing oxygen, nutrients, for the proliferating cells to form granulation tissue (Tsourdi et al.,2013).

The remodeling phase is considered the last step of tissue remodeling leading to skin recovery. During the remodeling phase, immature ECM and collagen type III

are degraded by MMPs and replaced with collagen I (Zhao et al., 2016; Gilbert et al., 2016; Bielefeld et al., 2013). Subsequently, collagen fibers rearrange they lie closer together across tension lines, facilitating cross-linking and thus increases the tensile strength of the wound where unnecessary blood vessels and cells undergo apoptosis and replaced by normal skin tissue (Emanuelli et al., 2016; Baltzis et al., 2014) **(Figure 1.A).**

In chronic skin ulcers, reactive oxygen species (ROS) are the key players. Elevated levels of ROS cause oxidative damage in DNA, proteins and lipids leading to tissue damage (Donato-Trancoso et al., 2016). They induce inflammation, which in turn lead to epithelial dysfunction, decreased reperfusion, impaired angiogenesis resulting in poor ulcer healing (Blakytny et al., 2006). Also chronic skin ulcer is characterized by reduced levels of tissue inhibitors of MMPs (TIMPs), the proteins that inhibit MMPs activity and as a result elevated levels of matrix metalloproteinases accelerates tissue degradation (Baltzis et al., 2014; Amin et al., 2016). MMPs degrade the growth factors involved in the healing process as insulin growth factor (IGF-I), TGF- β1, and platelet derived growth factor (PDGF) which are crucial for the healing process, thus inhibiting ECM & re-epithelization (Falanga, 2005). High concentrations of ROS and low TGF-β1 expression level increases macrophage chemo attractant protein-1 (MCP-1) levels, which in turn attract greater numbers of macrophage leading to sustained inflammation (Blakytny et al., 2009). Impaired angiogenesis is seen in patients with chronic ulcers. An angiogenesis-promoting growth factor, hypoxia inducible factor-1α (HIF-1α) is induced in response to hypoxia resulting in the transcription of growth factors as VEGF which is important for angiogenesis. In chronic ulcers, HIF-1α is down regulated leading to low expression of VEGF as a result poor angiogenesis and impaired wound healing (Catrina et al., 2004) **(Figure 1.B)**.



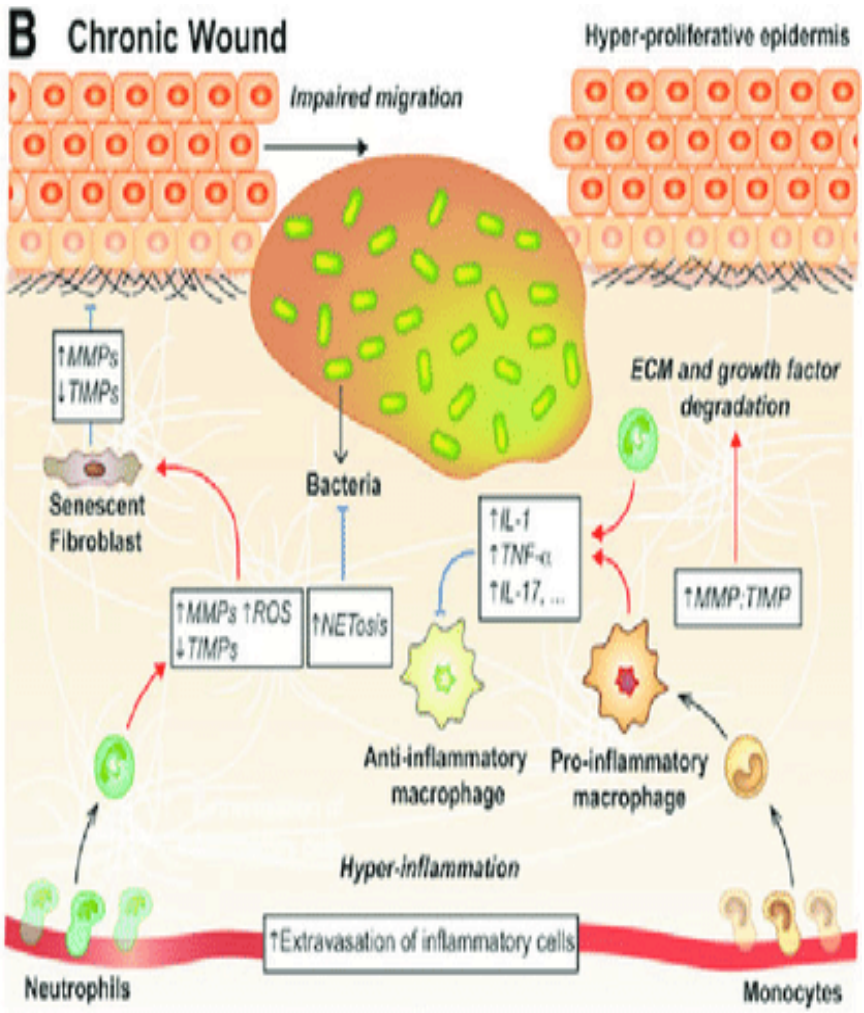


Figure 1 Overview for Mechanism of Healing in Acute and Chronic Wounds

A. Acute Wound. After acute injury circulating platelets are exposed to collagen leading to the activation of coagulation cascade, where fibrinogen is converted to fibrin leading to the formation of thrombus. Activated platelets produce pro-inflammatory cytokines and growth factors that employ inflammatory cells to the wound site to initiate the inflammatory, proliferative & remodeling phases. **B. Chronic Wound** is characterized by hyper inflammation where elevated levels of inflammatory cytokines as TNF alpha and IL-1 lead to inhibition of anti-inflammatory macrophages. Also, in chronic wounds, MMPs are elevated and reduced levels of TIMPs accelerate ECM and growth factors degradation all this leads to impaired healing process. Adapted from (Larouche et al., 2018)**.**

1.4 Types of Chronic wounds

Chronic skin ulcers have a complex etiology, with a diverse range of comorbidities making it difficult to study and find a therapeutic agent. Most chronic wounds fall into four main categories: arterial, venous, pressure, and diabetic ulcers **(Figure 2).** In the current study we will focus mainly on pressure ulcers in diabetes mellitus (DM).

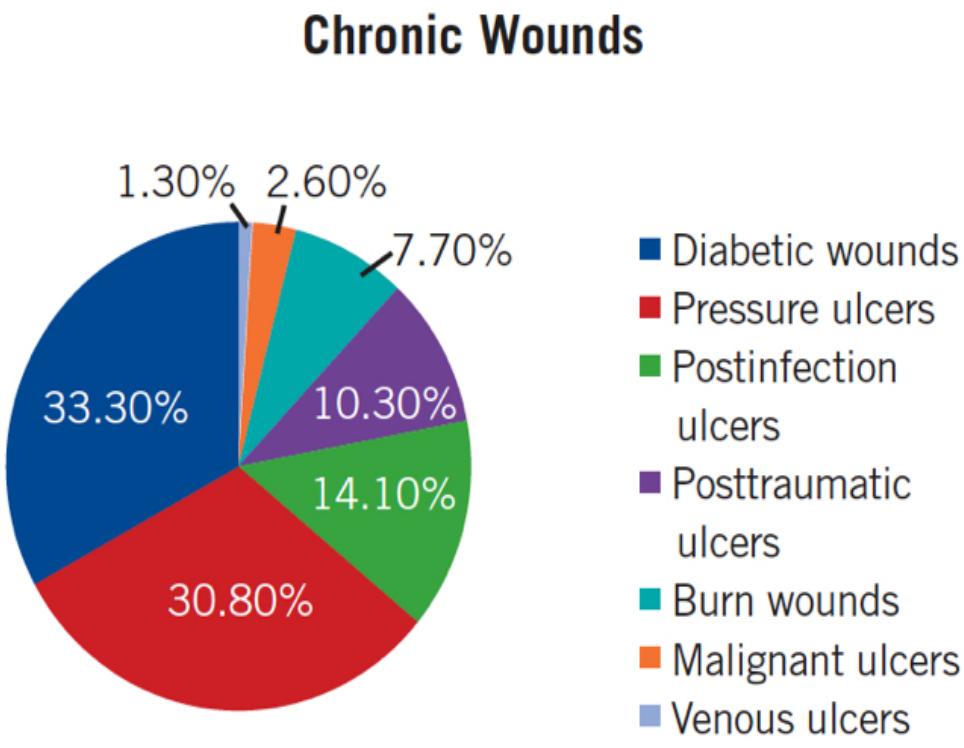


Figure 2 Common Causes of Chronic Wounds

Diabetic and Pressure Ulcers showed to be the most prevalent types of ulcers among all chronic wounds adapted from (Iyun et al, 2016).

1.4.1 Diabetic Ulcers

Diabetic Ulcers and foot infections are major complications affecting 15–20 % of diabetic patients' worldwide (Emanuelli et al., 2016; Jhamb et al., 2016). According to the International Diabetes Federation, in 2015 diabetes affected 415 million patients, with an expected increase 642 million by 2040 (Ogurtsova et al., 2017). Diabetic foot management costs nearly 9-13 billion USD annually on top of the management of DM itself (Raghav et al., 2018). Foot disorders include ulceration, infection and gangrene which are the main causes of hospitalization and in severe cases might lead to further disability due to amputation (Ray et al., 2005; Jhamb et al., 2016).

Hyperglycemia and peripheral neuropathy with impaired circulation, increase the risk of ulceration (Popov, 2010; Zhao, et. al., 2016). Moreover, diabetes related metabolic complications directly disrupt wound healing process (Baltzis et. al., 2014). The accumulation of advanced glycation end-products (AGEs) stimulates oxidative

stress, and disrupts the normal inflammatory cell function. Poor circulation and poor oxygenation are contributing factors to poor healing and chronicity of the wounds in diabetic patients (Berlanga-Acosta et al., 2013).

1.4.2 Pressure Ulcers (PU)

Pressure ulcers (Bed sores), also known decubitus ulcers, are the most common example of tissue necrosis (Roaf, 2006). Most pressure ulcers develop mainly in elderly bedridden patients (70%), following strokes, major orthopedic, and spinal cord injuries (Tubaishat et al., 2018; Grey et al., 2006). According to the Agency for Healthcare Research and Quality's (AHRQ) pressure ulcer affect 2.5 million patients per year in the US and cost $9.1-$11.6 billion per year. Skin surface over the bony prominences (e.g., hips, ankles, heels, coccyx, scapulae) are the most vulnerable areas (Baron et al., 2016 ; Mendoza-Garcia et al., 2015) **(Figure 3).** Those ulcers are painful and prone to infection, which may result sepsis or osteomyelitis (Mendoza-Mari Y et al., 2013). There are four main factors that are involved in pathogenesis of pressure ulcer which are; pressure, shear, friction, and moisture (Grey et al., 2006). Due to continuous pressure the blood supply to the skin is obstructed leading to poor circulation resulting in tissue death and an ulcer development **(Figure 4).**

According to the National Pressure Ulcer Advisory Panel (NPUAP), pressure ulcers are classified into 4 stages; stage 1: regions of intact skin with non-blanchable erythema. Stage 2: partial-thickness skin loss with exposed dermis, with pink to red viable wound area, and deeper tissues are not visible. Stage 3: full-thickness skin loss with adipose tissue seen in the ulcer. Stage 4: full-thickness skin and tissue loss with exposed muscle, tendon, ligament, cartilage or bone in the ulcer (The National Pressure Ulcer Advisory Panel -, European Pressure Ulcer Advisory Panel, and Pan-Pacific Pressure Injury Alliance, 2014) **(Figure 5).**

In the early stages, the patient has an intact nervous system, so the pressure is painful (stages 1 and 2), Once the epithelium is destroyed the ulcer may be relatively painless and can progress rapidly (stages 3 and 4) (Grey et al., 2006). The prevalence of high-grade pressure ulcers (grades 3 and 4) may reach 4% in elderly persons (Anders et al., 2010). Pressure ulcers may be prevented by changing patient position frequently (Nageswaran et al., 2015).

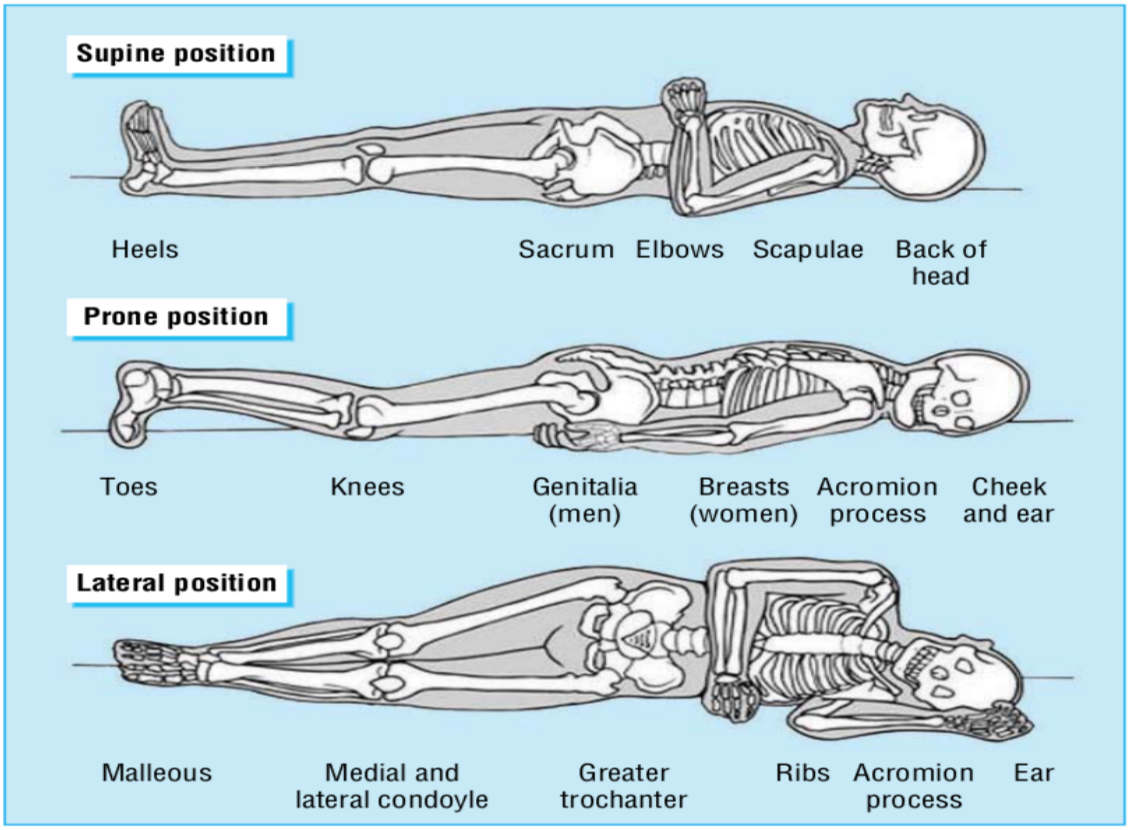


Figure 3 Common sites of pressure ulcer

Figure showing most bony prominences where skin ulcers most likely occur adapted from (Grey et al., 2006).

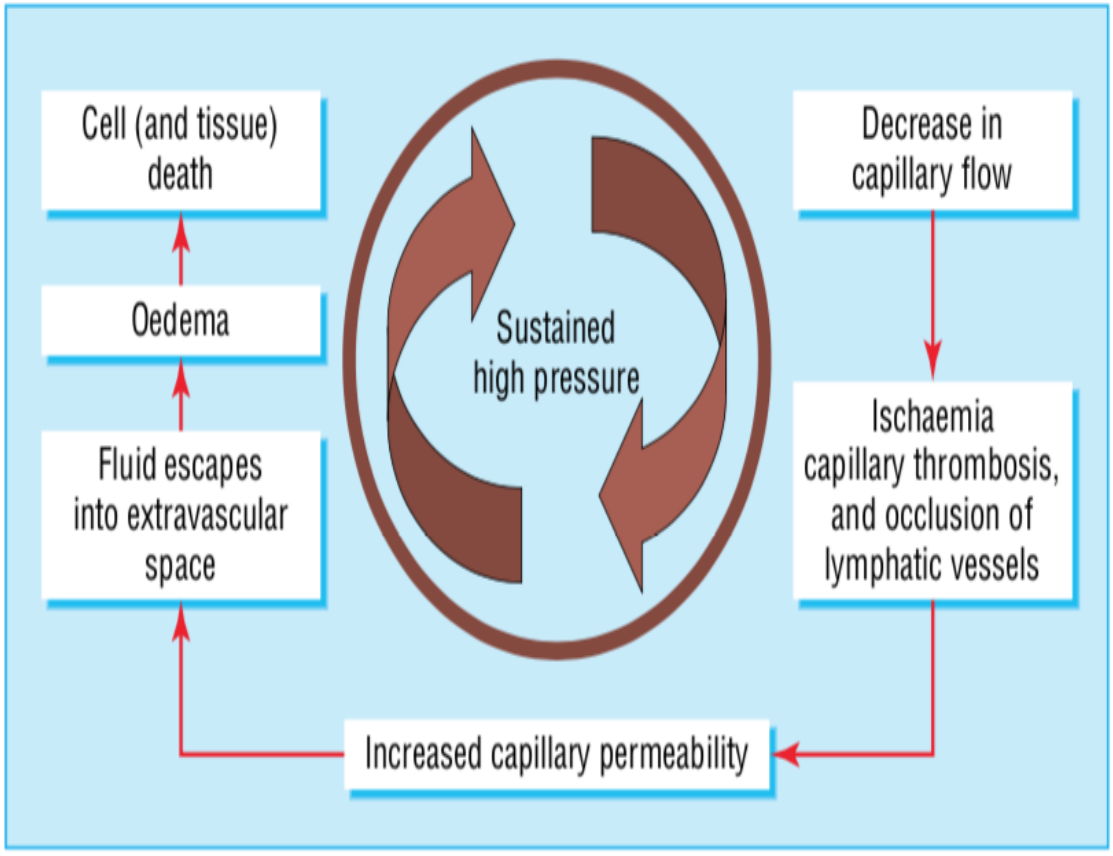


Figure 4 Pathophysiology of Pressure Ulcer

Diagram showing pathophysiology of pressure ulcer, sustained pressure leads to decrease of blood flow, resulting in ischemia and fluid escapes to extravascular spaces leading to edema and tissue death adapted from (Grey et al., 2006).

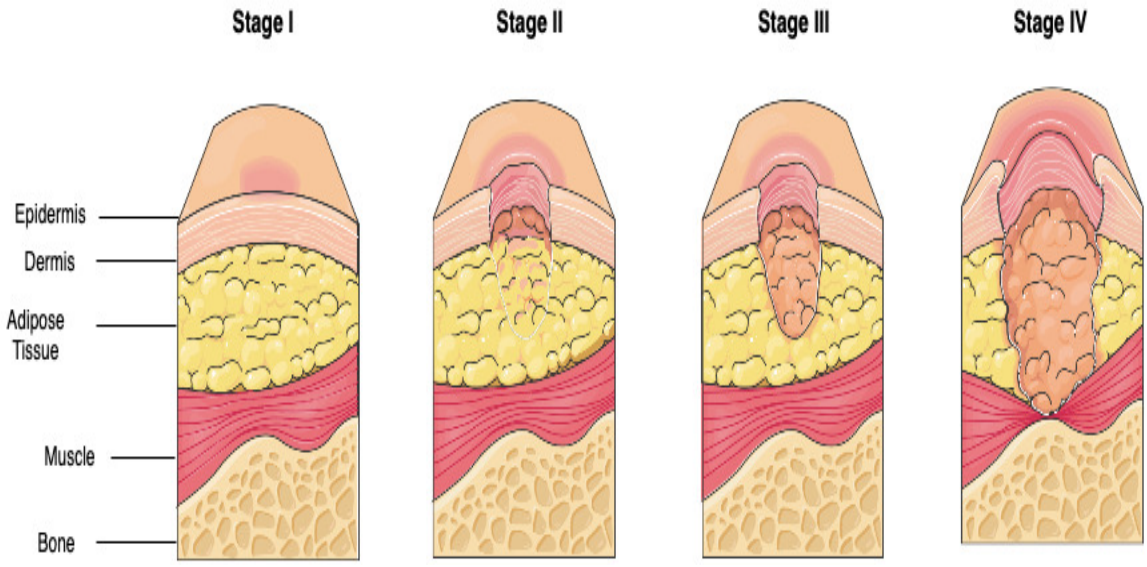


Figure 5 Stages of Pressure Ulcer

Stages of Pressure Ulcers according to NPUAP where; **Stage 1** Show intact skin non-blanchable erythema. **Stage 2** Partial-thickness skin loss with exposed dermis. **Stage 3** Full-thickness skin loss with exposed adipose tissue. **Stage 4** Full-thickness skin and tissue loss with exposed muscles, tendons. Adapted from (http://smart.servier.com/) with modifications.

1.4.3 Other types of Ulcers

Venous Leg Ulcer arise from chronic venous insufficiency in the lower limbs (Guest et al., 2018; Comerota et al., 2015). It causes a local rise in blood pressure, leading to leakage of macromolecules and red blood cells into the perivascular space. Subsequent edema and fibrosis decrease growth factors and oxygen diffusion, therefore, causing tissue ischemia (Guenin-Macé et al., 2014; Morton & Phillips, 2016).

Arterial Ulcers are less common than venous and diabetic ulcers, they occur because of arterial insufficiency and poor perfusion, leading to insufficient skin oxygenation, and tissue breakdown (Guenin-Macé et al., 2014).

Other types of skin ulceration include trauma, burn, and immune dysfunction, (Guenin-Macé et al., 2014; Tomioka et al., 2018).

1.5 Skin Ulcer Management

Skin ulcer management and prevention remain a challenge for healthcare (Sibbald et al., 2012). Preventive actions include; risk assessment, patient mobility and nutrition, skin care and regular pressure redistribution (Langemo et al., 2015). Once the skin is damaged action plans should take place, these might include;

1.5.1 Debridement

Debridement and removal of necrotic tissues to clean the wound and decrease infection (Leaper et al., 2011; Burtis et al., 2009).This can be achieved by surgical/ mechanical, or biological methods (Woo et al., 2015; Falabella, 2006). Biological debridement involve enzymes, although it may cause inflammation and slowing of the healing process (Falabella, 2006). Surgical or mechanical debridement are non-selective, and remove viable as well as necrotic tissues (Falabella, 2006). Other types of debridement, include Maggot debridement which involves larvae to remove only necrotic tissues (Sherman, 2009).

1.5.2 Antimicrobials

Antiseptics and topical or systemic antibiotics are the first line of treatment of skin ulcers to prevent infection, choice of antibiotic line should depend on culture and sensitivity results (Norman et al., 2016; Tsourdi et al., 2013). Extensive use of antibiotics in developing countries leads to antimicrobial resistance, and increases the risk of more dangerous types and resistant infections (Ayukekbong et al., 2017).

1.5.3 Topical preparations and Wound Dressings

Topical Preparations: including antimicrobials, antioxidants, growth factors and analgesics are used for wound care (S. Gupta et al.,2017). Although, easy to prepare and low cost, there are not convenient due to the need for multiple applications, and wound cover or dressing to protect the wound surface (Lipsky et al., 2009).

Wound Dressings have been developed to protect the wounds and accelerate healing (Han et al., 2017). Choice of dressing depends on the type and location of the wound and the quantity of exudates (S. Gupta et al., 2017). The table below shows examples of commercially available dressings with their advantages and disadvantages **(Table 1)**

Table 1: Examples of commercially available dressings used in wound treatment

Dressing Type	Commercial Examples	Comments	References
Gauze	Vaseline Gauze	• Inexpensive. • Cause drying. • May cause further damage on changing. • Need to be changed frequently.	(Han et al., 2017) (S. Gupta et al., 2017.)
Films	Bioclusive ® Blisterfilm® Tegaderm®	• Occlusive & Retains moisture. • For non-exudative wounds as it doesn't absorb exudates. • Protect against bacteria.	(Han et al., 2017)
Hydrogels	Nu-gel® Kikgel® Aqua-gel® Aquaform®	• Inexpensive. • Can be amorphous or sheets. • Permeable to Oxygen. • Protect from bacteria. • Have low tendency to absorb exudates. Useful for dry wounds. • Help in autolytic debridement.	(S. Gupta et al., 2017.) (Health Quality Ontario, 2009) (Sweeney et al., 2012) (Boatenget al., 2015)
Hydrocolloids	Aquacel ® DuoDERM® Tegasorb®	• Used for dry wound. • Occlusive, not used with exudative wound. • Not for infected wounds.	(Han et al., 2017) (Sweeney et al., 2012)
Foams	3M Adhesive Foam® Lyofoam®	• Synthetic polymers made of polyurethane (May cause allergy) and silicone. • They have absorptive capacity. • Protect against bacteria. • They minimize trauma during dressing changes.	(Han et al., 2017) (Sweeney et al., 2012)
Hydro fibers	Aquacel®	• Made of sodium carboxymethyl cellulose (CMC). • Have high tendency to absorb exudates. • Used for infected wounds. • They are inert dressing don't involve in the healing process. • Expensive Dressings.	(Sweeney et al., 2012) (Han et al., 2017)
Alginates	Algisite® Kaltostat®	• Do not adhere to the wound. • Highly absorbent. • Hemostatic. • Some patients may feel burning sensation this can be due to the rapid movement of fluid from wound into the dressing. • It doesn't protect against bacteria. • Not suitable for dry wounds.	(Han et al., 2017) (Sweeney et al., 2012) (Health Quality Ontario, 2009)

1.5.4 Skin Grafts & Substitutes

Autologous, full or partial thickness skin grafts have been used for non-healing chronic wounds (Han et al., 2017; Serena et al., 2015).

Recently, tissue-engineered skin substitutes gained importance due to their advantages, such as biodegradability, and their promotion of tissues growth, therefore, increasing healing rate and improving patient care (Boateng et al., 2015; Maarof et al., 2016). Some of those substitutes include; Omnigraft accelerates healing in diabetic foot and burns (Han et al., 2017). The main disadvantage of these scaffolds is their high cost (Han et al., 2017).

1.5.5 Growth Factors

Growth factors, such as, PDGF, fibroblast growth factor (FGF) and epidermal growth factor (EGF), are promising therapeutic agents in wound healing (Loh et al., 2013).

Several forms of growth factors have been studied in different types of chronic skin ulcers, with variable success, in addition to, their low bioavailability (Barrientos et al., 2014; Sweeney et al., 2012).

1.5.6 Vacuum Assisted Closure (VAC)

Vacuum Assisted Closure (VAC), or negative pressure is an effective, noninvasive adjunctive therapy that has been in use since 1997 to accelerate chronic skin ulcer healing (Han et al., 2017; Nain et al., 2011). Vacuum increases blood flow, enhances oxygenation, cellular growth and tissue repair (Schreiber, 2016), as well as enhancing wound contraction (Huang et al., 2014).

These devices limit the patients' mobility and are noisy. Changing the dressing and tube is usually painful and cause bleeding. VAC devices are also not recommended for cancer patients, spinal cord injuries or patients on anticoagulants (Schreiber, 2016).

1.5.7 Hyperbaric Oxygen therapy (HBOT)

High pressure aims to increase the oxygen concentration in the patient's blood and therefore, improving the oxygen supply to the wound. Although HBOT showed improvement in wound healing, there are still some doubts, and the high oxygen pressure may be harmful to the brain (Tuk et al., 2014; Van Neck et al., 2017; Han et al., 2017).

1.6 Phytomedicine in treatment of skin ulcers

Natural medicinal plant extracts have been widely used as topical applications for wound healing. *Aloe vera*, *Echinacea* , Chamomile, Ginseng, Ginkgo, Green tea and olive oil, as well as, many other plants were found to be effective in wound healing (Pazyar et al., 2014). Several research groups across the world showed that phytochemicals available in natural herbs might help in treating inflammatory conditions and might aid in wound healing and skin regeneration (Thangapazham et al., 2016). Phytochemicals protect the skin by suppressing free radicals and inhibition of nuclear factor kappa B (NF-κB) leading to reducing inflammation. Phytochemicals also affect other signaling pathways, including transforming growth factor-beta (*TGF*-β). Extensive research is needed to clarify the molecular targets and mechanisms of phytochemicals will lead to the development of effective formulations (Shah & Amini-Nik, 2017). In the current study, we will discuss how turmeric extract and oregano essential oil will help in wound healing.

Turmeric (the golden spice), is derived from the rhizome of *Curcuma longa.* Turmeric has shown antioxidant, anti-inflammatory, anticancer, antidepressant, anti-aging, antidiabetic, antimicrobial, wound healing effect **(Figure 6)** (S. C. Gupta et al., 2012). The main component of turmeric is curcumin, which was thought to be responsible for all biological activities. Recent studies identified new compounds other than curcumin. It became unclear that those activities are through curcumin or due to other compounds or synergistically of both. Those compounds showed to have anti-inflammatory and anticancer effect **(Figure 7)** (Aggarwal et al., 2013).

Curcumin was found to be effective in wound healing in diabetic and non-diabetic animal models and in animals subjected to γ-radiation (Jagetia et al., 2004) (Mani et al., 2008). Phan *et al.* (2001), showed that curcumin has inhibitory activity against hydrogen peroxide-induced oxidative damage in human keratinocytes and fibroblasts. Cheppudira *et al.* (2013), showed that curcumin could be a potential natural therapy to control severe pain associated with burn.

Turmeric contain different proteases that might aid in stopping bleeding (Shivalingu et al., 2015). Sarafian et al. (2015), proposed that turmeric micro-emulgel

may be considered a therapeutic option for many patients suffering from plaque psoriasis. Other research showed that turmeric extracts has a potential therapeutic role in spinal cord injuries (Kamel et al., 2017). Meizarini *et al*. (2018), showed that wound dressing consisting of a combination of zinc oxide and turmeric extract proved to be effective as an anti-inflammatory. Lone et al. (2018), showed that turmeric extract accelerated healing of dry socket alveolar osteitis following tooth extraction.

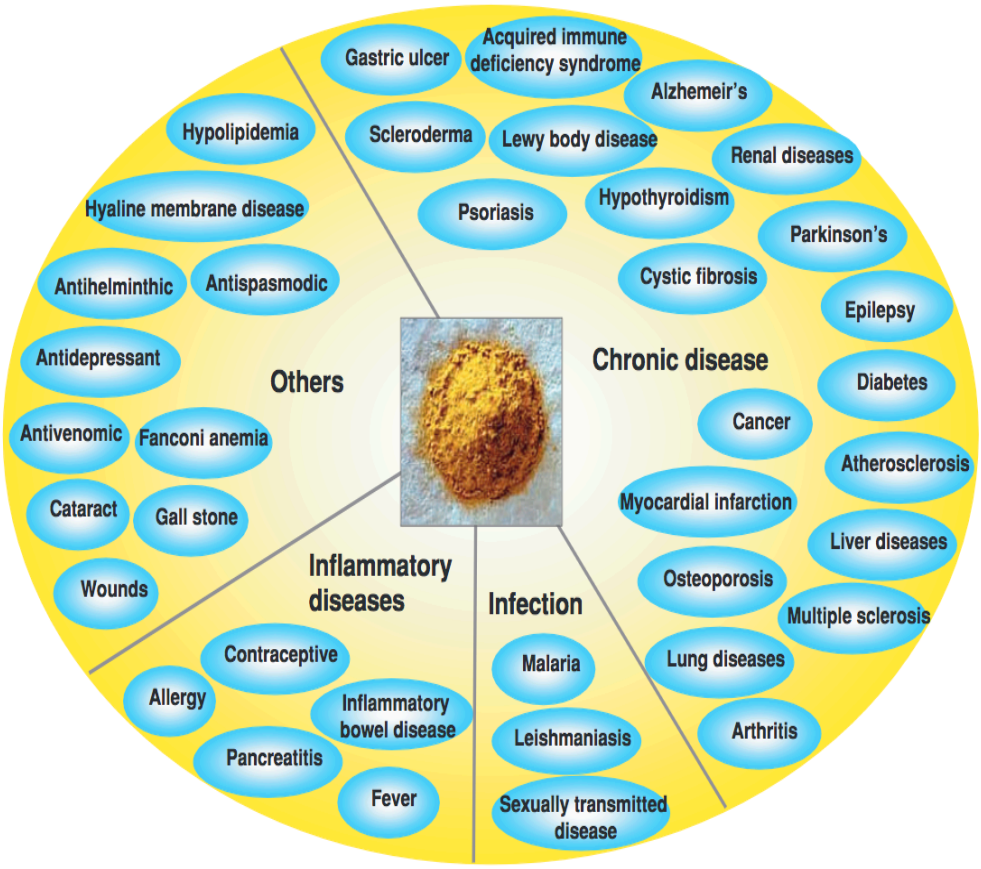


Figure 6 Medicinal Uses of Turmeric

Turmeric was found to be helpful in treatment of chronic diseases, such as psoriasis, inflammatory diseases as inflammatory bowel disease, and also proved to be helpful in wound healing adapted from (Aggarwal et al., 2007). Its low toxicity and side effects and its availability in large quantities in cheap prices make it a suitable therapeutic agent.

Curcumin has anti-inflammatory and anti-oxidative properties therefore, it may affect many molecular targets involved in inflammation, oxidative stress. It inhibits arachidonic acid metabolism, and downregulate enzymes as lipoxygenase (LOX), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS). As a result, it blocks the synthesis of prostaglandin (PG), and cytokines, e.g. interleukin (IL), and tumor necrosis factor (TNF). Curcumin also promotes the release of steroidal hormones from the adrenals. Further studies are needed to reveal the molecular targets of different Turmeric extract components **(Figure 8)** (Rao et al., 2007).

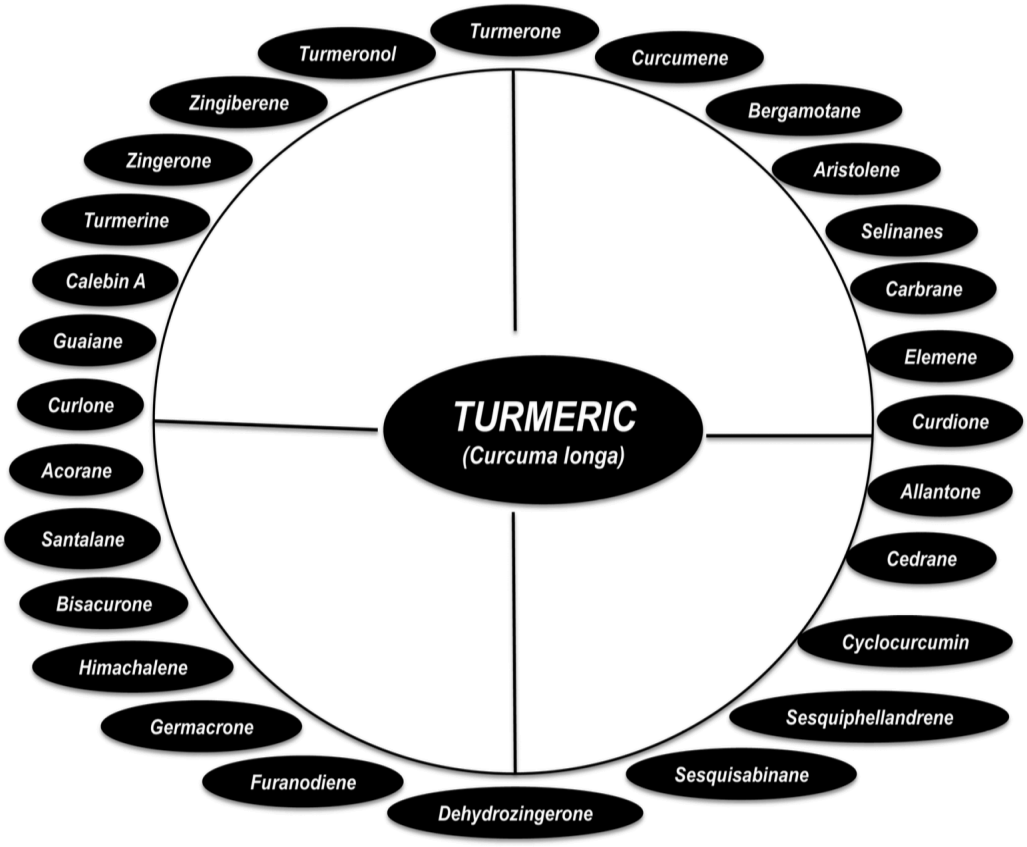


Figure 7 Non- Curcumin Components of Turmeric

Turmeric has a range of constituents other than curcumin that exert anti-inflammatory effect and thus help in wound healing adapted from (Aggarwal et al., 2013).

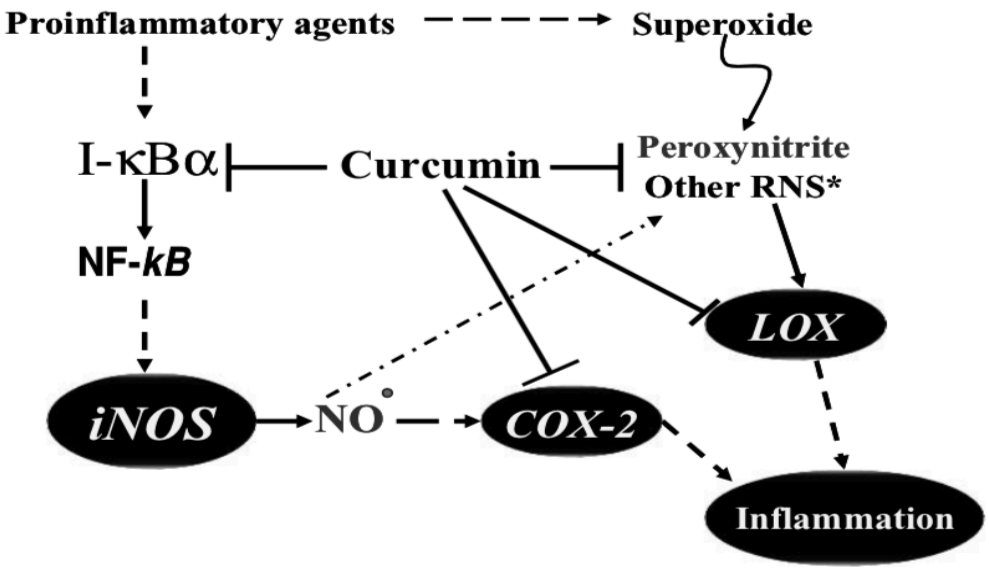


Figure 8 Anti-inflammatory & Anti-oxidant Mechanism of Curcumin

Curcumin suppresses inhibitory unit Iκ-Bα, which hinders subsequent nuclear translocation of the functionally active subunit of NF-κB, inhibiting the inducible nitric oxide synthase (iNOS) as a result inhibit nitric oxide leading to decreased expression of cyclooxygenase 2 (COX2) suppressing inflammation. Curcumin also inhibits lipoxygenases (LOX) resulting in suppressing leukotriene, which are inflammatory mediators. Adapted from (Rao et al., 2007).

Oregano is essential oil obtained from leaves of *Origanum vulgare* family *Lamiaceae* (Olmedo et al.,2014). The main components of oregano are the phenols isomers carvacrol and thymol, as well as their precursor monoterpenes γ-terpinene and p-cymene (Sakkas et al., 2017). Oregano shows antimicrobial, antifungal, anti-inflammatory and antioxidant effects (Ragi et al., 2011; Chun et al., 2005; Rosato et al., 2009). The antimicrobial effect was found to be at concentration less than 2% (Boateng et al., 2015).

1.7 Nanomaterial in Wound Care

Nanomaterial reduce drug toxicity, enhance solubility of hydrophobic drugs, increase drug penetration, provide controlled release of the drugs, increase their stability and protect them from being degraded (Goyal et al., 2016 ; Wang et al., 2011).

Nanofibers are considered one of the dressings that offer great advantages over current dressings. Electrospun nanofibers possess high surface area, porosity and have structure that mimic the ECM (Chen et al., 2017). Current research focuses on reducing inflammation and infection, therefore, creating positive environment for wound healing (Chen et al., 2017). Recent uses of nanofibers for wound healing include; loading growth factors to promote angiogenesis and accelerate wound healing rate, polyaniline-chitosan nanofibers to enhance cell attachment and proliferation, silver nanoparticles (AgNPs) incorporated in collagen nanofibers to decrease wound infection rates and accelerated closure (Moutsatsou et al., 2017; Rath et al., 2016; Xie et al., 2013).

Nanoparticles used for wound-healing applications are often made of polymers that have been used previously as wound dressings (Kalashnikova et al., 2015). According to literature there are different means for nanoparticles absorption through skin **(Figure 9)** (Palmer et al., 2016).

Chitosan is considered one of the most widely used biopolymers for nanoparticles preparation. This is due to its biodegradability, biocompatibility, low toxicity (Kamat et al., 2016). Chitosan has adhesive character the advantage that make them promising and wound healing agents this is beside their antibacterial and antifungal effect (Katas et al. 2013; Wang et al., 2011). Chitosan nanoparticles (ChNP) are synthesized using non-toxic solvents as they are soluble in acidic medium (Agnihotri et al., 2004). It was found that ChNP have higher antibacterial activity than chitosan and chitin. This is due to the spherical character of NP, and the positive charge of ChNP interact with the negatively charged surface of bacteria resulting in membrane disruption, leakage of intracellular components and cell death (Divya et al., 2017). All these benefits make them promising nanocarrier for drug delivery (Prabaharan, 2015). Chitosan nanoparticles were loaded on calcium alginate hydrogel to reduce inflammation and improve neovascularization (T. Wang et al., 2017), and were incorporated in polycaprolactone nanofibers to improve wound healing (Jung et al., 2015).

From our point of view, the only limitation of nanomaterials either nanofibers or nanoparticles is the difficulty of large-scale commercial production. More research is needed to move nanomaterials for wound healing from laboratory to the market (Zafar et al., 2016; Chen et al., 2017).

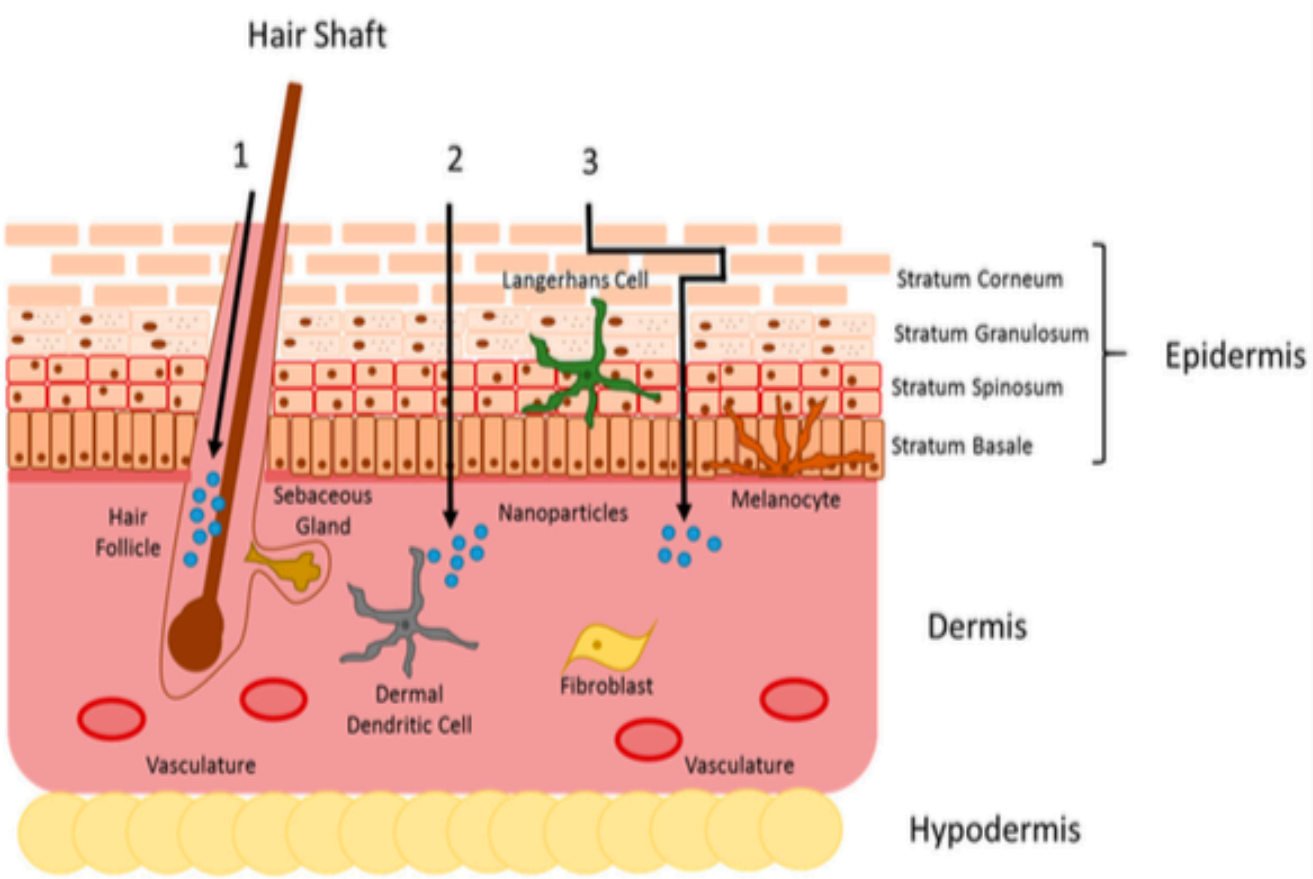


Figure 9 Mechanisms of Nanoparticle penetration through skin

Based on particle size, charge, morphology and polymer type, nanoparticles can penetrate skin through the **1) Appendageal route**, as hair follicles, sweat glands. **2) Intracellular route** through corneocytes **3) Intercellular route** were particles pass between corneocytes. Adapted from (Palmer et al., 2016).

1.8 Wound healing Models

Wound healing models are essential to study the pathogenesis of wound healing and to identify molecular targets, as well as, to test new therapeutic approaches (Sami et al., 2019; Ud-Din & Bayat, 2017). Wound models can be designed *in silico*, *in vitro*, *ex vivo* and *in vivo* using computational, cell culture, wound biopsies, and animal models (Wilhelm et al., 2017; Andrade et al., 2015; O'Dea et al., 2012). *In vitro* models are used to test new drugs, and scaffolds but do not provide a clear view of the biological interactions in a living organism, as they lack innervation and circulation that play

critical role in the healing process (Andrade et al., 2015; Wilhelm et al., 2017; Ud-Din & Bayat, 2017).

In vivo models are the most efficient wound healing models. They can either be human, small or large animals. Human model has the advantage of testing new drugs in clinical trials, their disadvantages include; the difficulty to obtain patients with chronic wounds and the lack of uniformity of the wound type and microbial composition (Ud-Din & Bayat, 2017). Animal models offer the best alternative. Large animals such as pigs have great advantages, as they have skin type similar to the human skin, and the main method of healing is through re-epithelization (Volk et al., 2013), although some wound contraction occurs in some sites. The disadvantage of using pigs is that they have significant high cost, and therefore hard to do replicates that provide statistical significance (R. Perez et al., 2008).

Rodents are the most commonly used animals for wound healing models due to their availability, low cost and small size, which makes it easy to include a relatively large number of animals, which provides statistical significance and decreases error (Trostrup et al., 2016). A great disadvantage of rodent use for chronic wounds is that they have unique panniculus carnosus layer, which causes rapid wound contraction (Wong et al., 2011), in addition to the significant differences in their immune systems compared to humans, which limits the usefulness of these models (Seaton et al., 2015).

In the current study, we describe a new model that mimics chronic pressure ulcer in human to address the shortcomings of other rodent wound models and overcome wound contraction due to the panniculus carnosus muscle.

Infected and chronic wounds are difficult to treat especially in diabetic patients due to bacterial resistance and excessive use of antibiotics. We describe a novel combination of antibacterial agents from Turmeric/ Oregano/ Chitosan nanoparticles.

1.9 Hypothesis

We hypothesize that our designed formula will be non-cytotoxic, anti-inflammatory, antioxidant, will clear wound infection, and improve wound healing, in diabetic animal model.

1.10 Objectives

Our objectives in the current study are;

1. Develop a deep pressure ulcer model in a diabetic animal and highlight the difference between excisional wound and pressure ulcer healing process in normal and diabetic animals.
2. Design a cost-effective formulations (ointment/amorphous hydrogel/ Nanofibers), for chronic skin ulcers using turmeric, oregano and chitosan nanoparticles.
3. *In vitro* and *in vivo* testing of the newly designed formulations, for cytotoxicity/ antibacterial, and wound healing efficacy.

Chapter 2 : Materials & Methods

Materials

Streptozotocin (Sigma- Aldrich, USA). Ketamine and Xylazine Hydrochloride, Ketoprofen, magnets (3 g weight, 15 mm diameter and 250 Gauss magnetic force). Citric acid, Sodium citrate buffer (Al-Nasr Chemicals, Egypt), 5% glucose solution (Al-Nasr Chemicals, Egypt). Chitosan Molecular weight 600,000 – 800,000 (Acros Organics, Belgium), Tripolyphosphate (TPP; Mistral chemicals UK). Glucometer (Free Style, Abbott, USA9), 99% acetic acid (Sigma- Aldrich, USA), Sodium Hydroxide (Al Nasr Chemicals), Deionized water (DI), Turmeric ethanolic extract (Herb pharm, USA), Turmeric CO_2 Organic Oil Extract (plant Therapy, USA), and Oregano Essential Oil (plant Therapy, USA), Difco Nutrient Broth, Difco Nutrient Agar (Thermo Fisher, German), *Staphylococcus aureus (S. aureus)*, *Escherichia coli* (*E. coli)*, petrolatum (Eva cosmetics), Polyvinyl Alcohol (PVA) "Mowiol 20-98" Mol. Wt = 125,000 (Sigma Aldrich, Germany). Dulbecco's Modified Eagles Media with L-glutamine (DMEM), Alkaline Phosphate buffer (PBS), trypsin & Pen/Strep (Lonza, Belgium), Dimethyl sulfoxide (DMSO; Sigma Aldrich), Acetic acid (Sigma Aldrich), Fetal Bovine Serum (FBS) (Life Science group, UK), Thiazolyl blue tetrazolium bromide (MTT; Serva Electrophores, Germany), Tegaderm patches (3M) FDA approved patches for diabetic foot ulcers (U.S. FDA Resources).

Methods

2.1 Development of Pressure Ulcer in a Diabetic Animal Model

Animal handling: Male Sprague-Dawley rats weighing ≈150-200 grams, were housed under standard 12 h light/ 12 h dark conditions with free access to water and food. All procedures were performed in compliance with the national institute of health (NIH) guidelines for the Care and Use of Laboratory Animals, and in compliance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC) of the October University for Modern Sciences and Arts (MSA) ethical committee.

Rats were anesthetized with intraperitoneal (I.P) injection of ketamine/Xylazine (ketamine 80-100 mg/kg, xylazine 10-12.5 mg/kg IP mg/kg), according to IACUC guidelines. Hair was clipped and rat skin was cleaned using Betadine®. Rats were

randomly divided into either control or experimental groups. The **first group** received a full thickness excisional wound (12-15 mm^2) extending through the panniculus carnosus using surgical scissors. In **second group** a sterilized magnet (3 g weight, 15 mm diameter and 250 Gauss magnetic force) was inserted deep to the panniculus carnosus muscle layer. An external magnet of the same dimensions was placed on the skin surface. Both magnets were kept until necrosis of the sandwiched layer of skin and both magnets fell producing an ulcer extending to the subcutaneous tissue.

A **third group** of animals were intraperitoneally injected with streptozocin (STZ) to induce diabetes according to the method described by (Furman et al., 2015) with modifications. After confirmation of diabetes, these animals also received a skin ulcer using the method described above.

Macroscopic clinical assessment and photographs of the wounds were performed at the time of surgery and on subsequent days. Wound evaluation using ImageJ® software was performed at regular time intervals. Parameters such as; depth, granulation tissue, and infection were assessed using scoring system adapted from (Martínez-De Jesús et al., 2010; Perez et al., 2010; Y. Huang et al., 2015; Strauss et al., 2016), with modifications. Each parameter received a score (zero= Healed, 1 minimal, 2 mild, 3 moderate, and 4 Severe).

Histopathological sections stained with hematoxylin and eosin (H&E) and masson trichrome stain, slides were examined and photographed using a BX51 light microscope (Olympus xc 30, Tokyo, Japan) and scored according to the scoring system originally described by (Gal et al., 2008).

2.2 Chitosan Nanoparticles

2.2.1 Preparation of Chitosan solution

Chitosan solution of concentrations (0.05%, 0.1%, 0.2%, 0.3% (w/v)) were prepared by stirring chitosan in an aqueous acetic acid solution (1% (v/v)) overnight at room temperature. The pH of Chitosan solution was adjusted to 4.8 using 1 N NaOH (Kheiri et al., 2017).

2.2.2 Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared through ionotropic gelation as described by (Calvo et al.,1997). Briefly, tripolyphosphate (TPP) solution of concentration 1 mg/ml was added dropwise to chitosan solution at ratio TPP : Chitosan (1:3) under vigorous magnetic stirring at room temperature 900 rpm for 30 minutes (Kheiri et al., 2017). Opalescent solution was formed as indicator for nanoparticles formation. Nanoparticles were collected by centrifugation (Eppendorf 5804 R) for two hours at speed 11,000 rpm and temperature 4^{o}C. Supernatants were discarded, and the particles were washed twice with deionized water and centrifuged for 15 minutes in each cycle. Nanoparticles (NPs) were finally re-dispersed in deionized water. Particles were kept in ice and sonicated for 3 minutes using probe sonicator (Branson Sonifier 150). Finally, particles were lyophilized (Biobase BK-FD10S) and stored at -20^{o}C for further use.

2.2.3 Nanoparticles Characterization

2.2.3.1 Hydrodynamic Size, Zeta Potential & Polydispersity Index

Nanoparticles (NPs) suspension was diluted with de-ionized (DI) water to a concentration 1 mg/ml before analysis. The hydrodynamic diameter (HD) and zeta potential (ZP), polydispersity index (PDI) were measured using a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). All samples were analyzed in triplicates at 25°C and results were expressed as mean ± standard deviation.

2.2.3.2 Morphological Assessment

Primary morphological analysis was performed visually using inverted light microscope (Olympus 1X70).

For advanced nanoparticles morphological analysis, a diluted drop of NP suspension (2 mg/ml) was spread on a glass slide and allowed to dry overnight at room temperature. Prior to imaging samples were coated with a fine gold layer using a gold sputter module for 90 seconds at 10 KV (JEOL JFC-1600 Auto fine coater, Japan). Samples were examined using scanning electron microscopy (SEM: FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany).

2.3 Antibacterial Test

Serial dilutions were used to evaluate the antibacterial activity of the chitosan nanoparticles & herbal extracts (turmeric ethanolic extract, turmeric CO_2 organic oil extract, and oregano essential oil), against gram positive and gram-negative bacterial strains, *Staphylococcus aureus* and *Escherichia coli* respectively. The concentration of chitosan nanoparticles tested were 5 mg/ml (0.5%), 10 mg/ml (1%), while the concentrations of extracts used were 0.1%, 0.5%, 1%, 5% & 10% of each extract.

Each bacterial strain was added to nutrient broth and kept overnight in shaker incubator (Innova 43). The optical density (OD) was adjusted to of 0.1 at wavelength of 625 nm using spectrophotometer (Ultrospec 3100 pro). Extracts and nanoparticles were sterilized under UV for 1.5 hours, samples were added to bacteria and incubated in shaker incubator overnight (Innova 43). The samples as well as the controls were serially diluted and spread on nutrient agar, which were then incubated overnight at 37°C. The experiment was performed three times in triplicates & the surviving colonies were counted and compared to the control. Results were expressed in % bacterial reduction according to the below equation;

% bacterial reduction = (1-T/C) *100

Where T is cfu/mL of test sample and C is cfu/ml of control.

2.4 Anti -Oxidant Assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay

The concept of this test depends on the reduction of the yellow tetrazolium salt to the purple crystals of formazan by antioxidant compounds (Muraina et al., 2009). Briefly; Tegaderm®, oregano, turmeric essential oil, and turmeric ethanolic extracts, were dissolved either in DMSO or DMEM to a final concentration of 1%, 5%, while chitosan nanoparticles were suspended at concentrations of 0.5%, 1% and incubated at 37°C in shaker incubator overnight. Herbal extracts and chitosan nanoparticles were placed in 96 well plate (Grenier Bio). 20 µl of 5 mg/ml of MTT was added to each well and the plate was incubated for 3 h at 37°C. Media were removed and DMSO was added to solubilize the formazan blue crystals. The optical absorbance was measured at 570 nm using a plate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany).

2.5 Ointment Formulation

Petrolatum was used as a vehicle to carry herbal extracts. Briefly; oregano, turmeric ethanolic extracts were levigated with petrolatum to form an ointment, concentrations were 1%, 5% respectively. Ointment preparation was stored at room temperature until use.

2.6 *In vivo* Pilot study

A pilot study to assess healing efficacy of turmeric and oregano was performed on pressure ulcer in diabetic and non-diabetic rats. Briefly, 5% turmeric ointment and 1% of oregano ointment was applied once a week. Macroscopic examination and scoring were done to determine their effect on the healing process. Parameters such as ulcer area, depth, granulation and inflammation, granulation and infection were investigated with the following scoring system originally described by Martínez-De Jesús, 2010, and Gupta & Kumar, 2015, with modifications: 0 = Healed; 1= minimal; 2= mild; 3= moderate; and 4= Severe.

2.7 Formulation Preparation

2.7.1 Ointment

Petrolatum was used as a vehicle to carry herbal extracts and chitosan nanoparticles. Briefly, oregano, turmeric ethanolic extracts and chitosan nanoparticles were levigated with petrolatum to form concentrations 1%, 5% and 1% of each item respectively.

2.7.2 Amorphous Hydrogel

Polyvinyl alcohol (PVA) of concentration 12% was prepared by dissolving PVA in deionized water in the autoclave at temperature 121^{o} C for 15 minutes. After cooling, herbal extracts of oregano, turmeric and chitosan nanoparticles were added to reach final concentrations of 1%, 5% and 1% respectively. The mixture was stirred at room temperature for 1 hour. The solution was filled in syringes and stored at room temperature for further use.

2.7.3 Hydrogel Sheet

The amorphous hydrogel was poured in a petri dish and physically cross-linked through repeated cycles of freeze-thawing (He et al., 2018; Kamoun et al., 2015). The plate was stored in -50^{o} C overnight, followed by 8 cycles of freeze-thawing were applied (1 hour freezing at -50^{o} C followed by thawing at room temperature for 30 minutes).

2.7.4 Xerogel

The hydrogel sheet was freeze dried (Biobase BK-FD10S) for 10 hours to obtain Xerogel (Niknia & Kadkhodaee, 2017).

2.7.5 Nanofibers preparation

Polyvinyl alcohol of concentration 12% was prepared by dissolving PVA in DI water for 3 hours at temperature 90^{o}C. After cooling, herbal extracts added to a final concentration of turmeric 5%, chitosan nanoparticles 1% and oregano 1%, and stirred for 1 hour at room temperature. The polymer was then finally electrospun by applying voltage 14, 16 & 18 KV and flow rate 0.8 ml/hr, 1.1 ml/hr & spinning distance 12 cm, needle diameter was 21G.

2.8 Nanofibers Characterization

The electrospun fibers were characterized morphologically using (SEM: FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany). Prior to imaging, samples were coated with fine gold layer using a gold sputter module for 90 seconds at 10 KV (JEOL JFC-1600 Auto fine coater, Japan). The fibers were checked for beads & the average nanofiber diameters were determined using ImageJ® software. For each of the developed nanofibrous mats, 100 fibers were measured, and the average diameter was calculated.

2.9 Antibacterial Test for the designed Formulas

The antibacterial activity of the preparations, using Tegaderm® as positive control was evaluated against both gram positive and gram-negative bacterial strains, *Staphylococcus aureus* and *Escherichia coli* respectively. Each strain was added to nutrient broth and kept overnight in shaker incubator (Innova 43). The optical density (OD) was adjusted to of 0.1 at wavelength of 625 nm using spectrophotometer (Ultrospec 3100 pro). The preparations were sterilized in ultraviolet radiation (UV) for 1.5 hours and incubated in shaker incubator overnight with the bacterial strains. The samples, as well as the controls were serially diluted and spread on nutrient agar which were then incubated at overnight at 37^{o}C. The experiment was performed in triplicates and the surviving colonies were counted and compared to the controls. Results were expressed in % bacterial reduction according to the following equation;

% bacterial reduction = (1-T/C) *100

Where T is cfu/mL of test sample and C is cfu/ml of control.

2.10 *In vitro* Biocompatibility

2.10.1 Cytotoxicity

Effects of the prepared formulas on cell viability was evaluated on of L929 cell line (ATCC®), using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The assay depends on the concept of reduction the yellow tetrazolium salt to the purple crystals of formazan, due to the effect of dehydrogenase enzymes that are released by the mitochondria of the living and active cells (Patravale et al., 2012). The number of viable cells present is directly proportional to the amount of purple formazan crystals developed.

Briefly, the prepared forms (ointment, hydrogel, nanofibers) as well as Tegaderm® (positive control) were sterilized by UV, and conditioned media were prepared by soaking different weights (10 mg, 20 mg) of the preparation in DMEM, containing , 5 % antibiotic Pen-Strept and 10% FBS for one day in shaker incubator (Innova 43) at 37° C.

Cells were seeded in clear 96-well plates (Grenier Bio) at a density of 5,000 cells/well and incubated 24 h to allow cell attachment. The conditioned media were filtered through 0.2µm syringe filters and added to the cells. The cells were then incubated with the conditioned medium of each formulation using Tegaderm® as positive control & cells without any treatment as negative control for 24 hours. Following incubation, the medium was removed and replaced with new one, MTT prepared according to manufactures instructions (Serva Electrophores, Germany) was added and cells were incubated for 3 h. Finally, the medium was removed and DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The test was repeated eight times in triplicates for each preparation. Cell viability (%) was calculated based on the following equation:

$$Survival\ rate\ \% = (\text{Ab}\ sample_{570\ nm} - \text{Ab}\ blank_{570\ nm}) / (\text{Ab}\ control_{570\ nm} - \text{Ab}\ blank_{570\ nm}) \times 100$$

Where Ab sample is the sample absorbance, Ab blank is the absorbance of blank, Ab control is the absorbance of the control.

2.10.2 Cell Morphological Examination

Following incubation with treatment as described above, the cells morphology was examined using inverted microscope (Olympus 1X70).

2.11 In vivo Testing for the designed Preparations

2.11.1 Macroscopic Examination

In vivo study was done to assess healing efficacy of the designed formulations on diabetic pressure ulcer model (described previously) in comparison to Tegaderm® as positive control. All formulations were applied once a week. Macroscopic (clinical) examination was performed to evaluate wound healing.

2.11.2 Histological Examination

At days 10, and 15 of treatment, rats were euthanized with an overdose of pentobarbital (Thiopental sodium) 75 mg/kg, IP, and skin samples collected and fixed in 10% paraformaldehyde, paraffin embedded, and 5 µ sections were cut and stained with hematoxylin and eosin to assess inflammatory cells and granulation tissue, collagen deposition was assessed using masson trichrome staining (Olympus xc30. Tokyo. Japan).

Semi-quantitative method was used to evaluate following histological processes and structures: re-epithelization, polymorphonuclear leucocytes (PMNL), fibroblasts, new vessels, and new collagen. Sections were evaluated according to the scale: 0, 1, 2, 3, 4. Parameters for histological assessment were adapted from (Gal et al., 2008).

Table 2: The semi-quantitative evaluation of histological sections

(ST – Surrounding Tissue, i.e. tissue out of GT; DL – Demarcation Line; SCT – Subcutaneous Tissue; GT – Granulation Tissue).

Scale	Epithelization	PMNL	Fibroblasts	New vessels	Collagen
0	Thickness of cut edges	absent	absent	Absent	absent
1	Migration of cells (< 50%)	mild ST	mild ST	mild-SCT	minimal-GT
2	Migration of cells (≥ 50%)	mild DL/GT	mild-GT	mild-GT	mild-GT
3	Bridging the excision	moderate DL/GT	moderate-GT	moderate-GT	moderate-GT
4	keratinization	marked DL/GT	marked-GT	marked-GT	marked-GT

2.12 Statistical Analysis

The statistical analysis were done using GraphPad PRISM software version 8.02 (Graphpad Inc., San Diego, CA, USA). All data were expressed in mean ± standard deviation. The tests used to test significance were multiple t- test, one way analysis of variance test (ANOVA) & 2- way ANOVA. In all analysis, *P-value* < 0.05 was considered statistically significant. Post hoc tests were done to confirm where differences occurred between groups.

Chapter 3 : Results & Discussion

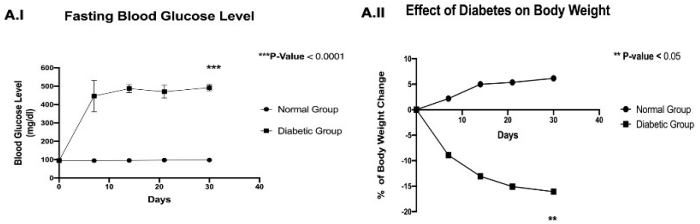
3.1 Development of Pressure Ulcer in a Diabetic Animal Model

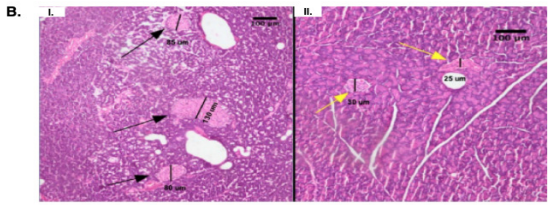
Based on our results, pressure ulcers showed significant differences compared to excisional wounds. The produced ulcer using the magnet method was a full thickness skin ulcer extending to the subcutaneous tissue, similar to an ulcer of the third degree that develops in human.

The pressure ulcer produced showed impaired healing and high infection rates, as well as the development of fibrous connective tissue that impairs the healing process. These ulcers provide an excellent model to study chronic wound healing. In diabetic rats the ulcers showed significant changes due to the diabetic pathological changes in the skin as well as impairment of the healing process **(Figure 10).**

Our conclusion was, that the diabetic pressure ulcer model is very useful to study the impaired healing process, and to study the effect of new therapeutics for chronic wounds (DG Sami et al, submitted 2019).

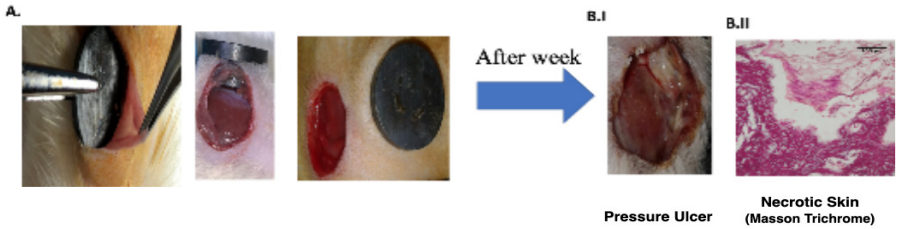
1. Diabetes Confirmation

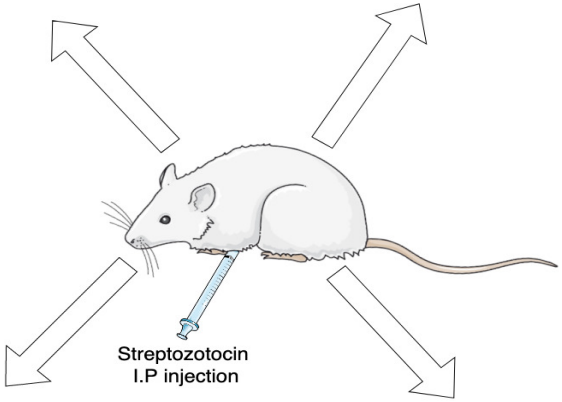




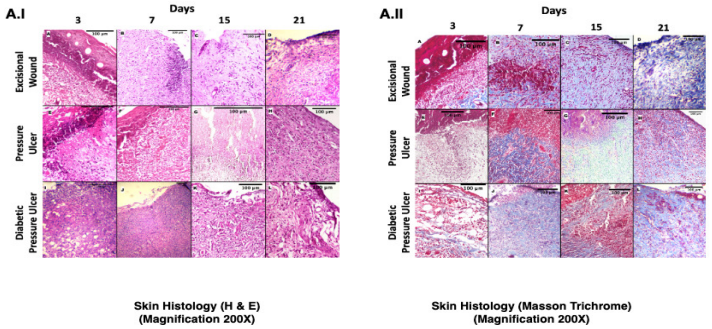
Pancreas Histology (H & E)

2. Development of Pressure Ulcer





4. Microscopic Examination



Skin Histology (H & E)
(Magnification 200X)

Skin Histology (Masson Trichrome)
(Magnification 200X)

3. Macroscopic Assessment

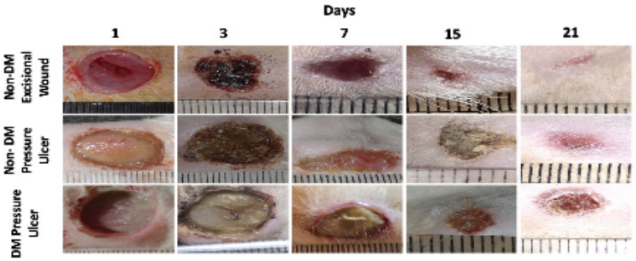


Figure 10 Graphical representation for Development Of Pressure Ulcer in Rodents

Rats were injected intraperitoneally with Streptozotocin. **1.) Diabetes was confirmed** through **A.I) Fasting Blood Glucose level** as diabetic group showed significant increase in blood glucose level if compared to the non-diabetic group that showed normal levels. **A.II) On Body Weight** diabetic group showed significant reduction in body weight if compared to the non-diabetic group. **B.) Hematoxylin & Eosin of Pancreas** showed destruction of islets of Langerhans in diabetic group while non-diabetic group showed normal islets **2.) Pressure Ulcer was developed** through implanting magnet below the panniculus carnosus muscle and another magnet was applied above the skin while the other side served as excisional wound in both non-diabetic & diabetic groups. **B.I)** After a week both magnets fell leaving pressure ulcer. **B.II) Masson Trichrome stain for the necrotic skin (sandwiched skin between magnets)** showed sloughing of epidermal layer and hyalinosis of dermal layer. **3.) Macroscopic Assessment** was done to compare between excisional wound & pressure ulcer in non-diabetic rats & Pressure ulcer in diabetic rats. Pressure Ulcer showed impaired healing if compared to excisional wound that was completely healed by day 21. Diabetic Pressure Ulcer didn't show healing by day 21.**4) Microscopic Examination of Skin Biopsies A.I) Hematoxylin & Eosin stain** to assess degree of inflammation between groups. **A.II) Masson Trichrome stain** to assess collagen formation between groups. (Adapted from DG Sami et al., 2019 submitted for Publication).

3.2 Chitosan Nanoparticles Characterization

3.2.1 Hydrodynamic Size, Zeta Potential & Polydispersity Index

The mean particle size for chitosan nanoparticles showed a significant increase with increasing concentrations of chitosan from 0.1% to 0.3% (p-value < 0.0001) **(Figure 11).** At a concentration of 0.05 %, chitosan particle size was very high (around 5000 nm). This might be due to insufficient chitosan to react with TPP (results not displayed). This is consistent with *Liu & Gao et al. (2008),* who reported that the size of chitosan nanoparticles depends on different factors one of them was chitosan concentration.

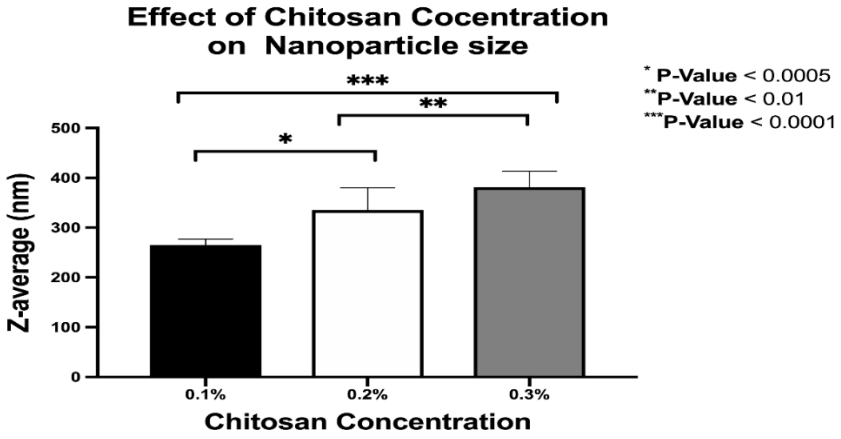


Figure 11 Chitosan Nanoparticles hydrodynamic average size

There is significant increase in average particle size with increase chitosan concentration from 0.1% to 0.3% (P-value < 0.0001; n= 3 in triplicates).

Zeta potential was measured to study the stability of particles. Particles with zeta potential greater than (>|30| mV) were considered stable, as particles exert electric repulsion to avoid its aggregation (Kheiri et al.,2017). In agreement with this concept, our particles formed at concentrations 0.1%, 0.2% & 0.3% showed high zeta potential suggesting their stability for longer period of time **(Table 3).**

Poly dispersity index (PDI) is an important parameter when assessing uniformity of nanoparticles in solution, larger PDI values indicates larger size distribution & may indicate also aggregation (Clayton et al., 2016). Samples with PDI less than 0.5 indicate narrow size distribution & high homogeneity (Al-Qushawi et al., 2016). Our results

showed that our samples are highly homogenous with PDI of 0.3 for concentrations 0.1%, 0.28 for concertation 0.2% & 0.26 for 0.3% **(Table 3).**

It is notable that the hydrodynamic diameter (*Z*-average) of the particles measured by dynamic light scattering (DLS) is higher than the size estimated from microscopy because of the high swelling capacity of chitosan. Therefore, microscopic evaluation through scanning electron microscopy (SEM) or transmission electron microscopy (TEM) is necessary to determine actual particle size and asses its morphology (Kheiri et al.,2017).

Table 3: Effect of Chitosan Concentration on Hydrodynamic Size, Zeta Potential & Polydispersity Index

Chitosan Conc. / **Parameter**	**0.1%**	**0.2%**	**0.3%**
Z- average (nm)	265 ±11	335 ±43	381 ±30
Zeta Potential (mv)	+51±3	+49±7	+54±2
PDI	0.3	0.28	0.26

According to literature, there is no specific particle size for skin penetration as nanoparticles can penetrate skin through various mechanisms (Zhang et al., 2013). Previous research showed that smaller particles showed more skin penetration compared to large particle size that was trapped on the surface in inflamed skin model (Try et al., 2016). Retinol encapsulated chitosan nanoparticles of sizes ranges from 50-200 nm were used for treatment of skin wrinkles, acne (Kim et al., 2006). Also, chitosan encapsulating acyclovir with average size 350-700 nm for treatment of herpes was tested *in vitro* (Hasanovic et al., 2009). Chitosan nanoparticles with small particle size showed more antibacterial activity than large ones, due to increase in the surface areas which would be in direct contact with the bacteria (Katas et al., 2011).

Based on this data and results of hydrodynamic size, we selected chitosan nanoparticles formed at concentration 0.1% as they showed least particle size and stability (zeta potential) for further testing **(Figure 12).**

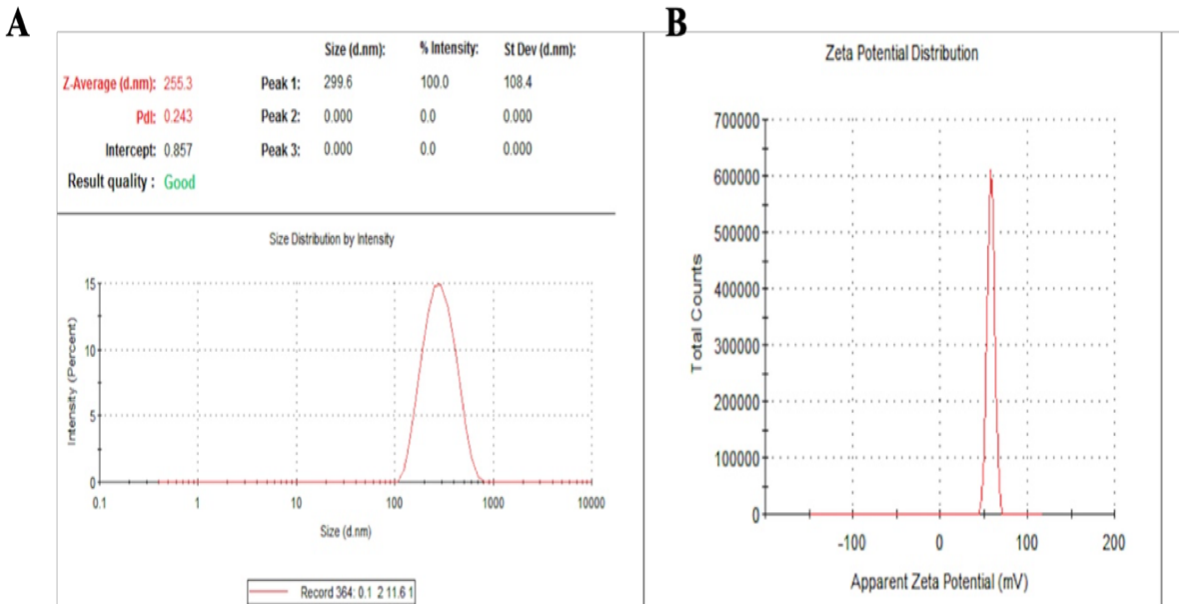


Figure 12 0.1 % Chitosan Nanoparticles average size, PDI & stability.

Average Particle size distribution of 0.1% chitosan nanoparticles was 255 nm, in homogenous (monodisperse) solution PDI 0.2. **B)** The results showed particles of high stability with zeta potential (+51 mv).

3.2.2 Morphology Characterization

Morphology of nanoparticles under inverted microscope with average size 400-500 µm, this size was not accurate as the particles were swollen **(Figure 13).** Therefore, further assessment of morphology using SEM was important to give accurate insight about the actual morphology, size & aggregates (Singh et al., 2016). SEM revealed spherical uniform nanoparticles with average diameter 77 nm & almost no aggregates **(Figure 14).**

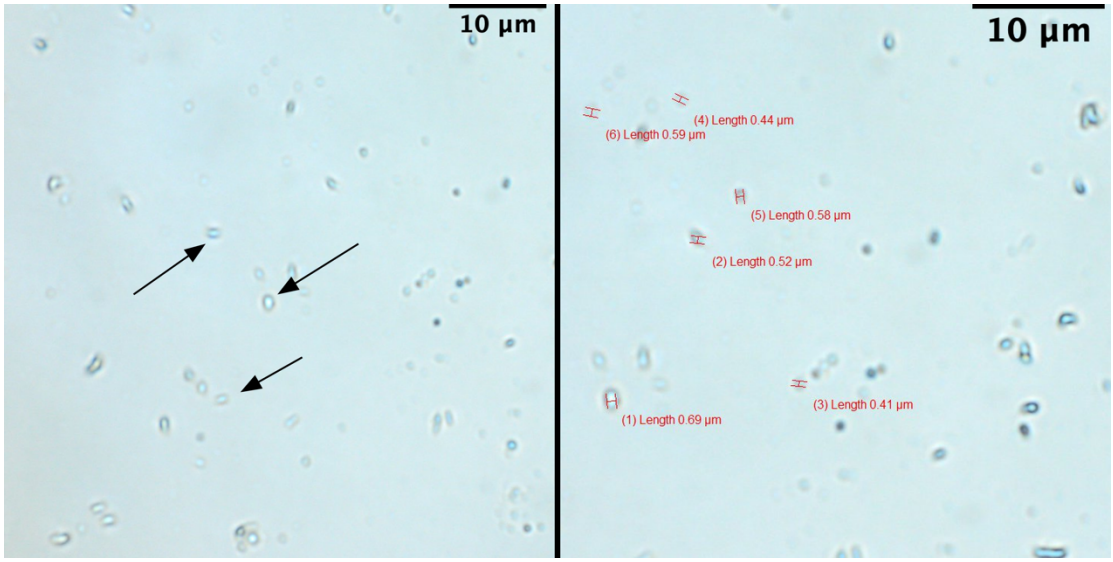


Figure 13 Morphology of Chitosan nanoparticles under light microscope

(Magnification 100 X)

A) Rounded chitosan nanoparticles (Black arrows). **B)** The average size of nanoparticles under inverted microscope was 400-500 µm.

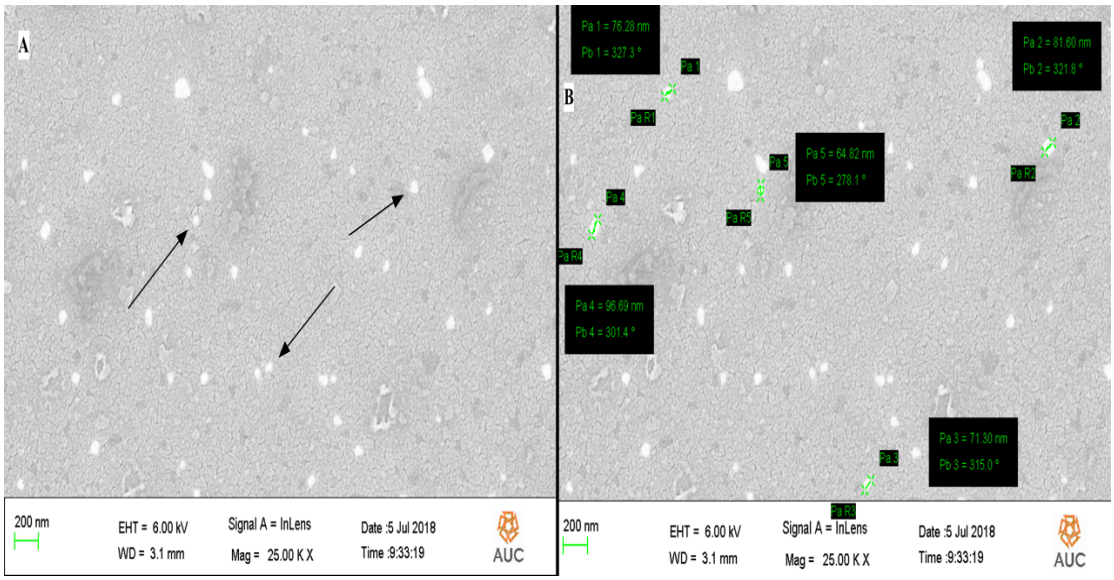


Figure 14 Morphology of Chitosan nanoparticles through SEM

A) Rounded chitosan nanoparticles of almost uniform size (Black arrows) with no aggregates can be seen. **B)** The average size of nanoparticles through SEM showed to be nearly 77 nm.

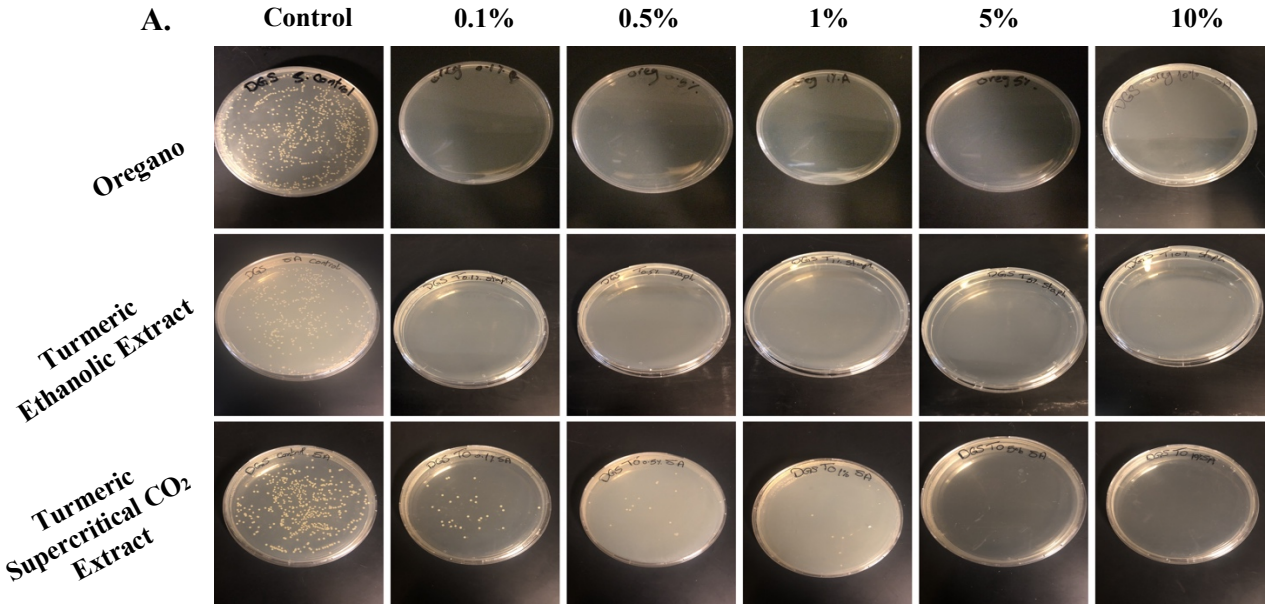
3.3 Antibacterial Test

Oregano essential oil was found to be very potent against both gram positive and gram-negative bacteria at a concentration of 0.1%, it showed 100% bactericidal effect **(Figure 15, 16).** This agrees with previous reports who found oregano to be powerful at concentration less than 2% (Hammer et al., 1999). It was hypothesized that essential oils of oregano cause disruption of bacterial cell membrane, blocking their enzyme system & disrupt ion exchange leading to cell death (Sakkas et al., 2017).

Turmeric ethanolic extract, caused total bacterial inhibition at concentrations of 0.1% against *S. aureus*, and 5% against *E. coli*. To exclude the effect of ethanol, turmeric oil supercritical CO_2 extract was used. Total inhibition of both *S. aureus* & *E.coli* at 5% was observed ***(Figure 15, 16).***

Turmeric exerts its antibacterial activity through disruption of bacterial membrane (Tyagi et al., 2015). It was noted that *E. coli* were less sensitive to turmeric extracts compared to *S. aureus.* This agrees with previous reports, that showed Gram negative *(E. coli)* were less susceptible due to the presence of lipopolysaccharide in their outer membrane (Th et al., 2010).

ChNP showed increase in bacterial inhibition with increasing concentration from 0.5% to 1%. They were found to be more potent against gram positive rather than gram negative ***(Figure 17).*** Divya et al., (2017) also reported that ChNP showed more inhibition on *S. aureus* than *E. coli*.



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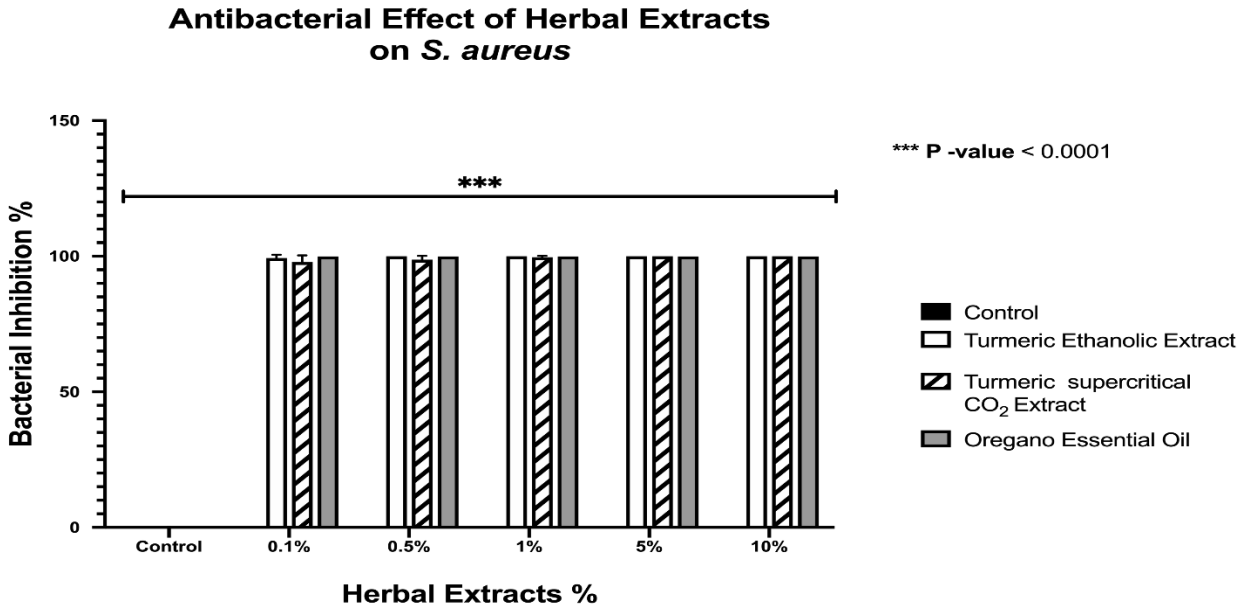
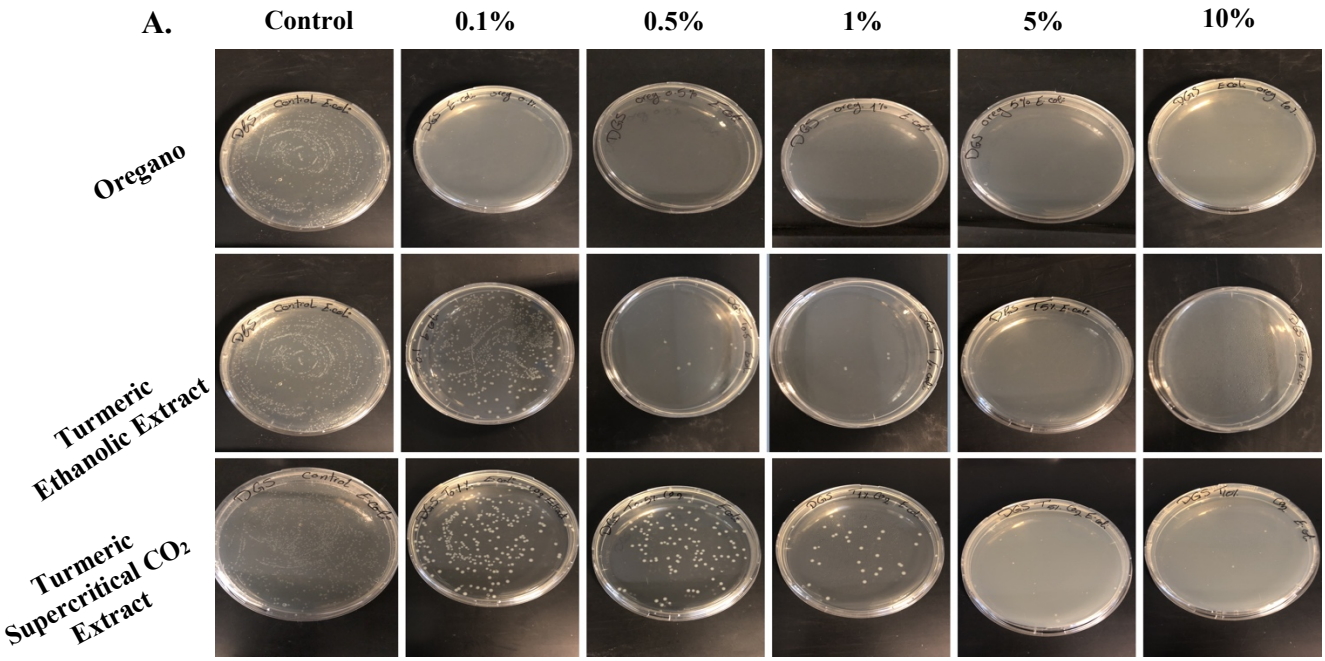


Figure 15 Effect of Oregano, Turmeric Extracts against S. aureus

A. Oregano & turmeric extracts (ethanolic / supercritical CO_2) showed antibacterial effect against *S. aureus* at very low concentration (0.1%). **B. Graphical representation of the results** expressed in % bacterial reduction **(P<0.0001 ; n = 3 in triplicates).**



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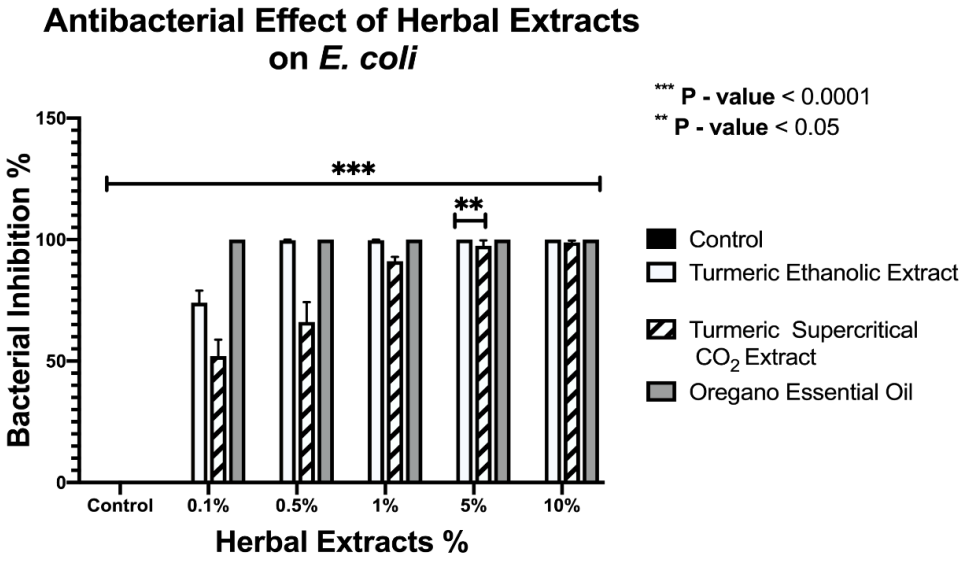
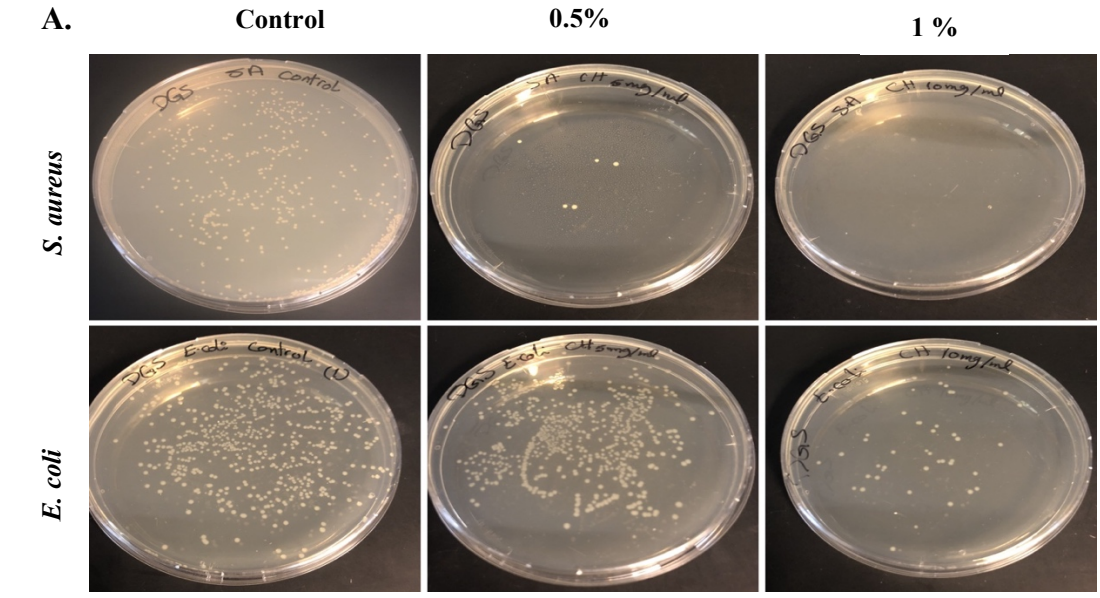


Figure 16 Effect of Oregano, Turmeric Extracts against E. coli

A. Oregano showed antibacterial effect against *E. coli* at very low concentration (0.1%). Turmeric ethanolic extract was more potent than turmeric oil, both showed total bacterial inhibition at concentration 5%. **B. Graphical representation of the results** expressed in % bacterial reduction **(P<0.0001 ; n=3 in triplicates).**



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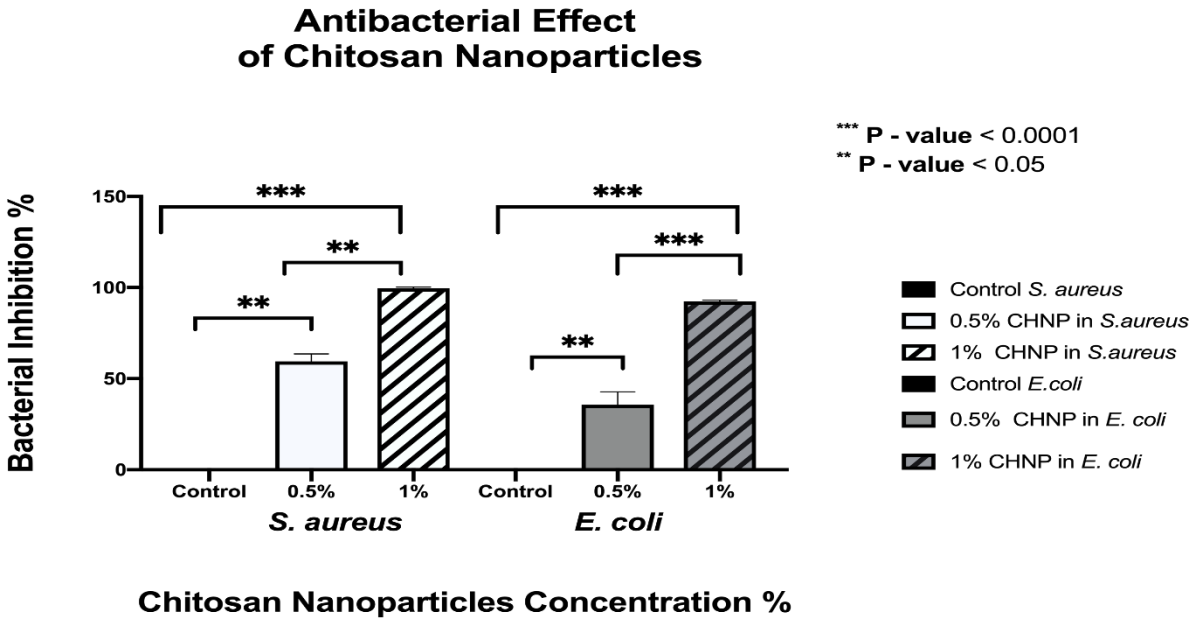


Figure 17 Effect of Chitosan nanoparticles on Gram positive & Gram-Negative Bacteria

A. Chitosan nanoparticles showed significant increase in antibacterial activity with increased concentration from 0.5% to 1%, in both gram positive (*S. aureus*) and gram negative (*E. coli*). Chitosan nanoparticles were found to be more effective on Gram positive more than Gram negative bacteria **(P<0.0001). B. Graphical representation of the results** expressed in % bacterial reduction **(P<0.0001).**

3.4 Anti - Oxidant Assay

Oregano showed significant increased antioxidant activity with increasing the concentration. This agreed with previous literature that proved that the antioxidant effect of oregano was due to the presence of the high phenolic content (Chun et al.2005; Stanojević et al., 2018). Turmeric ethanolic extract showed more antioxidant power than that of turmeric extracted by supercritical CO_2 (p-value < 0.0001). This suggests that the curcumin and the phenolic content might be higher in the ethanolic extracts. This hypothesis agreed with previous reports that ethanol extraction enhanced phenol and curcumin extraction (Martinez-Correa et al., 2017). Chitosan NP did not show significant antioxidant activity at different concentrations **(Figure 18)**. This is in agreement with previous reports that found free Chitosan NP did not exhibit antioxidant effects. Only after encapsulation with Quercetin chitosan showed high antioxidant effect through scavenging of 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) radical (Y. Zhang et al., 2008).

When the herbal extracts were suspended in DMEM media, significant increase in MTT reduction was observed (P-value < 0.0001). This can be due to the interaction between the phenolic groups in the extracts with the nutrients in the media that enhanced the MTT reduction to give its formazan blue color **(Figure 18).** Previous study showed that flavonoids in media influenced MTT reduction in the absence of cells (Talorete et al., 2006).

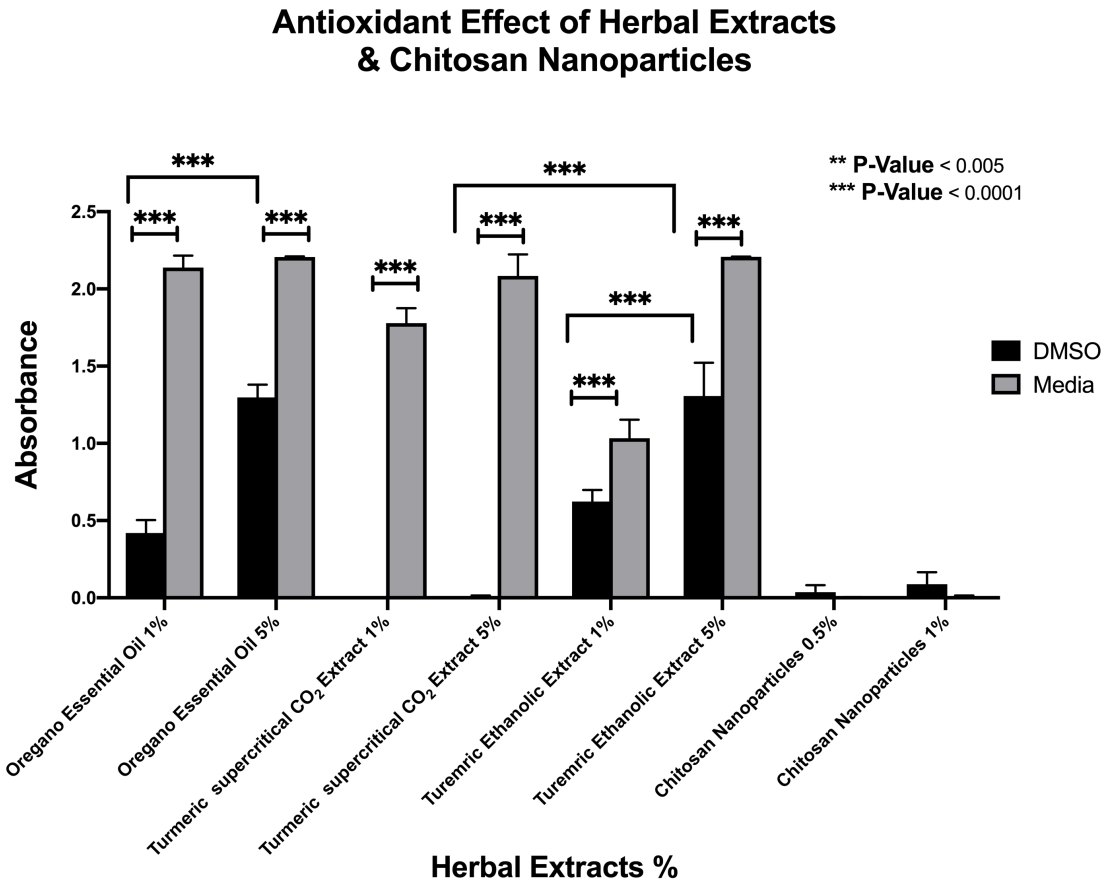


Figure 18 Antioxidant Effect of Herbs, ChNPs

Oregano showed significant increase in antioxidant activity with increase in the concentration. Turmeric ethanolic extract 5% was more potent than turmeric supercritical CO_2 extract (P -value < 0.0001). Turmeric ethanolic extract 5% was more potent than turmeric ethanolic extract 1% (P -value < 0.0001). ChNPs showed no significant antioxidant activity. Media enhanced MTT reduction in the presence of phenolic compounds of the herbs (P-value < 0.0001).

From the results of the antibacterial and antioxidant assays, we decided to use oregano at concentration of 1%, turmeric ethanolic extract at a concentration of 5% & ChNPs at a concentration 1%.

Upon testing the above combination for their antioxidant activity against Tegaderm® wound dressings. We found that our combination showed significant anti-oxidant activity if compared to Tegaderm® that did not show any antioxidant power (P-value < 0.0001) **(Figure 19).** Oregano and turmeric extracts are rich in phenolic compounds

that have great effect as antioxidants (Bordoloi et al., 2016; Nakatani et al., 2000). Tegaderm® is calcium alginate dressing with silver. To date there is no test to assess its antioxidant activity. Our results reveal that Tegaderm® has no antioxidant effect. This can be due to the fact that silver act as strong oxidizing agent so, it didn't affect MTT (Syper et al, 1967 ; Pubchem).

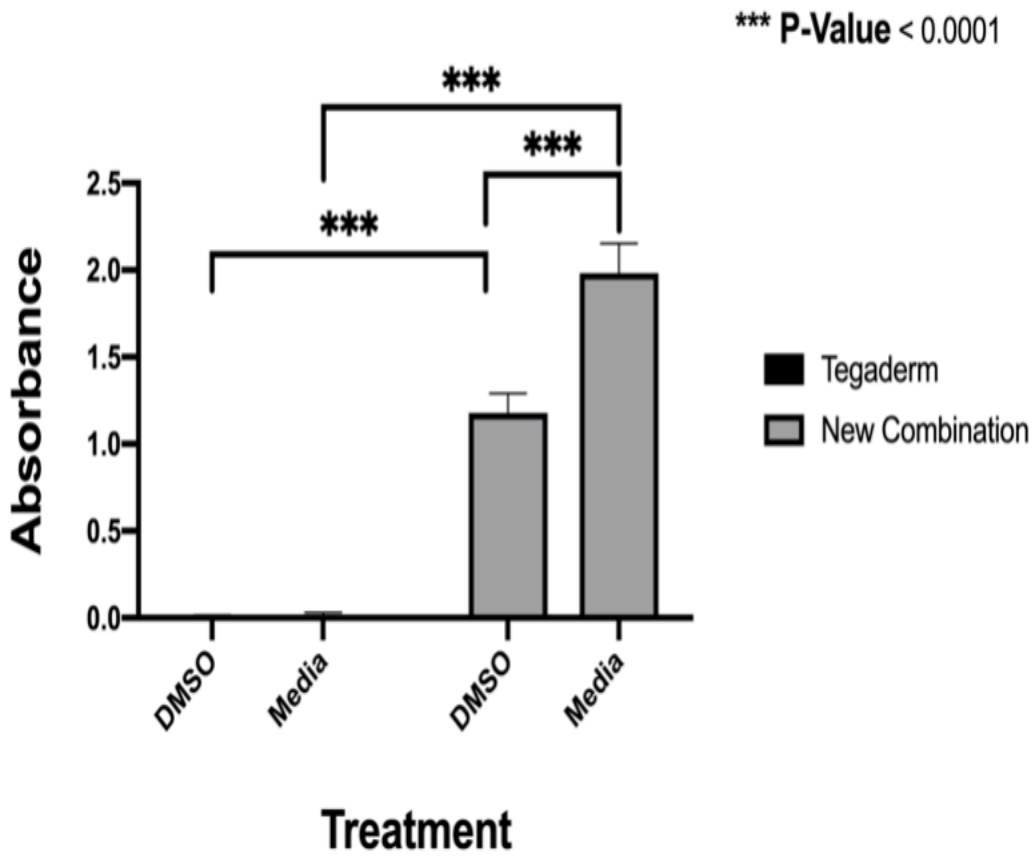


Figure 19 Antioxidant Effect of Combination

Combination of 1% oregano, 5% turmeric ethanolic extract & 1% ChNP showed significant antioxidant activity if compared to Tegaderm® that didn't have any oxidant power (P-value < 0.0001).

3.5 *In vivo* Testing

3.5.1 Pilot study for Herbs Macroscopic Evaluation

Turmeric ointments (5%) showed improvement in the pressure ulcer healing process in both diabetic and non-diabetic rats. Granulation tissue was greatly enhanced with complete healing achieved by day 10 in non-diabetic rats, while complete healing was achieved by day 15 in diabetic rats.

Although oregano 0.1% showed great antibacterial effect *in vitro* on both gram positive and gram-negative bacteria, this concentration was not effective as an anti-inflammatory on wounds. Therefore, the concentration was increased to 1% only to avoid irritation. This concentration was found to be potent anti-bacterial *In vivo*. It also dramatically improved granulation tissue formation with complete closure of the ulcer achieved by day 15 in non-diabetic animals, while in the diabetic rats healing was achieved by day 21 **(Figure 20)**.

One previous study showed the effect of using 3% oregano ointment for wound healing in patients with surgical excision. The study revealed significant reduction in bacterial infection on post-surgical wounds and improved the scar appearance compared to petrolatum (Ragi et al., 2011).

Turmeric extract 5 % ointment showed significant improvement in granulation and decreased infection, compared to no treatment groups in both diabetic and non-diabetic animals, achieving full wound closure by day 15 **(Figure 20).** Many researchers used curcumin to asses wound healing, a recent study showed that ointment containing 5% & 10% curcumin ethanolic extracts showed improved wound healing against an aspirin retarded wound healing process (Pawar et al., 2015). Other studies showed that curcumin applied in pluronic F127 gel, accelerated wound closure in mice (Yen et al., 2018). Petrolatum containing 5% turmeric ethanolic extracts enhanced healing at the episiotomy incision site in primiparous women. It decreased the period of healing from 14 days to10 days (Tara et al.,2009).

Our findings showed that, both oregano 1% and turmeric 5% ointments had advanced reduction in the healing time, and improved granulation & decreased infection in both diabetic and non-diabetic rats. However, oregano scored better in clearing infection compared to turmeric. On the other hand, turmeric enhanced the granulation tissue

formation more than oregano **(Figure 21).** To date, there is no comparative study conducted between oregano and turmeric to assess their healing activity.

Recent studies showed that chitosan nanoparticle loaded calcium alginate hydrogel demonstrated significant antibacterial activity & accelerated wound healing *in vivo* (T. Wang et al., 2018).

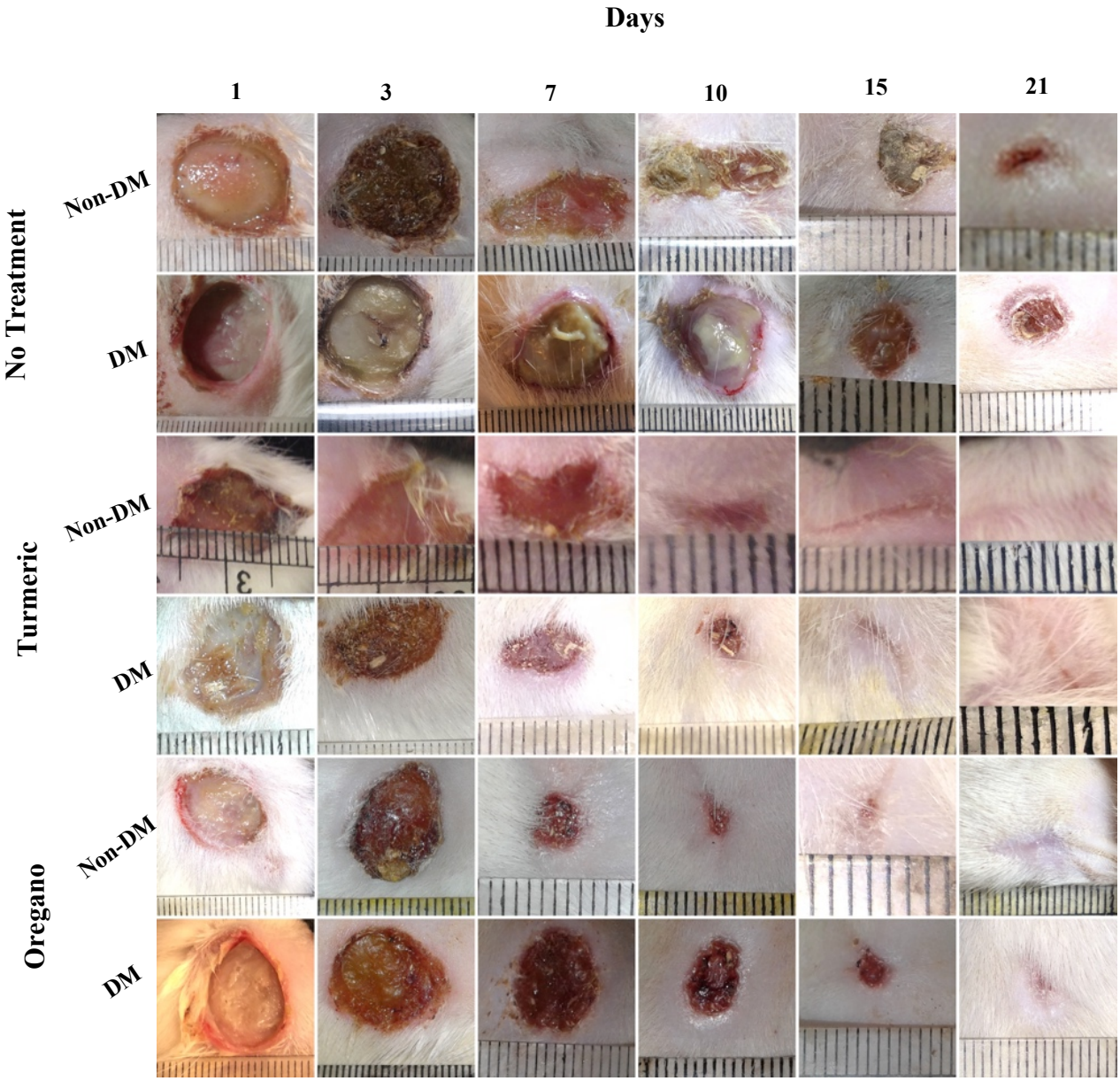


Figure 20 Photographic images to the extent of wound healing activity of Herbal extracts.

Untreated group, the diabetic group showed higher infection rates, more crusty and poor wound healing compared to the non-diabetic group. Turmeric treatment group showed complete healing by day 15 in both non-diabetic & diabetic groups. Oregano treatment group showed low infection, with complete healing by day 15 in non-diabetic group and day 21 in the diabetic group.

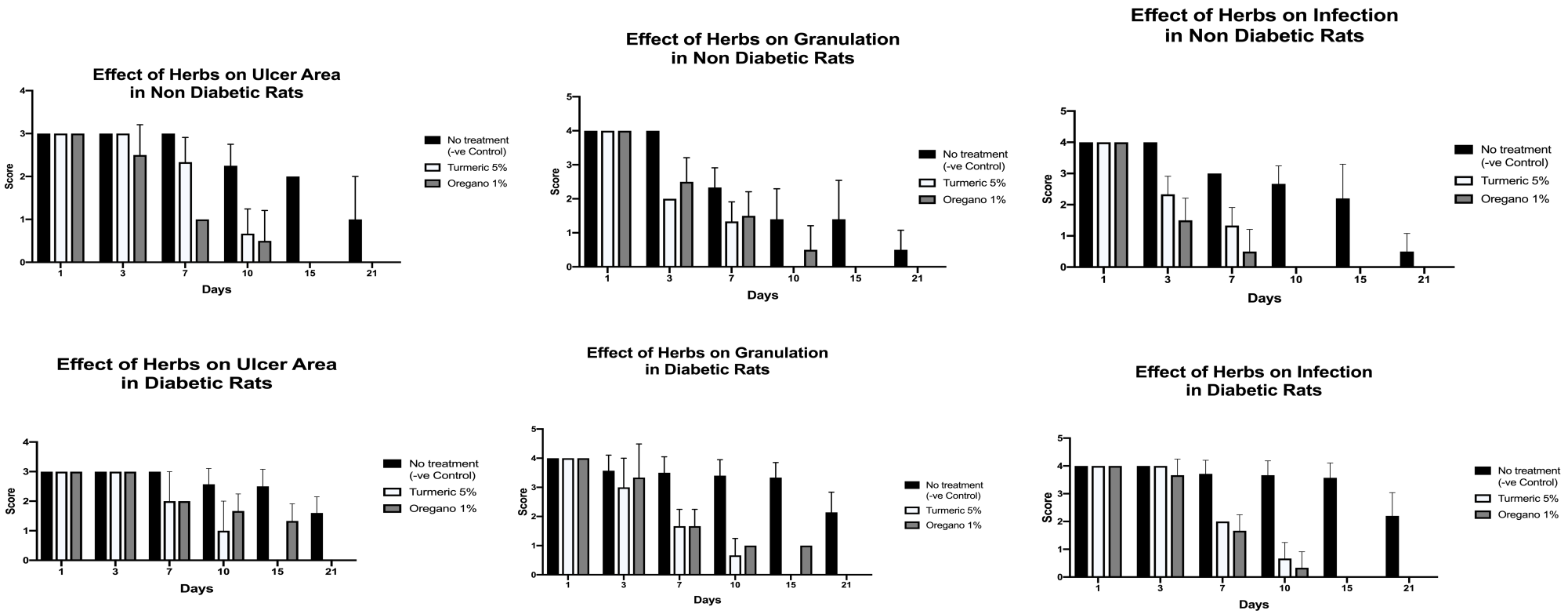


Figure 21 Effect of Herbs on Pressure Ulcer in Non-Diabetic and Diabetic Rats

Both 5% Turmeric & 1% Oregano reduced ulcer area in both models compared to the control group (No treatment). Turmeric 5 % had effect on granulation tissue macroscopically compared to the control group (No treatment). Oregano 1 % reduced infection macroscopically compared to the control group (No treatment).

3.6 Preparation Selection

The formulas that were selected for further studies were the ointment, amorphous hydrogel & nanofibers. The hydrogel sheet & xerogel were excluded from the study. The hydrogel sheet was tested *in vivo* and it created a dry tough layer that impaired the healing process (results not shown) (parhi et al, 2017; Kumar & Han, 2017). After the hydrogel sheet was dried through lyophilization to form xerogel, the product shrank, became very dry & lost its elasticity. Previous studies showed that upon PVA lyophilization deformation happened, collapse of the pores which affect negatively cell growth, therefore, limiting the use of xerogels (Yabin Zhang et al., 2015; Annabi et al., 2010).

3.7 Nanofibers Characterization

Based on the results of SEM, we chose to work with flow rate 0.8 ml/ hr as higher flow rates showed formation of beads **(Figure 22)**. According to the literature, lower flow rates were more suitable as the solvent will have enough time for evaporation (Annabi et al., 2010). On analyzing the average fiber diameter of different voltages using ImageJ® software, we decided to use 18KV, as it showed high frequency distribution of narrow fiber diameters & the fiber diameter mean was 211 nm **(Figure 23).** This can be attributed to the high level of jet stretching in relationship to increased charge repulsion within the jet and a strong external electric field (Pillay et al., 2013).

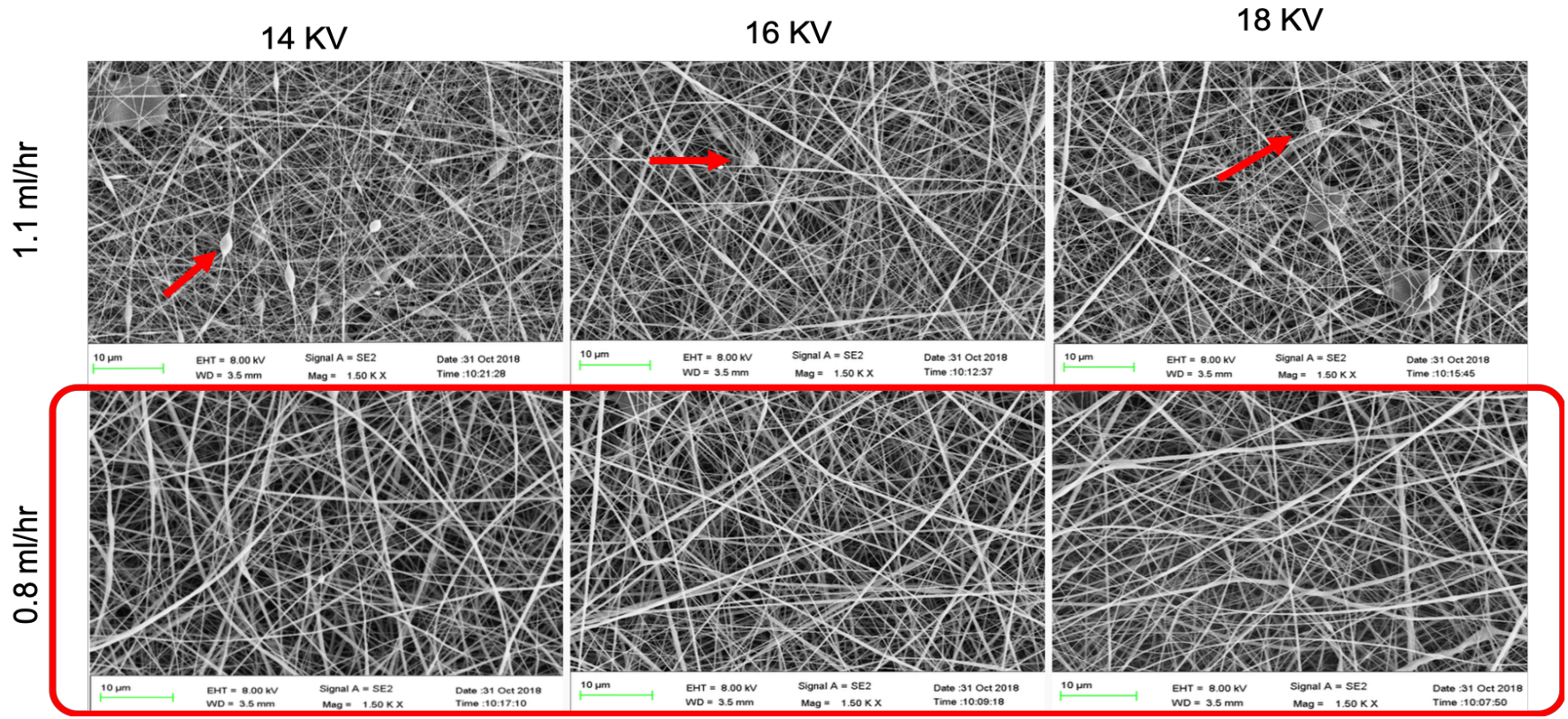


Figure 22 Effect of Flow rate on fiber Morphology

High flow rate 1.1 ml/hr resulted in the formation of beads (arrow), unlike the lower rate 0.8 ml/hr (red box) that produced uniform smooth fibers.

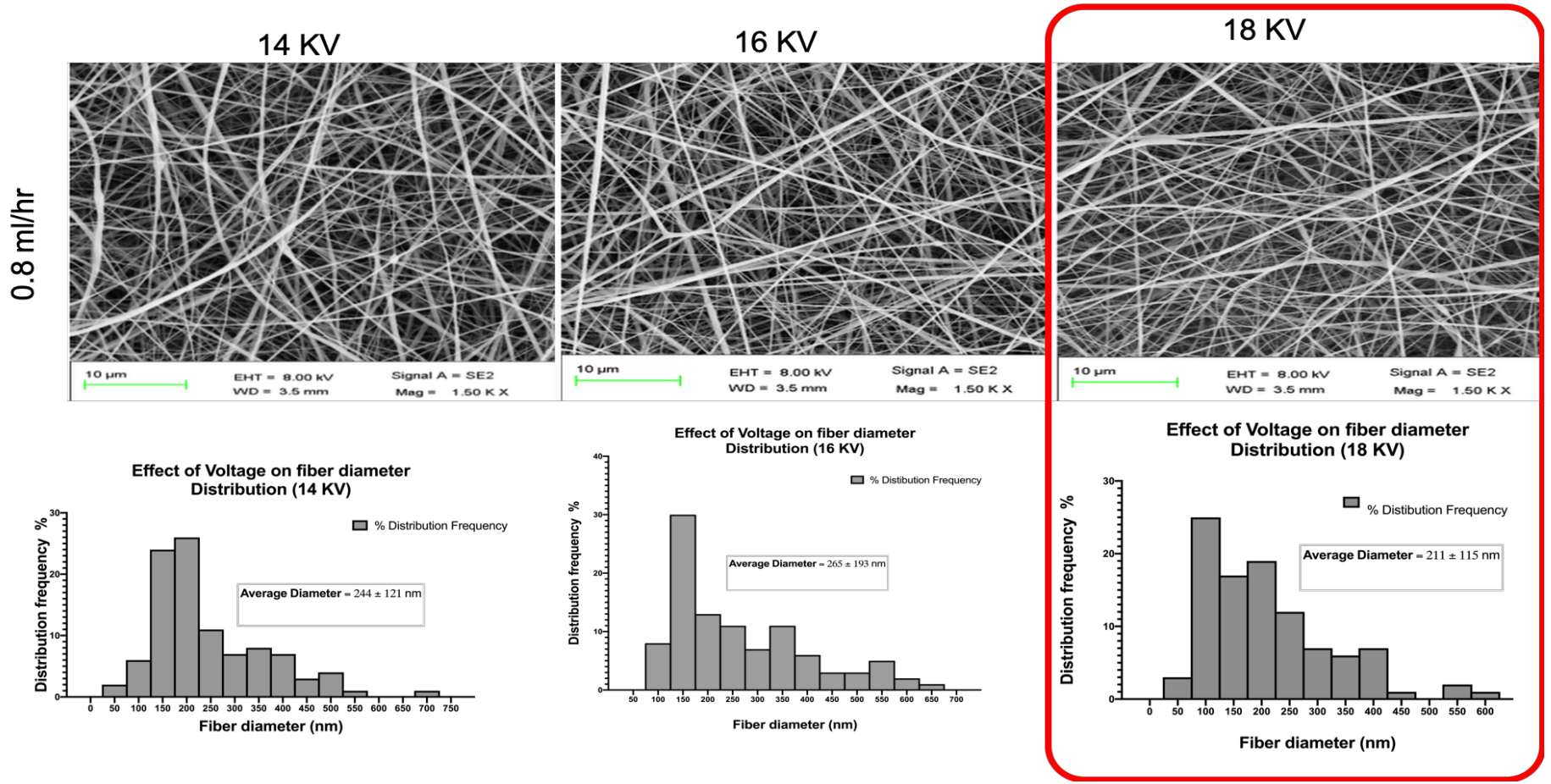


Figure 23 Effect of Different Voltages on fiber diameter distribution

Higher voltage (18 KV) resulted in increase in the frequency distribution of the narrow fiber diameter (average Diameter= 211± 115 nm) (red box).

3.8 Antibacterial Test for the designed Formulations

According to the antibacterial results, our formulations in ointment, hydrogel & nanofibers showed significant antibacterial effect on both gram positive & gram-negative bacteria (P-value < 0.0001). They showed antibacterial activity the same as the positive control (Tegaderm®). Although nanofibers had antibacterial effect, it was lower than that of the ointment & hydrogel **(Figure 24).** This can be due to the sustained release the nanofibers provide for the incorporated extracts (Weng & Xie, 2015). Another hypothesis is that electrospinning decreased the efficacy due to the volatility of the herb extracts. In agreement with previous studies, free petrolatum and free PVA did not show any antibacterial effect on both strains (Kawai & Hu, 2009; Hu & Wang, 2016; Gemeda et al., 2018).

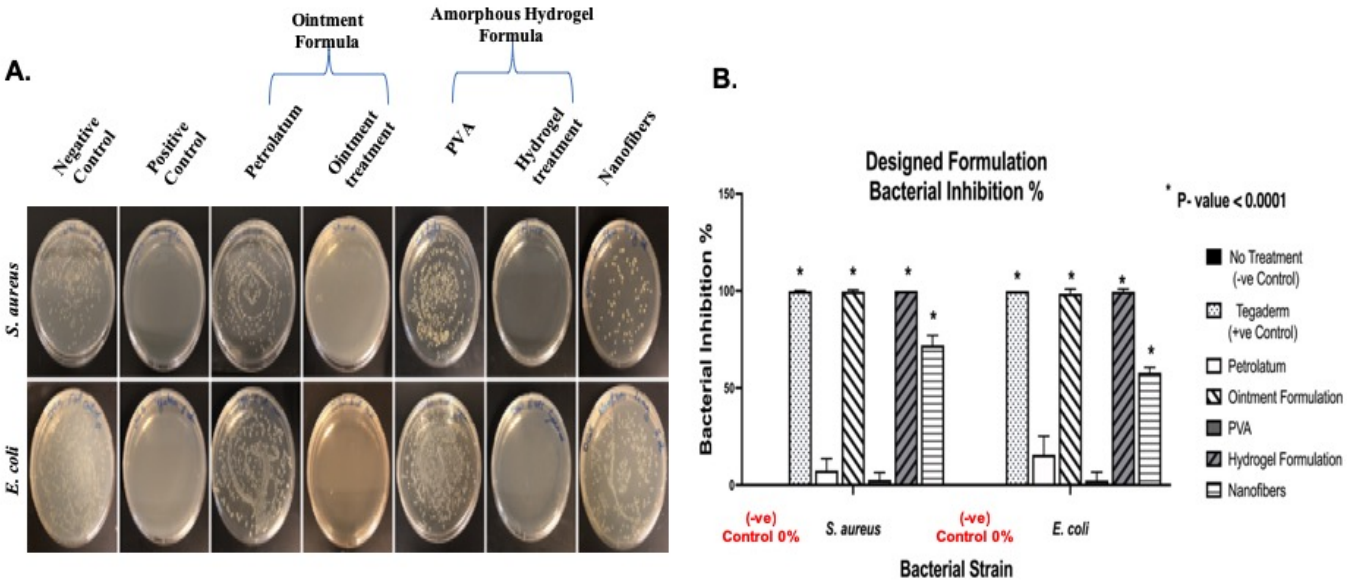


Figure 24 Effect of different forms on Gram Positive and Gram-Negative Bacteria

A. Bacterial Panel: Treatment ointment, hydrogel showed significant reduction on both *S. aureus* & *E. coli* as Tegaderm (positive control). Nanofibers also showed significant bacterial reduction in both strains but less than the ointment & hydrogel. Pure petrolatum & PVA didn't show any antibacterial effect. **B**. **Graphical representation** for the results (*P-value < 0.0001 ; n=3 in triplicates).

3.9 In Vitro Biocompatibility

3.9.1 Cytotoxicity

Cell viability (MTT) assay of L929 cells was performed to assess the cytotoxicity of the preparations on fibroblast cell line. Tegaderm® was used as a positive control, and cells without any treatment as negative control **(Figure 25).** Quantitative determination by MTT method showed that ointment, and hydrogel were not cytotoxic, the two formulations led to a significant cell viability increase compared with viability of the no treatment cells (negative control) (p-value < 0.0001). Tegaderm® was significantly toxic causing 90% cell death (p-value < 0.0001). The high cytotoxic effect of Tegaderm® could be attributed to the silver that the dressing contains. Previous studies showed that silver based dressing exhibited significant cytotoxic effects on both cultured keratinocytes and fibroblasts (Burd et al., 2007; Hiro et al., 2012).

Nanofibers, on the other hand showed almost 50% reduction in cell survival rate in a concentration dependent manner (p-value < 0.05) **(Figure 25).**The significant reduction in cell viability of nanofibers compared to the ointment, and hydrogel (p-value < 0.0001) can be attributed to reduction of particle size to nanoscale, which makes them more cytotoxic than microscale particles (Bhattacharya et al., 2012;Sahu et al., 2016). According to literature, the reduction in particle size leads to increase in the surface area which makes the number of molecules more exposed and thus show high reactivity. As a result, Reactive Oxygen Species (ROS) are produced which cause oxidative stress, inflammation and consequently result in DNA and protein damage (Khalili Fard et al., 2015).

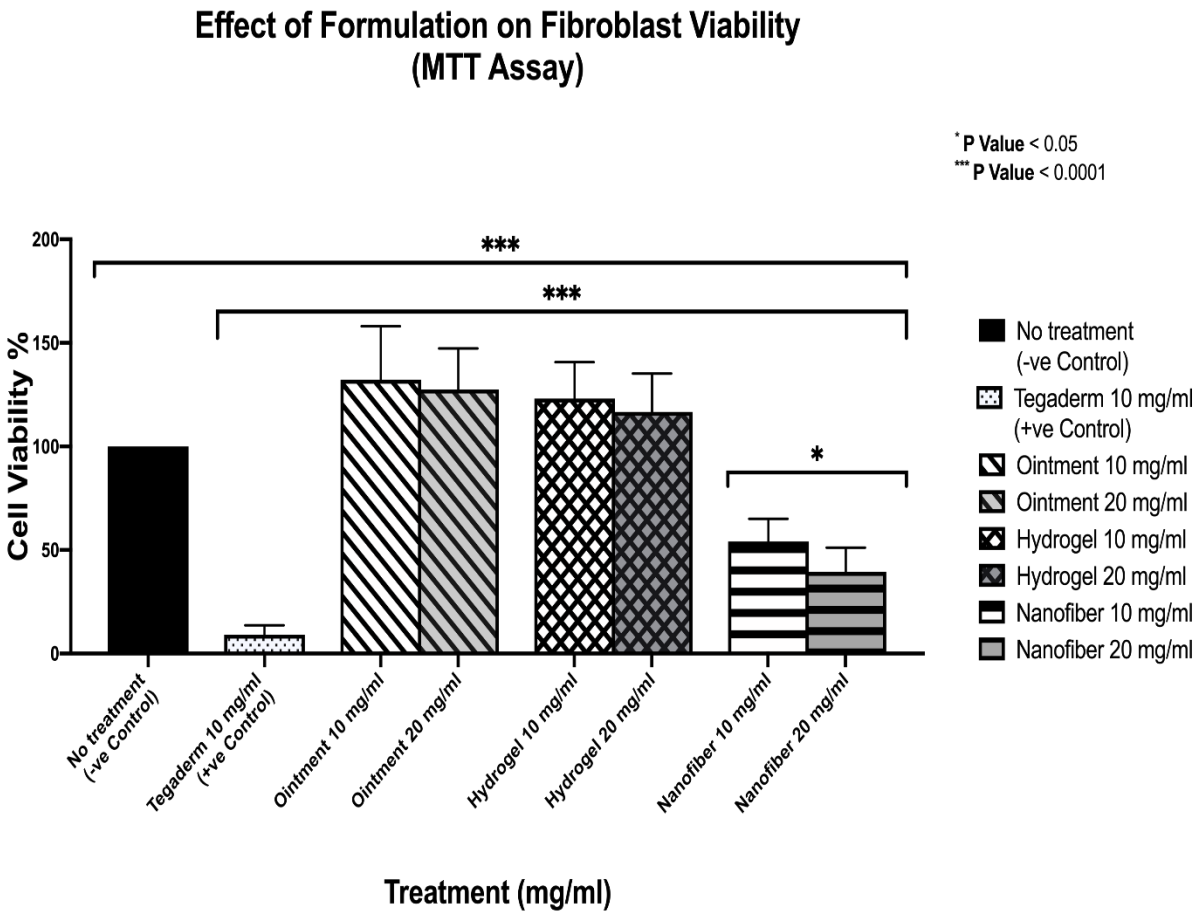


Figure 25 Effect of Different treatment forms on L929 cell Viability

Tegaderm treated cells (+ve control) showed significant reduction in cell viability if compared to untreated cells (P-Value < 0.0001). Both ointment and hydrogels showed significant increase in cell viability if compared to untreated cells (P-Value < 0.0001). All formulations had significant better viability than Tegaderm treated cells (P-Value < 0.0001). Nanofibers showed significant reduction in cell viability compared to the negative control (P-Value < 0.0001). Nanofibers showed significant reduction in cell viability in concentration dependent manner (P-Value < 0.05; n=8 in triplicates).

3.9.2 Cell Morphology

Microscopic examination of the L929 cell cells under microscope 24 hrs post-treatment, showed significant changes in cell morphology. Untreated cells (negative control) showed a majority of spindle shaped cells adherent to the culture plate **(Figure 26.A).** Tegaderm treated cells appeared rounded, and shrunken cells with no proliferation **(Figure 26.B).** Ointment and hydrogel treated cells at different concentrations showed normal spindle shaped cells with increased proliferation **(Figure 26.C-F).** Nanofiber (10 mg/ml) treated cells showed changes in the morphology, with some cells appeared rounded with reduced proliferation **(Figure 26.G).** Nanofiber (20 mg/ml) treated cells showed severe changes and most cells appeared rounded and shrunken with reduction in proliferation **(Figure 26.H).** Results of cell morphological assessment agree with cell viability MTT assay results.

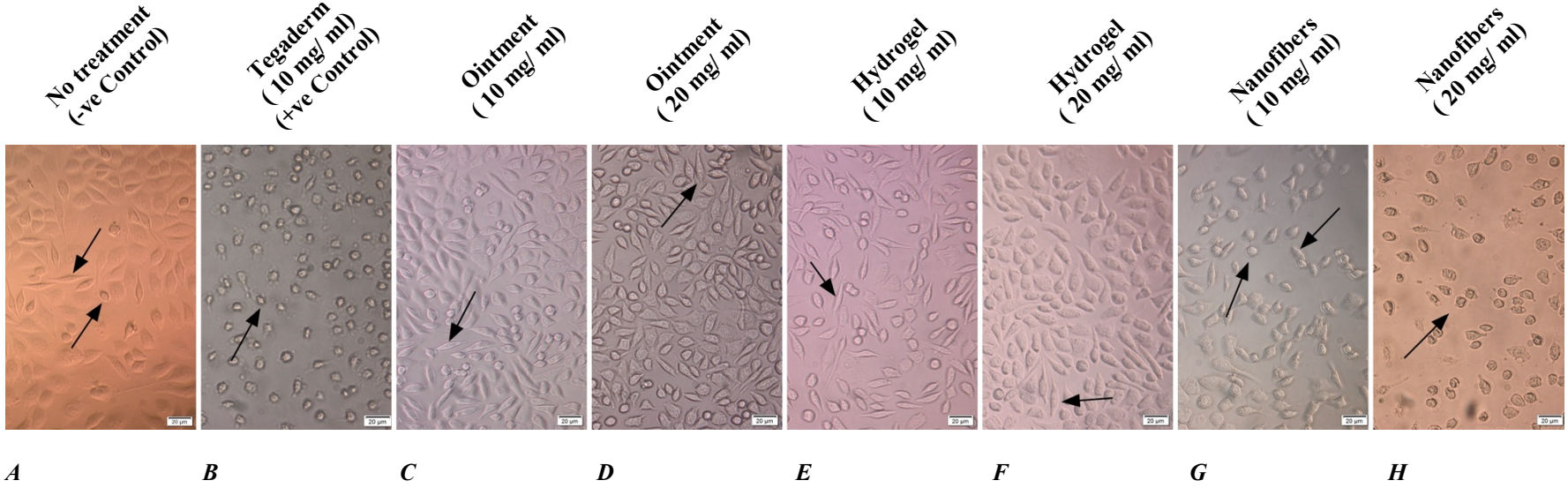


Figure 26 Effect of Different treatment forms on mouse Fibroblast (L929) cell line

Morphology of fibroblast cell line (L929) after 24 hours

A) Control culture (-ve control) showed spindle shaped & rounded cells (arrow); **B)** Cells treated with Tegaderm (+ve control) showed rounded damaged cells (arrow); Cells treated with ointment combination (10 mg/ml) **(C)** and (20 mg/ml) **(D)** showed normal cell morphology; Cells treated with Hydrogel combination (10 mg/ml) **(E)** and (20 mg/ml) **(F)** showed normal cell morphology; Cells treated with Nanofibers (10 mg/ml) **(G)** showed altered cell morphology (arrow); while those treated with Nanofibers (20 mg/ml) showed rounded cells (**H,** arrow). (**Magnification X200**) (n=8 in triplicates)

3.10 *In Vivo* Testing for the designed Formulations

3.10.1 Macroscopic Examination

Macroscopic (clinical) assessment of pressure ulcer in diabetic rats, showed that Tegaderm® caused poor healing and high infection compared to the other groups tested, by day 21 the ulcer size was relatively smaller compared to the control **(Figure 27).** Previous studies, showed that silver containing dressing prevented fibroblast proliferation, which impaired wound healing (Burd et al., 2007). Our *in vitro* assessment also supported this finding **(Figure 26.B).**

Amorphous hydrogel, according to some authors encourages autolytic debridement of necrotic tissue. They work through rehydrating hard eschar and slough tissue and thus promote the healing process (Dabiri et al., 2016). Our current results showed the opposite, animal group treated with hydrogel showed a high rate of infection, very poor healing and by day 21 the wound area was almost similar to that of the control group **(Figure 27)**. This can be attributed to the high-water content in the hydrogel, that made it rich environment for growth of bacteria. Recent studies showed that amorphous hydrogels exhibit poor bacterial barrier (Kamoun et al, 2017).

Animals treated with either ointment or Nanofibers showed very good healing, and low infection, compared to the control, complete wound closure occurred by day 15 while the untreated group till day 21 didn't show complete healing **(Figure 27).**

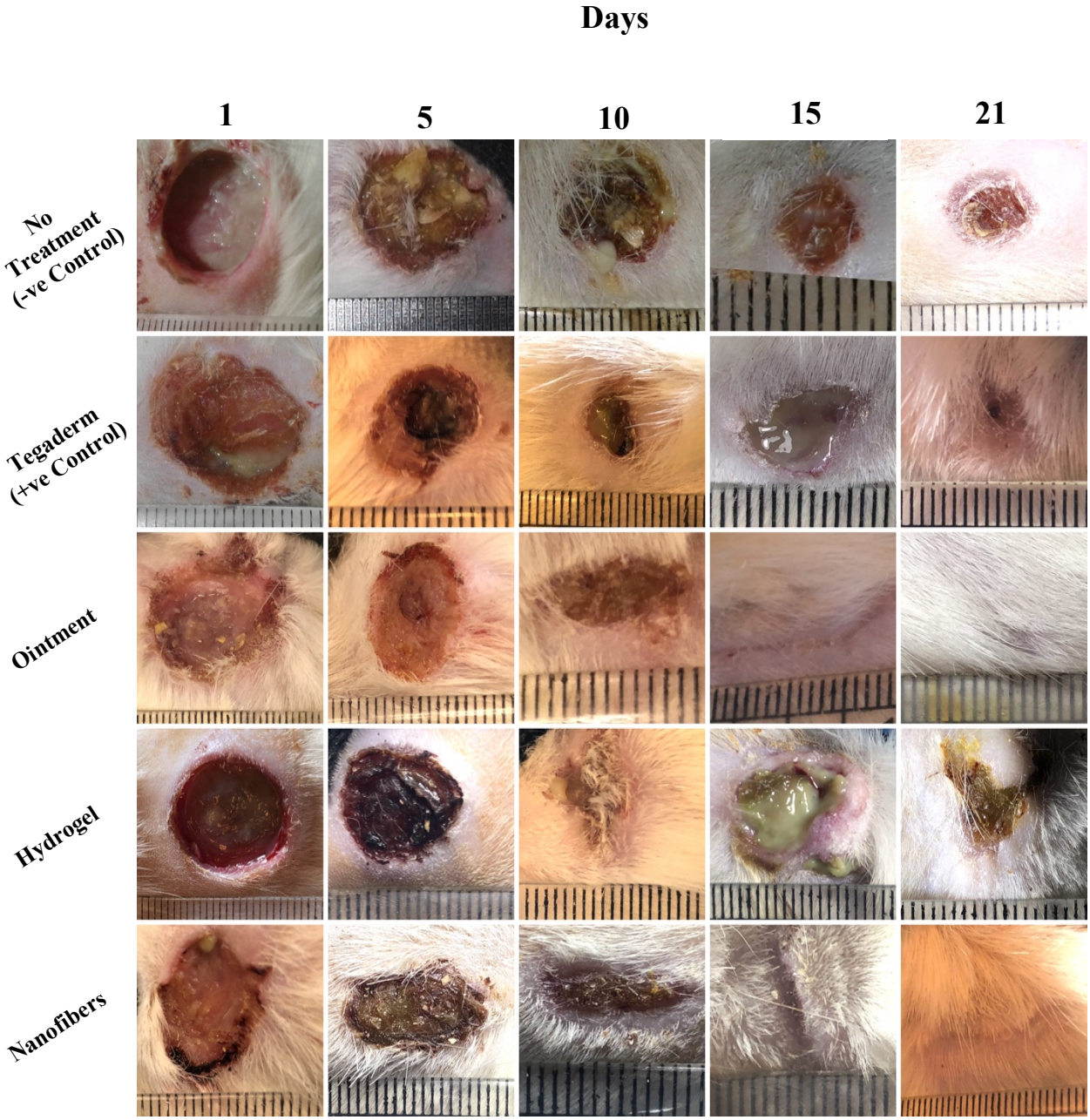


Figure 27 Macroscopic Assessment for the effect of Different Treatment forms on Diabetic Pressure Ulcer

Control group (no treatment) showed high infection, high fibrous connective tissue, and no healing by day 21. Tegaderm (+ ve control) and hydrogel treated rats showed poor healing and high infection. By day 21 Tegaderm treated rats showed smaller wound area compared to the no treatment group. Animals treated with ointment and those treated with nanofibers showed complete healing by day 15 (n= 4-6).

3.10.2 Histological Examination

10 Days following treatment, ointment & nanofibers treated groups showed migration of basal epithelial cells **(Figure 28. C, E).** While untreated group, Tegaderm® treated group and hydrogel treated group showed thickening of epidermal layer with necrotic tissue **(Figure 28. A, B, D).** Masson trichrome staining showed moderate mature collagen formation in ointment & nanofibers treated groups if compared to other groups **(Figure 29 C, E).**

At day 15 following treatment, histological evaluation of the treatment groups showed that in the **no treatment** (-ve control) group**,** the gap was covered by thick layer of necrotic tissue, and inflammatory cell infiltration was evident. The dermal layer showed new blood vessel formation (angiogenesis) and fibroblast proliferation that produced moderate pattern of granulation tissue **(Figure 28.F).** Masson's trichrome stained section showed moderate mature collagen fibers **(Figure 29.F).**

Tegaderm treated group **(+ ve control),** showed a gap covered by a thick layer of necrotic tissue. Inflammatory cells infiltration was seen as focal aggregation in epidermal and dermal layers. The dermal layer showed new blood vessel formation (angiogenesis) and fibroblast proliferation to produce moderate pattern of granulation tissue **(Figure 28.G).** Masson's trichrome stained tissue section showed moderate mature collagen fibers **(Figure 29.G).**

In the **ointment treatment** group**,** the gap was covered by keratinization epithelial cell layer with fewer numbers of inflammatory cells infiltrating mainly macrophages. The dermal layer showed new blood vessel formation and fibroblast proliferation to produce moderate pattern of granulation tissue **(Figure 28.H).** Masson's trichrome stained tissue section showed marked mature collagen fibers **(Figure 29.H).**

Hydrogel treatment group showed a gap with thickening of epidermis by necrotic tissues at its cut edges with mild inflammatory cells infiltration mainly macrophages. The dermal layer showed angiogenesis and fibroblast proliferation to produce marked pattern of granulation tissue **(Figure 28.I).** Masson's trichrome stained tissue section showed marked mature collagen fibers score **(Figure 29.I).**

Nanofibers treatment showed a gap covered by non-keratinization epithelial layer with few numbers of inflammatory cells infiltration mainly macrophages. The dermal layer showed angiogenesis and fibroblast proliferation producing a moderate pattern of

granulation tissue **(Figure 28.J).** Masson's trichrome stained tissue section showed marked mature collagen fibers score **(Figure 29.J)**.

According to histological scoring results at day 15, ointment and nanofibers showed best results compared to the negative and positive control. Better scores went to the ointment as it showed keratinization, low infection and decreased angiogenesis which were signs of complete healing **(Table 4).** This can be due to the rapid release of the herbal extracts and chitosan nanoparticles from the ointment, while nanofibers provide a slower sustained release. A previous study showed that, the release of vitamin-loaded as-spun fiber mats showed gradual release over the test periods, while the corresponding cast films exhibited a burst release of the vitamins (Taepaiboon et al., 2007).

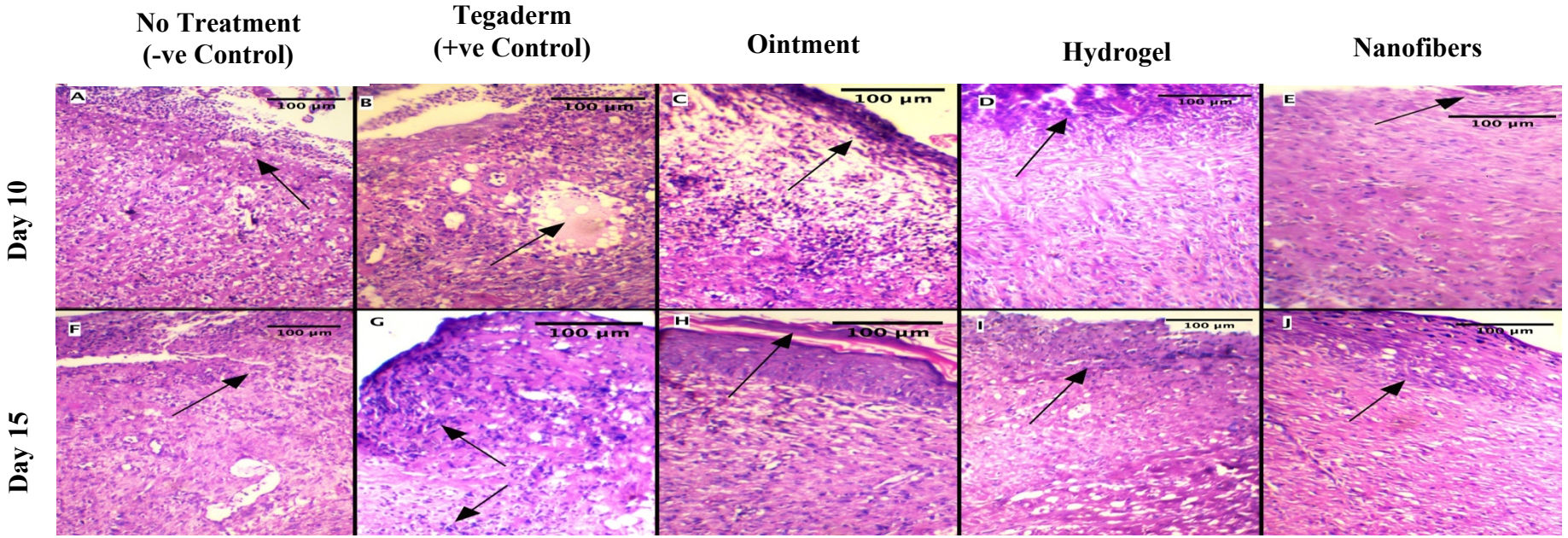


Figure 28 Effects of Different treatment forms on Diabetic Pressure Ulcer (H&E)

10 Days post treatment the **no treatment diabetic ulcer** skin tissue sections **(A)** showed thickening of epidermis by necrotic tissues (**arrow**). **Tegaderm treated group (B)** showed angiogenesis accompanied with fibrin thrombi (**arrow**). **Ointment treated group** showed **(C)** bridging of ulcer by epithelial cells **(arrow)**. The **Hydrogel treatment group (D)** showed necrotic tissues at its cut edges (**arrow**). The **Nanofibers treatment Group (E)** showed migration of basal epithelial cells (< 50%), (**arrow**).

15 Days post treatment the **no treatment diabetic ulcer (F)** showed moderate pattern of granulation tissue **(arrow)**. The **Tegaderm treated group (G)** showed focal aggregation of Inflammatory cells **(arrow). Ointment treated group (H)** showed keratinization epithelial cell layer (**arrow**). **Hydrogel treatment (I)** showed mild inflammatory cells infiltration (**arrow**). And the **Nanofibers treated Group (J)** showed non-keratinization epithelial layer (**arrow**). *Magnification (X 200) (n=4-6).*

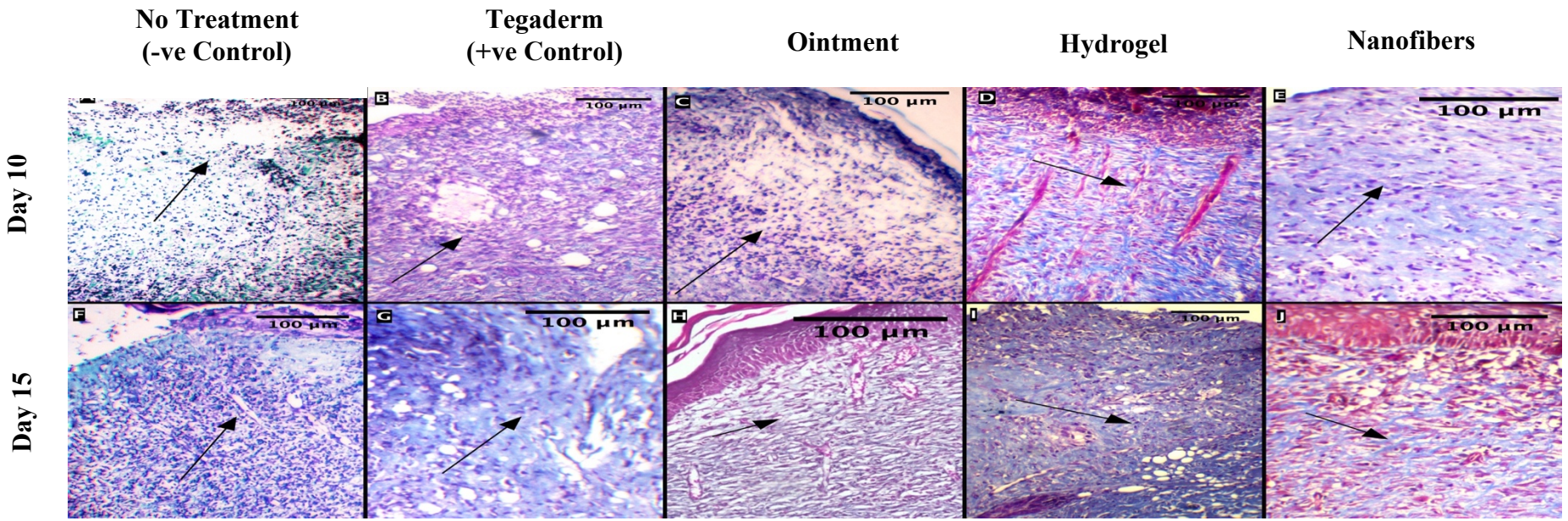


Figure 29 Effects of Different treatment forms on Diabetic Pressure Ulcer using Masson Trichrome

10 Days post treatment, the **no treatment diabetic ulcer (A)** and **hydrogel treatment group (D)** showed minimal mature collagen fibers **(arrow).** While, the **Tegaderm treated group (B)** showed mild mature collagen (**arrow**). Both **ointment treated (C)** and **nanofibers treated (E)** groups showed moderate mature collagen fibers (**arrows).**

At Day 15 the **no treatment diabetic ulcer (F)** and the **Tegaderm treated (G)** groups showed moderate mature collagen fibers **(arrows)**. While, the **ointment treated group (H)** and the **nanofibers treatment (J)** showed marked mature collagen fibers (**arrows).** The **hydrogel treatment group (I)** also showed marked mature collagen fibers (**arrow**). *Magnification (X 200) (n=4-6).*

Table 4 Semi-quantitative Histological Scoring at Days 10, 15

Group/ Day	Epithelization		PMNL		Fibroblasts		New vessels		Collagen	
	10	15	10	15	10	15	10	15	10	15
No Treatment (-ve Control)	0	0	4	3	1	3	2	2	1	3
Tegaderm (+ve Control)	0	0	4	3	1	3	3	3	3	3
Ointment	3	**4**	2	**1**	3	3	3	**1**	3	**4**
Hydrogel	0	0	4	2	3	3	4	2	2	3
Nanofiber	1	**3**	2	**1**	3	3	2	2	4	**4**

Scoring results showed that ointment and nanofibers treatments had dramatic effect on re-epithelization with the ointment showing better scores. The inflammatory infiltration PMNL were greatly reduced in the ointment, and nanofibers treated groups compared to the other groups. Angiogenesis was notably reduced in the ointment treated group which indicates complete healing. All formulas; ointment, Nanofibers & hydrogel enhanced collagen formation.

Chapter 4 : Conclusion & Future Perspectives

In conclusion, bed sores & diabetic skin infections are considered burden to healthcare & economies. In order to avoid excessive use of antibiotics and bacterial resistance, natural herbs provide good alternative as antibacterial and wound healing agents. From our results we found that turmeric, oregano extracts provide promising antibacterial agents, possess anti-inflammatory effect and act as natural antioxidants.

The antibacterial and antioxidant tests showed that turmeric ethanolic extract 5%, oregano essential oil 1% & chitosan nanoparticles 1% had the most antibacterial & antioxidant effects. Ointments were formulated of each herb individually. An *in vivo* pilot study was conducted on diabetic and non-diabetic rats with pressure ulcer. Results showed that turmeric 5% ointment successfully healed the ulcer in both diabetic and non-diabetic rats by day 15. The oregano 1% ointment achieved complete healing by day 15 in the non-diabetic group while in the diabetic group was achieved by day 21.

The above concentrations were incorporated in different forms (ointment, amorphous hydrogel & nanofibers) and were tested in the current study in comparison to commercially available wound dressing Tegaderm® (FDA approved dressing for treatment of leg ulcers). The best formula was found to be ointment followed by nanofibers. The prepared formulas showed significant antibacterial effects. On testing the formulas on L929 mouse fibroblast cell lines, ointments and hydrogels were non-cytotoxic and enhanced significantly cell proliferation. Nanofibers showed almost 50% reduction in cell viability while Tegaderm® were significantly toxic (90%) to the cells. *In vivo* results showed complete wound closure in 15 days, which suggest a great potential for further clinical applications for these formulations in chronic wounds and diabetic ulcers.

Future Directions

Encapsulation of turmeric and oregano extracts in chitosan nanoparticles will be of great value as they will provide sustained released for the extracts, protect them for oxidation. This is beside that they will protect the volatile oils from being degraded and as a result increasing their shelf life and retaining their function. Other advantage of encapsulation can be overcoming the stain that turmeric causes which limits its usage. Also, those formulated encapsulated nanoparticles can be embedded in spray form or biodegradable wound dressings to provide protective effect to the ulcer beside its healing effect.

References

Aggarwal, B. B., Sundaram, C., Malani, N., & Ichikawa, H. (2007). CURCUMIN: THE INDIAN SOLID GOLD. In The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease (pp. 1–75). Springer, Boston, MA. https://doi.org/10.1007/978-0-387-46401-5_1.

Aggarwal, B. B., Yuan, W., Li, S., & Gupta, S. C. (2013). Curcumin-free turmeric exhibits anti-inflammatory and anticancer activities: Identification of novel components of turmeric. Molecular Nutrition & Food Research, 57(9), 1529–1542. https://doi.org/10.1002/mnfr.201200838.

Agnihotri, S. A., Mallikarjuna, N. N., & Aminabhavi, T. M. (2004). Recent advances on chitosan-based micro- and nanoparticles in drug delivery. Journal of Controlled Release, 100(1), 5–28. https://doi.org/10.1016/j.jconrel.2004.08.010.

Al-Qushawi, A., Rassouli, A., Atyabi, F., Peighambari, S. M., Esfandyari-Manesh, M., Shams, G. R., & Yazdani, A. (2016). Preparation and Characterization of Three Tilmicosin-loaded Lipid Nanoparticles: Physicochemical Properties and in-vitro Antibacterial Activities. Iranian Journal of Pharmaceutical Research : IJPR, 15(4), 663–676.

Amin, N., & Doupis, J. (2016). Diabetic foot disease: From the evaluation of the "foot at risk" to the novel diabetic ulcer treatment modalities. World Journal of Diabetes, 7(7), 153–164. https://doi.org/10.4239/wjd.v7.i7.153.

Anders, J., Heinemann, A., Leffmann, C., Leutenegger, M., Pröfener, F., & von Renteln-Kruse, W. (2010). Decubitus Ulcers: Pathophysiology and Primary Prevention. Deutsches Ärzteblatt International, 107(21), 371–382. https://doi.org/10.3238/arztebl.2010.0371.

Andrade, T. A., Aguiar, A. F., Guedes, F. A., Leite, M. N., Caetano, G. F., Coelho, E. B., … Frade, M. A. (2015). Ex vivo Model of Human Skin (hOSEC) as Alternative to Animal use for Cosmetic Tests. Procedia Engineering, 110, 67–73. https://doi.org/10.1016/j.proeng.2015.07.011.

Annabi, N., Nichol, J. W., Zhong, X., Ji, C., Koshy, S., Khademhosseini, A., & Dehghani, F. (2010). Controlling the Porosity and Microarchitecture of Hydrogels for Tissue Engineering. Tissue Engineering. Part B, Reviews, 16(4), 371–383. https://doi.org/10.1089/ten.teb.2009.0639.

Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrobial Resistance and Infection Control, 6. https://doi.org/10.1186/s13756-017-0208x.

Baltzis, D., Eleftheriadou, I., & Veves, A. (2014). Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights. Advances in Therapy, 31(8), 817–836. https://doi.org/10.1007/s12325-014-0140-x.

Baron, J., Swaine, J., Presseau, J., Aspinall, A., Jaglal, S., White, B., … Grimshaw, J. (2016). Self-management interventions to improve skin care for pressure ulcer prevention in people with spinal cord injuries: a systematic review protocol. Systematic Reviews, 5, 150. https://doi.org/10.1186/s13643-016-0323-4.

Barrientos, S., Brem, H., Stojadinovic, O., & Tomic-Canic, M. (n.d.). Clinical application of growth factors and cytokines in wound healing. Wound Repair and Regeneration, 22(5), 569–578. https://doi.org/10.1111/wrr.12205.

Berlanga-Acosta, J., Schultz, G. S., López-Mola, E., Guillen-Nieto, G., García-Siverio, M., & Herrera-Martínez, L. (2013). Glucose Toxic Effects on Granulation Tissue Productive Cells: The Diabetics' Impaired Healing

[Research article]. https://doi.org/10.1155/2013/256043.

Bhattacharya, K., Hoffmann, E., Schins, R. F. P., Boertz, J., Prantl, E.-M., Alink, G. M., … Dopp, E. (2012). Comparison of micro- and nanoscale Fe+3-containing (Hematite) particles for their toxicological properties in human lung cells in vitro. Toxicological Sciences: An Official Journal of the Society of Toxicology, 126(1), 173–182. https://doi.org/10.1093/toxsci/kfs014.

Bielefeld, K. A., Amini-Nik, S., & Alman, B. A. (2013). Cutaneous wound healing: recruiting developmental pathways for regeneration. Cellular and Molecular Life Sciences: CMLS, 70(12), 2059–2081. https://doi.org/10.1007/s00018-012-1152-9.

Blakytny, R., & Jude, E. (2006). The molecular biology of chronic wounds and delayed healing in diabetes. Diabetic Medicine, 23(6), 594–608. https://doi.org/10.1111/j.1464-5491.2006.01773.x.

Blakytny, Robert, & Jude, E. B. (2009). Altered Molecular Mechanisms of Diabetic Foot Ulcers. The International Journal of Lower Extremity Wounds, 8(2), 95–104. https://doi.org/10.1177/1534734609337151.

Boateng, J., & Catanzano, O. (2015). Advanced Therapeutic Dressings for Effective Wound Healing—A Review. Journal of Pharmaceutical Sciences, 104(11), 3653–3680. https://doi.org/10.1002/jps.24610.

Bordoloi, M., Bordoloi, P. K., Dutta, P. P., Singh, V., Nath, S., Narzary, B., … Barua, I. C. (2016). Studies on some edible herbs: Antioxidant activity, phenolic content, mineral content and antifungal properties. Journal of Functional Foods, 23, 220–229. https://doi.org/10.1016/j.jff.2016.02.028.

Burd, A., Kwok, C. H., Hung, S. C., Chan, H. S., Gu, H., Lam, W. K., & Huang, L. (2007). A comparative study of the cytotoxicity of silver-based dressings in

monolayer cell, tissue explant, and animal models. Wound Repair and Regeneration, 15(1), 94–104. https://doi.org/10.1111/j.1524-475X.2006.00190.x.

Burtis, D. B., & Dobbs, M. R. (2009). CHAPTER 38 - Tetanus Toxin. In Clinical Neurotoxicology (pp. 427–435). Philadelphia: W.B. Saunders. https://doi.org/10.1016/B978-032305260-3.50044-7.

Calvo, P., Remuñan-López, C., Vila-Jato, J. L., & Alonso, M. J. (1997). Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharmaceutical Research, 14(10), 1431–1436.

Catrina, S.-B., Okamoto, K., Pereira, T., Brismar, K., & Poellinger, L. (2004). Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. Diabetes, 53(12), 3226–3232.

Chen, S., Liu, B., Carlson, M. A., Gombart, A. F., Reilly, D. A., & Xie, J. (2017). Recent advances in electrospun nanofibers for wound healing. Nanomedicine, 12(11), 1335–1352. https://doi.org/10.2217/nnm-2017-0017.

Cheppudira, B., Fowler, M., McGhee, L., Greer, A., Mares, A., Petz, L., … Clifford, J. L. (2013). Curcumin: a novel therapeutic for burn pain and wound healing. Expert Opinion on Investigational Drugs, 22(10), 1295–1303. https://doi.org/10.1517/13543784.2013.825249.

Chun, S.-S., Vattem, D. A., Lin, Y.-T., & Shetty, K. (2005). Phenolic antioxidants from clonal oregano (Origanum vulgare) with antimicrobial activity against Helicobacter pylori. Process Biochemistry, 40(2), 809–816. https://doi.org/10.1016/j.procbio.2004.02.018.

Clayton, K. N., Salameh, J. W., Wereley, S. T., & Kinzer-Ursem, T. L. (2016).

Physical characterization of nanoparticle size and surface modification using particle scatteringdiffusometry.Biomicrofluidics,10(5).https://doi.org/10.1063/1.4962992.

Comerota, A., & Lurie, F. (2015). Pathogenesis of venous ulcer. Seminars in Vascular Surgery, 28(1), 6–14. https://doi.org/10.1053/j.semvascsurg.2015.07.003.

Dabiri, G., Damstetter, E., & Phillips, T. (2016). Choosing a Wound Dressing Based on Common Wound Characteristics. Advances in Wound Care, 5(1), 32–41. https://doi.org/10.1089/wound.2014.0586.

Divya, K., Vijayan, S., George, T. K., & Jisha, M. S. (2017). Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. Fibers and Polymers, 18(2), 221–230. https://doi.org/10.1007/s12221-017-6690-1.

Donato-Trancoso, A., Monte-Alto-Costa, A., & Romana-Souza, B. (2016). Olive oil-induced reduction of oxidative damage and inflammation promotes wound healing of pressure ulcers in mice. Journal of Dermatological Science, 83(1), 60–69. https://doi.org/10.1016/j.jdermsci.2016.03.012.

Emanuelli, T., Burgeiro, A., & Carvalho, E. (2016). Effects of insulin on the skin: possible healing benefits for diabetic foot ulcers. Archives of Dermatological Research, 308(10), 677–694. https://doi.org/10.1007/s00403-016-1686-z.

Escandon, J., Vivas, A. C., Tang, J., Rowland, K. J., & Kirsner, R. S. (2011). High mortality in patients with chronic wounds. Wound Repair and Regeneration: Official Publication of the Wound Healing Society [and] the European Tissue Repair Society, 19(4), 526–528. https://doi.org/10.1111/j.1524-475X.2011.00699.x.

Falabella, A. F. (2006). Debridement and wound bed preparation. Dermatologic

Therapy, 19(6), 317–325. https://doi.org/10.1111/j.1529-8019.2006.00090.x.

Falanga, V. (2005). Wound healing and its impairment in the diabetic foot. The Lancet, 366(9498), 1736–1743. https://doi.org/10.1016/S0140-6736(05)67700-8.

Furman, B. L. (2015). Streptozotocin-Induced Diabetic Models in Mice and Rats. Current Protocols in Pharmacology, 70, 5.47.1-20. https://doi.org/10.1002/0471141755.ph0547s70.

Gainza, G., Villullas, S., Pedraz, J. L., Hernandez, R. M., & Igartua, M. (2015). Advances in drug delivery systems (DDSs) to release growth factors for wound healing and skin regeneration. Nanomedicine: Nanotechnology, Biology, and Medicine, 11(6), 1551–1573. https://doi.org/10.1016/j.nano.2015.03.002.

Gal, P., Kilik, R, Mokry, M., Vidinsky, B., Vasilenko, T., … Lenhardt, L. (2008). Simple method of open skin wound healing model in corticosteroid-treated and diabetic rats: standardization of semi-quantitative and quantitative histological assessments. Veterinární Medicína, 53(No. 12), 652–659. https://doi.org/10.17221/1973-VETMED.

Gemeda, N., Tadele, A., Lemma, H., Girma, B., Addis, G., Tesfaye, B., … Debella, A. (2018). Development, Characterization, and Evaluation of Novel Broad-Spectrum Antimicrobial Topical Formulations from Cymbopogon martini (Roxb.) W. Watson Essential Oil. https://doi.org/10.1155/2018/9812093.

Gilbert, R. W. D., Vickaryous, M. K., & Viloria-Petit, A. M. (2016). Signalling by Transforming Growth Factor Beta Isoforms in Wound Healing and Tissue Regeneration. Journal of Developmental Biology, 4(2). https://doi.org/10.3390/jdb4020021.

Goyal, R., Macri, L. K., Kaplan, H. M., & Kohn, J. (2016). Nanoparticles and nanofibers for topical drug delivery. Journal of Controlled Release : Official Journal of the Controlled Release Society, 240, 77–92. https://doi.org/10.1016/j.jconrel.2015.10.049.

Grey, J. E., Harding, K. G., & Enoch, S. (2006). Pressure ulcers. BMJ : British Medical Journal, 332(7539), 472–475.

Guenin-Macé, L., Oldenburg, R., Chrétien, F., & Demangel, C. (2014). Pathogenesis of skin ulcers: lessons from the Mycobacterium ulcerans and Leishmania spp. pathogens. Cellular and Molecular Life Sciences, 71(13), 2443–2450. https://doi.org/10.1007/s00018-014-1561-z.

Guest, J. F., Ayoub, N., McIlwraith, T., Uchegbu, I., Gerrish, A., Weidlich, D., … Vowden, P. (n.d.). Health economic burden that different wound types impose on the UK's National Health Service. International Wound Journal, 14(2), 322–330. https://doi.org/10.1111/iwj.12603.

Guest, J. F., Fuller, G. W., & Vowden, P. (2018). Venous leg ulcer management in clinical practice in the UK: costs and outcomes. International Wound Journal, 15(1), 29–37. https://doi.org/10.1111/iwj.12814.

Gupta, A., & Kumar, P. (2015). Assessment of the histological state of the healing wound. 2015, 2(5), 239–242.

Gupta, S., Black, J., II, L., & Niezgoda, J. (n.d.). Management of Chronic Wounds:, 20.

Gupta, S. C., Sung, B., Kim, J. H., Prasad, S., Li, S., & Aggarwal, B. B. (2012). Multitargeting by turmeric, the golden spice: From kitchen to clinic. Molecular Nutrition & Food Research, 57(9), 1510–1528. https://doi.org/10.1002/mnfr.201100741.

Hameedaldeen, A., Liu, J., Batres, A., Graves, G. S., & Graves, D. T. (2014). FOXO1, TGF-β Regulation and Wound Healing. International Journal of Molecular Sciences, 15(9), 16257–16269. https://doi.org/10.3390/ijms150916257.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86(6), 985–990. https://doi.org/10.1046/j.1365-2672.1999.00780.x.

Han, G., & Ceilley, R. (2017). Chronic Wound Healing: A Review of Current Management and Treatments. Advances in Therapy, 34(3), 599–610. https://doi.org/10.1007/s12325-017-0478-y

Hasanovic, A., Zehl, M., Reznicek, G., & Valenta, C. (2009). Chitosan-tripolyphosphate nanoparticles as a possible skin drug delivery system for aciclovir with enhanced stability. Journal of Pharmacy and Pharmacology, 61(12), 1609–1616. https://doi.org/10.1211/jpp.61.12.0004.

He, F., Jiao, H., Tian, Y., Zhao, L., Liao, X., Fan, Z., & Liu, B. (2018). Facile and large-scale synthesis of curcumin/PVA hydrogel: effectively kill bacteria and accelerate cutaneous wound healing in the rat. Journal of Biomaterials Science. Polymer Edition, 29(4), 325–343. https://doi.org/10.1080/09205063.2017.1417002.

Health Quality Ontario. (2009). Management of chronic pressure ulcers: an evidence-based analysis. Ontario Health Technology Assessment Series, 9(3), 1–203.

Herber, O. R., Schnepp, W., & Rieger, M. A. (2007). A systematic review on the impact of leg ulceration on patients' quality of life. Health and Quality of Life Outcomes, 5, 44. https://doi.org/10.1186/1477-7525-5-44.

Hiro, M. E., Pierpont, Y. N., Ko, F., Wright, T. E., Robson, M. C., & Payne, W. G.

(2012). Comparative Evaluation of Silver-Containing Antimicrobial Dressings on In Vitro and In Vivo Processes of Wound Healing. Eplasty, 12. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3471607.

Hu, D., & Wang, L. (2016). Physical and antibacterial properties of polyvinyl alcohol films reinforced with quaternized cellulose. Journal of Applied Polymer Science, 133(25). https://doi.org/10.1002/app.43552.

Huang, C., Leavitt, T., Bayer, L. R., & Orgill, D. P. (2014). Effect of negative pressure wound therapy on wound healing. Current Problems in Surgery, 51(7), 301–331. https://doi.org/10.1067/j.cpsurg.2014.04.001.

Huang, Y., Xie, T., Cao, Y., Wu, M., Yu, L., Lu, S., … Ruan, H. (2015). Comparison of two classification systems in predicting the outcome of diabetic foot ulcers: the Wagner grade and the Saint Elian Wound score systems. Wound Repair and Regeneration: Official Publication of the Wound Healing Society [and] the European Tissue Repair Society, 23(3), 379–385. https://doi.org/10.1111/wrr.12289.

Information, N. C. for B., Pike, U. S. N. L. of M. 8600 R., MD, B., & Usa, 20894. (2018). What are the treatment options for chronic wounds? Institute for Quality and Efficiency in Health Care (IQWiG). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK326436.

Iyun, A. O., Ademola, S. A., Olawoye, O. A., Michael, A. I., & Oluwatosin, O. M. (2016). Point Prevalence of Chronic Wounds at a Tertiary Hospital in Nigeria. Wounds: A Compendium of Clinical Research and Practice, 28(2), 57–62.

Jagetia, G. C., & Rajanikant, G. K. (2004). Role of curcumin, a naturally occurring phenolic compound of turmeric in accelerating the repair of excision wound, in mice whole-body exposed to various doses of γ-radiation. Journal of

Surgical Research, 120(1), 127–138. https://doi.org/10.1016/j.jss.2003.12.003.

Jhamb, S., Vangaveti, V. N., & Malabu, U. H. (2016). Genetic and molecular basis of diabetic foot ulcers: Clinical review. Journal of Tissue Viability, 25(4), 229–236. https://doi.org/10.1016/j.jtv.2016.06.005.

Jung, S.-M., Yoon, G. H., Lee, H. C., & Shin, H. S. (2015). Chitosan nanoparticle/PCL nanofiber composite for wound dressing and drug delivery. Journal of Biomaterials Science, Polymer Edition, 26(4), 252–263. https://doi.org/10.1080/09205063.2014.996699.

Kalashnikova, I., Das, S., & Seal, S. (2015). Nanomaterials for wound healing: scope and advancement. Nanomedicine, 10(16), 2593–2612. https://doi.org/10.2217/nnm.15.82.

Kamat, V., Bodas, D., & Paknikar, K. (2016). Chitosan nanoparticles synthesis caught in action using microdroplet reactions. Scientific Reports, 6, 22260. https://doi.org/10.1038/srep22260.

Kamoun, E. A., Chen, X., Mohy Eldin, M. S., & Kenawy, E.-R. S. (2015). Crosslinked poly(vinyl alcohol) hydrogels for wound dressing applications: A review of remarkably blended polymers. Arabian Journal of Chemistry, 8(1), 1–14. https://doi.org/10.1016/j.arabjc.2014.07.005.

Kamoun, E. A., Kenawy, E.-R. S., & Chen, X. (2017). A review on polymeric hydrogel membranes for wound dressing applications: PVA-based hydrogel dressings. Journal of Advanced Research, 8(3), 217–233. https://doi.org/10.1016/j.jare.2017.01.005.

Kapp, S., Miller, C., & Santamaria, N. (n.d.). The quality of life of people who have chronic wounds and who self-treat. Journal of Clinical Nursing, 27(1–2), 182–192. https://doi.org/10.1111/jocn.13870.

Katas, H., Mohamad, A., & Zin, N. M. (2011). Physicochemical effects of chitosan-tripolyphosphate nanoparticles on antibacterial activity against gram-positive and gram-negative bacteria. Journal of Medical Sciences, 11(4), 192–197. https://doi.org/10.3923/jms.2011.192.197.

Katas, H., Raja, M. A. G., & Lam, K. L. (2013). Development of Chitosan Nanoparticles as a Stable Drug Delivery System for Protein/siRNA [Research article]. https://doi.org/10.1155/2013/146320.

Kawai, F., & Hu, X. (2009). Biochemistry of microbial polyvinyl alcohol degradation. Applied Microbiology and Biotechnology, 84(2), 227–237. https://doi.org/10.1007/s00253-009-2113-6.

Khalili Fard, J., Jafari, S., & Eghbal, M. A. (2015). A Review of Molecular Mechanisms Involved in Toxicity of Nanoparticles. Advanced Pharmaceutical Bulletin, 5(4), 447–454. https://doi.org/10.15171/apb.2015.061.

Kheiri, A., Moosawi Jorf, S. A., Malihipour, A., Saremi, H., & Nikkhah, M. (2017a). Synthesis and characterization of chitosan nanoparticles and their effect on Fusarium head blight and oxidative activity in wheat. International Journal of Biological Macromolecules, 102, 526–538. https://doi.org/10.1016/j.ijbiomac.2017.04.034.

Khodaeian, M., Enayati, S., Tabatabaei-Malazy, O., & Amoli, M. M. (2015). Association between Genetic Variants and Diabetes Mellitus in Iranian Populations: A Systematic Review of Observational Studies. Journal of Diabetes Research, 2015, 585917. https://doi.org/10.1155/2015/585917.

Kim, D.-G., Jeong, Y.-I., Choi, C., Roh, S.-H., Kang, S.-K., Jang, M.-K., & Nah, J.-W. (2006). Retinol-encapsulated low molecular water-soluble chitosan nanoparticles. International Journal of Pharmaceutics, 319(1), 130–138.

https://doi.org/10.1016/j.ijpharm.2006.03.040.

Kumar, A., & Han, S. S. (2017). PVA-based hydrogels for tissue engineering: A review. International Journal of Polymeric Materials and Polymeric Biomaterials, 66(4), 159–182. https://doi.org/10.1080/00914037.2016.1190930

Langemo, D., Haesler, E., Naylor, W., Tippett, A., & Young, T. (2015). Evidence-based guidelines for pressure ulcer management at the end of life. International Journal of Palliative Nursing, 21(5), 225–232. https://doi.org/10.12968/ijpn.2015.21.5.225.

Larouche, J., Sheoran, S., Maruyama, K., & Martino, M. M. (2018). Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets. Advances in Wound Care, 7(7), 209–231. https://doi.org/10.1089/wound.2017.0761.

Leaper, D. J., Meaume, S., Apelqvist, J., Teot, L., & Gottrup, F. (2011). 24 - Debridement methods of non-viable tissue in wounds. In D. Farrar (Ed.), Advanced Wound Repair Therapies (pp. 606–632). Woodhead Publishing. https://doi.org/10.1533/9780857093301.5.606.

Lipsky, B. A., & Hoey, C. (2009). Topical Antimicrobial Therapy for Treating Chronic Wounds. Clinical Infectious Diseases, 49(10), 1541–1549. https://doi.org/10.1086/644732.

Liu, H., & Gao, C. (2008). Preparation and properties of ionically cross-linked chitosan nanoparticles. Polymers for Advanced Technologies, 20(7), 613–619. https://doi.org/10.1002/pat.1306.

Loh, C., Loh, A., & Lim, P. (2013). The efficacy of topical growth factors in wound healing – Which one is better? International Journal of Surgery, 11(8), 684–

685. https://doi.org/10.1016/j.ijsu.2013.06.527.

Lone, P. A., Ahmed, S. wakeel, Prasad, V., & Ahmed, B. (2018). Role of turmeric in management of alveolar osteitis (dry socket): A randomised clinical study. Journal of Oral Biology and Craniofacial Research, 8(1), 44–47. https://doi.org/10.1016/j.jobcr.2017.08.005.

Maarof, M., Law, J. X., Chowdhury, S. R., Khairoji, K. A., Saim, A. B., & Idrus, R. B. H. (2016). Secretion of wound healing mediators by single and bi-layer skin substitutes. Cytotechnology, 68(5), 1873–1884. https://doi.org/10.1007/s10616-015-9940-3.

Mani, H., Sidhu, G. S., Kumari, R., Gaddipati, J. P., Seth, P., & Maheshwari, R. K. (2008). Curcumin differentially regulates TGF-β1, its receptors and nitric oxide synthase during impaired wound healing. BioFactors, 16(1–2), 29–43. https://doi.org/10.1002/biof.5520160104.

Martinez-Correa, H. A., Paula, J. T., Kayano, A. C. A. V., Queiroga, C. L., Magalhães, P. M., Costa, F. T. M., & Cabral, F. A. (2017). Composition and antimalarial activity of extracts of Curcuma longa L. obtained by a combination of extraction processes using supercritical CO2, ethanol and water as solvents. The Journal of Supercritical Fluids, 119, 122–129. https://doi.org/10.1016/j.supflu.2016.08.017.

Martínez-De Jesús, F. R. (2010a). A checklist system to score healing progress of diabetic foot ulcers. The International Journal of Lower Extremity Wounds, 9(2), 74–83. https://doi.org/10.1177/1534734610371594.

Martínez-De Jesús, F. R. (2010b). A checklist system to score healing progress of diabetic foot ulcers. The International Journal of Lower Extremity Wounds, 9(2), 74–83. https://doi.org/10.1177/1534734610371594.

Meizarini, A., Aryati, Riawan, W., & Puteri, A. (2018). Anti-inflammatory properties of a wound dressing combination of zinc oxide and turmeric extract. Veterinary World, 11(1), 25–29. https://doi.org/10.14202/vetworld.2018.25-29.

Mendoza-Garcia, J., Sebastian, A., Alonso-Rasgado, T., & Bayat, A. (2015). Ex vivo evaluation of the effect of photodynamic therapy on skin scars and striae distensae. Photodermatology, Photoimmunology & Photomedicine, 31(5), 239–251. https://doi.org/10.1111/phpp.12180.

Mendoza-Mari Y, Valdés-Pérez C, second, Rodríguez-Corrales E, & Suárez-Alba J. (2013). Histological and Transcriptional Expression differences between Diabetic Foot and Pressure Ulcers. Journal of Diabetes & Metabolism, 4(8), 1–10. https://doi.org/10.4172/2155-6156.1000296.

Morton, L. M., & Phillips, T. J. (2016). Wound healing and treating wounds: Differential diagnosis and evaluation of chronic wounds. Journal of the American Academy of Dermatology, 74(4), 589–605; quiz 605–606. https://doi.org/10.1016/j.jaad.2015.08.068.

Moutsatsou, P., Coopman, K., & Georgiadou, S. (2017). Biocompatibility Assessment of Conducting PANI/Chitosan Nanofibers for Wound Healing Applications. Polymers (20734360), 9(12), 1–23. https://doi.org/10.3390/polym9120687.

Muraina, I. A., Suleiman, M. M., & Eloff, J. N. (2009). Can MTT be used to quantify the antioxidant activity of plant extracts? Phytomedicine, 16(6), 665–668. https://doi.org/10.1016/j.phymed.2008.11.005.

Nageswaran, S., Vijayakumar, R., & Sivarasu, S. (2015). Design of mechanical interface to re-distribute excess pressure to prevent the formation of decubitus ulcers in bed ridden patients. In 2015 37th Annual International Conference of

the IEEE Engineering in Medicine and Biology Society (EMBC) (pp. 1021–1024). https://doi.org/10.1109/EMBC.2015.7318538.

Nain, P. S., Uppal, S. K., Garg, R., Bajaj, K., & Garg, S. (2011). Role of Negative Pressure Wound Therapy in Healing of Diabetic Foot Ulcers. Journal of Surgical Technique and Case Report, 3(1), 17–22. https://doi.org/10.4103/2006-8808.78466.

Nakatani, N. (2000). Phenolic antioxidants from herbs and spices. Biofactors, 13(1–4), 141. https://doi.org/10.1002/biof.5520130123.

Niknia, N., & Kadkhodaee, R. (2017). Gum tragacanth-polyvinyl alcohol cryogel and xerogel blends for oral delivery of silymarin: Structural characterization and mucoadhesive property. Carbohydrate Polymers, 177, 315–323. https://doi.org/10.1016/j.carbpol.2017.08.110.

Norman, G., Dumville, J. C., Moore, Z. E., Tanner, J., Christie, J., & Goto, S. (2016). Antibiotics and antiseptics for pressure ulcers. In The Cochrane Library. John Wiley & Sons, Ltd. https://doi.org/10.1002/14651858.CD011586.pub2.

O'Dea, R., Byrne, H., & Waters, S. (2012). Continuum Modelling of In Vitro Tissue Engineering: A Review. In L. Geris (Ed.), Computational Modeling in Tissue Engineering (Vol. 10, pp. 229–266). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/8415_2012_140.

Ogurtsova, K., Fernandes, J. D. da R., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N. H., … Makaroff, L. E. (2017). IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Research and Clinical Practice, 128, 40–50. https://doi.org/10.1016/j.diabres.2017.03.024.

Olmedo, R., Nepote, V., & Grosso, N. R. (2014). Antioxidant activity of fractions from oregano essential oils obtained by molecular distillation. Food

Chemistry, 156, 212–219. https://doi.org/10.1016/j.foodchem.2014.01.087.

Palmer, B. C., & DeLouise, L. A. (2016). Nanoparticle-Enabled Transdermal Drug Delivery Systems for Enhanced Dose Control and Tissue Targeting. Molecules (Basel, Switzerland), 21(12). https://doi.org/10.3390/molecules21121719.

parhi, R. (2017). Cross-Linked Hydrogel for Pharmaceutical Applications: A Review. Advanced Pharmaceutical Bulletin, 7(4), 515–530. https://doi.org/10.15171/apb.2017.064.

Patravale, V., Dandekar, P., & Jain, R. (2012). 4 - Nanotoxicology: evaluating toxicity potential of drug-nanoparticles. In V. Patravale, P. Dandekar, & R. Jain (Eds.), Nanoparticulate Drug Delivery (pp. 123–155). Woodhead Publishing. https://doi.org/10.1533/9781908818195.123.

Pawar, R. S., Toppo, F. A., Mandloi, A. S., & Shaikh, S. (2015). Exploring the role of curcumin containing ethanolic extract obtained from Curcuma longa (rhizomes) against retardation of wound healing process by aspirin. Indian Journal of Pharmacology, 47(2), 160–166. https://doi.org/10.4103/0253-7613.153422.

Pazyar, N., Yaghoobi, R., Rafiee, E., Mehrabian, A., & Feily, A. (2014). Skin wound healing and phytomedicine: a review. Skin Pharmacology and Physiology, 27(6), 303–310. https://doi.org/10.1159/000357477.

Perez, D., Bramkamp, M., Exe, C., von Ruden, C., & Ziegler, A. (2010). Modern wound care for the poor: a randomized clinical trial comparing the vacuum system with conventional saline-soaked gauze dressings. American Journal of Surgery, 199(1), 14–20. https://doi.org/10.1016/j.amjsurg.2008.12.029.

Perez, R., & Davis, S. C. (2008). Relevance of animal models for wound healing.

Wounds: A Compendium of Clinical Research and Practice, 20(1), 3–8.

Phan, T.-T., See, P., Lee, S.-T., & Chan, S.-Y. (2001). Protective Effects of Curcumin against Oxidative Damage on Skin Cells In Vitro: Its Implication for Wound Healing. Journal of Trauma and Acute Care Surgery, 51(5), 927.

Pillay, V., Dott, C., Choonara, Y. E., Tyagi, C., Tomar, L., Kumar, P., … Ndesendo, V. M. K. (2013). A Review of the Effect of Processing Variables on the Fabrication of Electrospun Nanofibers for Drug Delivery Applications [Research article]. https://doi.org/10.1155/2013/789289.

pmhdev. (2015). What are the treatment options for chronic wounds? PubMed Health. Retrieved from https://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0079411.

Popov, D. (2010). Endothelial cell dysfunction in hyperglycemia: Phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical outcomes. International Journal of Diabetes Mellitus, 2(3), 189–195. https://doi.org/10.1016/j.ijdm.2010.09.002.

Prabaharan, M. (2015). Chitosan-based nanoparticles for tumor-targeted drug delivery. International Journal of Biological Macromolecules, 72, 1313–1322. https://doi.org/10.1016/j.ijbiomac.2014.10.052.

Pubchem. (n.d.). Silver nitrate. Retrieved February 1, 2019, from https://pubchem.ncbi.nlm.nih.gov/compound/24470.

Raghav, A., Khan, Z. A., Labala, R. K., Ahmad, J., Noor, S., & Mishra, B. K. (2018). Financial burden of diabetic foot ulcers to world: a progressive topic to discuss always. Therapeutic Advances in Endocrinology and Metabolism, 9(1), 29–31. https://doi.org/10.1177/2042018817744513.

Ragi, J., Pappert, A., Rao, B., Havkin-Frenkel, D., & Milgraum, S. (2011). Oregano extract ointment for wound healing: a randomized, double-blind, petrolatum-

controlled study evaluating efficacy. Journal of Drugs in Dermatology: JDD, 10(10), 1168–1172.

Rahman, G. A., Adigun, I. A., & Fadeyi, A. (2010). Epidemiology, etiology, and treatment of chronic leg ulcer: Experience with sixty patients. Annals of African Medicine, 9(1), 1. https://doi.org/10.4103/1596-3519.62615.

Rao, C. V. (2007). REGULATION OF COX AND LOX BY CURCUMIN. In B. B. Aggarwal, Y.-J. Surh, & S. Shishodia (Eds.), The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease (pp. 213–226). Boston, MA: Springer US. https://doi.org/10.1007/978-0-387-46401-5_9.

Rath, G., Hussain, T., Chauhan, G., Garg, T., & Goyal, A. K. (2016). Collagen nanofiber containing silver nanoparticles for improved wound-healing applications. Journal of Drug Targeting, 24(6), 520–529. https://doi.org/10.3109/1061186X.2015.1095922.

Ray, J. A., Valentine, W. J., Secnik, K., Oglesby, A. K., Cordony, A., Gordois, A., … Palmer, A. J. (2005). Review of the cost of diabetes complications in Australia, Canada, France, Germany, Italy and Spain. Current Medical Research and Opinion, 21(10), 1617–1629. https://doi.org/10.1185/030079905X65349.

Roaf, R. (2006). The causation and prevention of bed sores. Journal of Tissue Viability, 16(2), 6–8. https://doi.org/10.1016/S0965-206X(06)62002-0.

Rosato, A., Vitali, C., Piarulli, M., Mazzotta, M., Argentieri, M. P., & Mallamaci, R. (2009). In vitro synergic efficacy of the combination of Nystatin with the essential oils of Origanum vulgare and Pelargonium graveolens against some Candida species. Phytomedicine: International Journal of Phytotherapy and Phytopharmacology, 16(10), 972–975.

https://doi.org/10.1016/j.phymed.2009.02.011.

Sahu, D., Kannan, G. M., Tailang, M., & Vijayaraghavan, R. (2016). In Vitro Cytotoxicity of Nanoparticles: A Comparison between Particle Size and Cell Type [Research article]. https://doi.org/10.1155/2016/4023852.

Sakkas, H., & Papadopoulou, C. (2017). Antimicrobial Activity of Basil, Oregano, and Thyme Essential Oils. Journal of Microbiology and Biotechnology, 27(3), 429–438. https://doi.org/10.4014/jmb.1608.08024.

Sami, D. G., Heiba, H. H., & Abdellatif, A. (2019). Wound healing models: A systematic review of animal and non-animal models. Wound Medicine, 24(1), 8–17. https://doi.org/10.1016/j.wndm.2018.12.001.

Sarafian, G., Afshar, M., Mansouri, P., Asgarpanah, J., Raoufinejad, K., & Rajabi, M. (2015). Topical Turmeric Microemulgel in the Management of Plaque Psoriasis; A Clinical Evaluation. Iranian Journal of Pharmaceutical Research : IJPR, 14(3), 865–876.

Schreiber, M. L. (2016). Evidence-Based Practice. Negative Pressure Wound Therapy. MEDSURG Nursing, 25(6), 425–428.

Seaton, M., Hocking, A., & Gibran, N. S. (2015). Porcine Models of Cutaneous Wound Healing. ILAR Journal, 56(1), 127–138. https://doi.org/10.1093/ilar/ilv016.

Serena, T. E. (2015). The increasing role of epidermal grafting utilizing a novel harvesting system in chronic wounds. Wounds: A Compendium of Clinical Research and Practice, 27(2), 26–30.

Shah, A., & Amini-Nik, S. (2017). The Role of Phytochemicals in the Inflammatory Phase of Wound Healing. International Journal of Molecular Sciences, 18(5). https://doi.org/10.3390/ijms18051068.

Sherman, R. A. (2009). Maggot Therapy Takes Us Back to the Future of Wound Care: New and Improved Maggot Therapy for the 21st Century. Journal of Diabetes Science and Technology, 3(2), 336–344.

Shivalingu, B. R., Vivek, H. K., Nafeesa, Z., Priya, B. S., & Swamy, S. N. (2015). Comparative analysis of procoagulant and fibrinogenolytic activity of crude protease fractions of turmeric species. Journal of Ethnopharmacology, 172, 261–264. https://doi.org/10.1016/j.jep.2015.06.018.

Sibbald, R. G., Goodman, L., Norton, L., Krasner, D. L., & Ayello, E. A. (2012). Prevention and treatment of pressure ulcers. Skin Therapy Letter, 17(8), 4–7.

Singh, A. K. (2016). Chapter 4 - Experimental Methodologies for the Characterization of Nanoparticles. In A. K. Singh (Ed.), Engineered Nanoparticles (pp. 125–170). Boston: Academic Press. https://doi.org/10.1016/B978-0-12-801406-6.00004-2.

Stanojević, L. P., Stanojević, J. S., Cvetković, D. J., & Ilić, D. P. (2018). Antioxidant activity of oregano essential oil (Origanum vulgare L.). Biologica Nyssana, 7(2). Retrieved from http://journal.pmf.ni.ac.rs/bionys/index.php/bionys/article/view/160.

Strauss, M. B., Moon, H., Busch, J. A., Jones, C. K., Nhan, L., Miller, S., & Le, P. N. (2016). Reliability Assessment of an Innovative Wound Score. Wounds : A Compendium of Clinical Research and Practice, 28(6), 206–213.

Sweeney, I. R., Miraftab, M., & Collyer, G. (2012). A critical review of modern and emerging absorbent dressings used to treat exuding wounds. International Wound Journal, 9(6), 601–612. https://doi.org/10.1111/j.1742-481X.2011.00923.x

Syper, L. (1967). Silver (II) as an oxidant for organic compounds. Tetrahedron

Letters, 8(42), 4193–4198. https://doi.org/10.1016/S0040-4039(01)89720-3.

Taepaiboon, P., Rungsardthong, U., & Supaphol, P. (2007). Vitamin-loaded electrospun cellulose acetate nanofiber mats as transdermal and dermal therapeutic agents of vitamin A acid and vitamin E. European Journal of Pharmaceutics and Biopharmaceutics, 67(2), 387–397. https://doi.org/10.1016/j.ejpb.2007.03.018.

Talorete, T. P. N., Bouaziz, M., Sayadi, S., & Isoda, H. (2006). Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells. Cytotechnology, 52(3), 189–198. https://doi.org/10.1007/s10616-007-9057-4.

Tara, F., Golmakani, N., Motlagh, E. R., Assili, J., & Shakeri, M. (2009). P284 The effects of turmeric (Curcuma longa L) ointment on healing of episiotomy site in primiparous women. International Journal of Gynecology & Obstetrics, 107(S2), S493–S494. https://doi.org/10.1016/S0020-7292(09)61774-4.

Th, N., Cuc, K., Dung, T. T. K., & Cuong, P. V. (n.d.). ANTIMICROBIAL EFFECT OF TURMERIC OIL (CURCUMA LONGA L.), 9.

Thangapazham, R. L., Sharad, S., & Maheshwari, R. K. (2016). Phytochemicals in Wound Healing. Advances in Wound Care, 5(5), 230–241. https://doi.org/10.1089/wound.2013.0505.

Tomioka, T., Soma, K., Sato, Y., Miura, K., & Endo, A. (2018). S. Gupta. Auris Nasus Larynx, 45(5), 1130–1134. https://doi.org/10.1016/j.anl.2018.04.004.

Trøstrup, H., Thomsen, K., Calum, H., Høiby, N., & Moser, C. (2016, November 1). Animal models of chronic wound care: the application of biofilms in clinical research. https://doi.org/10.2147/CWCMR.S84361.

Trujillo, A. N., Kesl, S. L., Sherwood, J., Wu, M., & Gould, L. J. (2015). Demonstration of the Rat Ischemic Skin Wound Model. Journal of Visualized

Experiments : JoVE, (98). https://doi.org/10.3791/52637.

Try, C., Moulari, B., Béduneau, A., Fantini, O., Pin, D., Pellequer, Y., & Lamprecht, A. (2016). Size dependent skin penetration of nanoparticles in murine and porcine dermatitis models. European Journal of Pharmaceutics and Biopharmaceutics, 100, 101–108. https://doi.org/10.1016/j.ejpb.2016.01.002

Tsourdi, E., Barthel, A., Rietzsch, H., Reichel, A., & Bornstein, S. R. (2013). Current Aspects in the Pathophysiology and Treatment of Chronic Wounds in Diabetes Mellitus. https://doi.org/10.1155/2013/385641.

Tsourdi, E., Barthel, A., Rietzsch, H., Reichel, A., & Bornstein, S. R. (2013b). Current aspects in the pathophysiology and treatment of chronic wounds in diabetes mellitus. BioMed Research International, 2013, 385641. https://doi.org/10.1155/2013/385641.

Tubaishat, A., Papanikolaou, P., Anthony, D., & Habiballah, L. (2018). Pressure Ulcers Prevalence in the Acute Care Setting: A Systematic Review, 2000-2015. Clinical Nursing Research, 27(6), 643–659. https://doi.org/10.1177/1054773817705541.

Tuk, B., Tong, M., Fijneman, E. M. G., & van Neck, J. W. (2014). Hyperbaric Oxygen Therapy to Treat Diabetes Impaired Wound Healing in Rats. PLoS ONE, 9(10), 1–8. https://doi.org/10.1371/journal.pone.0108533.

Tyagi, P., Singh, M., Kumari, H., Kumari, A., & Mukhopadhyay, K. (2015). Bactericidal Activity of Curcumin I Is Associated with Damaging of Bacterial Membrane. PLoS ONE, 10(3). https://doi.org/10.1371/journal.pone.0121313.

Ud-Din, S., & Bayat, A. (2017). Non-animal models of wound healing in cutaneous repair: In silico, in vitro, ex vivo, and in vivo models of wounds and scars in human skin. Wound Repair and Regeneration: Official Publication of the

Wound Healing Society [and] the European Tissue Repair Society, 25(2), 164–176. https://doi.org/10.1111/wrr.12513.

van Neck, J. W., Tuk, B., Fijneman, E. M. G., Redeker, J. J., Talahatu, E. M., & Tong, M. (2017). Hyperbaric oxygen therapy for wound healing in diabetic rats: Varying efficacy after a clinically-based protocol. PLoS ONE, 12(5), 1–16. https://doi.org/10.1371/journal.pone.0177766.

Volk, S. W., & Bohling, M. W. (2013). Comparative wound healing--are the small animal veterinarian's clinical patients an improved translational model for human wound healing research? Wound Repair and Regeneration: Official Publication of the Wound Healing Society and the European Tissue Repair Society, 21(3), 372–381. https://doi.org/10.1111/wrr.12049.

Wang, J. J., Zeng, Z. W., Xiao, R. Z., Xie, T., Zhou, G. L., Zhan, X. R., & Wang, S. L. (2011). Recent advances of chitosan nanoparticles as drug carriers. International Journal of Nanomedicine, 6, 765–774. https://doi.org/10.2147/IJN.S17296.

Wang, T., Zheng, Y., Shen, Y., Shi, Y., Li, F., Su, C., & Zhao, L. (2017). Chitosan nanoparticles loaded hydrogels promote skin wound healing through the modulation of reactive oxygen species. Artificial Cells, Nanomedicine, and Biotechnology, 0(0), 1–12. https://doi.org/10.1080/21691401.2017.1415212.

Wang, T., Zheng, Y., Shen, Y., Shi, Y., Li, F., Su, C., & Zhao, L. (2018). Chitosan nanoparticles loaded hydrogels promote skin wound healing through the modulation of reactive oxygen species. Artificial Cells, Nanomedicine, and Biotechnology,46(sup1),138–149. https://doi.org/10.1080/21691401.2017.1415212.

Weng, L., & Xie, J. (2015). Smart Electrospun Nanofibers for Controlled Drug

Release: Recent Advances and New Perspectives. Current Pharmaceutical Design, 21(15), 1944–1959.

Wilhelm, K.-P., Wilhelm, D., & Bielfeldt, S. (2017). Models of wound healing: an emphasis on clinical studies. Skin Research and Technology: Official Journal of International Society for Bioengineering and the Skin (ISBS) and International Society for Digital Imaging of Skin (ISDIS) and International Society for Skin Imaging (ISSI), 23(1), 3–12. https://doi.org/10.1111/srt.12317

Wong, V. W., Sorkin, M., Glotzbach, J. P., Longaker, M. T., & Gurtner, G. C. (2011). Surgical Approaches to Create Murine Models of Human Wound Healing [Research article]. https://doi.org/10.1155/2011/969618.

Woo, K. Y., Keast, D., Parsons, N., Sibbald, R. G., & Mittmann, N. (2015). The cost of wound debridement: a Canadian perspective. International Wound Journal, 12(4), 402–407. https://doi.org/10.1111/iwj.12122.

Xie, Z., Paras, C. B., Weng, H., Punnakitikashem, P., Su, L.-C., Vu, K., … Nguyen, K. T. (2013). Dual growth factor releasing multi-functional nanofibers for wound healing. Acta Biomaterialia, 9(12), 9351–9359. https://doi.org/10.1016/j.actbio.2013.07.030.

Yen, Y.-H., Pu, C.-M., Liu, C.-W., Chen, Y.-C., Chen, Y.-C., Liang, C.-J., … Chen, Y.-L. (2018). Curcumin accelerates cutaneous wound healing via multiple biological actions: The involvement of TNF-α, MMP-9, α-SMA, and collagen. International Wound Journal, 15(4), 605–617. https://doi.org/10.1111/iwj.12904.

Zafar, M., Najeeb, S., Khurshid, Z., Vazirzadeh, M., Zohaib, S., Najeeb, B., & Sefat, F. (2016). Potential of Electrospun Nanofibers for Biomedical and Dental

Applications. Materials, 9(2). https://doi.org/10.3390/ma9020073.

Zargham, S., Bazgir, S., Tavakoli, A., Rashidi, A. S., & Damerchely, R. (2012). The Effect of Flow Rate on Morphology and Deposition Area of Electrospun Nylon 6 Nanofiber. Journal of Engineered Fibers and Fabrics, 7(4), 8.

Zhang, Yabin, Ye, L., Cui, M., Yang, B., Li, J., Sun, H., & Yao, F. (2015). Physically crosslinked poly(vinyl alcohol)–carrageenan composite hydrogels: pore structure stability and cell adhesive ability. RSC Advances, 5(95), 78180–78191. https://doi.org/10.1039/C5RA11331H.

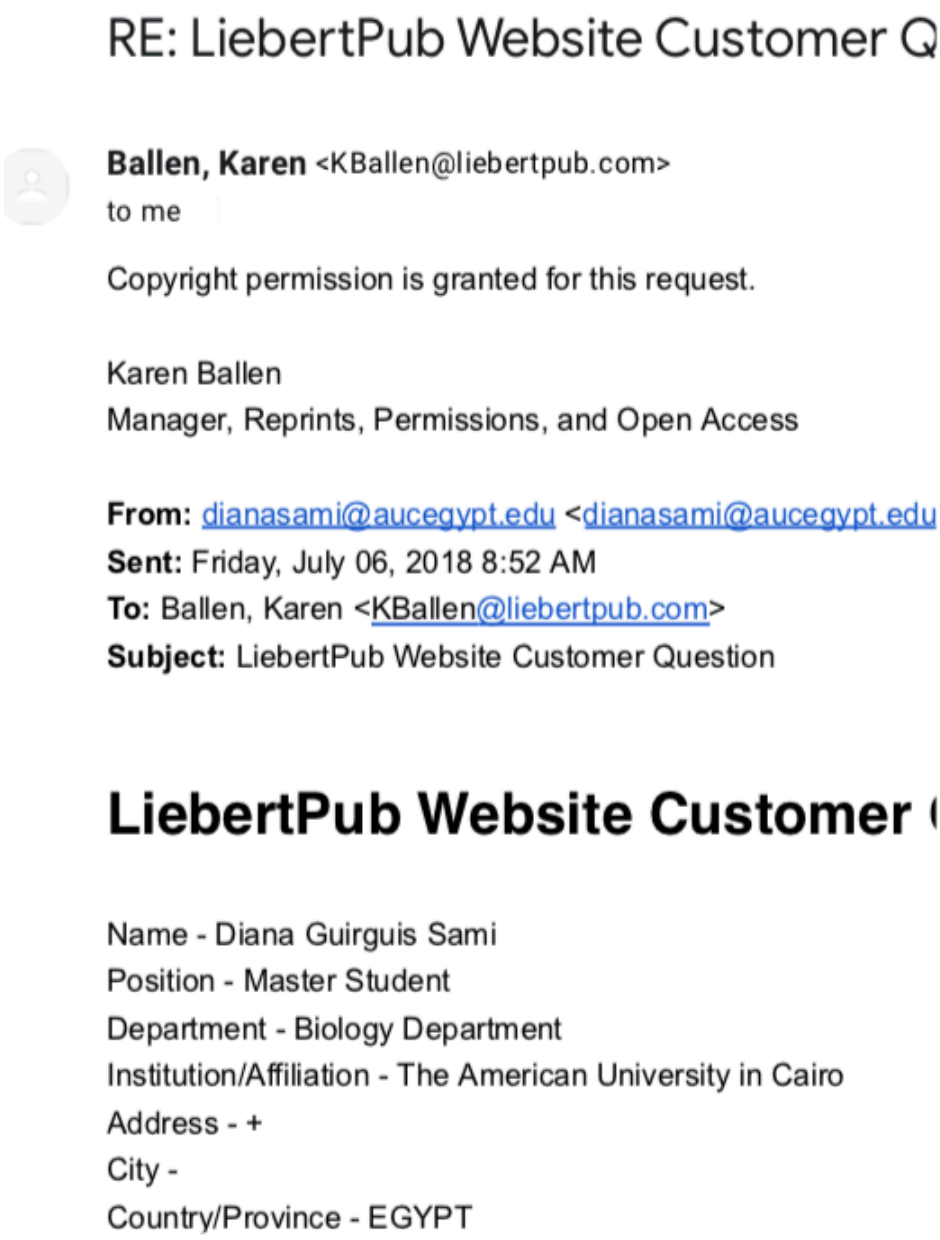
Zhang, Yuying, Yang, Y., Tang, K., Hu, X., & Zou, G. (2008). Physicochemical characterization and antioxidant activity of quercetin-loaded chitosan nanoparticles. Journal of Applied Polymer Science, 107(2), 891–897. https://doi.org/10.1002/app.26402.

Zhang, Z., Tsai, P.-C., Ramezanli, T., & Michniak-Kohn, B. B. (2013). Polymeric nanoparticles-based topical delivery systems for the treatment of dermatological diseases. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology, 5(3), 205–218. https://doi.org/10.1002/wnan.1211.

Zhao, R., Liang, H., Clarke, E., Jackson, C., & Xue, M. (2016). Inflammation in Chronic Wounds. International Journal of Molecular Sciences, 17(12), 2085. https://doi.org/10.3390/ijms17122085.

Appendix

Figure 1



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Institution/Affiliation - The American University in Cairo
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Country/Province - EGYPT

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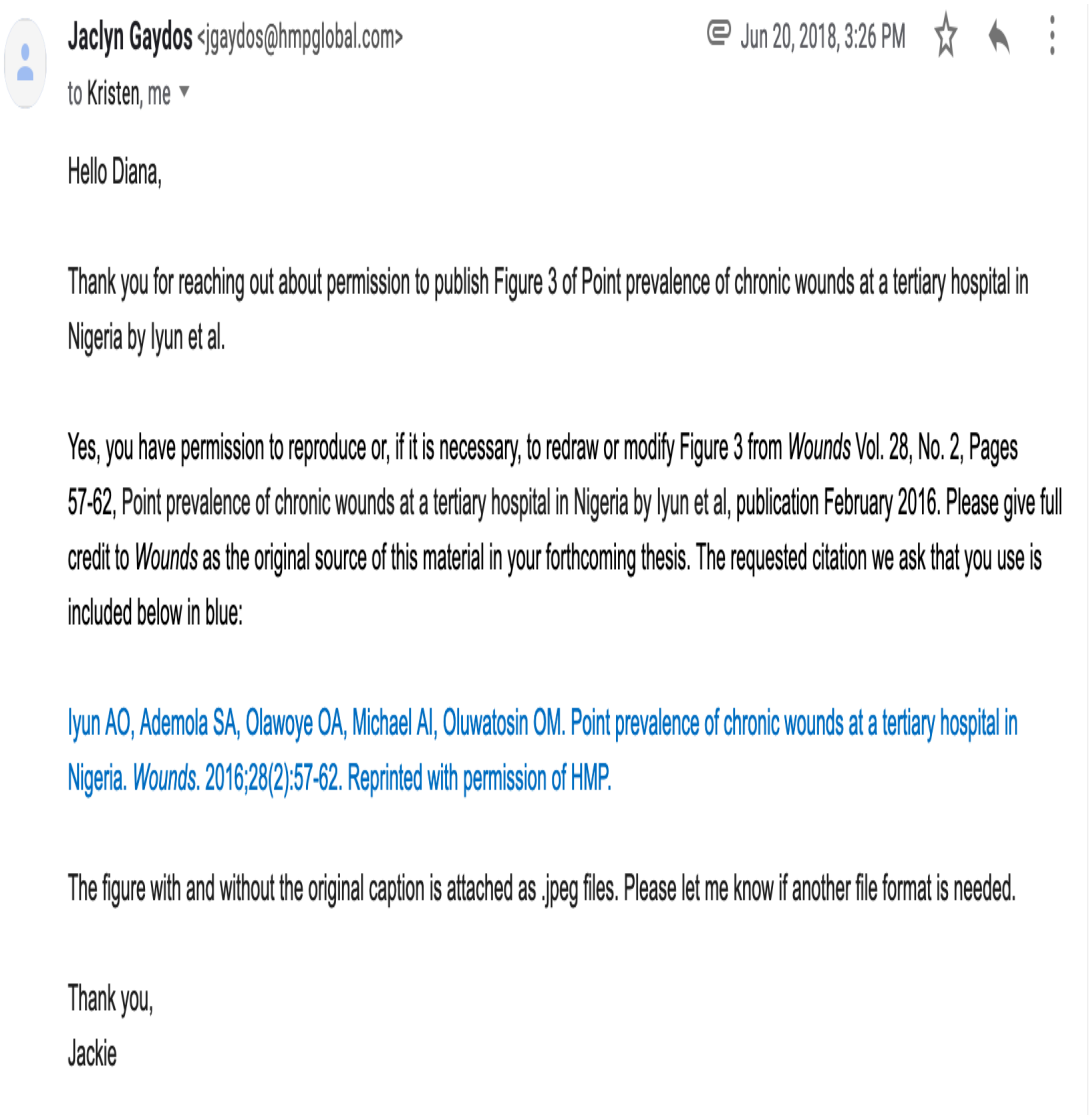

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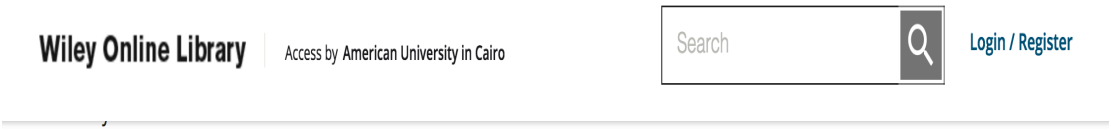

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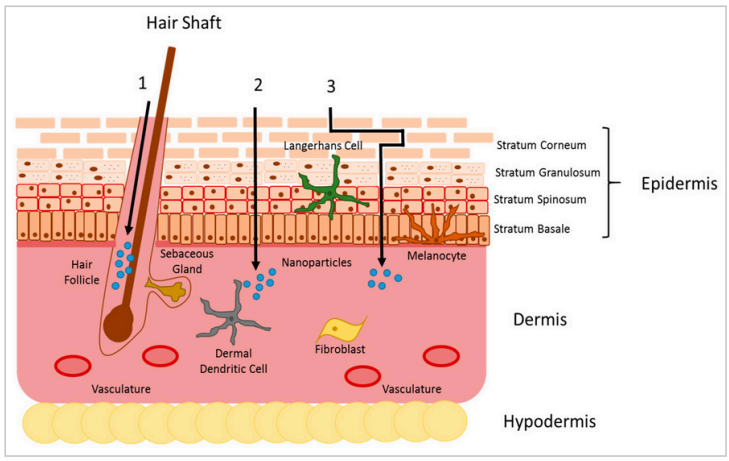

Figure 1

Plant Therapy <cs@plantherapy.com> Mar 28
to me

Type your response ABOVE THIS LINE to reply

Diana Guirguis Sami
Subject: Oregano oil

MAR 27, 2018 | 04:03PM MDT
Sarah replied:

Thank you for contacting Plant Therapy. All of our Oregano is extracted using our Steam Distilled method and now we do not add any additives or adulterants to our oils.I hope this has helped! Please let us know if there is anything else we can do to further assist you.We hope you have a wonderful day!

Tricia (Plant Therapy)
Jul 14, 7:23 AM MDT

Hello Diana,

Our Tumeric CO2 extract organic oil, does not have ethanol or other solvents in them.

The only oils that might have a trace of a solvent (not even a high enough amount to make it into our GC/MS reports), would be the solvent extracted oils such as Rose or Jasmine Absolute.

I hope that this helps. Have a great weekend!