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THE EFFECT OF EXTRACELLULAR VESICLES OF RHUS CORIARIA L. ON WOUND HEALING

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ABSTRACT

THE EFFECT OF EXTRACELLULAR VESICLES OF RHUS CORIARIA L. ON WOUND HEALING

Wounds are injuries, disruptions, or damage to living tissue that occur as a direct result of trauma or due to underlying disease processes such as diabetes, venous/arterial insufficiency, or immunological diseases. Wounds are classified as acute and chronic. Acute wounds are injuries that usually heal within 3-4 weeks. During this time, chronic wounds do not heal. Wound healing is a complex biological and physiological process in which the body uses two different mechanisms to heal, including tissue regeneration and tissue repair. The history of wound healing treatment dates back to ancient times. Medicinal plants have been used in wound treatment among the people in Turkey for many years. *Rhus coriaria* L. (Anacardiaceae), known as sumac, is traditionally used in the treatment of wounds and burns in the Southeastern Anatolia Region of Turkey, especially in Gaziantep. The wound healing effect of sumac extract has been well documented in previous studies, but sumac extracellular vesicles have not been studied yet. Therefore, the objectives of this study are to develop an optimized protocol for the isolation and identification of extracellular vesicles from sumac plant and then evaluate the wound healing effect of SECVs based on *in vitro* tests including cell viability and proliferation, cell migration, wound healing assay, tube formation assay, and gene expression analysis. According to the results, it is observed that SECVs enhance cell migration, proliferation, and healing capacity of wound-associated cells. On the other hand, it is found that SECVs do not induce the angiogenesis of the endothelial cells. Furthermore, qPCR results showed that genes related cell migration and anti inflammation were upregulated as a result of SECV treatment. Therefore, all the results indicate that SECVs have a promising potential in terms of providing successful wound healing process. In conclusion, the presented study will be a valuable precursor in searching SECVs and the potential effects on wound healing. With new developing techniques, the mechanisms might understood better and the application of SECVs' effect might reinforced via *in vivo* studies.

ÖZET

RHUS CORIARIA L.' DEN ELDE EDİLEN EKSTRASELÜLER VEZİKÜLLERİN YARA KAPANMASI ÜZERİNDEKİ ETKİSİ

Yaralar, travmanın doğrudan bir sonucu olarak veya diyabet, venöz/arteriyel yetmezlik , immünolojik hastalıklar gibi altta yatan hastalık süreçleri nedeniyle meydana gelen yaralanmalar, aksamalar, canlı dokuda meydana gelen hasarlardır. Yaralar akut ve kronik olarak sınıflandırılır. Akut yaralar genellikle 3-4 hafta içinde iyileşen yaralanmalardır. Bu süre zarfında kronik yaralar iyileşmez. Yara iyileşmesi, vücudun iyileşmek için doku rejenerasyonu ve doku onarımı dahil olmak üzere iki farklı mekanizmayı kullandığı karmaşık bir biyolojik ve fizyolojik süreçtir. Yara iyileştirme tedavisinin tarihi çok eskilere dayanmaktadır. Türkiye'de halk arasında yara tedavisinde uzun yıllardan beri şifalı bitkiler kullanılmaktadır. Sumak olarak bilinen *Rhus coriaria L.* (Anacardiaceae), Türkiye'nin Güneydoğu Anadolu Bölgesi'nde, özellikle de Gaziantep'te geleneksel olarak yara ve yanık tedavisinde kullanılmaktadır. Sumak ekstraktının yara iyileştirici etkisi önceki çalışmalarda belgelenmiştir, ancak sumak ekstraselüler vezikülleri henüz araştırılmamıştır. Bu nedenle, bu çalışmanın amaçları sumak bitkisinden hücre dışı keseciklerin izolasyonu ve tanımlanması için bir protokol geliştirmek ve daha sonra hücre canlılığı ve çoğalması, hücre göçü gibi *in vitro* testlere dayanarak SECV'lerin yara iyileştirme etkisini değerlendirmektir. Tez kapsamında yarık deneyi, hücre göçü testi, hücre toksisite testi, anjiyogenez testi ve gen ekspresyon profilleme yapılmıştır. Sonuçlara göre SECV'lerin hücre göçünü çoğalmasını ve yara iyileşmesini tetiklediği; Öte yandan sumak EV'ler anjiyogenezde çok önemli bir rol oynamadığı gözlemlenmiştir. Gen ekspresyonu analiziyle hücre göçü ve anti-inflammasyonu artırdığı sonuçları bulunmuştur. Deneyler sumak EV'lerin potansiyel bir yara iyileştirme destekleyicisi olduğunu göstermiştir. Sonuç olarak, sumak EV'lerinin yara kapanmasında umut verici olduğu çıkarımı yapılmıştır. Bu çalışma, sumaktan türetilen EV'lerin ve yara iyileşmesi üzerindeki potansiyel etkilerin araştırılmasında öncü bir çalışma olarak görülebilir. Bu çalışmaya *in vivo* çalışmalar eklenebilir ve gelecekte mekanizmaların aydınlatılmasıyla daha yeni yaklaşımlarla geliştirilebilir.

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Beginnings are excited, while ends are sad. The end of this thesis felt me both of these feelings together.

Cheers to new beginnings! To be continued

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LIST OF ABBREVIATIONS

αSMA	Alpha-smooth muscle actin
ATP	Adenosine triphosphate
ATP5B	ATP synthase F1 subunit beta
BSA	Bovine serum albumin
cDNA	Complementary DNA
COL1A1	Collagen, type I, alpha 1
COL3A1	Collagen, type III, alpha 1 chain
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethly sulfoxide
DMEM	Dulbeccos's Modified Eagle Medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EV	Extracellular vesicle
FBR	Fibrinogen beta chain
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
HaCaT	Human immortalized keratinocytes
HDF	Human dermal fibroblast
HPA	Hypothalamic pituitary adrenal
HUVEC	Human umbilical vein endothelial cells
H2O2	Hydrogen peroxide
ICC	Immunocycochemistry
ILV	Intraluminal vesicles
IL1	Interleukin 1
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
Ki67	Antigen Kiel 67
MetS	Metabolic syndrome
miRNA	Micro RNA
mL	Mililiter

MMP9	Matrix metalloproteinase
mRNA	Messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mTOR	Mammalian target of rapamycin
Nf-Kß	Nuclear factor kappa B
Nrf2	Nuclear factor erythroid 2-related factor 2
NTA	Nanoparticle tracking analysis
PA	Phosphatidyl acid
PBS	Phosphate-buffered saline
PBST	PBS+ 0.1% Tween 20 mixture
PDGF	Platelet derived growth factor
PEN1	Penetration I
PFA	Paraformaldehyde
PSA	Penicillin, streptomycin, and amphotericin B
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SECV	Sumac extracellular vesicles
siRNA	Small interfering RNA
sRNA	Small RNAs
SNS	Sympathetic nervous system
TEM	Transmission electron microscopy
TGFß1	Transforming growth factor ß 1
TGFß3	Transforming growth factor ß 3
TGN	The trans-Golgi network
TNFα	Tumor necrosis factor alpha
TSP1	Thrombospondin 1
UC	Ultracentrifuge
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

A wound is a situation that occurs in the body tissues in different ways. Such as cuts, abrasions, burns, surgical operations, lacerations, pressure injuries, etc. Wounds are assessed by their size, depth, location, color, pain, and so on. Their healing period and method change according to these criteria. To speed up the wound healing period, wound care is important by avoiding infections on the wound and minimizing scars [1]. A minor cut tends to heal in a few days with careful control and orchestration over cell migration and the right amounts of innervation, inflammation, and angiogenesis. On the other side, it could take many weeks for a major surgery wound to recover, and it can leave a noticeable scar.

Wound healing is a miracle for the body to recover itself. This miracle sometimes takes plenty of time, while sometimes it happens immediately. The wounds separate into two groups depending on the causes, healing time, and process. Acute wounds are the wounds that happen suddenly as a result of trauma, injury, cuts, abrasions, or burns, and so they stay for a short time on the skin or underlying tissues. Acute wounds need a little bit of time to heal in the normal phases of wound healing. These normal phases are: homeostasis (which is blood clotting), inflammation, proliferation (which is forming new tissues), and lastly, remodeling [2]. On the other hand, chronic wounds form in more complex ways, and according to this, healing time elongates. Chronic wounds become chronic and need more time and effort to recover themselves. Causes of chronic wounds are more serious than acute wounds, such as vascular insufficiency, diabetes, ischemia, cancer, and autoimmune disorders [3].

The aim of the wound healing process is recovery, renewal, and getting rid of unwanted scars and inflammation. In this study, the processes and new technologies are observed.

1.1. ANATOMY OF SKIN

The skin, the largest organ of the body, covers the outer surface and acts as a barrier between external elements and internal organs. It functions as a filter to protect the internal system from harmful external particles. The skin is divided into two main layers: the dermis (inner layer) and the epidermis (outer layer). Both layers are crucial for protection and the healing process [4].

The term "epidermis" originates from the Greek prefix "epi-" meaning "upon" and "derma" meaning "skin." Therefore, "epidermis" literally translates to "upon the skin." The epidermis performs critical functions such as protecting internal organs from external threats and preventing water loss. The epidermis itself comprises five distinct layers: Stratum Basale, Stratum Spinosum, Stratum Granulosum, Stratum Lucidum, and Stratum Corneum, arranged from the innermost to the outermost layer, as illustrated in Figure 1.1 [5,9].

The Stratum Basale, also known as the basal layer or stratum germinativum, is the deepest layer of the epidermis. It consists of a single sheet of cuboidal keratinocytes that actively divide through mitosis. Being the closest to the dermis, the Stratum Basale is separated from it by the basal lamina. This layer also contains melanocytes, which are responsible for producing pigments that determine skin color [6].

The Stratum Spinosum, situated above the Stratum Basale, includes structures called desmosomes that facilitate cell junctions and provide strength. Desmosomes are connected with polygonal-shaped keratinocytes to ensure cellular resilience. Additionally, this layer is involved in the production of keratinocytes and antimicrobial peptides, which serve protective functions.

The Stratum Granulosum is the central layer among the five layers of the epidermis. In this layer, keratinocytes undergo differentiation and transform into diamond-shaped lamellar granules and keratohyalin granules. This layer produces lipids that help maintain skin hydration and structural support through the accumulation of keratohyalin [7].

The Stratum Lucidum is a layer of the epidermis found in hairless parts of the body, such as the palms of the hands and the soles of the feet. In this layer, keratinocytes are transformed into keratohyalin, forming a clear, thin layer. This layer is responsible for enhancing the durability and thickness of the skin against pressure.

The Stratum Corneum is the outermost layer of the epidermis, composed of dead keratinocytes known as anucleate squamous cells. All the underlying layers are covered by the Stratum Corneum [8].

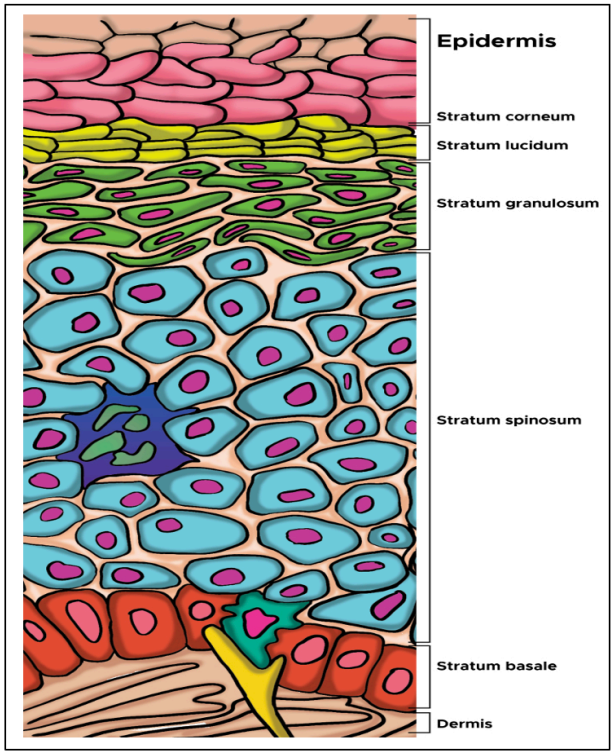

Figure 1.1. Illustration about epidermal layers and dermis location [9].

Since, epidermal layer examined in detail, parentheses might open about the epidermis cells. The epidermis includes epithelial cells, Langerhans cells, Merkel cells, keratinocytes, and melanocytes. Despite the presence of these diverse cell types, blood vessels are absent in the epidermal layer. Figure 1.2 illustrates the detailed components of the epidermal and dermal layers [14].

Keratinocytes are the most abundant cell type in the epidermis, as previously mentioned. They produce fibrous proteins known as keratins, which support skin structure and lipid secretion [10].

Melanocytes, another type of epithelial cell, are responsible for producing skin color pigments. They synthesize melanin, which not only imparts color to the skin but also acts as a filter against UV light, thereby protecting the skin from its harmful effects [11].

Langerhans cells, also referred to as dendritic cells, are specialized for the immune response of the skin. These cells capture antigens attempting to penetrate the skin and are responsible for their uptake and transfer to lymph nodes when compatible. Langerhans cells generate an immune response against invaders, pathogens, and allergens.

Merkel cells, located in the stratum basale layer (the innermost part of the epidermis), function as sensory receptors. These oval-shaped cells are connected with desmosomes by keratinocytes in nerve endings. They are involved in tactile sensation and form the surface of sensitive sensory areas of the body, such as fingerprints, palms, and soles [12].

The dermis, the second major skin layer, is situated beneath the epidermis. It contains components embedded in the extracellular matrix (ECM), such as fibroblasts, elastin fibrils, and collagens. The dermis regulates body temperature through vasodilation and vasoconstriction processes. Additionally, it functions as a reservoir, supplying nourishment and oxygen to sustain cell viability. The dermis contains blood vessels, hair follicles, sweat glands, nerve endings, collagen, and elastin fibrils. It is essential for maintaining cellular shape, thermoregulation, elasticity, and providing a scaffold for tissue repair [13].

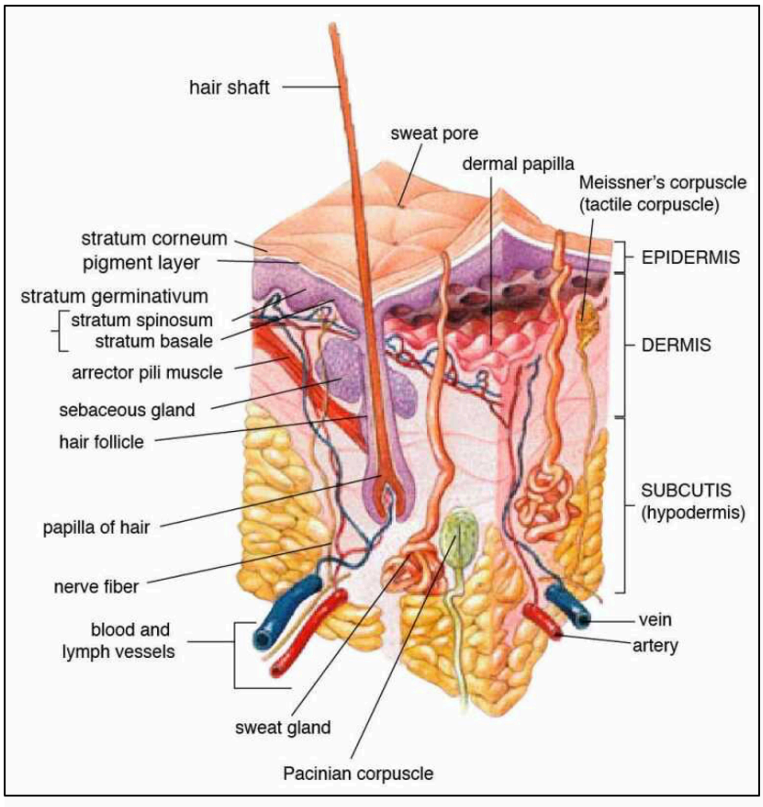

Figure 1.2. Skin and components [14].

Epidermis and dermis layers are come together to make the skin anatomy with these unique functions.

1.2. WOUND HEALING PROCESS

A wound is an abnormality that occurs in or on the skin due to various causes such as accidents, burns, surgical operations, and immune diseases. Wound healing is a series of events in which the body repairs the wound. The healing process begins immediately after the wound occurs, and its duration, as well as the extent of scarring, depends on the wound's location, depth, and severity. This healing process involves the collaboration of signal molecules, cells, and extracellular matrix (ECM) components. If a wound does not heal within three months, it is often classified as a chronic wound. Chronic wounds tend to persist in the affected area. The primary objective of wound healing is to restore the integrity of the epithelial barrier and correct dysfunctions to enable normal skin functions. The healing period involves a series of complex procedures in the following order: hemostasis, inflammation, proliferation, and remodeling [15,16].

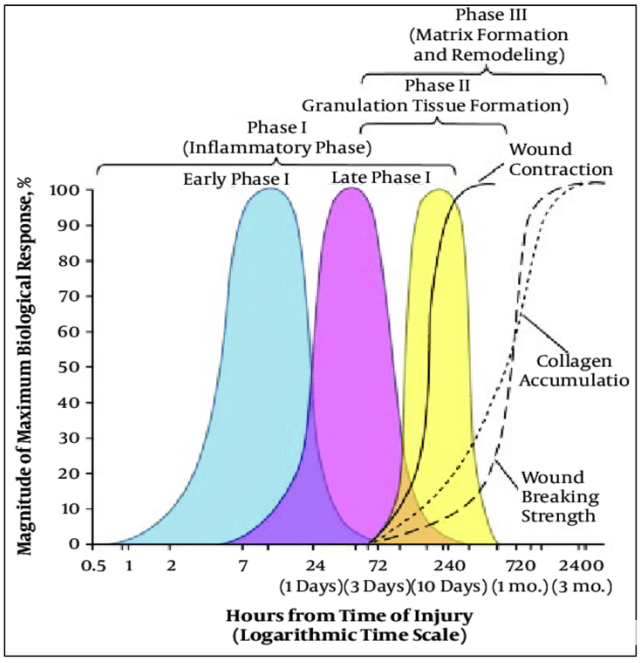

Figure 1.3. Timetable of wound [16].

1.2.1. Homeostasis

The wound healing process begins with the occurrence of a wound. Hemostasis is the initial stage of this process. The primary objective at this stage is to control bleeding. Blood vessels constrict, leading to vasoconstriction, and coagulation occurs as a result. This minimizes blood loss by reducing bleeding. Platelet activation occurs immediately, directing collagen

fibers to the wound area [17]. This activation continues with the involvement of fibrinogens and clotting factors to form a plug and deactivate the clot, thereby achieving coagulation. Once the hemostatic plug is formed to stop the clotting, the thrombin protein is produced to induce platelet contraction. This aims to enclose the wound area and cover it with a very thin layer [18]. Fibrins provide strength to the clot, preventing blood loss and protecting inner tissues. Through the activation of intrinsic and extrinsic coagulation pathways, platelets bind to collagen. During the hemostasis phase, cytokines are released and play a crucial role in ECM decomposition, chemotaxis, and angiogenesis. These cytokines include vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), whose gene expression will be examined in subsequent sections. Hemostasis can be defined as the introductory step to inflammation. Cellular debris and dead cells are collected by inflammatory cells such as neutrophils and macrophages, which migrate to the wound area [19].

1.2.2. Inflammation

At the end of the hemostasis phase, the inflammatory phase begins in the wound area by collecting dead cells. Prostaglandin and histamine cytokines are released from inflammatory mast cells to enhance capillary permeability in preparation for cell migration. Neutrophils engulf and digest foreign molecules within the first 48 hours. In addition to mast cells, larger inflammatory cells such as macrophages migrate to the wound site and interact with TGFβ, PDGF, FGF, and EGF [20]. The common feature of these cytokines is that they are all growth factors, and issues with their release can delay wound healing.

However, these growth cytokines alone are not sufficient. Additional cytokines are released, including tumor necrosis factor-alpha (TNFα), interleukin-1 (IL1), and interleukin-6 (IL6) [21]. These cytokines are responsible for activating endothelial cells and fibroblast cells, promoting ECM structure production, and initiating the production of new blood vessels (angiogenesis). As part of the repair mechanism, vasodilation increases the number of blood vessels and the amount of free oxygen around the wound site. Interleukin-10 (IL10) is also released to aid in the remodeling phase [22,23].

1.2.3. Proliferative Phase

As previously mentioned, collagen and ECM components are generated by fibroblasts. These cells migrate to the wound site and surround the area to facilitate repair. Upon arrival at the wound site, fibroblasts begin proliferating and producing collagen. As fibroblasts proliferate and produce collagen, new fibrils are deposited in the wound area, replacing the clotting components produced earlier [25]. The fibers can be envisioned as the structural framework, akin to a skeleton in the body, providing structural integrity.

During the proliferation phase, new blood vessels are formed through angiogenesis to support the essential needs of the cells, such as oxygen and nourishment [22,26]. Epithelial cells migrate to the wound site to form new epithelial tissues. This migration occurs with the scattering of collagen type IV and laminin. Additionally, proteoglycans, glycoproteins, and elastin are produced to rebuild the ECM [27].

1.2.3.1. Re-epithelization

During the proliferative phase, the process of epithelialization is repeated to achieve wound closure. This step, known as re-epithelialization, involves the restoration of the outermost surface of the skin. The migration of epithelial cells to the wound site occurs immediately after tissue damage. Keratinocytes, which are among the most crucial cells for wound closure, are responsible for forming a new layer over the wound [28]. Epithelial cells migrate to the wound site and undergo a series of cell divisions and proliferations. This results in the generation of new daughter cells that cover the wound area. The stabilization of the newly formed epithelial structure is supported by the basement membrane. This process continues until the entire wound area is covered and closed [29].

1.2.3.2. Angiogenesis

The term "angiogenesis" refers to the formation of new blood vessels. Angiogenesis occurs after the re-epithelialization phase has taken place at the wound site. The purpose of the increased number of vessels is to enhance and supply fundamental needs such as oxygen and nourishment to the wound area. The stimulation and angiogenesis processes are mediated by the release of signals from VEGF, FGF, and angiopoietin [25,30]. A process known as "sprouting angiogenesis" occurs with the help of these signals, involving the repeated activation of angiogenesis events. As endothelial cells arrive at the wound site, new branches

of vessels grow and spread throughout the area. Subsequently, pericytes become involved. These cells enclose blood vessels as the angiogenesis process continues, ensuring the integrity of the blood vessel walls and contributing to the growth and maturation of blood vessels [31].

Smooth muscle cells provide contractile assistance and stabilization to the blood vessels. Consequently, new blood vessels are formed. These newly created blood vessels may integrate into the pre-existing circulatory system and become a part of it. They begin by forming small connections with neighboring capillaries, venules, and arteries. Angiogenesis is a crucial event in the wound healing process [32].

1.2.3.3.Granulation

The granulation phase is another critical stage of wound closure. During this phase, the granulation of endothelial cells forms a new layer over the wound. Regeneration and wound healing approach completion with the presence of highly vascularized cells in the granulation step. Granulation occurs during the proliferation phase, following hemostasis and inflammation. In this part of angiogenesis, fibroblast proliferation results in the formation of new ECM components, collagen fibers, fibronectins, and glycosaminoglycans. These products facilitate the generation of blood vessels through granulation during proliferation [33].

Macrophages also play a significant role in the granulation step of proliferation by releasing growth factors and cytokines. Over time, the granulated tissue develops and becomes ready for the remodeling phase. The collagens produced by fibroblasts enhance cellular strength and elasticity [34]. Over-granulated tissues are broken down by proteolytic enzymes such as MMPs. The granulation event imparts a pinkish or reddish hue to the tissues and is characterized by a moist appearance. Granulation promotes epithelialization, proliferation, and migration activities essential for wound closure.

1.2.3.4.Contraction

Contraction in wound healing represents the final step of the proliferation phase. Contraction refers to the reduction of the wound size by drawing the wound edges together. As previously mentioned, myofibroblasts are derived from the differentiation of fibroblasts. These

myofibroblasts provide contractile energy through their contractile proteins, such as alpha-smooth muscle actin (αSMA) [35].

Myofibroblasts also support cytoplasmic processes and exert strain on neighboring tissues to reduce the wound tissue area. Contraction is influenced by several factors, including the size and texture of the surrounding tissue, the strength of the wound edges, and the elasticity of the skin. The use of external supports, such as surgical sutures and medical staples, helps apply pressure to deeper wounds, making contraction more effective. The hemostasis, inflammation, and proliferation steps are all significantly enhanced by the contraction phase, leading to a diminished wound site [36].

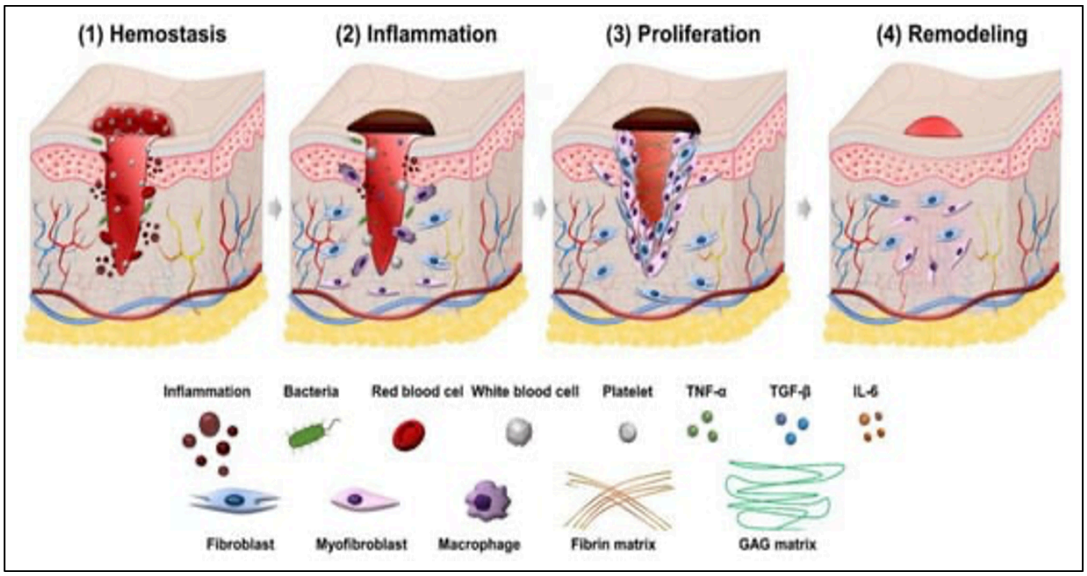

Figure 1.4. Wound healing stages on skin sections [37].

1.2.4. Remodelling

The remodeling of a wound begins on the eighth day post-injury and may continue for up to one year. Remodeling is the final phase following hemostasis, inflammation, and proliferation. This phase involves the re-arrangement and optimization of the properties acquired thus far, ensuring optimal strength, stability, and flexibility [28,38]. During remodeling, fibroblasts undergo maturation and differentiation, depositing collagen and other structural components to enhance stability and function. Collagen aligns to restore the function of newly formed tissue and integrate the structures [36,39].

In addition to collagen fibers, elastin, proteoglycans, and glycoproteins also undergo differentiation during remodeling [17,18]. ECM constituent remodeling is facilitated by matrix metalloproteinases (MMPs). In cases of over-granulation, excess tissue is removed, and type III collagen (COL3) is replaced with type I collagen (COL1) to create a tighter scar [37]. For better remodeling outcomes, medical and cosmetic interventions may be applied.

It is important to note that angiogenesis continues during remodeling, ensuring that newly formed vessels provide the necessary nutrients and oxygen to the tissues [31]. The duration of the remodeling phase depends on factors such as wound depth, size, extent of damage, quality of medical support, and the patient's adherence to wound care. Key characteristics of the remodeling phase include collagen fiber maturation, ECM alignment, reduction in scar size, sustained angiogenesis, and continuous tissue maturation [40].

1.3. Factors Effecting Wound Healing Process

1.3.1. Local Factors in Wound Healing

Local factors significantly influence wound healing. The wound site is affected by environmental factors such as oxygenation and the presence of infections in tissue care. The wound healing process depends largely on the quality of these environmental conditions. While these factors can sometimes promote wound closure, they can also impede it under certain circumstances [41].

1.3.1.1. Oxygenation

In cellular mechanisms, oxygen molecules play a crucial role. ATP, which are the energy packets of cells, are activated through oxygen stimulation. Oxygenation is vital in all stages of wound healing as it prevents infection, aids in angiogenesis, promotes keratinocyte involution and migration to the wound area, supports the formation of new epithelial cells during re-epithelialization, enhances fibroblast and collagen production, and ultimately contributes to wound contraction [42].

Polymorphonuclear leukocytes, which regulate the production of superoxide to destroy pathogens, are also influenced by oxygen levels. The excessive consumption of oxygen by normal living cells can deplete the oxygen available in the environment, leading to hypoxia. To mitigate this, effective systemic factors such as young age and the absence of diabetes improve blood circulation, delivering oxygen to hypoxic sites [43]. A wound site with

insufficient oxygen fails to heal properly, and intermittent oxygenation of the environment results in delayed healing.

Keratinocytes, macrophages, and fibroblasts are stimulated under hypoxic conditions. In the presence of a hypoxic environment, factors such as PDGF, TGF-β, VEGF, and TNF-α facilitate cell proliferation, angiogenesis, migration, and chemotaxis [44]. During the normal wound healing process, reactive oxygen species (ROS) like hydrogen peroxide (H2O2) and superoxide (O2) act as signaling molecules to promote cell motility, angiogenesis, and PDGF cytokine signal transduction [45]. ROS serve a beneficial role in both oxygen-sufficient and oxygen-deficient environments, but excessive ROS can cause damage. Therefore, maintaining an optimal oxygen level is crucial for a successful wound healing process [46].

1.3.1.2. Infections

When an infection occurs, micro-organisms naturally present on the skin surface can penetrate deeper layers. Infections can be categorized as contaminated infection, colonized infection, local infection & critical colonization, and expansive invasive infection. The distinction between contamination and colonization lies in the presence of replicative and non-replicative micro-organisms on the wound [47]. Replicative micro-organisms are found in colonized infections, whereas non-replicative micro-organisms are present in contaminated areas. In local infection & critical colonization, moderate tissue reactions begin, and micro-organisms enter replicative stages. Invasive infections use the tissue as a host to invade and propagate micro-organisms.

As previously mentioned, inflammation is a crucial step in wound healing, involving the removal of unwanted contaminated micro-organisms. However, inflammation can be delayed when microbial aggregates are present [48]. IL10 and TNFα can extend the inflammatory phase due to both bacteria and endotoxins. Prolonged inflammation can hinder wound healing, potentially resulting in the wound becoming chronic.

Additionally, the prolongation of this phase leads to the accumulation of matrix metalloproteinases (MMPs), resulting in the degradation of ECM components. Simultaneously, the levels of natural protease inhibitors diminish. This imbalance in proteases causes dysfunction in the growth factors produced in the chronic wound area [49,50].

1.3.2. Systematic Factors in Wound Healing

Wound healing is a complex series of events that begin with coagulation and conclude with contraction. Various types of cells and tissues play roles in this sequence of events. While successful cellular processes are crucial, external factors can significantly influence the quality of the resulting tissue. These factors can either promote wound closure under favourable conditions or diminish the quality of closure when conditions are suboptimal. Factors that affect wound healing quality include the patient's age, nutritional status, presence of chronic diseases, medication usage, smoking and alcohol intake, obesity, hormonal imbalances, stress, and genetic factors [51,52,53].

1.3.2.1. Age

According to the World Health Organization (WHO), individuals over the age of sixty are classified as elderly [54]. In advanced age, the rate of cell proliferation decreases, and telomeres shorten. Consequently, the time required for wound closure lengthens due to the slower process. As people age, their capacity for renewal and regeneration declines. Aging affects immune cell activity and cytokine release, leading to delayed immune responses compared to younger individuals. This delay in immune response results in a slower healing mechanism. Collagen production diminishes, making the ECM structure less durable. Additionally, blood vessels narrow, reducing blood flow in elderly individuals. The lack of oxygen, as previously mentioned, occurs with aging cells and diminished vascular flow. Elderly individuals often develop chronic diseases over the years, complicating the circulatory system with conditions such as hypertension, cardiovascular diseases, and metabolic imbalances. As people age, their skin undergoes changes, including reduced elasticity and decreased integrity due to lower production of collagen and elastin [55].

1.3.2.2. Stress and Bad Habits

Stress is a key factor in nearly all diseases. Numerous studies have demonstrated that the neuroendocrine imbalance stimulated by stress results in the deterioration of health. Stress indirectly affects the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). Stress triggers the release of stress hormones via the HPA axis and SNS, leading to elevated levels of cortisol, glucocorticoids, and catecholamines. Cortisol, in particular, disrupts the production mechanism of cytokines such as IL-1, IL-6, and TNF-α.

This imbalance results in an inadequate immune response to infections, leading to impaired wound healing.

In some cases, stress may either boost the immune response or cause dysregulation of inflammation, delaying the immune response. In both scenarios, the normal wound healing process is incomplete. Stress also affects collagen production, resulting in weak wound structures. The migration and proliferation of keratinocytes, fibroblasts, and endothelial cells are restricted under stress. Additionally, poor dietary habits, lack of physical activity, tobacco use, and alcohol consumption further delay or even prevent wound healing [56].

Stress is also known to be a major contributor to a wide range of health dysfunctions, including diabetes, cancer, and obesity.

1.3.2.3. Diabetes

Diabetes affects wound healing in several significant ways. Complications include impaired oxygenation, distorted angiogenesis, insufficient perfusion, and ischemia. Ischemia extends the inflammatory phase due to excessive reactive oxygen species (ROS). In chronic diabetic wounds, elevated levels of matrix metalloproteinases (MMPs) lead to the degradation of structural components and delay tissue repair. Diabetes causes a series of issues, including impaired phagocytosis and chemotaxis of neutrophils. These deficiencies transform diabetic wounds into acute wounds on the skin.

Neovascularization and angiogenesis are also adversely affected by diabetes. Abnormal levels of VEGF, angiopoietin-1, and angiopoietin-2 result in insufficient or even prevented angiogenesis. Reduced infiltration of leukocytes and neuropeptides leads to inadequate wound healing. Changes in the basement membrane alter membrane permeability and the delivery of nutrients to the wound area. Collectively, these factors contribute to the development of chronic wounds associated with diabetes [57].

1.4. TISSUE ENGINEERING ON WOUND HEALING

As technology advances, new approaches emerge in science. In modern science, the official investigation of wound healing studies began in the early 20th century. During that period, researchers explored the wound healing mechanism, cellular processes, and influential factors. Biomaterials, biofilms, biological scaffolds, hydrogels, stem cells, and, more recently, extracellular vesicles (EVs) have been utilized to enhance wound healing. Tissue

engineering aims to mimic damaged tissue using synthetic products and intersecting technology with cells [58].

Extracellular vesicles (EVs) represent a new frontier in wound repair. Various methods have proven successful in treating wounds, but the advantage of EVs lies in their natural production by multicellular organisms. Studies have demonstrated that EVs possess inherent healing capabilities. Moreover, EVs can be engineered and sequenced for enhanced functionality [59]. In the subsequent section, EVs will be defined in greater detail. Given that EVs act as intercellular cargo, engineering studies can leverage this unique property.

EVs play a significant role in the hemostasis, inflammation, proliferation, and angiogenesis phases of wound healing.

1.4.1. Plant Extracellular Vesicles

EVs are naturally produced by cells and function as cargo and signal carriers. They are surrounded by a phospholipid bilayer and can contain proteins, nucleic acids, lipids, and metabolites. Their primary role is to transfer signals both intercellularly and intracellularly. Over time, it has been well established that EVs exhibit special properties and biological activities, such as reducing pathological events and inducing therapeutic healing in conditions like cancer, trauma, injuries, and infections. Due to their low immunogenicity and high biocompatibility, EVs are considered promising agents for new drug delivery systems [59].

Animal-derived EVs have been used in clinical settings for a long time. However, the production of animal-derived EVs is expensive and yields only minor amounts. Conversely, plant-derived EVs offer a viable alternative. Edible plant-derived EVs, in particular, are becoming popular due to their large-scale production from plant extract sap and their typically lower toxicity on cells. Recent studies have discussed the tissue regenerative, anti-inflammatory, anti-cancer, and anti-tumor effects of plant-derived EVs [59,60].

1.4.1.1. Molecular Properties

Plant-derived extracellular vesicles (EVs) have a membranous structure with a lipid bilayer acting as a skeleton and containing several active compounds inside. The composition of EVs is similar to that of animal-derived ones, featuring cytoplasmic structures, nucleic acids,

and lipids. Plant-derived EVs contain proteins, lipids, nucleic acids (miRNAs, siRNAs), and carbohydrates in descending order of abundance.

Proteins determine the functionality of EVs and differentiate them for specific uses. The two types of plant-derived EV proteins are transmembrane and plasma membrane-related proteins. These proteins play a significant role in the internalization of plant-derived EVs into mammalian tissues. Aquaporins maintain membrane stability, and heat shock proteins help regulate temperature. Plasma membrane-related proteins support ubiquitin, metabolic enzymes, and signal transduction, while cytosolic proteins aid in remodeling and pathogen destruction. The protein composition of plant-derived EVs varies based on the plant type [60].

Lipids in plant-derived EVs contribute to stability, integrity, and function. The lipid bilayer comprises phospholipids and glycerol lipids. Phosphatidic acid (PA) plays crucial roles in membrane fusion and division, as well as protein regulation during endocytosis. EVs containing PA impact growth, mTOR activity, and proliferation. Notably, plant-derived EVs lack cholesterol in their layers [61].

Nucleic acids in plant-derived EVs regulate biological properties based on their RNA content. These EVs contain significant amounts of messenger RNA (mRNA), small RNAs (sRNA), and microRNAs (miRNA). Plant-derived EVs can transfer RNA components between cells, with mRNA and miRNA acting as regulators for RNA signal receptor cells, potentially altering cellular morphology and functions. sRNA serves as a link between interacting cells [62].

Other metabolites, including secondary metabolites, exist in plant-derived EVs and exhibit unique properties. These secondary metabolites include nitrogenous and phenolic compounds and terpenoids. Nitrogenous metabolites are derived from protein monomers and play specific roles in EV function. Despite the known benefits, many properties of the metabolites within plant-derived EVs remain unexplored [63].

1.4.1.2. Biogenesis

EVs are a group of vesicles formed during the process of endosome maturation and membrane dynamics. They can be classified based on their biosynthesis into exosomes, microvesicles, apoptotic bodies, ectosomes, and migrasomes [63].

Exosome biogenesis involves the formation of multivesicular bodies (MVBs) and intraluminal vesicles (ILVs). Exosomes range in size from 30-200 nm in diameter. Microvesicles are generated through plasma membrane dynamics and are larger than exosomes, ranging from 100-1000 nm in diameter. Apoptotic bodies originate during programmed cell death (apoptosis) and are the largest group of EVs, measuring 500-5000 nm in diameter. They function as regulators of macrophages and homeostasis in wound healing.

Initially, exosomes were considered cellular waste because early studies on plant-derived EVs identified the PEN1 protein, which is associated with high stress levels and inflammation against pathogens [64]. EVs play a crucial role in the immune defense of plants. The origination of plant-derived EVs is thought to involve the transformation of the clathrin-coated tubular network (TGN) in the Golgi apparatus [63,64].

Despite extensive research, some aspects of the origination of plant-derived EVs remain unclear.

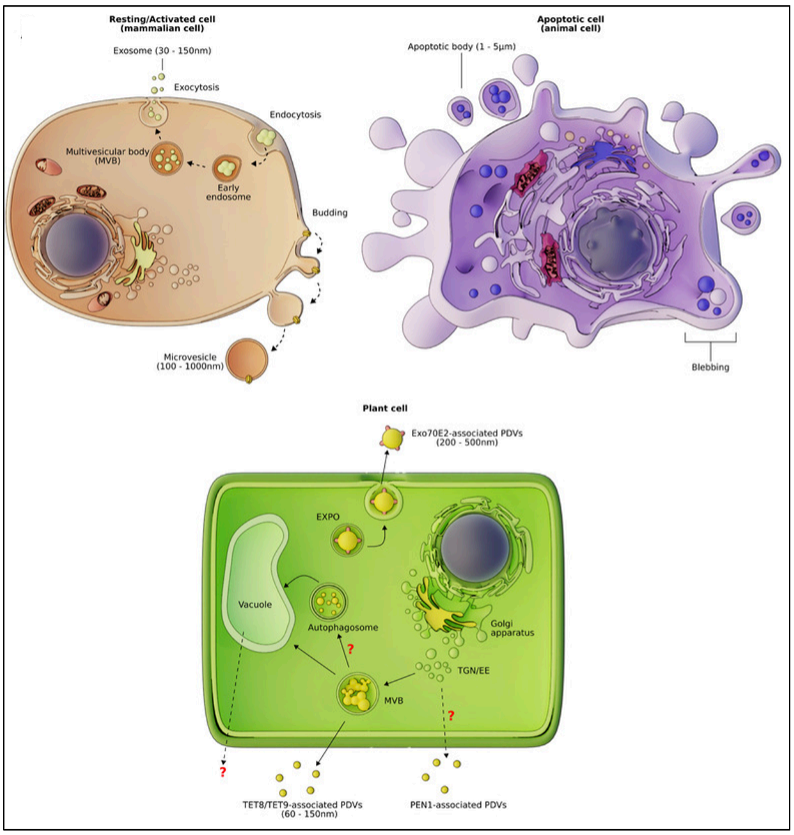

Figure 1.5. Biogenesis of EVs [63].

1.4.1.3. Extracellular Vesicles as Natural Helpers on Wound Healing

In the hemostasis stage, blood clotting and coagulation processes address the wound. For better understanding, this event can be likened to ambulance and emergency medical technicians arriving at the scene to provide first aid and control the wound. Problems during this stage can lead to a series of complications in the wound healing process and circulatory issues. Insufficient clotting results in blood loss due to excessive bleeding. Historically, certain plants have been used to stop bleeding, including Moringa oleifera, cactus, beet (Beta vulgaris), and various Aloe species [65,66]. Plant-based therapeutics have been applied and continue to be explored. Humanity has long sought cures in the plant kingdom and traditional medicine. However, there is limited information about the role of plant-based EVs in hemostasis.

In the second stage of wound healing, inflammation is influenced by plant-derived EVs, as shown in various studies. Prolonged inflammation can lead to chronic wounds and tissue damage. In such situations, anti-inflammatory agents can be utilized. Homeostatic redox balance, regulated by plant-derived EVs, is essential for initiating the wound healing process. Additionally, reactive oxygen species (ROS) are necessary for wound healing, but excessive ROS can result in non-healed wounds. Plant-derived EVs help maintain this balance as antioxidants, ROS diminisher, apoptotic preventer, and protectors against colitis [67]. Given these significant roles, it can be said that plant-derived EVs are crucial in managing wound inflammation.

In the continuation of wound healing, the next stage is proliferation, involving the rebuilding of epithelial tissue, re-epithelialization, formation of new blood vessels (angiogenesis), and granulation. Plant-derived EVs have properties that enhance cell viability, induce cell migration, and diminish ROS. Various studies have investigated the proliferative effects of plant-derived EVs on wound healing. There is still much to learn about the proliferation capacity of plant-derived EVs [68].

In the maturation or remodeling phase of wound healing, collagen type III (COL3) is replaced by collagen type I (COL1), and cross-linking formation is required for wound closure. Herbs are often used as agents to reduce keloids and scars. However, there are no definitive studies proving the role of plant-derived EVs in the remodeling phase.

1.4.1.4. Therapeutic effects of Plant EVs

Extracellular vesicles (EVs) are emerging as promising therapeutic agents. Numerous journals publish findings daily about their potential in treatment. EVs are beginning to surpass stem cell-based therapies due to their superior biocompatibility and lower immunogenicity. While mammalian-derived EVs have been utilized for their regenerative properties, attention has gradually shifted toward plant-derived EVs. These can be produced in large quantities from plant juices.

In *Citrus limon L*, EVs have demonstrated antioxidative effects and reduced ROS production in mammary cells [69].

EVs derived from *Vaccinium myrtillus* play a role in the Nrf2 pathway, protecting the wound healing mechanism from TNF-α-induced inflammation [70].

Curcuma longa-derived EVs are involved in the Nf-κB pathway and are responsible for alleviating colitis inflammation and promoting intestinal wound closure [71].

Similarly, ginger-derived EVs promote intestinal wound closure and reduce lung inflammation [72].

1.4.2. *Rhus coriaria L.* (Sumac)

Sumac is a spice commonly used in Middle Eastern and Mediterranean cuisine. *Rhus coriaria L.*, known as "sumac" in everyday life, originates from the *Rhus* genus and has a rich historical background. Historically, sumac has been used by Middle Eastern residents as a medicinal herb. Contemporary scientific research on sumac often references its historical usage. In some studies, the effects of sumac extracts have been investigated. For instance, one study tested the anti-cancer effects of sumac extract, finding that the secondary metabolites in sumac act as inhibitory agents against various human cancer cell lines [73]. Another study explored the supplementary effects of sumac extract in managing metabolic syndrome (MetS), demonstrating that sumac-based supplements effectively reduced systolic blood pressure and stabilized triglyceride levels, suggesting potential benefits as a supportive supplement [74].

As of now, the potential of sumac-derived extracellular vesicles (EVs) has not been extensively researched. Previous studies on sumac extract have shown promising results in

diminishing oxidative stress, indicating the potential for new therapeutic agents [75]. Given that EVs represent a novel area for transmitting signals of desired plant cells, the effects of sumac-derived EVs were explored in this thesis. The approach to wound healing using sumac-derived EVs and the series of experiments conducted are detailed in the procedures below.

1.5. AIM OF THE STUDY

Throughout human history, individuals have sustained injuries for various reasons. Wounds that occur accidentally, as side effects of diseases such as diabetes, from surgical procedures, bedsores, abrasions, and scars are not initially health-threatening. However, if wound care is not properly maintained, infections can develop, potentially leading to chronic wounds. With advancements in technology, new drug therapy candidates have been invented. One such candidate is extracellular vesicles (EVs), which have gained popularity due to their cargo transition activity. This concept has led to the idea of combining EV technology with curative traditional herbs.

As part of this innovative approach, sumac-derived EVs have been isolated for the first time, as far as we know. In this study, we optimized, isolated, characterized, measured toxicity activity, observed scratch closure and migratory events, assessed tube formation, and conducted mRNA gene expression profiling of skin cell lines HaCaT, HUVEC, and HDF. The aim of this study was to test the role of sumac-derived EVs in wound closure events through a series of mentioned experiments.

2. MATERIALS and METHODS

2.1. MATERIALS

Table 2.1. Instrument Used in Experiments

	Instrument	Brand & Cat. no
1	Laminar Flow Cabin, Heal Force Class II Biosafety Cabinet A2	Class II BSC
2	CO_2 Incubator	InVitro Cell ES NU-5800, NuAire
3	Centrifuge	Sigma, 3-18 KS
4	Blender	Waring, 8011 EB
5	Invert Microscope	Nikon, TS100, Netherlands
6	Nanosight (NTA)	Malvern, NS300
7	Scanning Electron Microscopy (SEM)	Zeiss, EVO LS 10
8	Flouroscence Inverted Microscope	Zeiss, Axio Vert.A1
9	-80 Freezer	New Brunswick, Germany
10	Varioskan LUX	Thermo Scientific, VL0LA0D0
11	NanoDrop Spectrophotometers	Thermo Scientific, 2000
12	Elisa Microplate reader	Bio-techELx800, USA
13	Thermal Cycler	BioradMycycler 1709703, USA
14	CFX96 Real-Time PCR	Bio-Rad, C1000 Touch, USA
15	Vortex	Dragon Lab, MX-F, China
16	Incubate Shaker	New Brunswick Innova, USA
17	Magnetic Stirrer	Heidolph MR, Germany

Table 2.2. Equipment Used in Experiments

	Item	**Brand & Cat. no**
1	Filter 0.22 µm and 0.45 µm	TPP, Switzerland
2	Graduated Cylinder	Isolab, Germany
3	Hemacytometer	Sigma Aldrich, Z359629, USA
4	Coverslip	Sigma, Z375357, USA
5	Cryovial	091.11.102, Isolab, Germany
6	Cell culture flasks	T-25, T-75, T-150, TPP Switzerland
7	Serological Pipettes	5,10,25,50 mL CAPP, Germany
8	Micropipettes	2231300002, Germany
9	Microcentrifuge-tubes	1.5, 2 mL, 078.03.002, 078.03.003, Isolab, Germany
10	Falcon tubes	15 and 50 mL, Isolab, Germany
11	Micropipette tips	301-03-051, Axygen, USA
12	Fetal Bovine Serum	Gibco, 10500064
13	PSA	Gibco, 15240062
14	Trypsin-EDTA (0.25 percent)	Gibco, 25200056
15	Dimethyl Sulfoxide	Santa Cruz Biotechnology sc-202581, USA
16	Phosphate Buffered Saline	10010023, Thermo Fisher Scientific, USA
17	6 well culture plate	92006, TPP, Germany
18	12 well culture plate	92012, TPP, Germany
19	24 well culture plate	92024, TPP, Germany
20	96 well culture plate	92096, TPP, Germany
21	Dulbecco's Modified Eagle's Medium, high glucose	Gibco 41966, Thermo Fisher Scientific, USA

22	Amicon Ultracentrifugal Filter, 10 kDa	Millipore, UFC9010
23	Matrigel	#356230, Corning
24	Bovine Serum Albumin %10	#37520, Thermo Fisher Scientific, USA
25	Tween 20	#BS100, Biosharp Life Sciences
26	Triton X	#A16046.AP, Thermo Fisher Scientific, USA
27	DAPI	#D1306, Thermo Fisher Scientific, USA
28	COL1A1 primary antibody	ab11439, Abcam, USA
29	Elastin primary antibody	Ab23747, Abcam, USA
30	Laminin primary antibody	ab11575, Abcam, USA
31	Fibronectin primary antibody	Ab2413, Abcam, USA
32	AF555, secondary antibody	Ab406412, Abcam, USA

Table 2.3. Kits and Assays Used in Experiments

	Kit & Assay	**Brand & Cat. no**
1	MTS Assay	G111A, Promega
2	Hoechst 33342 Trihydrate	ThermoScientific, H3570
3	High Pure RNA Isolation Kit	Roche, 11828665001
4	SuperScript™ Double-Stranded cDNA Synthesis Kit	ThermoScientific, 11917020
5	CytoSelect™ 24-Well Wound Healing Assay	CellBioLabs, CBA-120
6	CytoSelect™ 24-Well Cell Migration Assay	CellBioLabs, CBA-100
7	iTaq™ Universal SYBR® Green Supermix, 200 x 20 µl rxns, 2 ml (2 x 1 ml)	Biorad, 1725120

Table 2.4. Cell Lineages Used in Experiments

	Name	**Brand & Cat no**
1	HDF, Primary human fibroblast	PCS 201-012
2	HUVEC, Primary Umbilical Vein Endothelial Cells	CRL-1730
3	HACAT, Human Keratinocyte	CLS 300493

2.2. METHODOLOGY

2.2.1. Cell Culture

To conduct an experimental series of wound healing assays, Human Dermal Fibroblast cells (HDF), Human Umbilical Cord Endothelial Cells (HUVECs), and Human Immortalized Keratinocytes (HaCaT) cells were used to model the skin from the inside out. All three cell lines were cultured under humidified incubator conditions at 37 °C with 5% CO_2.

The cell culture conditions involved using complete Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose (#41966052, Invitrogen, Gibco, United Kingdom). To complete the DMEM (4.5 g/L), 1% (v/v) Penicillin-Streptomycin-Amphotericin (PSA) mixture (#15240062, Invitrogen, Gibco, United Kingdom) was added as an antibiotic solution, and 10% Fetal Bovine Serum (FBS, #10500-064, Gibco, United Kingdom) was included as an essential growth factor.

2.2.2. Cell Thawing

To initiate a cell culture, the desired cells were retrieved from the liquid nitrogen storage tank and thawed. Simultaneously, the culture medium was warmed to 37 °C in a bead bath. The cell solution was transferred to a 15 mL Falcon tube, and 5 mL of warmed culture media was added to the cell solution drop by drop, very slowly, to avoid stressing the cells. Gentle pipetting was performed, and the Falcon tube was placed into the cell culture centrifuge. The centrifuge was operated for 5 minutes at 300 g. The resulting pellet, composed of cells, was separated from the supernatant, which consisted of DMSO solution and cell medium. The supernatant was discarded, and the pellet was resuspended in fresh media and transferred to a new flask for growth. A media change was performed the next day.

2.2.3. Cell Passaging

Cell passaging for the selected cell lines is performed when the cells reach approximately 80% confluency. Once 80% confluency is observed under the microscope, the passage should be carried out. The cell monolayer is washed with 1X PBS. Trypsin-EDTA 0.025% is added to the flask to cleave the arginine-lysine bonds between the cells and the flask surface, and the flask is incubated for 3 minutes. After 3-4 minutes, the cells are checked under the microscope to ensure they are detaching from the surface. The trypsin-EDTA is neutralized with an equal amount of cell medium (1:1), and the mixture is collected in a Falcon tube for centrifugation. The tube is centrifuged for 5 minutes at 300 g, and the supernatant is discarded. The pellet is resuspended in fresh media, adjusted based on the pellet size, and the desired number of cells is transferred to new flasks for co-culturing.

2.2.4. Cell Counting

After cell passaging steps done in an order, the time for cell counting. The cell pellet which taken from centrifuge is dissolved with fresh media, this added media is depend on the amount of the pellet. This dissolving media amount should be accurate because this amount is affected the cell number. The volume of fresh media is a parameter on cell counting. Cell counting can be done by manual, with hemacytometer. Hemacytometer have different horizontal and vertical lined regions, at the centre of the hemacytometer, the horizontal and vertical part crossed. The centre intersection part is look like checkered notebook paper. 10 µl of dissolved cell solution taken and put between the hemacytometer and lamella. The cells seen this part is counting under microscope at 4x magnification. Cell number is found by a multiplication. Since we counted cells, we have a dissolve coefficient, we need one more 10^4 coefficient because of the hemacytometer coefficient. We have an equation to calculate the total number of cells as; 10^4 x dissolution coefficient x counted cells on hemacytometer = total number of cells.

2.2.5. Cell Cryopreservation

Cell cryopreservation is necessary for storing cell lines during passaging. In cell cryopreservation, cells are frozen in a cryovial with dimethyl sulfoxide (DMSO) as a cryoprotectant. After centrifugation, the cells are resuspended in fresh medium, and 900 µl

of the cell solution is mixed with 100 µl of DMSO reagent in a cryovial, achieving a final concentration of 10% DMSO.

Once DMSO is added, it is important to act quickly. The Mr. Frosty freezing container should be prepared beforehand. The cells are placed inside the Mr. Frosty box, which is then transferred to a –80 °C freezer, followed by storage in liquid nitrogen. The Mr. Frosty box allows for gradual freezing by decreasing the temperature by 1 °C per minute.

2.2.6. Extracellular Vesicle Isolation and Characterization

2.2.6.1. Rhus coriaria L. Extracellular Vesicle Isolation by Ultracentrifugation (UC) Preparation

Sumac is harvested only in august month of the year. Sumac obtained from local fruit-vegetable market from Gaziantep, Turkey in august, 2023. After sumac taken, sumac juice is obtained by diluting deionized water and sumac (100 gr sumac + 400 mL dH2O). Sumac is splited with water in commercial blender. Removal of seeds and pulp done by the filter paper until only sumac juice is left. Sumac juice preparation done and ready to centrifuge steps. Sumac juice placed in falcon tubes and put inside the centrifugation device swing-out rotor mutually equal amounts. Sumac juice centrifuged 3600 g x 10 minutes x 4 °C x 9/9 acceleration/deceleration. Since there is a pellet, the supernatant is transferred in a new falcon tube and the process is repeated until the pellet is removed. For sumac, two sets of centrifugations are enough to remove the pellet. The rotor is changed with fixed-angle rotor. Sumac juice is transferred in new falcons and centrifuged at 18,000 g x 15 minutes x 4 °C x 9/9 acceleration/deceleration. The supernatant is collected and filtered 0,45 µm pore sized filter and prepared for ultracentrifuge [76].

2.2.6.2. Isolation by Sucrose Ultracentrifugation Method:

To obtain, sucrose is weighed 3.42 gr and 7,5 mL deionized water is mixed. 10 mL of 1M sucrose is acquired. Sumac is divided into 8,5 mL per matte UC tubes and 1,5 mL 1M sucrose is added at the bottom of the tube with low gravity mode of pipettor. The aim of this is to see two different phases in tube. The sumac juice on the top and the sucrose is on the bottom. After this, UC device set at open top mode x 101,000 g x 70 minutes x 20 °C.

The phases are seen same as before ultracentrifuge, but exosomes are depleted in the sucrose part, the bottom of the tubes. Then supernatant part is removed, and sucrose part is collected

in a new falcon tube. Ultrafiltration steps should done. Ultrafiltration is done by Amicon® Ultra Centrifugal Filter (#UFC905024). Result of ultracentrifuge is 4-6 mL sucrose exosome mixture, this amount is completed to 15 mL with deionised water, in a tube and transferred in Amicon® ultrafiltration tube. Tube is placed in centrifuge and set at 3600 g x 10 minutes x 15 °C x 9/9 acceleration/deceleration [76]. The aim of this is, to remove sucrose, the mixture should dilute at least 1000-fold. The result is scaled with pipet and to continue, the result should be between 200-400 µl. Centrifuge process is repeated until the result is between 200-400 µl as mentioned. In sumac, 2 repeats provide the 200-400 µl range. This result is mixed with 3 mL sterile PBS as a final addiction. The mixture is filtered with 0,2 µm filter and other components removed. The exosome is ready for characterization.

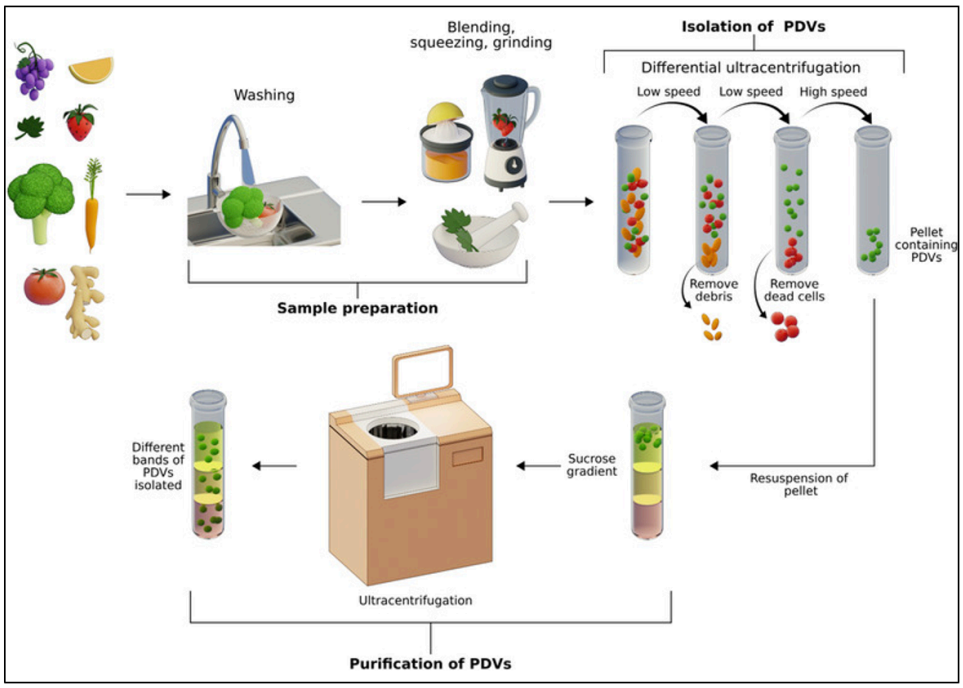

Figure 2.1. Illustration of the plant derived EVs isolation by UC method [76].

2.2.7. *Rhus coriaria L.* Characterization by Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) is a powerful technique to see and record the movements of particles. Unique particles make a random motion in the fluid called Brownian motion. The laser beam provides to observe particles in this scattering. Nanosight NS300 (Malvern Instruments, Amesbury, UK) is used in 488 nm laser and a high-resolution camera inside. This camera record 10 seconds videos as mentioned laser principle.

To apply; EVs is diluted 1/500 ratio with deionised water. The device is set according to manufacturer's protocol. The analysis is done by the nanoparticle track analysis (NTA) software 3.3.301.

2.2.8. *Rhus coriaria L.* Characterization by Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a powerful method to visualize particles in detail. TEM analysis is a need to characterize exosomes because TEM allows the information about; size, shape, layer structure and amount of exosomes.

TEM is important in exosome characterization, because TEM provides to see micro-sized particles in nanometer level resolution. This property allows high quality and detailed image. TEM analysis also gives information about ultrastructural properties like lipid composition, membrane structure and content. With TEM analysis, distribution of lipids, proteins and nucleic content can be determined too. Briefly, 1:1 ratio of sumac EVs and 2% paraformaldehyde (PFA) mixed gently. Mixture incubated at room temperature for 5 minutes. Then prepared for the scan by the JEM-2100Plus, JEOL microscope with 200 kV.

2.2.9. Effects *of Rhus coriaria L.* Derived EVs on Cell Viability

Cell viability assay is a key step in experiment to determine the non-toxic but effective dose of exosomes. In cell viability assay, the chosen salt is measure the proliferation, metabolic activity and thus viability of cells in a colorimetric way. In this thesis, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) salt chosen as reagent. Cell viability of HaCaT, HUVEC and HDF cells analysed. The protocol follows as; $5x10^3$ cells/per well were seeded in 96 well plate in 5 doses and positive & negative controls. Cells were seeded in humidified incubator conditions as 5% CO_2, 37 °C for 24h. Next day, sumac EVs given with the doses of 6,65E+09 p/mL, 6,65E+08 p/mL, 6,65E+07 p/mL, 6,65E+06 p/mL, 6,65E+05 p/mL. Positive control contains 20% DMSO and negative control is directly complete cell culture medium. Next day 24 hours after the exosomes given, 10% MTS reagent and PBS-Glucose mixture is added on cells and incubated 1 hour in incubator conditions and without light. After 1 hour pass, the cell viability was determined at 490 nm in ELISA plate reader.

The doses of exosomes are determined by the help of NTA and MTS assay, non-toxic, effective dose selected and applied in following experiments.

2.2.10. Cell Proliferation Assay

Cell proliferation assay principle is depending on the comparison of cell numbers before and after giving exosome. Cell proliferation assay measure the division of cells. In the protocol, it optimised as $5x10^4$ cells for HaCaT and HUVEC. These number of cells were seeded on 6 well plate and incubated overnight at humidified incubator conditions. Next day, selected two doses of exosomes given to cells with 2% FBS-medium. After 24h later, one plate of cells were passaged and counted with hemacytometer. So the difference calculated and it gave an information about the proliferation of cells.

2.2.11. Wound Healing Assay

Wound healing assay, also called scratch assay is a key step to understand the scars and their healing. These assays play an important role in proving the closure of the wound and with these, closure demonstrate easily by microscope. Wound healing done with the help of a kit by CytoSelect™ Wound Healing Assay Kit (Cell Biolabs, Inc., San Diego, USA). The kit contains 24 wells and 4-sectioned inserts to create a wound on wells, the basic principle of kit is, the cells were seeded on wells with inserts and a little pressure is applied on inserts to create the wound. According to manufacturer's protocol, 0.5-1.0 x 10^6 cells/ml cell suspension with 2% FBS-medium added on inserts and waited overnight at humidified incubator conditions. Next day, inserts removed carefully without twisting or touching the walls of wells to begin scratch assay. Wells washed with PBS and selected exosome doses are prepared and given to cells. After EVs given, images taken under light microscope as 0^{th} hour. The following 12h, 16h, and 24h images taken as manual assay. The calculation and comparison part is the same with manual assay.

The wound distance calculated by the ImageJ program as the formula below;

$$\text{Wound Closure (\%)} = (1 - \text{Wound width at 14h} / \text{Initial wound width at 0h}) \times 100$$

14^{th} h width represents the last image and 0^{th} h width represents the initial wound image.

2.2.12. Migration Assay

Cell migration is also a critic part of wound healing. It is expected that, the healthy cells migrate towards the wound to repair it. Cell migration consists of different molecular and cellular mechanisms together. Some of them are follows; chemotaxis, reorganise of cellular

skeleton, proteolysis, remodelling of ECM etc. It is important to understand these mechanisms reorganisation to resolve the problems in current technologies. Here in this study, a kit by CytoSelectTM 24-well Cell Migration and Invasion Assay kit (Cell Biolabs Inc., San Diego, CA, USA) is used to model cellular migration on HUVEC. Kit contains 24 well plates and polycarbonate membrane inserts. 24 well plate wells' act as the outer layer of skin and the inserts which is placed in wells acts as the inner layer of skin. In the protocol, 0.5-1.0 x 10^6 cells/ml cell in serum free medium is prepared and seeded inside the insert, and the 2% FBS-medium added between the insert and wells. There are control wells and the selected dose of exosome applied wells in this set-up. And the rest is then applied according to manufacturer's protocol. In the end, the whole samples transferred to 96 well-plate and measured with microplate reader at OD 560 nm.

2.2.13. Tube Formation Assay

One of the most popular *in vitro* tests for angiogenesis is tube formation. In the experiment, endothelial cells are plated onto a material that resembles a basement membrane; the cells grow into tubules on this substrate. Many of these tubules have a lumen, and the cells form close connections with each other and the cell matrix. In this study, Matrigel used as a basement membrane to cover the slide surface. The protocol follows as; 55 µL Matrigel loaded per well of 96 well plate. 30-minute incubation done in humidified incubator conditions. At the same time, HUVEC cells were washed and trypsinated. The 2% FBS cell culture medium prepared and selected dose for sumac EVs mixed with medium. Then 5x10^4 cells counted for each well and cells prepared with sumac EVs. The prepared cells loaded on the Matrigel coated 96 well plate and 4-20h incubation period begin. In this experiment, 5.5h incubation enough to work Matrigel coating and tube formation activity. The images taken by Zeiss fluorescence microscopy at 5X magnification.

2.2.14. RNA Isolation

RNA isolation is a method to separate RNA by liquid separation. RNA isolation is needed to conduct experiments such as RT-PCR followed; sequencing, RNAse protection assays, reverse-transcription polymerase-chain reaction (RT-PCR) etc. In the study, HaCaTs and HUVECs were seeded 200.000 cell/per well on 6 well plate and incubated overnight with 2% FBS-medium. Next day, cells washed with PBS and scratch is created with 200 µL tip like in wound healing assay. After scratching, exosomes and control groups prepared and

left overnight at incubator conditions. 24h later, cells washed with PBS, cells resuspended with trypsin, cells collected, and the pellet prepared for RNA isolation. The isolation part applied according to manufacturer's protocol. In the end, obtained RNA diluted with RNAse free water and RNA concentration measured with Nanodrop (Nanodrop 2000, Thermo Fischer).

2.2.15. Complementary DNA Isolation

Since RNA isolated, the following step must be complementary DNA synthesis. In researches, RNA cannot be able to use by itself, otherwise the obtained RNA then transformed to complementary DNA. The complementary DNA is exactly completes the original RNA sequence. cDNA is preferred because of its stability, accessibility, efficiency when compared to RNA. In this, isolated RNAs transformed to cDNA by the kit (cat. no. 11483188001; Sigma-Aldrich; Merck KGaA). Synthesis done according to manufacturer's protocol.

2.2.16. Gene Expression Analysis by Real Time Polymerase Chain Reaction

To search the effects of exosomes on wound healing, gene-based research must apply. These gene expression analysis done by RT-PCR. The master mix prepared according to Table 2.5. In this study, primers selected according to the properties like; inflammatory genes (interleukins 6-8-10; IL6-IL8-IL10), ECM formation genes (type 1 and type 3 collagen; COL1A1 and COL3A1, transforming growth factor beta 1 and 3; TGFß1 and TGFß3, matrix metalloproteinase 9; MMP9, fibrinogen beta chain; FBR), proliferatory genes (marker of proliferation; Ki67, platelet derived growth factor; PDGF) angiogenesis genes (vascular endothelial growth factor; VEGF, fibroblast growth factor 2; FGF2). In this study, PCR reactions done by SYBR Green method by the kit (#4368706, Applied Biosystems, Thermo Scientific, USA).

Table 2.5. RT-PCR Master Mix

Reagents in Reaction	**Volume of Reagent**
Nuclease Free Water	2 µl
Forward Primer	0,5 µl
Reverse Primer	0,5 µl
cDNA Template	2 µl
2x SYBR Green Mix	5 µl

In Table 2.5. amounts of components for SYBR Green mix per one reaction showed. This calculation done with considering the replicates and margin of errors. With these reaction, mRNA levels of genes measured and so, gene expression level evaluated. In table 2.6. the PCR conditions shown.

Table 2.6. qPCR Conditions

Phase	**Repeats**	**Time**	**Temperature**
Initiation	1	30 seconds	95 °C
Denaturation	35	5 seconds	95 °C
Annealing	35	20 seconds	58 °C
Extension	35	20 seconds	72 °C
Final Extension	1		72 °C
Melt Curve	60	5 seconds	0.5 °C/cycle 65-95 degree
Hold	∞	5 minute	4 °C

The reaction mixes prepared according to Table 2.6. placed in PCR plate with the reference of ATP5B gene and the other primers. The reaction loaded on the CFX96 Touch System device and the cycles at Table 2.6. applied. In Table 2.7. the primer sequences for RT-PCR shown. PCR analysis done by the CFX Manager 3.1 software (Bio-Rad Laboratories, CA, USA) and normalized to the mRNA level of ATP5B.

Table 2.7. Primers Used in qPCR Analysis (R: reverse primer, F: forward primer).

Gene	Sequence		References
ATP5B	F	GTCTTCACAGGTCATATGGGGA	[77]
	R	ATGGGTCCCACCATATAGAAGG	
IL6	F	ACTCACCTCTTCAGAACGAATTG	[78]
	R	CCATCTTTGGAAGGTTCAGGTTG	
IL8	F	ACTGAGAGTGATTGAGAGTGGAC	
	R	AACCCTCTGCACCCAGTTTTC	
IL10	F	ATCCAAGACAACACTACTAA	
	R	TAAATATCCTCAAAGTTCC	
COL1A1	F	CCACGCATGAGCGGACCCTAA	
	R	ATTGGTGGGATGTCTTCGTCTTGG	
TGFß1	F	TACCTGAACCCGTGTTGCTCTC	
	R	GTTGCTGAGGTATCGCCAGGAA	
MMP9	F	AGACCTGGGCAGATTCCAAAC	
	R	CGGCAAGTCTTCCGAGTAGT	
FBR	F	CCACCCCCATAAGGCATAGG	
	R	GTAGGGGTCAAAGCACGAGTCATC	
KI67	F	AAAGAAGCCTGTGGGCGAA	
	R	TTGAGCACTCTGTAGGGTCG	
VEGF	F	AGGGCAGAATCATCACGAAGT	
	R	AGGGTCTCGATTGGATGGCA	
TGFß3	F	TGGCTGTTGAGAAGAGAGTCC	
	R	TCATCCTCATTGTCCACGCC	
COL3A1	F	TGGTCTGCAAGGAATGCCTGGA	
	R	TCTTTCCCTGGGACACCATCAG	
PDGF	F	GCAAGACCAGGACGGTCATTT	
	R	GGCACTTGACACTGCTCGT	
TSP1	F	AGACTCCGCATCGCAAAGG	
	R	TCACCACGTTGTTGTCAAGGG	
FGF2	F	GGTGAAACCCCGTCTCTACA	[79]
	R	TCTGTTGCCTAGGCTGGACT	

2.2.17. Immunocytochemistry

Immunocytochemistry (ICC) is a technique used to detect and visualize specific proteins or antigens in cells. The method relies on antibodies that specifically bind to target proteins, and it uses labeled secondary antibodies for detection. In this experiment, 4x 10^4 cells/well seeded on 48 well plate. 24h later, scratch done and sumac EVs applied with selected doses immediately after. 24h later too, as the first day of ICC, cells were washed with 200 µl sterile PBS. After washing, cells fixed with 700 µL %4 PFA per well for 15 minutes at room temperature. Tween 20 is a non-ionic detergent that's frequently used in immunocytochemistry (ICC) and other laboratory procedures. After fixation, cells washed with PBS again. %0.1 Triton X-100 is added 150 µL per well for permeabilization for 5 minutes at room temperature. After this, 0.1% Tween 20 solution is prepared with PBS and obtaining PBST. The other washing steps will continue with PBST. Triton X-100 washed with PBST for 5 minutes. %1 BSA used as a blocking solution, 200 µL per well added and incubated for 1 hour at room temperature. Primary antibodies COL1A1, fibronectin, laminin and elastin diluted 1/200 ratio with %1BSA and PBST mixture. Primary antibodies added 300 µL per well and lefted overnight at +4 °C. At the second day of ICC, primary antibodies collected and wells washed with PBST for 3 times per 5 minutes. Secondary rabbit antibody is prepared 1/200 ratio and applied on cells 300 µL per well for 1h at room temperature again. After 1h later, secondary antibody is collected and washed with PBST for 1 time. 1/1000 ratio of DAPI dye is prepared in PBST and applied on cells 300 µL per well for 5 minutes at dark room temperature. DAPI is removed and wells washed with PBST for 3 times. Sterile PBS is added to see the images under light microscope.

3. RESULTS

3.1. *Rhus coriaria L.* EV Characterization and Size Distribution by Nanoparticle Tracking Analysis (NTA)

Sumac EVs isolated by the ultracentrifuge method. When this protocol followed correctly, the characterization might be the next steps. The size range of EVs is 30-200 nm in diameter. Smaller of bigger sizes are different categories (such as microvesicles: up to 1000 nm, apoptotic bodies: 1000-5000 nm).

The traditional and most used characterization method for EV particles is nanoparticle tracking analysis (NTA). NTA investigates the parameters of EVs; the size, concentration, visualization, particle purify, dynamical behaviours, and qualification control. The randomized movement of EVs called Brownian motion, generally a zigzag direction motion, is the diffusion of EVs in solution. EVs make a Brownian move in solutions, this allows to determine the size of EVs by that tracking. NTA also might analyse the concentration of EVs by the filtration of laser beam inside. NTA gives a result with the particle unit per volume of EVs inside. Figure 3.1 below represents the size distribution analysis of sumac EVs with NTA.

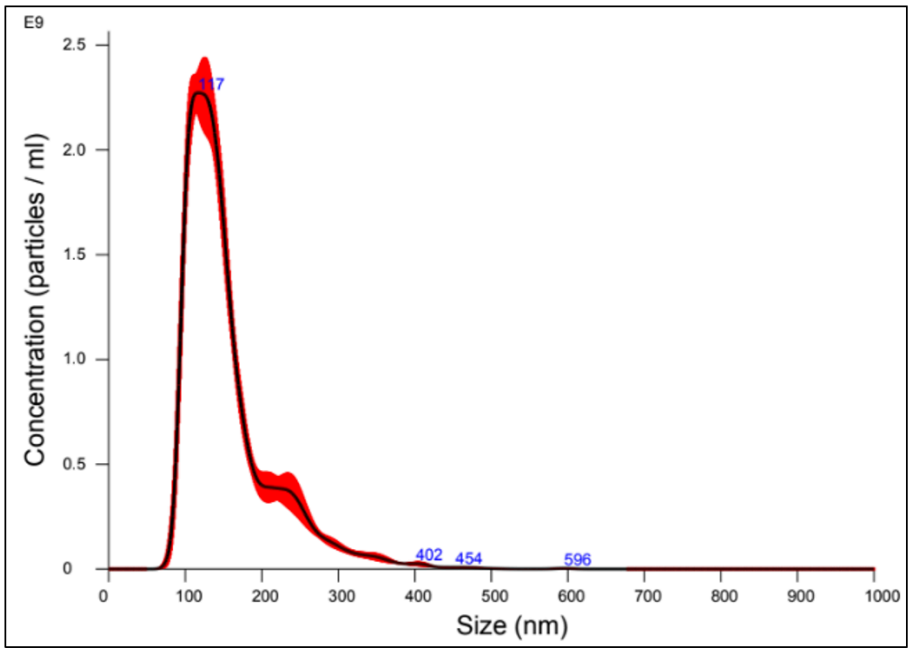

Figure 3.1. Sumac EVs size distribution analysis by NTA. All the values were showed as means ± standard deviation SD.

3.2. *Rhus coriaria L.* EV Characterization by Transmission Electron Microscopy (TEM)

In other method for EV characterization, transmission electron microscopy (TEM) used with its high resolute - negative dying property in this study Figure 3.2. TEM allows structural particle and composition analysis in EVs. The other parameters that TEM might search are; morphological shape, size distribution, integrity of membrane structure, internal content, purity. In TEM, EVs viewed as spherical shaped, or cup shaped on its lipid layer. TEM allows the quality and the size of EVs. In TEM, the EVs which have normal complete membrane reflects as an intact particle, while the degraded membraned EVs appear with low integrity. With high resolution TEM, the EV content or cargo molecules might seem too.

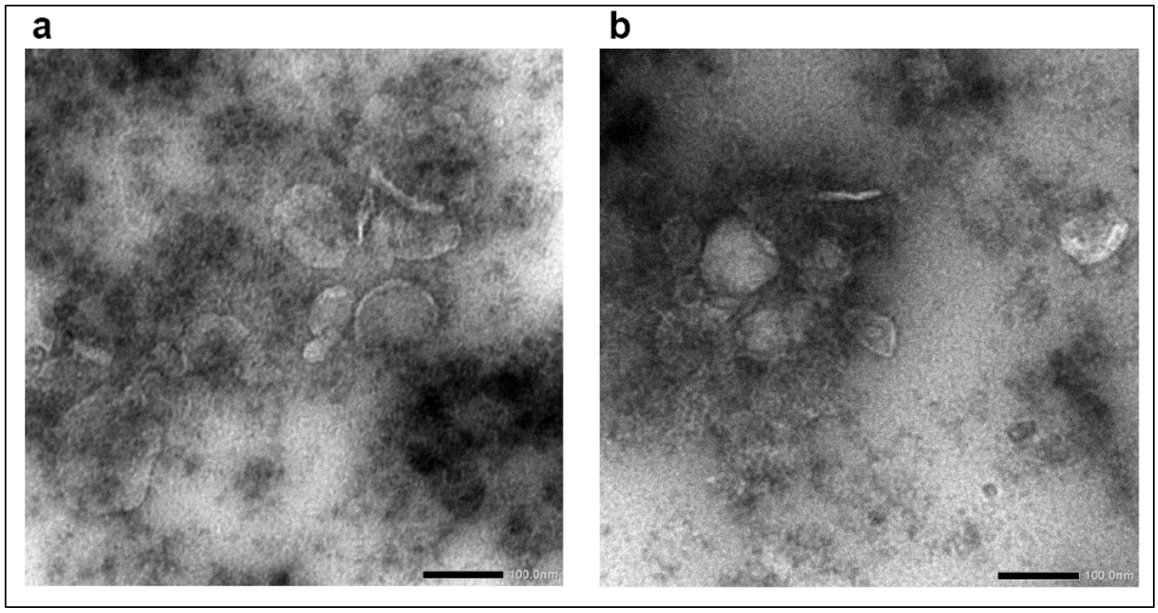

Figure 3.2. Sumac EVs in high resolution TEM image. (a) and (b) shows sumac derived EVs' images taken from different points. Scale bar = 100 nm.

As known, the sumac EVs didn't searched before, so the comparison of this results couldn't do. But when compared with other plant derived EVs, our results found proper refer to EV.

3.3. Cell Viability

MTS assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, is an assay to measure the cell proliferation. The viability of cells are measured in a colorimetric way. 5 doses are determined and calculated from the NTA results.

Cultured cells treated with EVs and incubated for 24 hours at humidified incubator conditions. After 24h, MTS reagent given to cell with the ratio of 1:10 via PBS glucose. Then, 1h incubation at dark and incubator conditions, the cell viability measured at the 490 nm OD.

MTS assay was applied to test the cell viability of sumac EVs. MTS provides colour change on plate and numerical values of viability. The results in Figure 3.3. shows comparison between positive control group containing 20% DMSO + media mixture and negative control group with only media; and sumac EV applied groups. The negative control groups' cell viability accepted as major (100%) and positive control groups' cell viability accepted as the minor. The expected result is, doses might give results between the negative and positive control groups. In wound healing, non-toxic optimal dose is searching. The doses 6,65E+09 p/mL and 6,65E+08 p/mL give similar effects with non-toxicity. Figure 3.3. demonstrates the MTS results for sumac EVs.

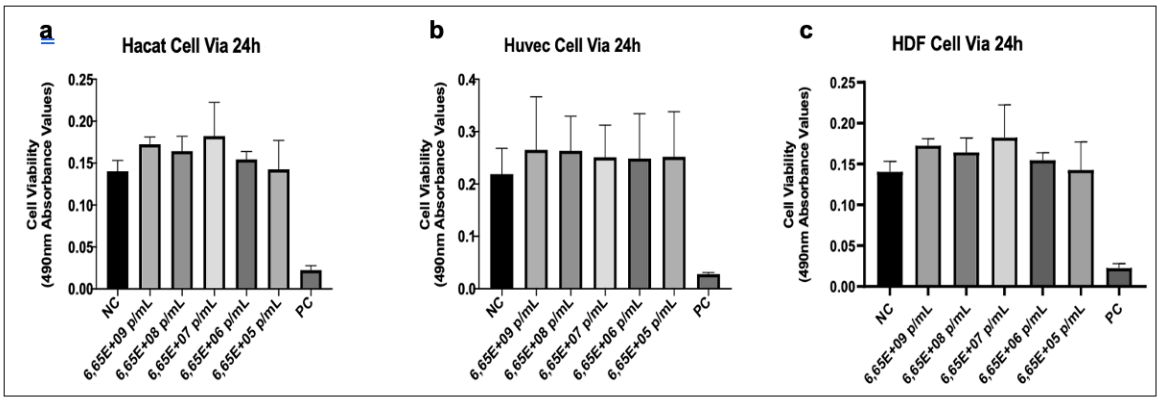

Figure 3.3. Cell viability results by MTS assay. (a) demonstrates effects of sumac EVs on HaCaT, (b) demonstrates effects of sumac EVs on HUVEC and (c) demonstrates the effects of sumac EVs on HDF cells viability measured with 5 different doses for 24h. NC: cell culture medium, PC: 20% DMSO+80% cell culture medium. All the values were showed as means ± standard deviation SD. All experiments conducted at least 3 independent replicates.

3.4. Cell Proliferation Assay

Cell proliferation assay, as it understands from the name, measures the cell proliferation rates of cells when treated with agents. At the beginning, 5x 10^5 cells/well seeded on 6 well

plate. Since the beginning number of cells were known, the control groups and EV applied group might compared in Figure 3.4. two doses were selected from the MTS results.

In the calculation plate, cells washed with PBS and trypsinated, after centrifuge, cells were counted under microscope.

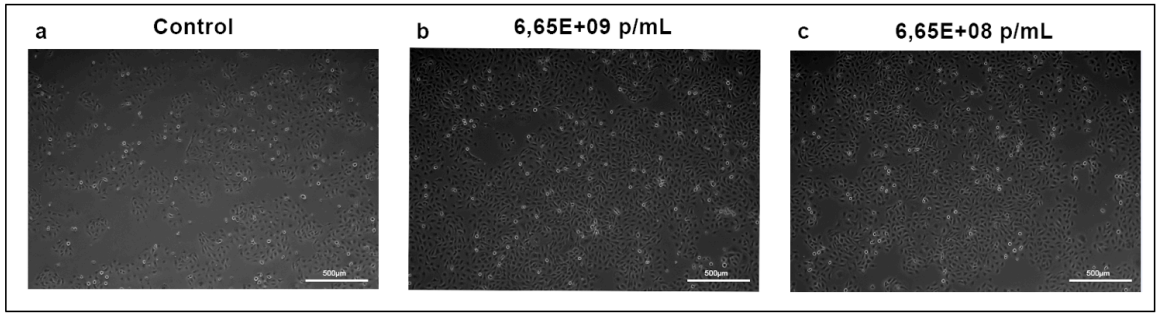

Figure 3.4. Proliferation rates of control group (a) and different doses of sumac EV treated groups (b,c) for HaCaT. (a) demonstrates the control group, (b) demonstrates the sumac EV dose at 6,65E+09 p/mL, and (c) demonstrates the sumac EV dose at 6,65E+08 p/mL. According to images, EV applied wells proliferate and cell number increased as seen. Scale bar = 500 μm. All experiments conducted at least 3 independent replicates.

3.5. Wound Healing Assay

In wound healing studies, scratch assay (also named wound healing assay) is a key experiment to prove the healing. The assay principle depends on creating a wound physically, apply treatment and take images in different hours. This assay is important on assessment of wound closure, disease modelling, searching therapeutic components. Also in scratch assay, tissue repair mechanism, wound closure inside and outside might searched.

Cells were seeded on 24 well plate with the inserts which create scratch. Next day inserts removed, and scratches created per well and EVs applied in selected two doses. Images took from the fluorescent microscopy at 5X magnification. Two doses selected from NTA and MTS assay applied. In Figure 3.5, left line from above to below are 0h (T0) of groups, middle line is 14h treated with EVs and right line represents the 16h treated with EVs. As seen, cell covers the wound site itself but when sumac EVs applied, there is a significant difference between control and treatment groups. In 16h, the wound is fully closed when treated with EVs but in control group, slit is still didn’t close.

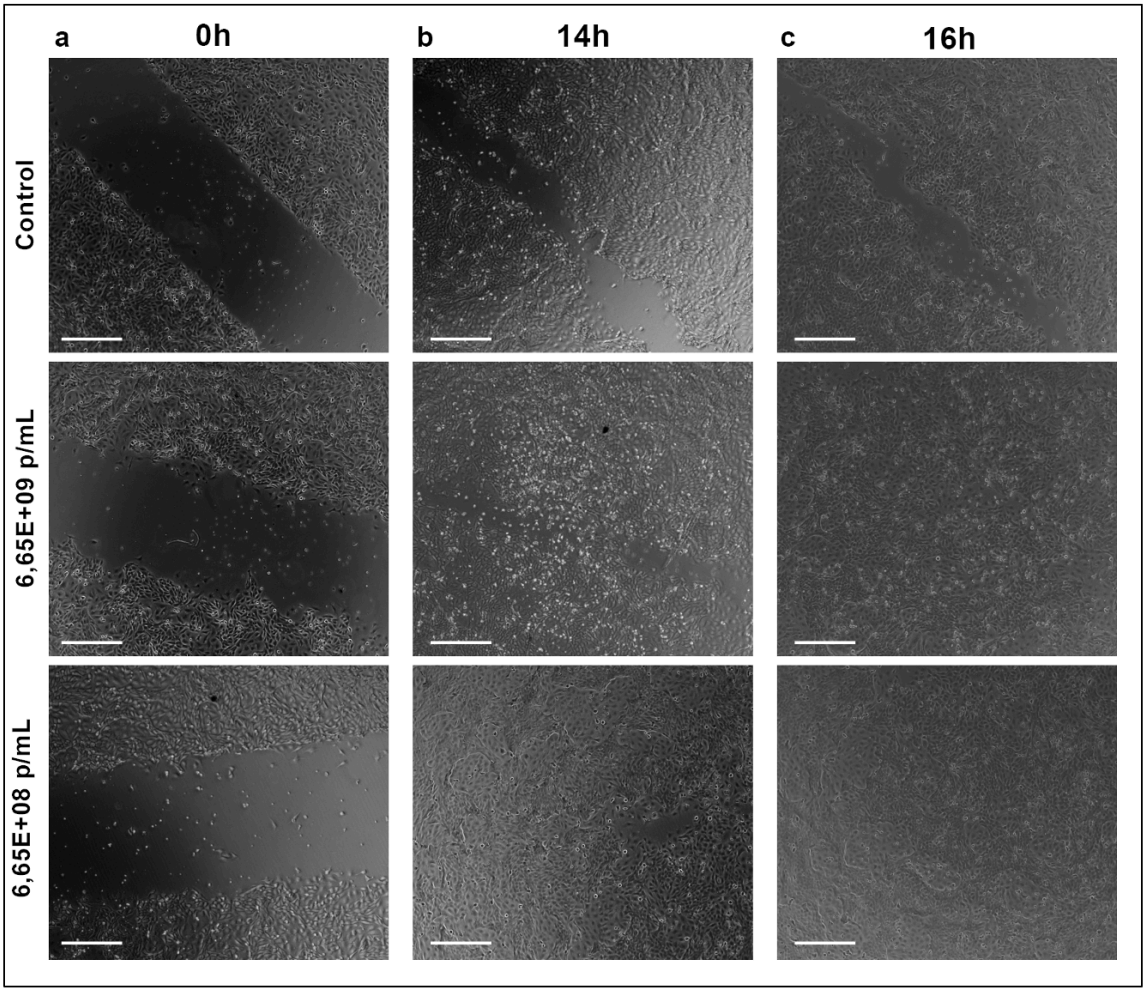

Figure 3.5. Wound healing scratch assay on HaCaT cell line. On the left side, starting from column (a) demonstrates the 0th hour on control and two doses of sumac EVs. On the middle side of figure starting from column (b) demonstrates the control and two different doses of sumac EV applied groups for 14th hour and on the right side of figure starting from column (c) demonstrates the control and two different doses of sumac EV applied groups for 16th hour. Scale bar = 500 μm. All experiments conducted at least 3 independent replicates.

3.6. Migration Assay

Cell migration assay, as named, searches for the migratory events of cells and cell motility affective factors. In this process, cells moved to the other side of insert to model skin migration. The inserts inside wells act as the layers of skin and the transition towards chemoattractant. In migration assay, chemotaxis mimics via the inserts placed in 24 well plate. Selected two doses and control groups were seeded and EVs given inside the insert

according to Figure 3.6. The outside of the insert contains complete medium. The results were dyed with 4X Lysis Buffer/CyQuant® GR and measured at 480/520 nm OD.

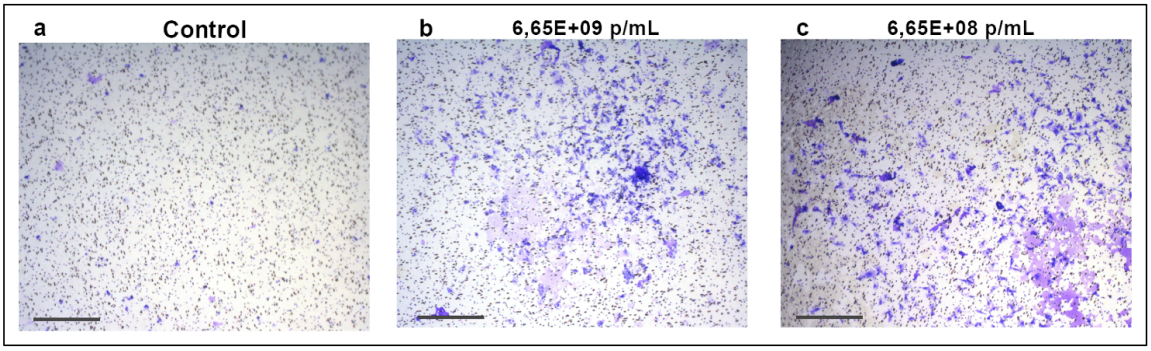

Figure 3.6. Migration of EV applied cells. (a) demonstrates the non-treated control group of HaCaT cells. (b) demonstrates the migration rate with sumac EV dose of 6,65E+09 p/mL and (c) demonstrates the migration rate with sumac EV dose 6,65E+08 p/mL. Scale bar = 500 µm. All experiments conducted at least 3 independent replicates.

The bluish-purple colour represents the migrated cells through chemoattractant. The significant difference appears between groups.

3.7. Tube Formation Assay

The tube creation assay is one of the most popular *in vitro* models for the angiogenesis rearrangement stage. The test assesses endothelial cells' capacity to form capillary-like structures when they are plated at sub confluent densities and given the proper extracellular matrix support. Inhibitors of tube development may find application in cancer and other disorders where tumors induce the formation of new blood vessels to obtain oxygen and nutrients and to grow larger than they first started. Figure 3.7. shows the tube formation on HUVEC control and sumac EV applied cells below.

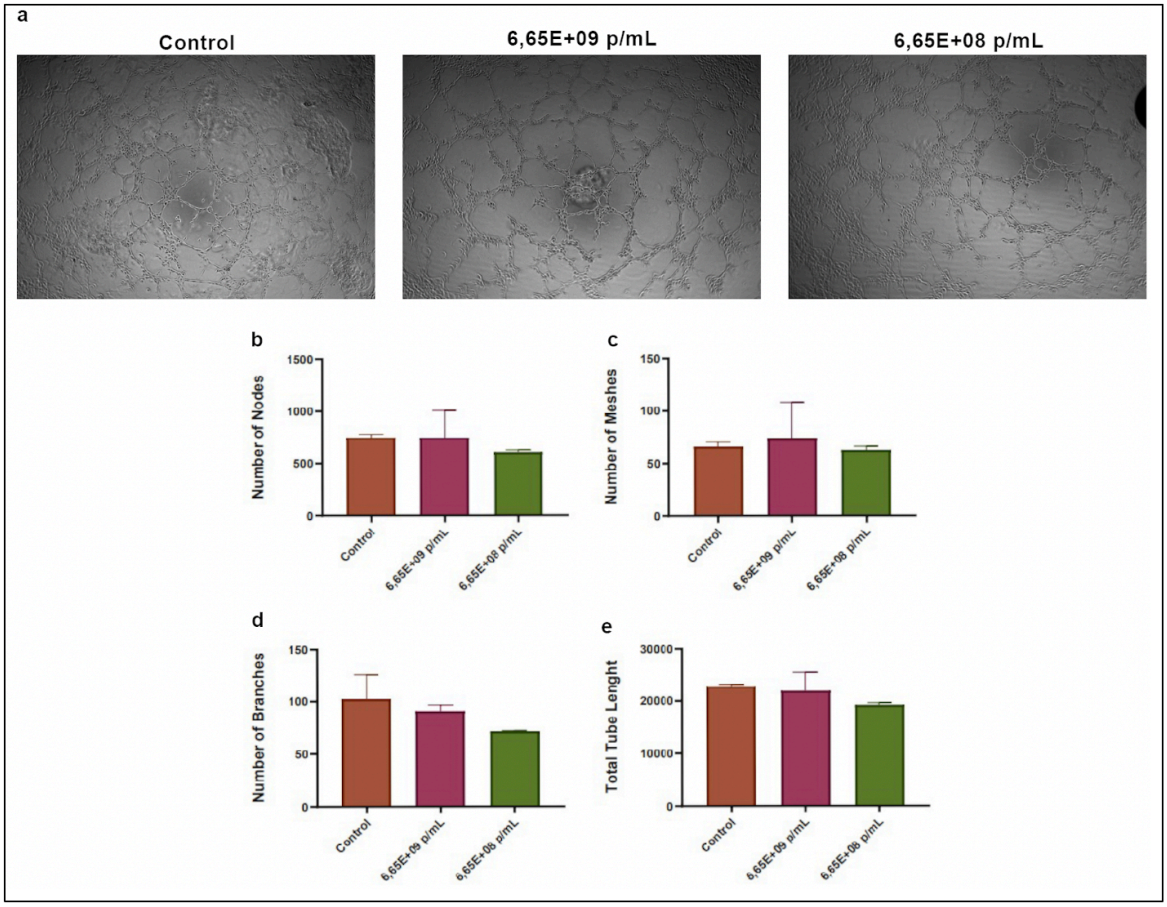

Figure 3.7. Tube formation assay images with control group and sumac EV treated groups (a) and graphical analysis with different properties (b-e). (left of a) shows the images of non-treated control group and (middle a) shows images of 5.5 hour 6,65E+09 p/mL sumac EV treated HUVEC and (right of a) shows images for 6,65E+08 p/mL sumac EV treated HUVEC. (b) images examines angiogenesis analysis with the number of nodes formed, (c) examines angiogenesis analysis with the number of meshes created, (d) examines angiogenesis analysis with number of branches formed,(e) examines angiogenesis analysis with the generated total tube length. Scale bar = 500 μm. All experiments conducted to the mean ± SD of at least 3 independent replicates.

3.8. Quantitative Polymerase Chain Reaction Assay (PCR)

The mRNA expression is a final and most reliable part of experiments. The gene expression levels, mRNA amounts are measured with the help of this test. The genes selected to make a gene expression profile for wound healing by sumac EVs. To search potential healing effects of sumac EVs, sumac EVs applied with selected two doses for 24h. The gene

expression profiling shown below Figure 3.8. and Figure 3.9. Gene expression profiling was a key step to determine the mRNA amounts of sumac EVs applied groups with comparison of non-treated control group. According to results, some genes expressed as promoters in wound healing stages while some are not affect in gene level. In HaCaT cell line, Col1A1, FBR, IL6, IL8, Ki67, VEGF. COL3A1, MMP9 genes are not directly express a significant effect with sumac EV treatment. However, TGF1, IL10, TGFß3 express a significant effect on gene level with sumac EV application. Also, in HUVECs IL8, IL10, VEGF, MMP9 didn't show an important effect when sumac EVs applied. The significant effects showed in IL6, FGF2, TSP1, TGFß3 genes when treated with sumac EVs.

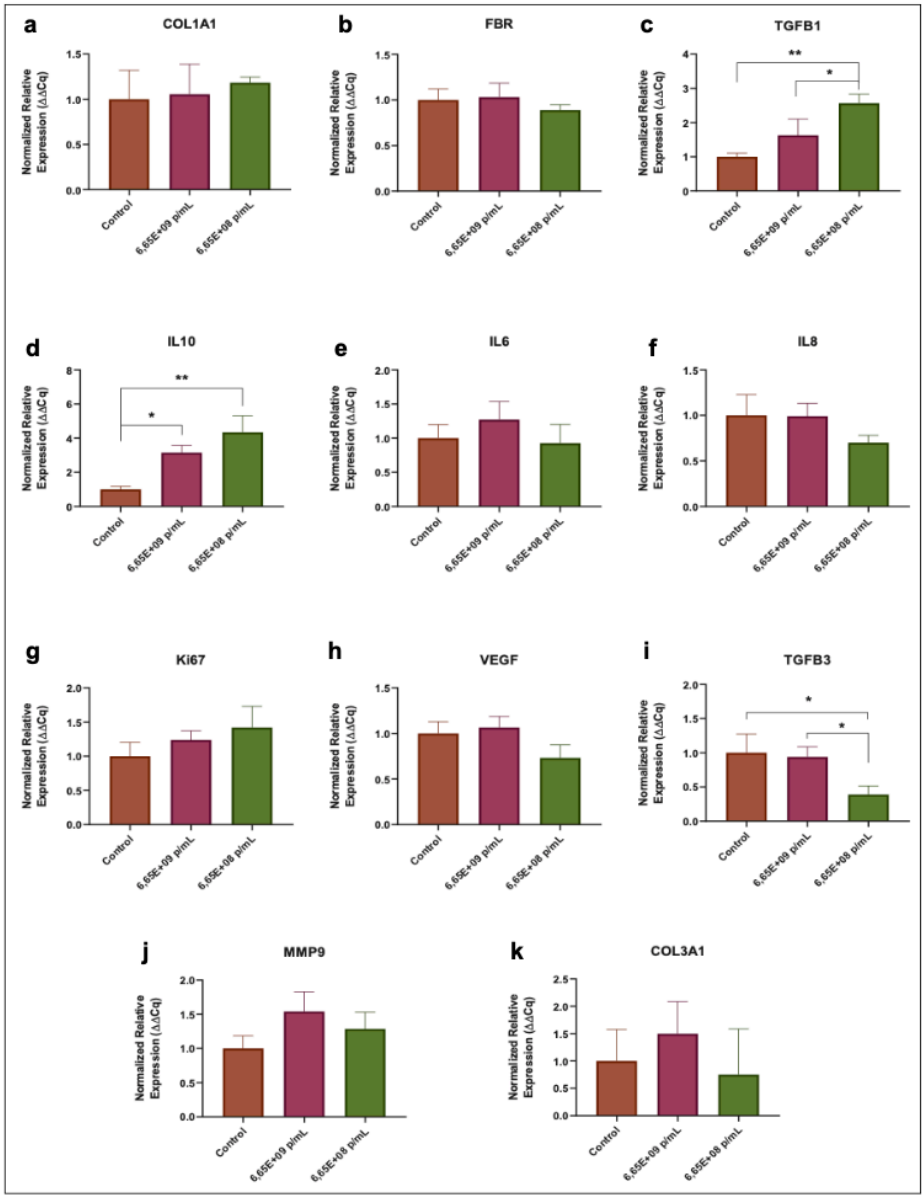

Figure 3.8. mRNA levels of Sumac EV applied HaCaT cells. In figure, ECM component gene expressions (a, b, k), cytokines and inflammatory gene expressions (c-f and i), proliferative phase gene expression (g), growth factor gene expressions (b,h) and remodelling gene expression (j) shown.

Abbreviations: COL1A1: Collagen type I alpha 1, FBR: fibronectin, TGFß1: Transforming growth factor beta 1, IL10: Interleukin-10, IL8: Interleukin-8, IL6: Interleukin-6, Ki67: Antigen Kiel 67, VEGF: Vascular endothelial growth factor, TGFß3: Transforming growth factor beta-3, MMP9: Matrix metalloproteinase-9, COL3A1: collagen type III, alpha-1 Control: non-treated HaCaT cells. All experiments conducted at least 3 independent replicates. *p<0.05, **p<0.01.

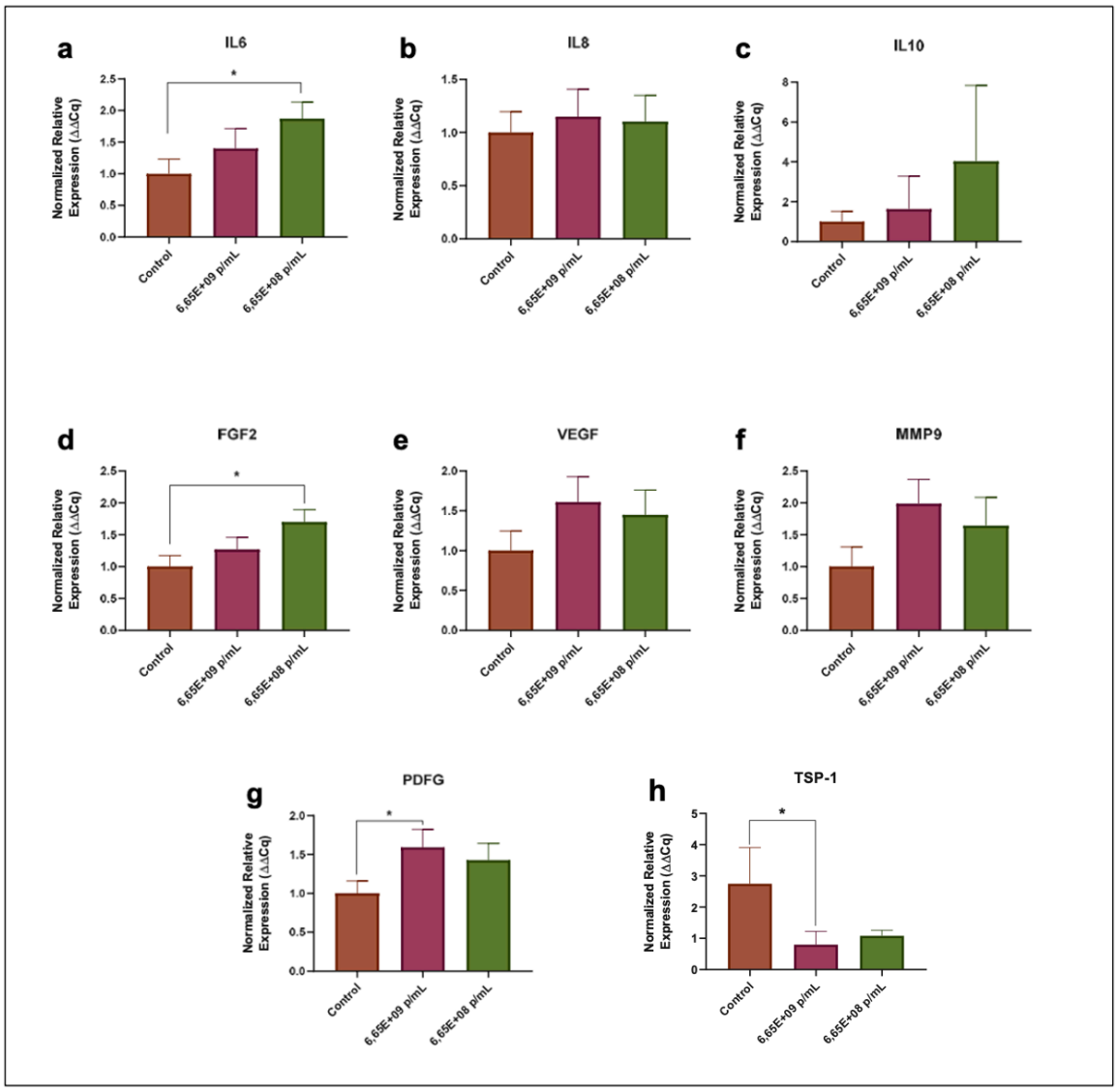

Figure 3.9. mRNA levels of Sumac EV applied HUVEC. Growth factor gene expressions (d,e,g), inflammatory genes (a-c) and matrix genes for remodelling (f-h) shown. Abbreviations: IL6: Interleukin-6, IL8: Interleukin-8, IL10: Interleukin-10, FGF2: Fibroblast growth factor 2, VEGF: Vascular endothelial growth factor, MMP9: Matrix metalloproteinase-9, PDFG: platelet-derived growth factors. TSP-1: Thrombospondins 1 Control: non-treated HUVEC. All experiments conducted at least 2 independent replicates. *$p<0.05$

3.9. Immunocytochemistry

Immunocytochemistry (ICC) is a laboratory technique used to visualize the presence and localization of specific proteins or antigens in cultured cells using antibodies. This method combines immunological and chemical aspects to provide detailed insights into the cellular processes involved in wound healing. Initially, samples are fixed to maintain cellular structure. They are then treated with blocking agents to prevent non-specific antibody binding. Following this, samples are incubated with primary antibodies, then with secondary antibodies. The resultant signal is visualized under a microscope for analysis. In this study, selected doses applied on scratch model and counterstaining done with DAPI dye. The primary antibodies used are Collagen I, Elastin, Laminin and Fibronectin. Results showed that, there are no significant change in COL1, Elastin and Laminin on negative control groups on the wound site. The sumac EV applied groups in COL1, Elastin and Laminin expressed a positive result on wound site. The wound area on sumac EV treated groups closed faster than control group. When searching for Fibronectin, sumac EV applied groups are recovered while control group scratch is still open. the Fibronectin expression is relatively increased at sumac EV applied groups when compared with control group.

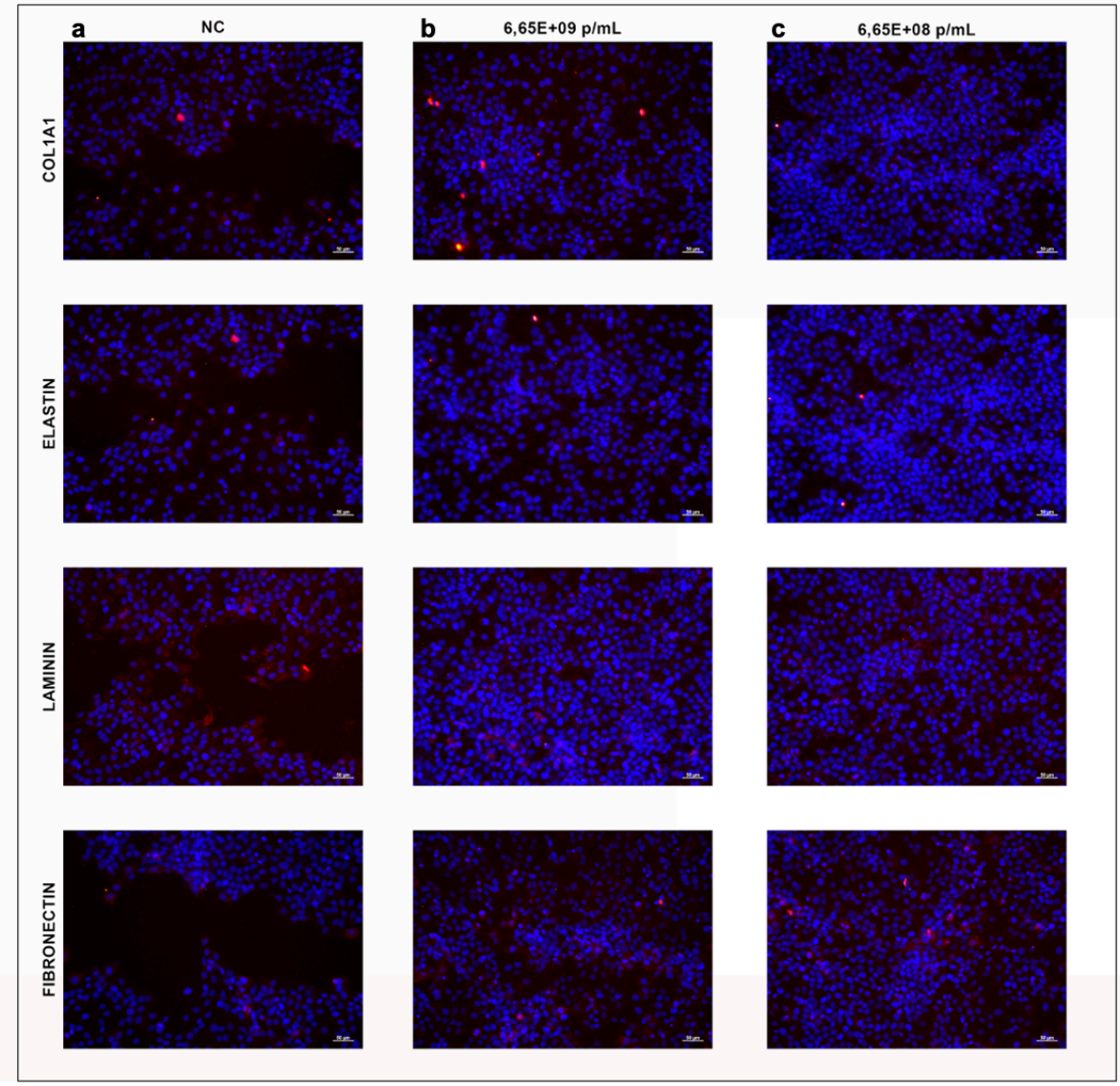

Figure 3.10. Immunoflouroscence analysis of control and Sumac EV treated groups of HaCaT. Negative control group a, 6,65E+09 p/mL group b, 6,65E+08 p/mL group c showed. Scale bar = 50 µm

4. DISCUSSION

Since the existence of humanity, people have dealt with wound healing. In earlier times, wounds were treated with plants to promote healing. Over time, drug formulations have been developed, inspired by nature. The skin, our largest organ, serves as the outer layer of our body. Infections and microbes located on the skin's outer layer can cause more significant problems if not treated properly. The skin is a permeable layer, and wound damage makes it more susceptible to environmental harmful factors. Issues on the outer layer of the skin can affect the internal layers, disrupting homeostasis.

With advancing technologies, it was realized that extracellular vesicles (EVs) are not mere cellular thresholds; they act as intercellular and intracellular messengers. Additionally, the small size of EVs allows them to diffuse through tissues. These properties suggest that EVs could be used in wound treatment. Their transport capacity, size, biocompatibility, and bioavailability make EVs a promising method for treating skin degradations. Various sources of EVs have been explored, with plant-derived EVs being particularly promising due to their nutritional compatibility. In general, the body is less likely to reject something edible. Studies on the use of plant-derived EVs in wound healing exist in the literature [80].

Wound healing agents can be classified based on their properties, such as antioxidant and anti-inflammatory effects. Compounds like myricetin, acteoside, lupeol, and bilirubin repair wounds through their anti-inflammatory characteristics [37]. Natural compounds such as curcumin, quercetin, and resveratrol have antioxidant properties for wound treatments. Honeybee products, propolis, and tannins exhibit antimicrobial activity in wound healing. These agents are important and effective in different ways. New therapeutic approaches arise with developing technology. Traditional plant therapies and techniques inspire the development of new treatments. Combining medicinal herbs with EVs opens a new door in wound healing. However, this does not imply that existing agents are insufficient for treating wounds. Wound healing studies are greatly influenced by advanced biotechnology [81].

In this study, EVs derived from the sumac plant (*Rhus coriaria L.*), native to the southeastern Anatolia region, were investigated as a wound healing agent. The idea for this study originated in Gaziantep city. Sumac has been well researched, and, as far as we know, no studies have focused on sumac-derived EVs. Sumac extracts have demonstrated various effects, including anticancer, antioxidant, antidiabetic, and antifungal properties. There are

limited studies on sumac and its wound healing effects [82]. Given the paucity of research on sumac extracts, this study focused on optimizing the production, characterization, toxicity, proliferation, scratch assay and migration activity, tube formation assay, RNA isolation, and gene expression profiling of sumac-derived EVs. The cell lines HaCaT, HUVEC, and HDF were used to model skin layers *in vitro*. Sumac-derived EVs were used as a therapeutic agent for wound healing on these cell lines and skin models. In this section, the results and inferences are discussed in detail.

Sumac juice was obtained using an optimized method (gr/mL) and sucrose cushion ultracentrifugation (UC) to isolate the desired EVs for investigating their effects on wound healing [83].

The first step of this experimental series is the characterization of sumac-derived EVs following isolation. Two techniques were employed to characterize the obtained sumac-derived particles and confirm their classification as EVs. Particle size was determined using nanoparticle tracking analysis (NTA). The NTA device tracks the motion of EV particles, and cameras inside the NTA follow the routes of EVs to calculate their sizes and concentrations by recording these movements. According to the International Society for Extracellular Vesicles (ISEV), exosomes range from 30-200 nm in diameter [84]. In this study, the size of sumac-derived EVs was determined to be approximately 155 nm. The results in Figure 3.1 demonstrate that sumac EVs are properly classified as EVs.

The characterization process continued with transmission electron microscopy (TEM) analysis to validate the results obtained from NTA. Exosome purification and characterization must be performed in tandem with molecular imaging techniques due to the growing interest in exosomes as biomarkers for treatments [85]. High-resolution imaging methods, such as electron microscopy, can differentiate between various sizes and morphologies of exosomes, which change based on their origin and function. In TEM, negative staining was used to visualize most exosomes. TEM analysis provided detailed morphology and size analysis of sumac derived EVs. Figure 3.2 shows that the morphology is well-defined. These two methods confirmed that the isolated particles are EVs. The next step was to conduct toxicity tests on skin layer cell lines.

The toxicity of sumac derived EVs must be assessed before use. Non-toxic but effective dose selection was performed using the MTS reagent. MTS is a salt that determines toxicity

through color changes. Before measuring the MTS values, the color changes indicate toxic doses when compared with the positive control (20% DMSO group). However, color changes alone are insufficient for dose determination; these changes must be measured and converted into numerical values to plot graphs and provide meaningful results. Five doses (6.65E+09 p/mL, 6.65E+08 p/mL, 6.65E+07 p/mL, 6.65E+06 p/mL, and 6.65E+05 p/mL) were prepared through serial dilutions of sumac EVs in cell culture medium. The doses were calculated from the NTA results. HaCaT, HUVEC, and HDF cell lines were seeded on a 96-well plate, and sumac EVs were applied for 24 hours. The results showed that two non-toxic doses (6.65E+09 p/mL and 6.65E+08 p/mL) could be used effectively for comparisons. In all three cell lines, at least four wells were used, and these processes were repeated at least three times to ensure reliability. Figure 3.3 shows that the two doses produced nearly the same effects when applied for 24 hours.

Another protocol was conducted to verify cell viability through a cell proliferation assay. This experiment compared the cell number differences between control (non-EV treated) and sumac EV-treated groups. Figure 3.4 clearly shows that the cell intensity differs between the control and EV-treated groups. This indicates that sumac-derived EV application promotes cell proliferation in the HaCaT cell line. The doubling time of cells with sumac EVs is reduced, and cell number and clusters increase exponentially. These results collectively prove that the selected EV doses are non-toxic and promote cell proliferation [86].

Furthermore, cell migration activities were measured. These tests investigated cell migration and wound healing capacity. Firstly, a wound healing scratch assay was conducted. The assay principle involves creating a wound on a 2D skin model and assessing the EVs' ability to seal the wound. The results were positive, indicating that wounds in the sumac EV-applied wells closed faster than those in the control group. This suggests that sumac EVs have a promising effect in developing new technologies for wound healing [87]. In Figure 3.5, it is clearly seen that wounds treated with sumac EVs closed nearly within 14 hours, unlike the control wounds, which had not yet closed even after 16 hours of the experiment. This visual experiment demonstrates the effective healing properties of sumac-derived EVs.

In addition, cell migration activity was tested. The HaCaT cell line was chosen for these scratch and migration activities. This time, a migration assay protocol was conducted. The insert-placed wells mimicked the inner and outer environments of the cell, with the inserts

representing the outer layer. The skin cell model was treated with EVs, with the inserts containing EVs while the outside environment did not. At the end of the experiment, the treated and non-treated cells were stained with 4X Lysis Buffer/CyQuant® GR [88]. The color changes clearly distinguished the results. The more intense the color, the more cells underwent migration. As expected, Figure 3.6 shows that sumac EVs have a significant effect on cell migration in the wound area [89].

When new tissue forms during the wound healing process, angiogenesis is an inevitable event. Angiogenesis refers to the formation of new blood vessels in the healing area. This process ensures the delivery of essential elements like nutrients and oxygen to the wound site, thereby accelerating tissue formation. A tube formation assay was conducted to mimic skin angiogenesis activity [25]. In Figure 3.7, sumac-derived EVs were applied to HUVEC cells, and the results showed that sumac EVs were not highly efficient in promoting angiogenesis.

Undoubtedly, gene expression profiling is one of the significant experiments in these setups. The selected genes for gene expression in wound healing are important components for interpreting the effects of sumac-derived EVs on wounds [90]. Gene expression was explored in HaCaT and HUVEC cells. The selected genes for HaCaT cells include COL1A1 (Collagen type I alpha 1), FBR (fibronectin), TGFß1 (Transforming growth factor beta 1), IL10 (Interleukin-10), IL8 (Interleukin-8), IL6 (Interleukin-6), Ki67 (Antigen Kiel 67), VEGF (Vascular endothelial growth factor), TGFß3 (Transforming growth factor beta-3), MMP9 (Matrix metalloproteinase-9), and COL3A1 (Collagen type III alpha-1). For HUVEC cells, the selected genes include IL6 (Interleukin-6), IL8 (Interleukin-8), IL10 (Interleukin-10), FGF2 (Fibroblast growth factor 2), VEGF (Vascular endothelial growth factor), MMP9 (Matrix metalloproteinase-9), PDGF (Platelet-derived growth factors), and TSP1 (Thrombospondin-1) [91,92]. In Figure 3.8, HaCaT gene expression profiling graphs are provided for treatments with 6.65E+09 p/mL and 6.65E+08 p/mL sumac EVs. Figure 3.9 presents the HUVEC gene expression profiling graphs under the same conditions.

FBR is an important gene responsible for cell migration, proliferation, and ECM remodelling. In the early inflammatory phase, FBR facilitates the recruitment of neutrophils and macrophages to the wound area. It also plays a crucial role in cell migratory and proliferative phases. The expression of the fibronectin gene is tightly regulated throughout various stages of wound healing and is essential for coordinating the cellular and molecular

processes necessary for successful tissue regeneration and repair. Dysregulation of fibronectin expression or function can lead to chronic wound formation and impaired wound healing. Therefore, understanding fibronectin's role in wound healing could significantly impact the development of medical treatments aimed at promoting tissue repair. As anticipated, sumac EVs upregulated FBR gene expression in HaCaT cells. Collagen, the primary structural protein in the extracellular matrix (ECM) of connective tissues, involves COL1A1 in its synthesis and structure [93].

As mentioned, FBR induces the expression of COL1A1, leading to increased collagen production. This upregulation of COL1A1 is related to the proliferation and migration of collagens participating in the newly formed ECM. If COL1A1 production diminishes, the wound might leave a scar, become infected, and turn into chronic or cancerous tissue. Numerous growth factors and cytokines, including IL6, PDGF, and TGFβ, are involved in wound healing and regulate COL1A1 gene expression. By triggering intracellular signalling pathways that control COL1A1 transcription and translation, these signalling molecules modify collagen synthesis and deposition. Generally, the synthesis and organization of COL1A1, which provide mechanical strength and structural support to the wound site throughout the healing process, depend on COL1A1 gene expression. To promote wound repair effectively and prevent pathological scarring, therapeutic techniques involving the modulation of collagen metabolism and COL1A1 expression must be developed. As expected, COL1A1 and COL3A1 levels in HaCaT cells increased in mRNA profiling, paralleling FBR expression [94].

The multifunctional cytokine TGFβ is essential for wound healing because it regulates several cellular functions related to tissue regeneration and repair. In the proliferative phase, TGFβ stimulates the proliferation and migration of fibroblasts, endothelial cells, and keratinocytes, which are involved in tissue healing. TGFβ stimulates the synthesis of ECM proteins like collagen and fibronectin, providing structural support and promoting granulation tissue development. MMPs are produced in response to TGFβ, and these MMPs facilitate ECM remodelling. In this study, sumac EV-treated cells induced TGFβ production at the wound site in both HaCaT and HUVEC cells. TGFβ gene expression plays a crucial role in coordinating the intricate cellular and molecular processes involved in wound healing. Inadequate TGFβ signal regulation can hinder the healing process and contribute to the emergence of chronic wounds or pathological fibrosis. Understanding TGFβ's role in

wound healing can significantly impact developing medical treatments that promote tissue regeneration and repair [95].

Through its ability to facilitate ECM remodelling and regulate the migration of different cell types involved in tissue repair, MMP-9 is an enzyme essential to wound healing. MMP-9 promotes the migration of these cells into the wound site, where they contribute to tissue regeneration and repair by breaking down the ECM and forming channels for cell migration. By destroying inflammatory mediators and ECM elements connected to the inflammatory response, MMP-9 aids in resolving inflammation. The results demonstrated an elevation of MMP-9 mRNA amounts in HaCaT and HUVEC cells treated with sumac EVs at a 6.65E+09 p/mL dose. Generally, the intricate processes involved in wound healing, including inflammation, ECM remodelling, angiogenesis, cell migration, and wound contraction, depend on the expression of the MMP-9 gene [96].

VEGF gene expression in wound healing research offers significant insights into the modulation of angiogenesis, the process by which pre-existing blood vessels divide into new ones. Researchers might clarify the temporal dynamics of VEGF expression at various stages of wound healing through analysis of VEGF gene expression. VEGF expression tends to peak during the proliferative and early inflammatory phases of wound healing. According to this information, Figures 3.8 and 3.9 show increased VEGF levels in the gene-based expression of HaCaT and HUVEC cells when a 6.65E+09 p/mL dose of sumac EVs was applied. Information regarding the degree of angiogenic activity in the wound tissue can be obtained through quantitative measurement of VEGF gene expression. Reduced VEGF expression may indicate compromised angiogenic responses and delayed wound healing, while higher levels of VEGF expression are generally linked to enhanced angiogenesis and improved wound healing outcomes [97].

Ki-67 is a protein marker for cellular proliferation, and monitoring the gene expression profile of this marker can reveal important information about how cells proliferate during wound repair. Ki-67 is not present in resting (G0) cells but is expressed in all active phases of the cell cycle (G1, S, G2, and mitosis). As a result, Ki-67 gene expression profiling is an accurate proxy for determining how vigorously the cells within the wound site proliferate. Low levels may indicate a decreased capacity for proliferative activity, while high levels of Ki-67 expression imply active cell proliferation. In this study, HaCaT cells treated with sumac EVs showed exponentially increased gene expression levels with higher doses. The

resolution of tissue injury and the advancement of wound healing can be associated with quantitative examination of Ki-67 gene expression levels. The wound microenvironment contains various growth factors, cytokines, and other signalling molecules that can influence Ki-67 gene expression [22,98]. Factors like FGF and TGFβ can increase cell proliferation and upregulate Ki-67 expression to support tissue repair and regeneration.

An essential component of grasping the molecular processes behind tissue repair and regeneration is the role of FGF in wound healing. It has been demonstrated that FGF-2 encourages the recruitment of inflammatory cells, such as neutrophils and macrophages, to the wound site. In this study, FGF2 gene expression levels of HUVEC cells were elevated more at a 6.65E+08 p/mL sumac EV dose. By removing contaminants and debris, FGF2 cells facilitate the healing of wounds. Angiogenesis (the creation of new blood vessels), epithelialization (the generation of re-epithelial cell layers), and granulation tissue are all facilitated by increased expression of FGF genes during the proliferation phase [99].

Another important component in the healing of wounds is PDGF, whose gene expression significantly impacts different phases of tissue regeneration and repair. PDGF induces macrophages to migrate to the wound site, where they help remove necrotic tissue and secrete growth factors essential for the healing process. Increased expression of the PDGF gene drives these cells to proliferate and create granulation tissue, necessary for tissue repair and wound closure. In this study, elevated PDGF gene expression levels were observed with sumac EVs on HUVEC cells. The expression of PDGF genes controls several elements of the wound healing process, such as angiogenesis, cell proliferation, the creation of the extracellular matrix, the recruitment of immune cells, and the resolution of inflammation [100].

TGFβ3 is a versatile cytokine vital for wound healing, especially for controlling fibrosis, inflammation, and tissue remodelling. In this study, TGFβ3 levels decreased when treated with sumac EVs on HaCaT cells. The reason might be that sumac EVs stimulate TGFβ3, preventing the synthesis of pro-inflammatory cytokines and chemokines, and thus might reduce the inflammatory response. TGFβ3 contributes to promoting a balanced inflammatory milieu that is favourable to healing at the wound site by suppressing the recruitment and activation of immune cells. TGFβ3 also facilitates the development of less fibrous, functional tissue during the healing process [101].

IL6, IL8, and IL10 play important roles in inflammation, immunological control, and tissue repair, and their gene expression can substantially affect several features of wound healing. By encouraging the migration and proliferation of endothelial cells, IL6 promotes angiogenesis, the development of new blood vessels. The polarization and activation of macrophages, which are essential for coordinating the inflammatory and resolution stages of wound healing, are mediated by IL6. In this study, HUVEC cells treated with sumac EVs showed elevated gene expression levels, with the highest gene expression level observed at a 6.65E+09 p/mL sumac EV dose. The shift in macrophage phenotype from pro-inflammatory (M1) to anti-inflammatory (M2), linked to tissue remodelling and healing, is facilitated by IL6 [102,103,104].

Neutrophils are strongly attracted to IL8, which facilitates their migration to the site of damage. By encouraging fibroblast migration and proliferation, IL8 affects fibroblast activity. In this study, IL8 levels in HUVEC cells with the selected two doses of sumac EVs were close to each other, while the HaCaT cells treated with the same doses showed diminished IL8 levels. This result suggests that the selected doses of sumac EVs may lead to the overexpression of interleukins and stimulate migratory or proliferative events more than expected [105].

During the healing process, fibroblasts play a role in collagen deposition and tissue remodelling. The strongly anti-inflammatory cytokine IL10 aids in immune response regulation and the resolution of inflammation. In this study, IL10 levels in both HaCaT and HUVEC cells indicated that sumac EVs induce gene expression levels. IL10 expression prevents excessive inflammation and tissue damage by opposing the pro-inflammatory effects of cytokines, including IL6 and IL8. Collagen deposition and the generation of pro-fibrotic mediators are inhibited by IL10. During wound healing, the expression of the genes encoding IL6, IL8, and IL10 affects angiogenesis, fibroblast activity, immunological control, and tissue repair [102].

To detect protein level of sumac derived EVs affects on cells, immunocytochemistry analysis done. In ICC, detection of positive staining is achieved using a molecular marker, which can either be fluorescent or chromogenic. ICC in wound healing studies provides critical insights into the molecular and cellular mechanisms driving the healing process. By visualizing and quantifying specific proteins, researchers can better understand the dynamics of wound repair and develop more effective treatments [106]. The technique is followed in

an order; cell culture preparation, fixation, permeabilization, blocking, primary antibody incubation COL1-Laminin-Elastin-Fibronectin, secondary rabbit antibody incubation, counterstaining and imaging is done in this study [107,108]. These structural protein expressions are quiet important in wound healing studies. Because these types of proteins have decisive roles on wound healing period. In one study, wheat exosomes used as a wound healing agent. ICC is applied to see the COL1 expression. These ECM protein expressions expected to elevate in wound healing. Like in wheat exosome study, COL1 expression is searched in this study [109]. The antibodies targeted Collagen I, Elastin, Laminin, and Fibronectin. The results indicated no significant changes in Collagen I, Elastin, and Laminin in the negative control groups at the wound site. In contrast, groups treated with sumac EV showed positive expression of Collagen I, Elastin, and Laminin at the wound site. The wound areas in the sumac EV-treated groups closed more quickly than those in the control group. For Fibronectin, the wounds in the sumac EV-treated groups healed, whereas the scratches in the control group remained open. Additionally, Fibronectin expression was relatively higher in the sumac EV-treated groups compared to the control group.

Overall, the detailed results show that when HaCaT, HUVEC, and HDF cell lines are treated with selected doses of sumac EVs, these EVs may serve as potential helpers and inducers of wound healing activity. Nevertheless, the studies could be broadened and developed by further research. *In vivo* studies might help understand the wound closure potential effects on actual skin in animal models such as rats or mice.

5. CONCLUSIONS

This study aimed to elucidate the potential of sumac-derived extracellular vesicles (EVs) in wound healing and their molecular mechanisms. A series of experiments were conducted to demonstrate the effects of these EVs. According to the scratch assay and migration assay, the results confirmed the wound closure activity of sumac EVs. Furthermore, sumac EVs were shown to promote cell proliferation without exhibiting toxic effects. This research represents the first study investigating the wound healing properties of sumac-derived EVs.

From the in vitro experiments, sumac EVs indicated they might be a promising new candidate as a supportive agent in wound healing. However, *in vivo* studies must be conducted to solidify these hypotheses. Future research should explore the pathways involved in wound healing to illuminate currently unanswered questions.

In conclusion, this study offers promising potential as a precursor for future research on the therapeutic effects of sumac-derived EVs. It can be further developed with *in vivo* studies to better understand the mechanisms involved, ultimately paving the way for new therapeutic approaches in wound healing.

REFERENCES

1. Ernstmeyer K, Christman E. Nursing Fundamentals. NCBI Bookshelf; 2021. https://www.ncbi.nlm.nih.gov/books/NBK591823/

2. Saleh K, Sönnergren HH. Control and treatment of infected wounds. Elsevier eBooks. 2016, p. 107–15.

3. Falanga V, Isseroff R R, Soulika A M, Romanelli M, Margolis D, Kapp S, Granick M, Harding K. Chronic wounds. Nature Reviews Disease Primers. 2022;8(1): 50.

4. Wong R, Geyer S, Weninger W, Guimberteau J, Wong JK. The dynamic anatomy and patterning of skin. Experimental Dermatology. 2015;25:92–8.

5. Lefèvre-Utile A, Braun C, Haftek M, Aubin F. Five Functional Aspects of the Epidermal Barrier. International Journal of Molecular Sciences. 2021;22:11676.

6. Abdo JM, Sopko NA, Milner SM. The applied anatomy of human skin: A model for regeneration. Wound Medicine. 2020;28:100179.

7. Wickett RR, Visscher MO. Structure and function of the epidermal barrier. American Journal of Infection Control. 2006;34:S98–110.

8. Hoath SB, Leahy DG. The Organization of Human Epidermis: Functional Epidermal Units and Phi Proportionality. The Journal of Investigative Dermatology. 2003;121:1440–6.

9. Yousef H, Alhajj M, Sharma S. Anatomy, Skin (Integument), Epidermis. [Updated 2022 Nov 14]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2024 Jan.

10. Das P, Mounika P, Yellurkar ML, Prasanna VS, Sarkar S, Velayutham R, et al. Keratinocytes: An Enigmatic Factor in Atopic Dermatitis. Cells. 2022;11:168

11. Cichorek M, Wachulska M, Stasiewicz A, Tymińska A. Skin melanocytes: biology and development. Postępy Dermatologii I Alergologii. 2013;1:30–41.

12. Schenk P. Melanocyten, Langerhanssche und Merkelsche Zellen in oralen Epithelien [Melanocytes, Langerhans and Merkel cells in oral epithelium]. Acta Oto-Laryngol. 1975;80(3-4):301-11.

13. Salazar J, Carmona T, Zacconi FC, Venegas-Yazigi D, Cabello-Verrugio C, Choi WI, et al. The Human Dermis as a Target of Nanoparticles for Treating Skin Conditions. Pharmaceutics. 2022;15:10.

14. Madireddy S, Crane JS. [Figure, Anatomy of the human skin Contributed by Wikimedia Commons, USGOV (Public Domain)] - StatPearls - NCBI Bookshelf. Available from: https://www.ncbi.nlm.nih.gov/books/NBK563245/figure/article-23283.image.f3/

15. Martin P, Nunan R. Cellular and molecular mechanisms of repair in acute and chronic wound healing. British Journal of Dermatology Supplement. 2015;173:370–8.

16. Hassanpour I, Naeini AT, Aminlari M, Nazifi S. Effect of Punicalagin Nanofibrous-Dressing on Tissue Total Antioxidant Capacity Index Through Wound Healing in Adult Wistar Rats. Majallah-i Taḥqīqāt-i ʿUlūm-i Pizishkī-i Zāhidān/Majallah-i Taḥqīqāt-i ʿUlūm-i Pizishkī-i Zāhidān/Zahedan Journal of Researches in Medical Sciences. 2019;In Press.

17. Broughton G, Janis JE, Attinger CE. The Basic Science of Wound Healing. Plastic and Reconstructive Surgery/PSEF CD Journals. 2006;117:12S-34S.

18. Janis JE, Harrison B. Wound Healing: Part I. Basic Science. Plastic and Reconstructive Surgery/PSEF CD Journals. 2016;138:9S-17S.

19. Li D, Landén NX. MicroRNAs in skin wound healing. European Journal of Dermatology/EJD European Journal of Dermatology. 2017;27:12–4.

20. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert Reviews in Molecular Medicine. 2011;13.

21. Albrecht L-J, Tauber SC, Merres J, Kress E, Stope MB, Jansen S, et al. Lack of Proinflammatory Cytokine Interleukin-6 or Tumor Necrosis Factor Receptor-1 Results in a Failure of the Innate Immune Response after Bacterial Meningitis. Mediators of Inflammation. 2016;2016:1–12.

22. Veith AP, Henderson K, Spencer A, Sligar AD, Baker AB. Therapeutic strategies for enhancing angiogenesis in wound healing. Advanced Drug Delivery Reviews. 2019;146:97–125.

23. Otrock Z, Mahfouz R, Makarem J, Shamseddine A. Understanding the biology of angiogenesis: Review of the most important molecular mechanisms. Blood Cells, Molecules, & Diseases/Blood Cells, Molecules & Diseases. 2007;39:212–20.

24. 24. Muire PJ, Thompson MA, Christy RJ, Natesan S. Advances in immunomodulation and immune engineering approaches to improve healing of extremity wounds. International Journal of Molecular Sciences. 2022;23:4074.

25. Li J, Zhang Y, Kirsner RS. Angiogenesis in wound repair: Angiogenic growth factors and the extracellular matrix. Microscopy Research and Technique. 2002;60:107–14.

26. Everts PA, Lana JF, Onishi K, Buford D, Peng J, Mahmood A, et al. Angiogenesis and Tissue Repair Depend on Platelet Dosing and Bioformulation Strategies Following Orthobiological Platelet-Rich Plasma Procedures: A Narrative Review. Biomedicines. 2023;11:1922.

27. Pardue EL, Ibrahim S, Ramamurthi A. Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering. Organogenesis. 2008;4:203–14.

28. Landén NX, Li D, Ståhle M. Transition from inflammation to proliferation: a critical step during wound healing. Cellular and Molecular Life Sciences. 2016;73:3861–85.

29. Gordon SR. Cell Migration along the Basement Membrane during Wound Repair. The Corneal Endothelium as a Model System. Studies in Mechanobiology, Tissue Engineering and Biomaterials, 2009, p. 43–84.

30. Tan ST, Dosan R. Lessons From Epithelialization: The Reason Behind Moist Wound Environment. Open Dermatology Journal. 2019;13:34–40.

31. Akbarian M, Bertassoni LE, Tayebi L. Biological aspects in controlling angiogenesis: current progress. Cellular and Molecular Life Sciences. 2022;79.

32. Bacakova L, Travnickova M, Filova E, Matějka R, Stepanovska J, Musilkova J, et al. The Role of Vascular Smooth Muscle Cells in the Physiology and Pathophysiology of Blood Vessels. InTech eBooks. 2018.

33. Alhajj M, Goyal A. Physiology, Granulation Tissue. StatPearls - NCBI Bookshelf 2022. https://www.ncbi.nlm.nih.gov/books/NBK554402/.

34. Yu Y, Yue Z, Xu M, Zhang M, Shen X, Ma Z, et al. Macrophages play a key role in tissue repair and regeneration. PeerJ. 2022;10:e14053.

35. Chitturi RT, Balasubramaniam AM, Parameswar RA, Kesavan G, Haris KT, Mohideen K. The role of myofibroblasts in wound healing, contraction and its clinical implications in cleft palate repair. J Int Oral Health. 2015;7(3):75-80.

36. Desmouliere A, Darby IA, Laverdet B, Bonté F. Fibroblasts and myofibroblasts in wound healing. Clinical, Cosmetic and Investigational Dermatology. 2014:301.

37. Trinh X-T, Long N-V, Van Anh LT, Nga PT, Giang NN, Chien PN, et al. A Comprehensive Review of Natural Compounds for Wound Healing: Targeting Bioactivity Perspective. International Journal of Molecular Sciences. 2022;23:9573.

38. Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound Healing: A Cellular Perspective. Physiological Reviews. 2019;99:665–706.

39. Smith PC, Martínez C, Martínez J, McCulloch CA. Role of Fibroblast Populations in Periodontal Wound Healing and Tissue Remodeling. Frontiers in Physiology. 2019;10.

40. Moretti L, Stalfort J, Barker TH, Abebayehu D. The interplay of fibroblasts, the extracellular matrix, and inflammation in scar formation. Journal of Biological Chemistry. 2022;298:101530.

41. Guo S, DiPietro LA. Factors Affecting Wound Healing. Journal of Dental Research. 2010;89:219–29.

42. Eisenbud DE. Oxygen in Wound Healing. Clinics in Plastic Surgery. 2012;39:293–310.

43. Younis I. Role of oxygen in wound healing. Journal of Wound Care. 2020;29:S4–10.

44. Gushiken LFS, Beserra FP, Bastos JK, Jackson CJ, Pellizzon CH. Cutaneous Wound Healing: An Update from Physiopathology to Current Therapies. Life. 2021;11:665.

45. Dunnill C, Patton T, Brennan J, Barrett J, Dryden M, Cooke J, et al. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. International Wound Journal. 2015;14:89–96.

46. Berdiaki A, Neagu M, Spyridaki I, Kuskov A, Perez S, Nikitovic D. Hyaluronan and Reactive Oxygen Species Signaling—Novel Cues from the Matrix? Antioxidants. 2023;12:824.

47. Ersanli C, Tzora A, Voidarou C, Skoufos S, Zeugolis DI, Skoufos I. Biodiversity of Skin Microbiota as an Important Biomarker for Wound Healing. Biology. 2023;12:1187.

48. Wynn M. The impact of infection on the four stages of acute wound healing: an overview. Wounds UK. 2021;17:6-90.

49. Maheswary T, Nurul AA, Fauzi MB. The Insights of Microbes' Roles in Wound Healing: A Comprehensive Review. Pharmaceutics. 2021;13:981.

50. Nirenjen S, Narayanan J, Tamilanban T, Subramaniyan V, Chitra V, Fuloria NK, et al. Exploring the contribution of pro-inflammatory cytokines to impaired wound healing in diabetes. Frontiers in Immunology. 2023;14.

51. Monika P, Chandraprabha MN, Rangarajan A, Waiker PV, Murthy KNC. Challenges in Healing Wound: Role of Complementary and Alternative Medicine. Frontiers in Nutrition. 2022;8.

52. Hess CT. Checklist for Factors Affecting Wound Healing. Advances in Skin & Wound Care. 2011;24:192.

53. Bielefeld KA, Amini-Nik S, Alman BA. Cutaneous wound healing: recruiting developmental pathways for regeneration. Cellular and Molecular Life Sciences. 2012;70:2059–81.

54. Ageing [Internet]. Geneva: World Health Organization. 2020 Feb 5 [cited 2024 Jun 5]. Available from: https://www.who.int/health-topics/ageing#tab=tab_1

55. Bär C, Blasco MA. Telomeres and telomerase as therapeutic targets to prevent and treat age-related diseases. F1000Research. 2016;5:89.

56. Hong H, Ji M, Lai D. Chronic Stress Effects on Tumor: Pathway and Mechanism. Frontiers in Oncology. 2021;11.

57. Burgess JL, Wyant WA, Abujamra BA, Kirsner RS, Jozic I. Diabetic Wound-Healing Science. Medicina. 2021;57:1072.

58. Nirenjen S, Narayanan J, Tamilanban T, Subramaniyan V, Chitra V, Fuloria NK, et al. Exploring the contribution of pro-inflammatory cytokines to impaired wound healing in diabetes. Frontiers in Immunology. 2023;14.

59. Ambrosone A, Barbulova A, Cappetta E, Cillo F, De Palma M, Ruocco M, et al. Plant Extracellular Vesicles: Current Landscape and Future Directions. Plants. 2023;12:4141.

60. Ali NB, Razis AFA, Ooi DJ, Chan KW, Ismail N, Foo JB. Theragnostic Applications of Mammal and Plant-Derived Extracellular Vesicles: Latest Findings, Current Technologies, and Prospects. Molecules/Molecules Online/Molecules Annual. 2022;27:3941.

61. Donoso-Quezada J, Ayala-Mar S, González-Valdez J. The role of lipids in exosome biology and intercellular communication: Function, analytics and applications. Traffic. 2021;22:204–20.

62. Hu Q, Hu Z, Yan X, Lu J, Wang C. Extracellular vesicles involved in growth regulation and metabolic modulation in Haematococcus pluvialis. Biotechnology for Biofuels and Bioproducts. 2024;17.

63. Lian MQ, Chng WH, Liang J, Yeo HQ, Lee CK, Belaid M, et al. Plant-derived extracellular vesicles: Recent advancements and current challenges on their use for biomedical applications. Journal of Extracellular Vesicles. 2022;11.

64. Oshchepkova A, Zenkova M, Vlassov V. Extracellular Vesicles for Therapeutic Nucleic Acid Delivery: Loading Strategies and Challenges. International Journal of Molecular Sciences. 2023;24:7287.

65. Alazaiza M, Albahnasawi A, Ali G, Bashir M, Nassani D, Maskari TA, et al. Application of Natural Coagulants for Pharmaceutical Removal from Water and Wastewater: A Review. Water. 2022;14:140.

66. Narauskaitė D, Vydmantaitė G, Rusteikaitė J, Sampath R, Rudaitytė A, Stašytė G, et al. Extracellular Vesicles in Skin Wound Healing. Pharmaceuticals. 2021;14:811.

67. Kim M, Jang H, Kim W, Kim D, Park JH. Therapeutic Applications of Plant-Derived Extracellular Vesicles as Antioxidants for Oxidative Stress-Related Diseases. Antioxidants. 2023;12:1286.

68. Yang C, Zhang W, Bai M, Luo Q, Zheng Q, Xu Y, et al. Edible plant-derived extracellular vesicles serve as promising therapeutic systems. Nano TransMed. 2023;2:100004.

69. Baldini N, Torreggiani E, Roncuzzi L, Perut F, Zini N, Avnet S. Exosome-like Nanovesicles Isolated from Citrus limon L. Exert Antioxidative Effect. Current Pharmaceutical Biotechnology. 2018;19:877–85.

70. De Robertis M, Sarra A, D'Oria V, Mura F, Bordi F, Postorino P, et al. Blueberry-Derived Exosome-Like Nanoparticles Counter the Response to TNF-α-Induced Change on Gene Expression in EA.hy926 Cells. Biomolecules. 2020;10:742.

71. Narauskaitė D, Vydmantaitė G, Rusteikaitė J, Sampath R, Rudaitytė A, Stašytė G, et al. Extracellular Vesicles in Skin Wound Healing. Pharmaceuticals. 2021;14:811.

72. Mu J, Zhuang X, Wang Q, Jiang H, Deng Z, Wang B, et al. Interspecies communication between plant and mouse gut host cells through edible plant derived exosome-like nanoparticles. Molecular Nutrition & Food Research. 2014;58:1561–73.

73. Gabr SA, Alghadir AH. Potential anticancer activities of Rhus coriaria(sumac) extract against human cancer cell lines. Bioscience Reports. 2021;41.

74. Mirenayat FS, Hajhashemy Z, Siavash M, Saneei P. Effects of sumac supplementation on metabolic markers in adults with metabolic syndrome: a triple-blinded randomized placebo-controlled cross-over clinical trial. Nutrition Journal. 2023;22.

75. Emanet M, Okuda M, Şen Ö, Lavarello C, Petretto A, Takeoka S, et al. Sumac (Rhus coriaria) Extract-Loaded Polymeric Nanosheets Efficiently Protect Human Dermal Fibroblasts from Oxidative Stress. ACS Applied Bio Materials. 2022;5:5901–10.

76. Cong M, Tan S, Li S, Gao L, Huang L, Zhang H-G, et al. Technology insight: Plant-derived vesicles—How far from the clinical biotherapeutics and therapeutic drug carriers? Advanced Drug Delivery Reviews. 2022;182:114108.

77. Amatori, Stefano, Giuseppe Persico, and Mirco Fanelli. Real-Time Quantitative PCR Array to Study Drug-Induced Changes of Gene Expression in Tumor Cell Lines. Journal of Cancer Metastasis and Treatment. 2017;90.

78. Wang, X. A PCR Primer Bank for Quantitative Gene Expression Analysis. Nucleic Acids Research. 2003;31.

79. Zhang, P., Baxter, J., Vinod, K., Tulenko, TN., Di Muzio, PJ. Endothelial differentiation of amniotic fluid-derived stem cells: synergism of biochemical and shear force stimuli. Stem Cells and Development. 2009;18.

80. Fan S-J, Chen J-Y, Tang C-H, Zhao Q-Y, Zhang J-M, Qin Y-C. Edible plant extracellular vesicles: An emerging tool for bioactives delivery. Frontiers in Immunology. 2022;13.

81. Shedoeva A, Leavesley D, Upton Z, Fan C. Wound Healing and the Use of Medicinal Plants. Evidence-Based Complementary and Alternative Medicine. 2019;2019:1–30.

82. Kubatka P, Kello M, Kajo K, Samec M, Liskova A, Jasek K, et al. Rhus coriaria L. (Sumac) Demonstrates Oncostatic Activity in the Therapeutic and Preventive Model of Breast Carcinoma. International Journal of Molecular Sciences. 2020;22:183.

83. Brennan K, Martin K, FitzGerald SP, O'Sullivan J, Wu Y, Blanco A, et al. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Scientific Reports. 2020;10.

84. Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. Journal of Extracellular Vesicles. 2024;13.

85. Jung MK, Mun JY. Sample Preparation and Imaging of Exosomes by Transmission Electron Microscopy. Journal of Visualized Experiments. 2018.

86. Hanson B, Vorobieva I, Zheng W, Conceição M, Lomonosova Y, Mäger I, et al. EV-mediated promotion of myogenic differentiation is dependent on dose, collection medium, and isolation method. Molecular Therapy Nucleic Acids. 2023;33:511–28.

87. Jonkman JEN, Cathcart JA, Xu F, Bartolini ME, Amon JE, Stevens KM, et al. An introduction to the wound healing assay using live-cell microscopy. Cell Adhesion & Migration. 2014;8:440–51.

88. Masuzzo P, Hulstaert N, Huyck L, Ampe C, Van Troys M, Martens L. CellMissy: a tool for management, storage and analysis of cell migration data produced in wound healing-like assays. Bioinformatics. 2013;29:2661–3.

89. Jones LJ, Gray M, Yue ST, Haugland RP, Singer VL. Sensitive determination of cell number using the CyQUANT® cell proliferation assay. Journal of Immunological Methods. 2001;254:85–98.

90. Deonarine K, Panelli MC, Stashower ME, Jin P, Smith K, Slade HB, et al. Gene expression profiling of cutaneous wound healing. Journal of Translational Medicine. 2007;5.

91. Ågren MS, Litman T, Eriksen JO, Schjerling P, Bzorek M, Gjerdrum LMR. Gene Expression Linked to Reepithelialization of Human Skin Wounds. International Journal of Molecular Sciences. 2022;23:15746.

92. Zhang W, Ling Y, Sun Y, Xiao F, Wang L. Extracellular Vesicles Derived from Mesenchymal Stem Cells Promote Wound Healing and Skin Regeneration by Modulating Multiple Cellular Changes: A Brief Review. Genes. 2023;14:1516.

93. Claeys L, Storoni S, Eekhoff M, Elting M, Wisse L, Pals G, et al. Collagen transport and related pathways in Osteogenesis Imperfecta. Human Genetics. 2021;140:1121–41.

94. Devos H, Zoidakis J, Roubelakis MG, Latosinska A, Vlahou A. Reviewing the Regulators of COL1A1. International Journal of Molecular Sciences. 2023;24:10004.

95. Liarte S, Bernabé-García Á, Rodríguez-Valiente M, Moraleda JM, Castellanos G, Nicolás FJ. Amniotic Membrane Restores Chronic Wound Features to Normal in a Keratinocyte TGF-β-Chronified Cell Model. International Journal of Molecular Sciences. 2023;24:6210.

96. Reiss MJ, Han Y-P, Garcia E, Goldberg M, Yu H, Garner WL. Matrix metalloproteinase-9 delays wound healing in a murine wound model. Surgery. 2010;147:295–302.

97. Tejedor S, Wågberg M, Correia C, Åvall K, Hölttä M, Hultin L, et al. The Combination of Vascular Endothelial Growth Factor A (VEGF-A) and Fibroblast Growth Factor 1 (FGF1) Modified mRNA Improves Wound Healing in Diabetic Mice: An Ex Vivo and In Vivo Investigation. Cells. 2024;13:414.

98. Eming SA, Murray PJ, Pearce EJ. Metabolic orchestration of the wound healing response. Cell Metabolism. 2021;33:1726–43.

99. Geng K, Wang J, Liu P, Tian X, Liu H, Wang X, et al. Electrical stimulation facilitates the angiogenesis of human umbilical vein endothelial cells through MAPK/ERK signaling pathway by stimulating FGF2 secretion. American Journal of Physiology Cell Physiology. 2019;317:C277–86.

100. Thapa RK, Margolis DJ, Kiick KL, Sullivan MO. Enhanced Wound Healing via Collagen-Turnover-Driven Transfer of PDGF-BB Gene in a Murine Wound Model. *ACS Applied Bio Materials.* 2020;3:3500–17

101. Janakiram NB, Valerio MS, Goldman SM, Dearth CL. The Role of the Inflammatory Response in Mediating Functional Recovery Following Composite Tissue Injuries. International Journal of Molecular Sciences. 2021;22:13552.

102. Fernández-Guarino M, Hernández-Bule ML, Bacci S. Cellular and Molecular Processes in Wound Healing. Biomedicines. 2023;11:2526.

103. Chen S, Saeed AFUH, Liu Q, Jiang Q, Xu H, Xiao GG, et al. Macrophages in immunoregulation and therapeutics. Signal Transduction and Targeted Therapy. 2023;8.

104. Kotwal GJ, Chien S. Macrophage Differentiation in Normal and Accelerated Wound Healing. Results and Problems in Cell Differentiation. 2017, p. 353–64.

105. Shou X, Wang Y, Jiang Q, Chen J, Liu Q. miR-126 promotes M1 to M2 macrophage phenotype switching via VEGFA and KLF4. PeerJ. 2023;11:e15180.

106. Margunato S, Taşlı PN, Aydın S, Karapınar Kazandağ M, Şahin F. In Vitro Evaluation of ProRoot MTA, Biodentine, and MM-MTA on Human Alveolar Bone Marrow Stem Cells in Terms of Biocompatibility and Mineralization. Journal of Endodontics. 2015 Oct;41(10):1646–52.

107. Ishihara J, Ishihara A, Fukunaga K, Sasaki K, White MJV, Briquez PS, et al. Laminin heparin-binding peptides bind to several growth factors and enhance diabetic wound healing. Nature Communications. 2018 Jun 4;9(1):2163

108. Lenselink EA. Role of fibronectin in normal wound healing. International Wound Journal. 2013 Jun 7;12(3):313–6.

109. Şahin F, Koçak P, Güneş MY, Özkan İ, Yıldırım E, Kala EY. In Vitro Wound Healing Activity of Wheat-Derived Nanovesicles. Applied Biochemistry and Biotechnology. 2018 Nov 26;188(2):381–94