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**Department of Cellular and Molecular Biology**

# END OF STUDY THESIS

In view of obtaining the Academic Master's Degree in Biological Sciences

Specialty: Applied Biochemistry

**UNDER DECISION1275**

**THEME**

# Wound healing effect of eggshell membrane enriched-ointment

Presented by:

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In front of the jury composed of:

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بسم الله الرحمن الرحيم

الحمد لله، محبةً وشكرًا وامتنانًا، الذي بفضله وكرمه وُفِّقنا، والحمد لله الذي لم نتجاوز طريقًا ولا نبذل جهدًا إلا بمعونته.

اليوم أنظر إلى حلم طال انتظاره، وقد أصبح حقيقةً أفخر بها. لم تكن الرحلة قصيرة، بل امتدت لسنوات، بدأتها بطموح، وأنهيتها بنجاح.

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"وآخر دعوانا أن الحمد لله رب العالمين".

***Hanane Logbi***

# إهـــــداء

بسم الله الرحمن الرحيم

الحمد لله، محبةً وشكرًا وامتنانًا، الذي بفضله وكرمه وُفِّقنا، والحمد لله الذي لم نتجاوز طريقًا ولا نبذل جهدًا إلا بمعونته.

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(عدنان، كوثر، رضا ، وسام، سنهوية ، محاسن ، يسرى، شاهيناز و نضال)

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**وئـــــام**

"وآخر دعوانا أن الحمد لله رب العالمين".

# ABSTRACTS

**Abstract**

The purpose of this study is to investigate the effects of eggshell membrane powder (ESM) and zinc-enriched plant seeds (LNS) on wound healing. Eggshell waste was selected and collected from confectionery shops in El Oued (Algeria). Colorimetric assays for the content determination of proteins and total sugars included the Bradford method and the Dubois method, respectively. Characterization techniques of Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) were used to study the structural and compositional properties of ESM and LNS powders. The study evaluated the biological effects *in vivo* by applying an ointment consisting of 50% ESM and 50% LNS mixed in with petroleum jelly. The ointment was applied once to the wounds of female *Wistar albino* rats. According to biochemical study, the protein content of ESM is higher—it reaches 88.94%—than that of LNS, which is just 7.40%. Total sugar content was also higher in ESM (7.25%) than in LNS (1.50%). FTIR spectroscopy verified the existence of protein-related functional groups (such as amides) in ESM, while LNS spectra revealed strong peaks attributable to polysaccharides and ester/phosphoryl linkages. Moreover, calcium carbonate in ESM gave rise to a crystalline structure in X-ray diffraction (XRD), whereas LNS showed an amorphous pattern that was in keeping with its carbohydrate and dietary fiber content. Furthermore, the results demonstrated that, in comparison to LNS alone or the control group, the ESM+LNS combination markedly accelerated wound closure. By day six, there was a noticeable synergistic impact as the wound healing rate in the ESM+LNS group increased to almost 75%, compared to 60% in the LNS group and just 23% in the control group. Additionally, histological sections demonstrated that the ESM+LNS group had nearly full skin repair, with collagen fibers rearranged and dermal appendages like hair follicles and sebaceous glands returning, characteristics not seen in the other groups. These results lend credence to the idea that ESM supplies a structural and bioactive basis that promotes tissue regeneration, while LNS contributes antioxidant and pro-angiogenic qualities.

**Keywords**: Eggshell membrane, Collagen, Zinc, Wound healing.

**الملخص**

الغرض من هذه الدراسة هو التحقيق في آثار مسحوق غشاء قشر البيض (ESM) وبذور النباتات الغنية بالزنك (LNS) على التئام الجروح .تم اختيار نفايات قشر البيض وجمعها من محلات الحلويات في الوادي (الجزائر) .تضمنت الاختبارات اللونية لتحديد محتوى البروتينات والسكريات الكلية طريقة برادفورد وطريقة دوبوا على التوالي .تم استخدام تقنيات توصيف مطيافية الأشعة تحت الحمراء لتحويل فورييه (FTIR) وحيود الأشعة السينية (XRD) لدراسة الخصائص البنيوية والتركيبية لمساحيق ESMو .LNS قامت الدراسة بتقييم التأثيرات البيولوجية في الجسم الحي من خلال وضع مرهم يتكون من 50٪ ESMو 50٪ LNSمخلوطًا بالفازلين .تم وضع المرهم مرة واحدة على جروح إناث فئران ويستار البيضاء .وفقًا للدراسة البيوكيميائية، فإن محتوى البروتين في ESM أعلى - يصل إلى 88.94٪ - من محتوىLNS ، والذي يبلغ 7.40٪ فقط .كان محتوى السكر الكلي أعلى أيضًا في (7.25٪) ESM منه في (1.50٪) LNS . أكد التحليل الطيفي FTIR وجود مجموعات وظيفية مرتبطة بالبروتين (مثل الأميدات) فيESM ، بينما كشفت أطياف LNS عن قمم قوية تُعزى إلى السكريات المتعددة وروابط الإستر / الفوسفوريل . علاوة على ذلك، أدى كربونات الكالسيوم في ESM إلى ظهور بنية بلورية في حيود الأشعة السينية(XRD) ، بينما أظهر LNS نمطًا غير متبلور يتماشى مع محتواه من الكربوهيدرات والألياف الغذائية .علاوة على ذلك، أظهرت النتائج أنه بالمقارنة مع LNS وحده أو مجموعة Control ، فإن تركيبة ESM + LNS سرّعت بشكل ملحوظ من التئام الجرح .بحلول اليوم السادس، كان هناك تأثير تآزري ملحوظ حيث زاد معدل التئام الجروح في مجموعة ESM + LNS إلى ما يقرب من 75٪، مقارنة بـ 60٪ في مجموعة LNS و 23٪ فقط في مجموعة Control بالإضافة إلى ذلك، أظهرت المقاطع النسيجية أن مجموعة ESM+LNS شهدت إصلاحًا جلديًا شبه كامل، مع إعادة ترتيب ألياف الكولاجين وعودة الزوائد الجلدية، مثل بصيلات الشعر والغدد الدهنية، وهي خصائص لم تُلاحظ في المجموعات الأخرى .تُعزز هذه النتائج فكرة أن ESM يوفر أساسًا هيكليًا وحيويًا يُعزز تجديد الأنسجة، بينما يُساهم LNS بخصائص مضادة للأكسدة ومحفزة لتكوين الأوعية الدموية.

**الكلمات المفتاحية** :غشاء قشور البيض، الكولاجين، الزنك، شفاء الجروح.

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# INTRODUCTION

Algeria produces large volumes of eggshell waste from food manufacturing, restaurants, and households, yet the majority is discarded as municipal waste without undergoing any form of bioconversion or upcycling (Ahmed et al., 2020). The lack of waste valorization policies and industrial-scale biotechnological platforms has hindered the advancement of bioactive products from ESM. Harnessing such resources not only addresses environmental concerns but also supports the development of local biomedical industries (Kethiri, 2025; King'ori, 2011).

Eggshell membrane (ESM), the thin biological layer located between the eggshell and egg white, is increasingly recognized as a promising natural biomaterial due to its unique structural and biochemical properties. It consists mainly of fibrous proteins such as collagens (type I, V, and X), as well as glycosaminoglycans (GAGs), hyaluronic acid, elastin, and bioactive peptides that are crucial for tissue regeneration and repair (Park et al., 2023; Ahmed et al., 2020; Rath et al., 2016). The matrix-like organization of these components gives ESM an architecture similar to the human extracellular matrix, making it a suitable candidate for nutritional supplement for treating joint pain, wound dressing and skin tissue engineering applications (Yaning, 2021; Park et al., 2018).

Wound healing is a complex and multistage process involving hemostasis, inflammation, proliferation, and tissue remodeling. An ideal wound healing material should be biocompatible, promote cell adhesion and proliferation, maintain a moist environment, and possess antimicrobial and anti-inflammatory properties (Alberts, 2025; Gupta et al., 2019). ESM naturally exhibits many of these features. It has been shown to accelerate epithelialization (Peng, 2025), stimulate fibroblast migration, and regulate cytokine production, all of which are essential for efficient wound closure (Berry, 2024; Giansanti et al., 2021). Moreover, studies have confirmed that ESM contains antimicrobial proteins such as lysozyme (Yang and Yan, 2025) and Ovo transferrin, which inhibit bacterial growth and reduce the risk of wound infection (Nimalaratne et al., 2015).

Zinc is an essential trace element that plays a vital role in accelerating wound healing through several biological mechanisms. It is involved in cell proliferation, protein and collagen synthesis, as well as stabilization of cell membrane structure. Zinc is also crucial for the activity of various enzymes associated with tissue regeneration. Additionally, it contributes to the regulation of the inflammatory response by helping reduce excessive inflammation and promoting a faster transition to the proliferative phase. Studies indicate that zinc deficiency leads to significant delays in wound healing, while its topical or oral application results in noticeable improvements in tissue repair and regeneration rates. For example, it is found that topical zinc application stimulated granulation tissue formation and accelerated re-epithelialization in animal models (Lansdown et al., 2007).

Furthermore, it has been confirmed that zinc acts as an antioxidant and modulator of the immune response during the stages of wound healing, highlighting its importance as a supportive agent in therapeutic topical formulations (Lin et al., 2018).

This paper aims to investigate the effect of eggshell membrane and a zinc-rich plant component on accelerating wound healing. This study was conducted in a laboratory setting, involving the application of the novel formulation to animal models and monitoring of the changes associated with the healing process.

The present document is structured into three chapters. The first chapter is about a review on eggshell membrane, collagen and wound healing. The second chapter note the material and methods used during the experimental steps of this study. The third chapter presents the results obtained followed by discussion. A general conclusion, along with some future perspectives concludes this work.

# CHAPTER I

# Literature review

This chapter present an overview about eggshell membrane, collagen, and wounds healing via the effect of zinc.

## I.1. Eggshell membrane

### I.1.1. Structure and morphology

The ESM is a fibrous structure that is located between the ES and the egg white (Xinhua et al., 2024; Nys et al., 2004). It is a biopolymeric fibrous net that is necessary for the formation of ES and that prevents the mineralization of the egg white from the inside while providing a non-mineralized platform for the outer mineralization of ES (Haddad et al., 2021; Rose et al., 2009; Fernandez et al., 2003). Fig 1 and 2 shows the structure of ESM. Fig1 is an artistic representation of the cross-sectional view of an ES, showing both the inner and outer ESM in the lower part. Fig 2 shows the location of the ESM in the entire egg. The upper inset displays a schematic view that describes all the substructures of the ESM, and a picture of the inner ESM is included in the lower inset. The ESM can be separated into three sections, as previously mentioned and shown in the upper inset of *(*Matej et al., 2014) Fig 2 the outer ESM, the inner ESM, and the LM. Each of these three structures has a unique morphology (Bonke et al., 2021).

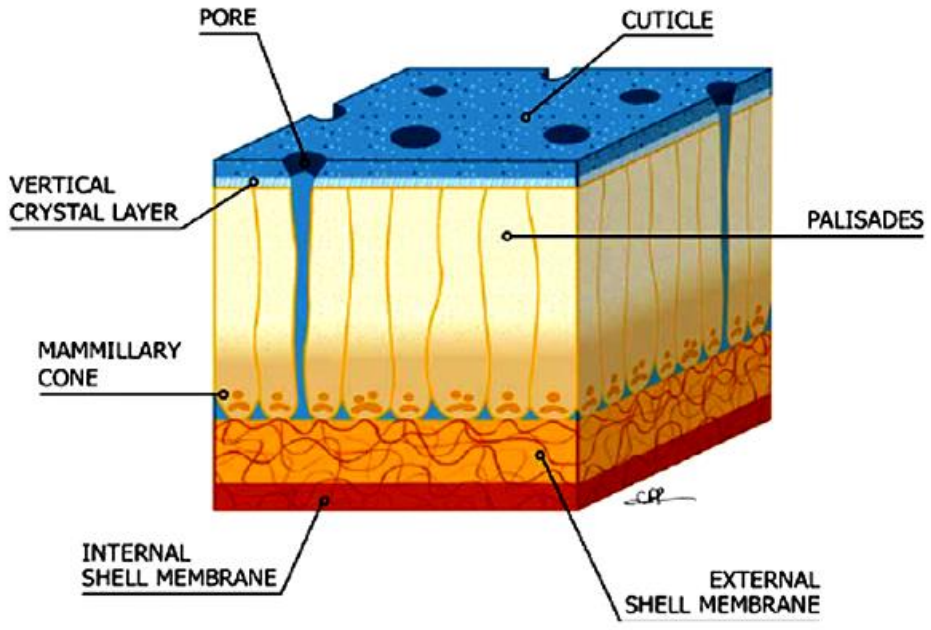


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**Figure 1.** Artistic rendition of cross-sectional view of chicken eggshell. The major morphological features are labelled (Maxwell et al., 2012)

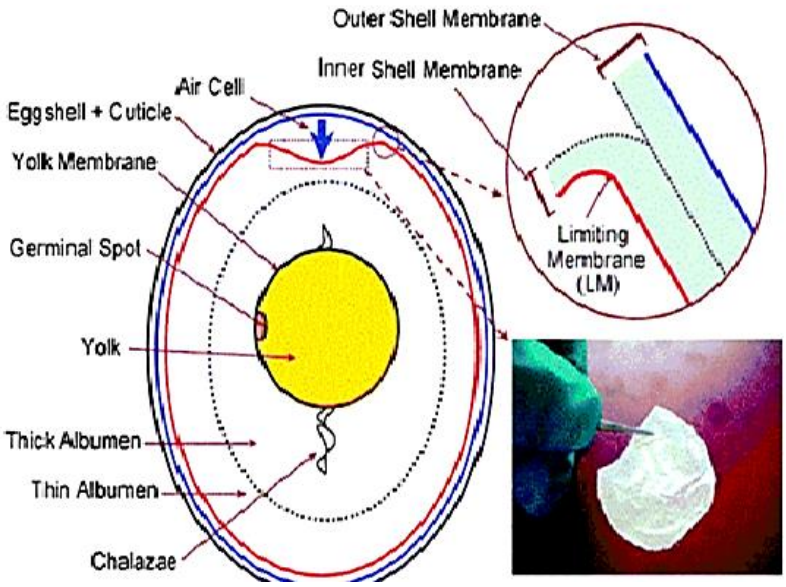



**Figure 2.** Structure of the egg upper inset substructure of the eggshell membrane; lower inset a photograph of the inner eggshell membrane (Bonke et al., 2021)

The outer ESM is just beneath the ES, and its fibers are 1–7 μm thick. The outer shell membrane's fibers reach into the shell's mammillary knobs. The entire outer membrane layer is between 50 and 70 μm thick. The air-filled area, the largest in the air cell, separates the inner ESM from the outer ESM Fig 2. The inner membrane's fibers have a diameter of 0.1 to 3 μm, which is less than that of the outer one. The inner membrane layer as a whole is also thinner, with a thickness of 15 to 26 μm. The outer membrane is interwoven with the inner ESM's fibers. The innermost, thinnest portion of the ESM that envelops the egg white is represented by the LM and appears as particles that fill the gaps between the inner membrane fibers several microns outward from the level at which the inner membrane fibers initially appear upon staining with fluorescein isothiocyanate (FITC) (Han et al., 2023; Wong and frank, 1997). Figure 3 displays scanning electron microscopy (SEM) pictures that illustrate the ESM's location within the ES as well as the morphology of its substructures. The fibers of the shell membrane are typically layered parallel to the egg's surface (Matej et al., 2014).

### I.1.2. Eggshell Biosynthesis/Formation

The formation of eggshell is a highly regulated biological process that exemplifies natural biomineralization. It involves the sequential deposition of organic matrices and calcium carbonate crystals to form a multilayered structure optimized for mechanical strength, gas exchange, and calcium supply to the developing embryo (Rodriguez et al., 2015). The eggshell consists of two main components: an organic scaffold formed by the inner and outer shell membranes (Hincke et

al., 2012). Advanced imaging techniques, such as scanning electron microscopy (SEM), have been essential in visualizing the fine structural organization of these layers and the intricate interactions between them (Hincke et al., 2000).

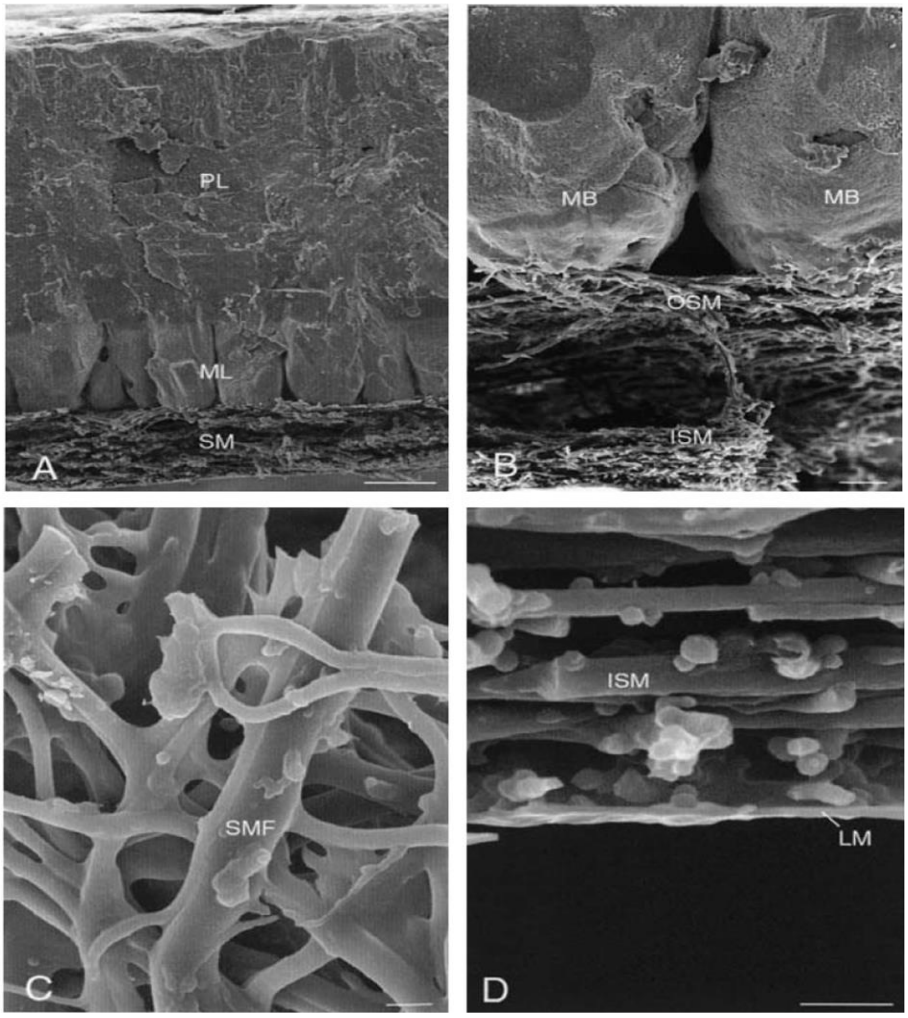



**Figure 3.** Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes (Shi et al.,2021;Rose et al.,2000); *(A) eggshell cross-fractured to reveal different layers of the eggshell (mammillary layer, ML and palisade layer, PL and the eggshell membrane, SM); (B) higher magnification of the ESM–mammillary body interface (mammillary bodies, MB, outer ESM, OSM, inner ESM, ISM); (C) enlargement of the shell membrane fibres, SMF to reveal their interwoven and coalescing nature; (D) inner aspect of the inner ESM, ISM demonstrating the limiting membrane, LM that surrounds the egg white. Scale bars: A = 50 μm ;B=20 μm; C and D=2 μm*

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been employed to visualize these structural features, as demonstrated in Fig. 4 a and 4 b (Torres et al., 1997; Hincke et al., 2000).

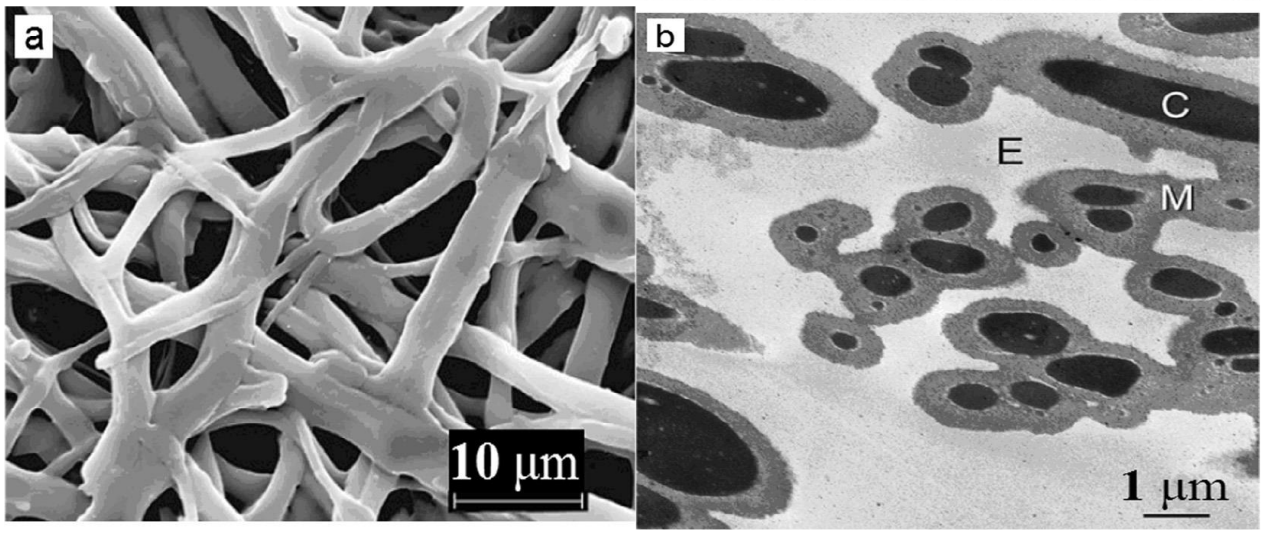



**Figure 4.** SEM (a) and stained TEM (b) image of the outer ESM (Matej et al., 2014) *C, core; M, mantle; E, extra fiber spaces*

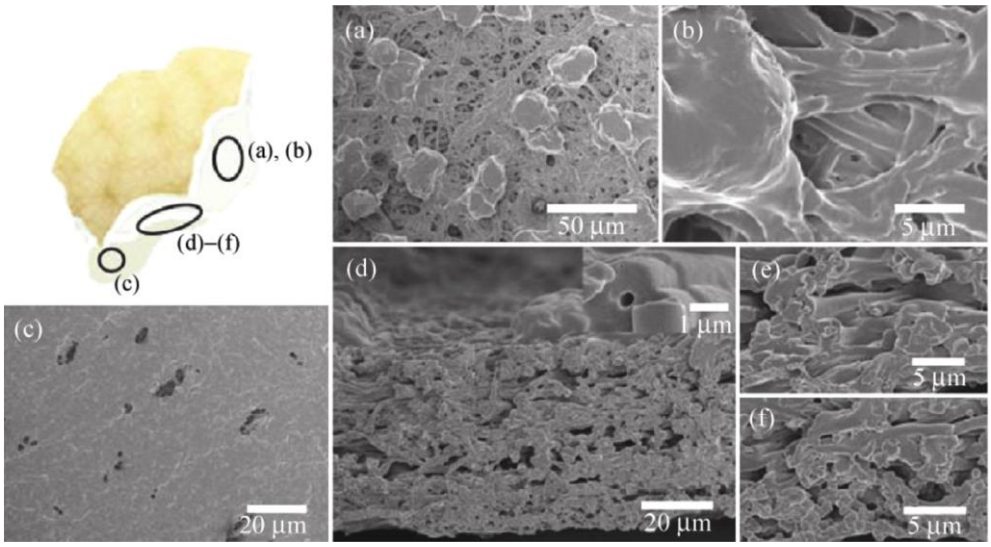



**Figure 5.** SEM images of the eggshell membrane*; (a,b) outer surface with many knobs; (c) smooth inside face (contacting the egg white) ;(d) cross section the inset is the section of a single fiber, exhibiting acore cortex structure with holes inside; (e) magnified image of the outer ESM;(f) magnified image of the inner ESM*

The decrease in the diameter of the fibers from the outer side to the inner side of the ESM was confirmed (Fig. 6) and fiber sizes of 2.5–5 µm at the surface of the outer ESM and 1.5–2 µm on the inner side of the inner ESM were observed (Mansilla et al., 2023; Zhou, 2010).

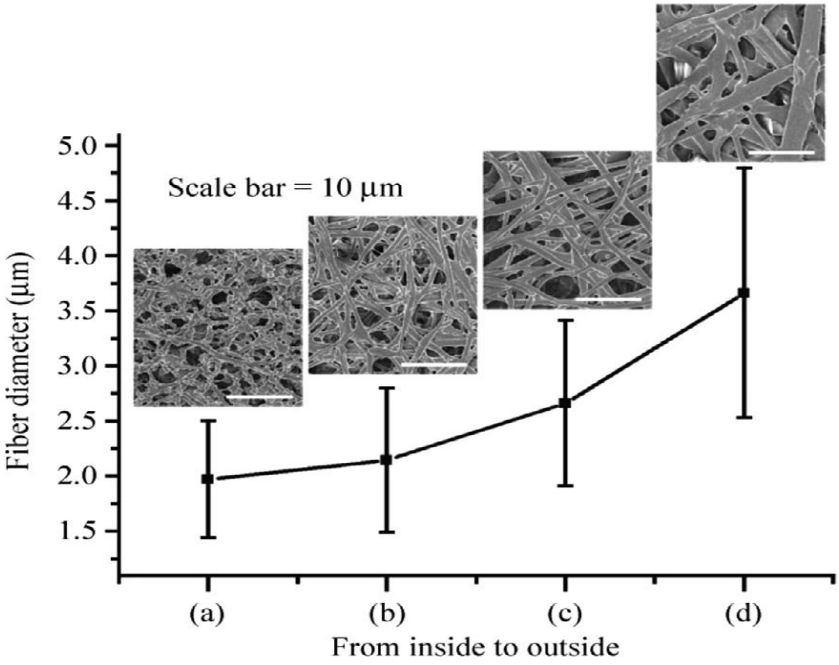



**Figure 6.** Diameter of the fiber membrane from the inside to the outside (Zhou and Wang, 2010); *(a) inner surface of the inner membrane; (b)outer surface of the inner membrane;(c) inner surface of the outer membrane;(d) outer surface of the outer membrane*

### I.1.3. Chemical Composition of ES & ESM

The carbonates, sulfates, and phosphates of calcium, magnesium, and organic materials are the main components of the ES. ES also contains traces of the elements Na, K, Mn, Fe, Cu, and Sr (Alok et al., 2016). Compared to ESM (1.358 g/cm³), ES has a density of roughly 2.53 g/cm³, which is substantially higher. Calcium carbonate (94%), organic matter (4%), calcium phosphate (1%), and magnesium carbonate (1%) are the main constituents of ES. Nearly 60% of ESM is made up of protein (collagen, 35%), glucosamine (10%), chondroitin, and hyaluronic acid (5%), with minor amounts of other inorganic elements like calcium, magnesium, silicon, and zinc. The basic side Chains of amino acids create positively charged spots on the membrane surface (ABDUL, 2022).

While the inner ESM contains both collagen I and collagen V, the outer ESM presents only collagen I. Another type of collagen, collagen X, is found in both the outer and inner layers of the ESM. Fibronectin, a dimeric form of glycoprotein with the function of activating or binding proteins, is another type of protein present in the ES (Hincke et al., 2012; Rose et al., 2012). Osteopontin, which contains numerous binding sites for cells and calcium as well as various serine/threonine phosphorylation sites, is also present in the ESM (Gautron et al., 2011; Pines et al., 2020). In addition to these various proteins, $CaCO_3$ minerals are also present in the ESM, along

with sialic acid, uronic acid, and small amounts of saccharides. The main chemical components of the eggshell membrane and their functions are summarized in Tab 1 (Yaning et al., 2021).

**Table 1.** Main chemical components of the eggshell membrane and their functions.

| **Main Components** | **Major Biochemical Functions** |
|---|---|
| Collagens | Optimum mechanical strength<br>Thermal stability<br>Wound healing<br>Osteocompatibility<br>Anchorage to nanohydroxyapatite<br>Biomineralization |
| Osteopontin | Affinity binding for hydroxyapatite and osteoblasts<br>Inhibitor of mineralization<br>Modulation of osteoclast differentiation<br>Recruitment of macrophages<br>Regulation of cytokine production<br>Inhibition of vascular calcification<br>Regulation of apatite crystal size and growth<br>Tissue remodeling |
| Fibronectin | Promotion of cell adhesion<br>Improving cell growth, migration, and differentiation<br>Wound healing |
| Keratin | Self-Assembly<br>Promotion of cell adhesion |
| Cysteine-rich eggshell membrane proteins (CREMPS) | Chromatin folding and compaction |
| Histones | Potent antimicrobial properties |
| Avian beta defensins | Promotion of innate defense system<br>Reinforcement of the antimicrobial defenses associated with the ESM |
| Ovocalyxin-36 | Potent antimicrobial properties<br>Positive immune-modulating effects |
| Apolipoproteins | Binding and transport of lipids |
| Protocadherin | Adhesion and differentiation functions |
| Chondroitin sulfate | Formation of porous hydrated gels<br>Immuno-inhibition property for articular cartilage repair<br>Binding $Ca^{2+}$<br>Molecules' migration through the matrix |
| Hyaluronic acid | Water-retaining property<br>Improving angiogenesis and tissue morphogenesis |

## I.2. Collagen

The most prevalent structural protein in all mammals is collagen. In many connective tissues, including skin, bones, cartilage, and tendons, it is a part of the extracellular matrix (Amirrah et al., 2022; Pillai et al., 2024). Three lengthy helicoidal chains of amino acid residues with nonhelical terminals at both ends make up naturally occurring collagen molecules. Collagens from different animals have at least 46 distinct polypeptide chains (Shoulders and Raines, 2009).

### I.2.1. Structure of collagen

Specific amino acid sequences that encode one or more triple-helical domains are present in all 28 subtypes of collagen. A distinctive repetition of the amino acids G-X-Y makes up triple-helical domains, where G always stands for glycine, X for proline, and Y for hydroxyproline. Three polypeptides, known as alpha chains, can be formed into a triple-helical collagen protein thanks to the favorable hydrogen bonding created by this repeating sequence (Tvaroška, 2024). The placement, size, and distribution of triple-helical domains determine the grouping of different forms of collagen. (Fig7 ) provides a summary of these groups. Three of the same alpha chain or three of distinct alpha chains can make up a collagen. For instance, type I collagen typically has two α1 and one α2 chains, type II collagen has three α1 chains, and type IX collagen has α1, α2, and α3 chains. Minor collagens, which make up less than 10% of the overall collagen content, have significant roles in collagenous tissues despite the fact that collagen types I and II are the most prevalent (Mahesh et al., 2022). The collagen network is strengthened and matured by the covalent enzymatic collagen crosslinks formed by collagen types I, II, III, V, XI, and IX. (Fig 7) shows the process by which these collagen crosslinks are formed (Jadach et al., 2024).

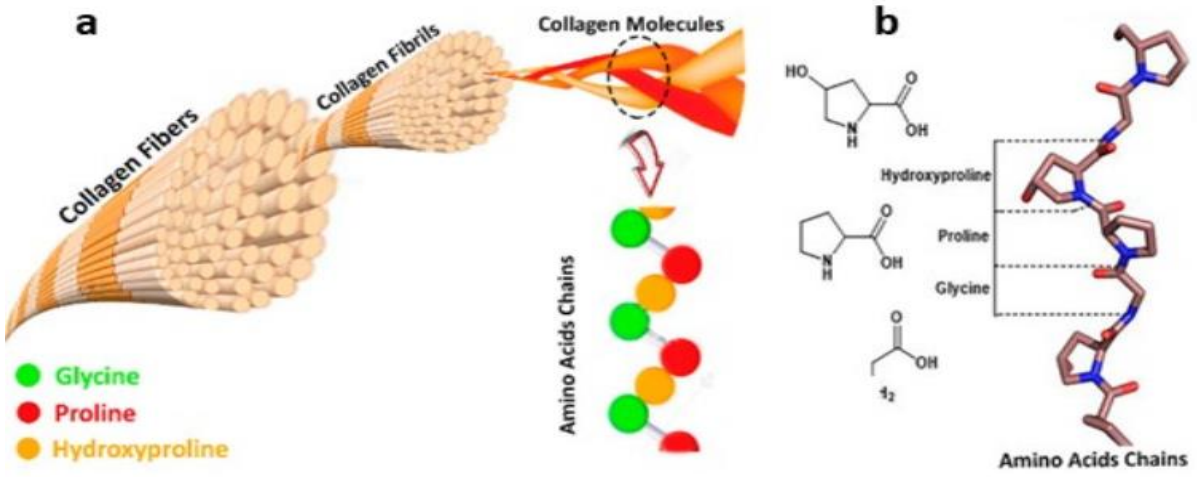



**Figure 7.** Structure of collagen fiber (a), and main constituent amino acids (b) (Jadach et al., 2024)

### I.2.2. Sources of collagen

Different collagen types are found in various tissues all over the body. Three of the most abundant collagen types are displayed for non-cartilage tissues (fig. 8.a), cartilage tissues (fig. 8.b), and temporomandibular joint (TMJ). Collagen plays several roles in diverse body parts, such as:

- Skin: Slows aging, improves hydration and elasticity, helps wound healing, and boosts tissue regeneration;
- Cardiovascular: Lowers blood pressure, glucose, cholesterol, improves metabolism and insulin secretion;
- Nervous System: Enhances memory, reduces stress and anxiety-related issues;
- Skeletal and Muscular: Strengthens bones, reduces osteoporosis, improves muscle strength and body composition;
- Gastrointestinal: Protects gut lining, boosts metabolism, improves gut flora;
- Immune System: Fights tumor cells, strengthens immunity, reduces allergic reactions (Jadach et al., 2024).

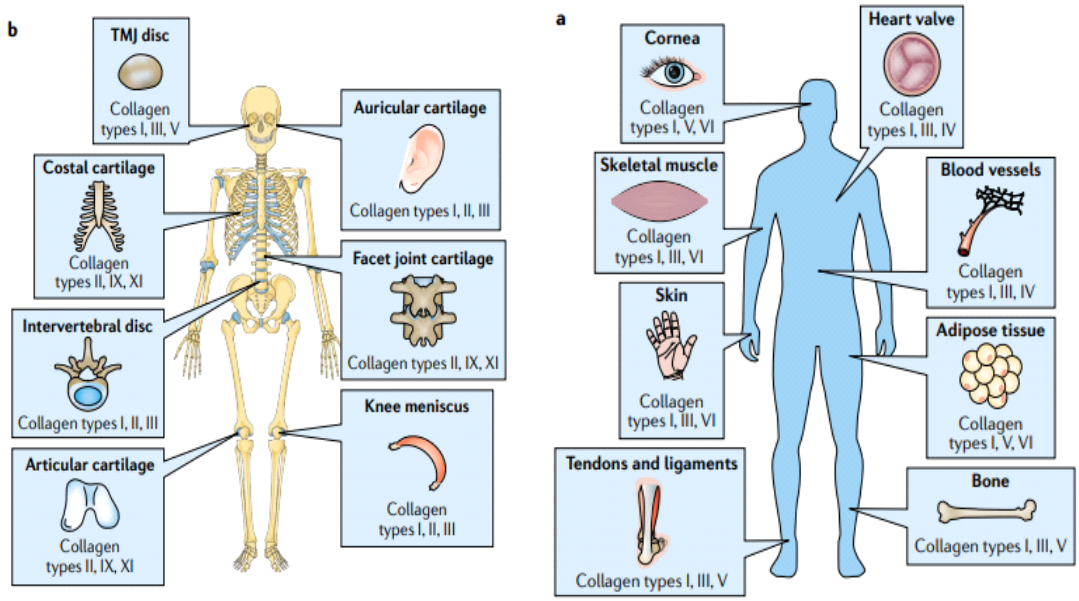



**Figure 8.** Collagen types of the human body (Benjamin et al., 2020)

The main source of fibrillar collagen type I is animal skin. Traditionally, collagen was isolated from the skin of animals such as cattle and swine. To these sources were added lately marine organisms, generally fish, which results in marine collagen. Even if it does not differ significantly from land collagen, it has certain commercial and technological advantages. It is a

known fact that in some countries, swine and cattle products are prohibited or difficult to access, so marine collagen represents an important source. Collagen is typically animal-derived. However, in the commercialization of natural products, a new collagen is used, and that is vegetable collagen. Such products are actually mixtures, blends of collagen with different ingredients of vegetable origin, or products based on soybean oil, which does not stand for the name of collagen (Cherim and Mustafa, 2019).

### I.2.3. Uses of collagen

#### I.2.3.1. Collagen in cosmetic industry

Collagen's biological function has been acknowledged, and its potential for usage in the clinical, pharmacological, and cosmetic domains is enormous (fig 9). The kinds I, II, and III are the main and most prevalent collagens; they create the structural fibrils of tissues, whilst the remaining kinds simply participate in the association of these fibrils with other collagens. The other forms of collagen have therefore not been utilized in cosmetic products (Jadach et al., 2024). Because of its excellent biocompatibility with the human body, type I collagen which forms fibrils is the most commonly employed in the production of products for a variety of cosmetic applications. Collagen that forms fibrils (type I, II, III, V, XI) is the primary basis for biomaterials used in tissue engineering. Additionally, collagen type I is a component of skin regeneration templates, dental composites, cosmetics, biodegradable matrices, and collagen shields in ophthalmology (Alina et al., 2020).

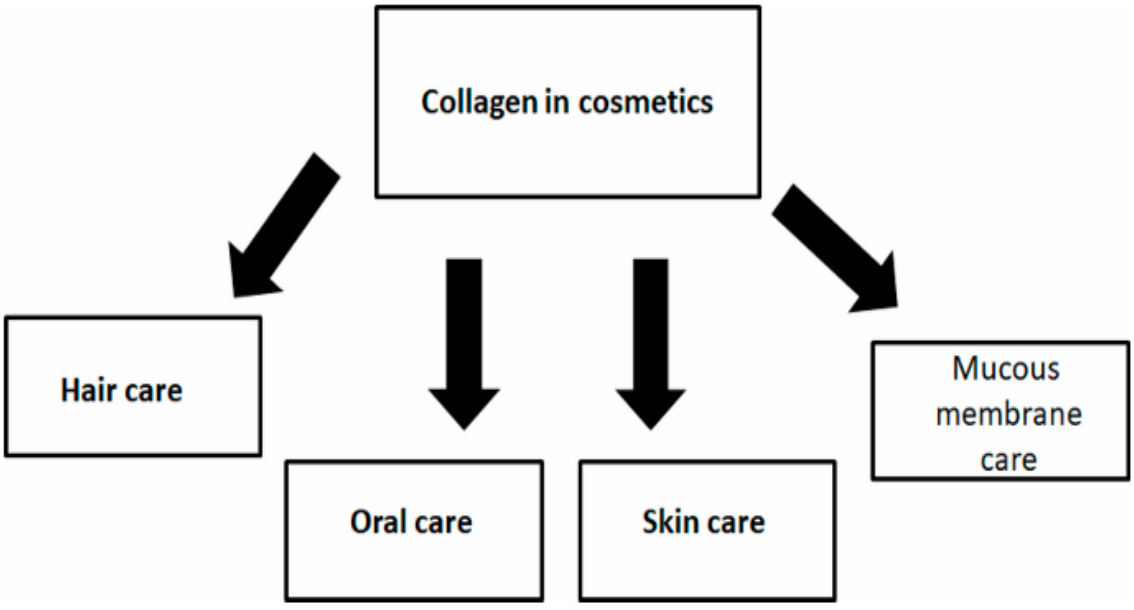



**Figure 9.** Application of collagen in cosmetics (Alina et al., 2020)

Moreover, skin aging is a complex biological process that affects several constituents of the skin (fig. 10) and hence its appearance. The two primary processes of skin aging, intrinsic and extrinsic (fig 11), are controlled, respectively, by genetic variations and by extrinsic components. Skin aging alterations are due to structural changes in the skin cells and in the texture of the dermal tissue because of the action of free radicals (reactive oxygen species, ROS) produced mostly by UV radiation and to a certain extent by cellular metabolism. ROS induce a molecular destruction and consequently the loss of biological functions. One effective strategy to managing the skin ageing process is adopting a healthy nutritional approach to life, maintaining a balanced diet and a good supply of food supplements. This can restore the homeostasis of macro and micronutrients and support the physiology of cells and tissues in the skin. Hydrolysed collagen, an increasingly popular nutraceutical, is composed of low molecular weight small peptides, which are easily digestible, absorbed and distributed in the human body. Numerous clinical trials have now been performed showing the efficacy and benefits of collagen peptides on skin properties, such as hydration, elasticity and reduction of wrinkles. As a result, hydrolysed collagen can be considered an important weapon in the everyday fight against skin ageing (Sibilla and Genovese, 2015 ).

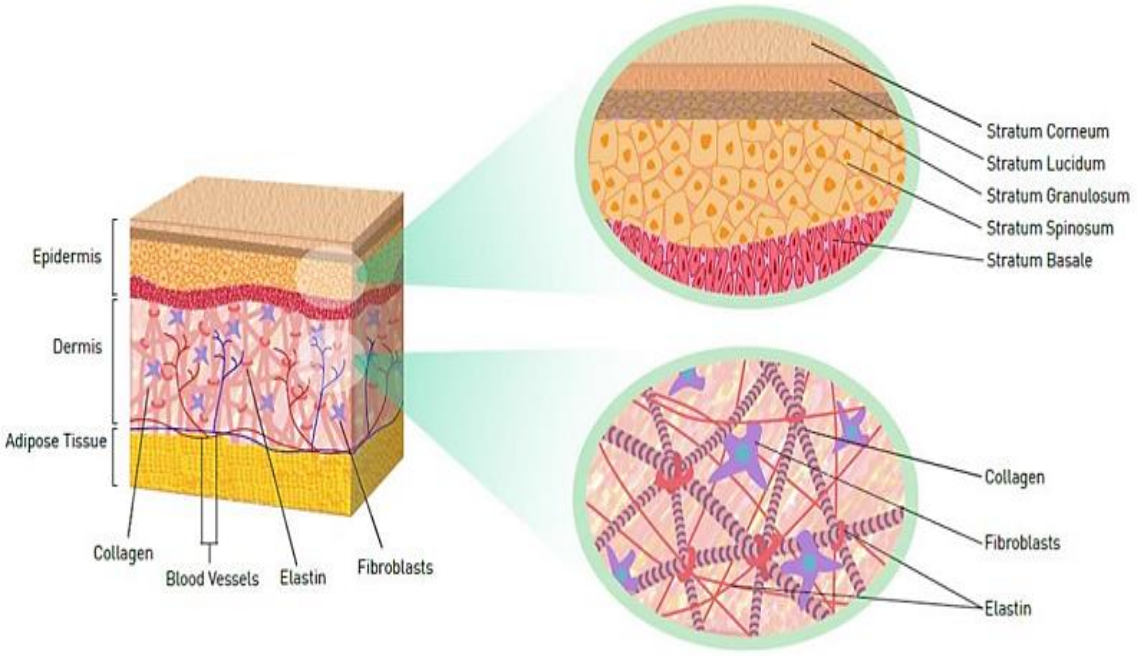



**Figure 10.** Structure of healthy skin (Sibilla and Genovese, 2015)

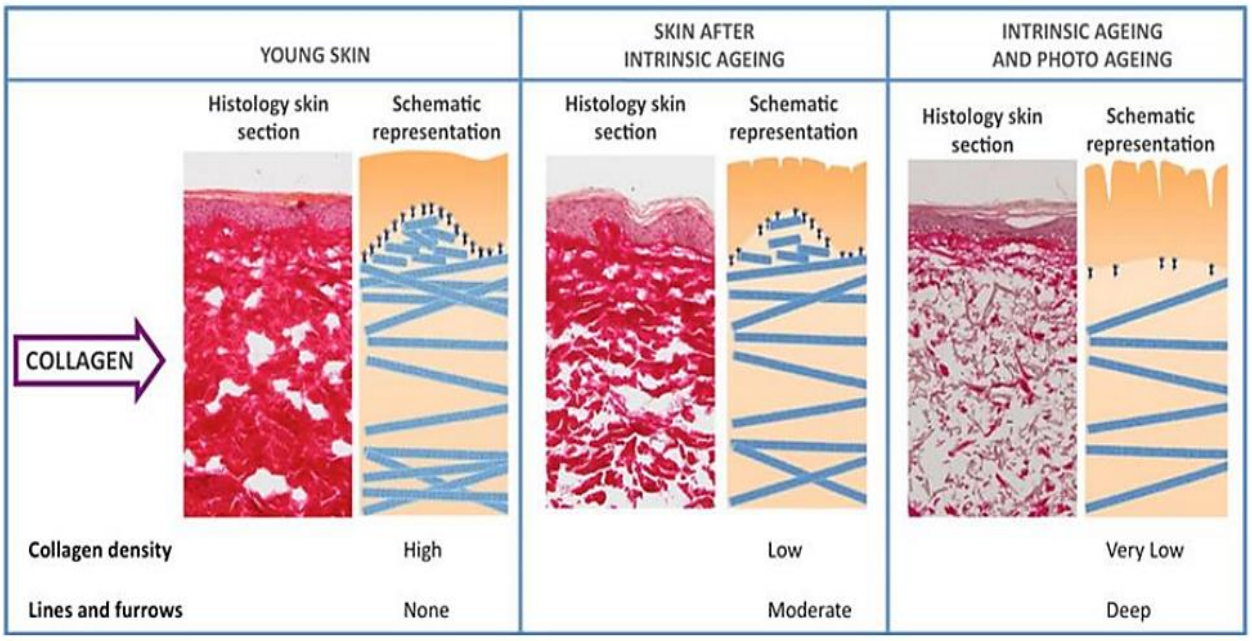



**Figure 11.** Collagen decreases after intrinsic and extrinsic aging (Sibilla and Genovese, 2015)

### I.2.3.2. Collagen for treating skin injuries

Collagen also plays an important role in regulating the regeneration of tissues, promoting cell adhesion, and cell proliferation. Previous studies show that collagen is closely involved in cell proliferation, differentiation, and migration; intercellular signal transmission; tissue formation; blood coagulation; and so on. Thus, it may participate in different stages of tissue repair. During hemostasis, collagen induces platelet activation and aggregation, thereby depositing fiber clots at the injured site. In the inflammatory stage, the activation of immune cells promotes the secretion of inflammatory cytokines, thus affecting the migration of fibroblasts, epithelial cells, and endothelial cells, and fibroblasts contribute to the deposition of collagen. Collagen degradation releases fragments that promote fibroblast proliferation and growth factor synthesis, leading to angiogenesis and re-epithelialization. In the mature remodeling phase, the extracellular matrix is remodeled to restore its tensile strength. Therefore, medical materials based on natural collagen are extensively used as an alternative option to traditional materials, which show non-immunogenicity, low sensitization, better biocompatibility and have promising clinical applications (Zaho et al., 2023).

### I.2.3.3. Eggshell membrane as a promising supplement to maintain bone health

Bone loss is a well-known phenomenon in the older population leading to increased bone fracture risk, morbidity, and mortality. Supplementation of eggshell membrane (ESM) is evaluated due to its possible application to prevent bone loss and usage in osteoporosis therapy. The similar organic chemical composition of ESM and human bone is described in detail as both mainly consist

of collagen type I, chondroitin sulfate, dermatan sulfate, hyaluronic acid and elastin (tab. 02). ESM and its components are reported to improve mineralization in bone tissue (fig. 12). In many studies ESM intake reduced pain in patients with joint disorders and reduced inflammatory processes. Additionally, ESM improved calcium uptake in human cells. These findings in comparison with a clinical pilot study reporting pain reduction in osteoporotic patients and increased osteoblast activity in *in vitro* assays support ESM to be a beneficial supplement for bone health (Fladerer et al., 2024; Wong et al., 2013).

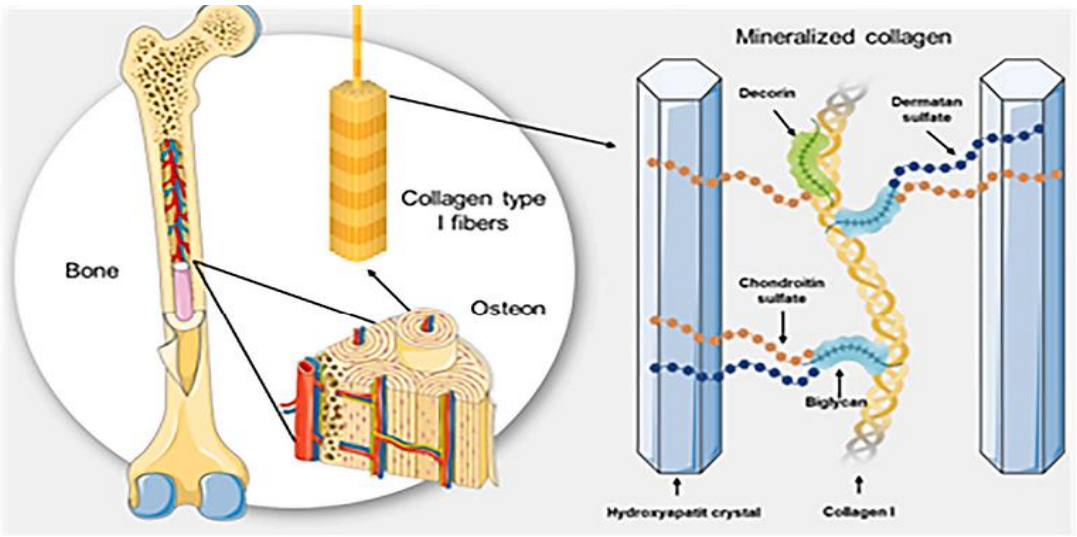



**Figure 12. S**tructural proteins for collagen type I mineralization (Fladerer et al., 2024)

**Table 2.** Comparison of organic chemical composition of bone and ESM **(**Fladerer and al., 2024)

| **Compound** | **Bone** | **ESM** |
|---|---|---|
| Collagens | Collage type I, III, V (ratio 10011) Chondroitin sulfate Dermatan sulfate Hyaluronic acid | Collage type I, V, X (ratio 10011) Chondroitin sulfate Dermatan sulfate Hyaluronic acid Keratan sulfate |
| GAGs | Chondroitin sulfate Dermatan sulfate Hyaluronic acid | Collage type I, V, X (ratio 10011) Chondroitin sulfate Dermatan sulfate Hyaluronic acid Keratan sulfate |
| Other proteins | Proteoglycans Elastin g-carboxyglutamic acid-containing proteins Small integrin-binding ligands N-linked glycoproteins Glycoproteins | Proteoglycans Elastin Egg white proteins Eggshell matrix Proteins |

## I.3. Human Skin

Human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue. The dermal epidermal junction is undulating in section; ridges of the epidermis, known as rete ridges, project into the dermis. The junction provides mechanical support for the epidermis and acts as a partial barrier against the exchange of cells and large molecules. Below the dermis is a fatty layer, the panniculus adiposus, usually designated as 'subcutaneous.' This is separated from the rest of the body by a vestigial layer of striated muscle, the panniculus carnosus (Proksch et al., 2008; Kueper et al., 2007).

There are two main kinds of human skin. Glabrous skin (non hairy skin), found on the palms and soles, is grooved on its surface by continuously alternating ridges and sulci in individually unique configurations known as dermatoglyphics. It is characterized by a thick epidermis divided into several well marked layers, including a compact stratum corneum, by the presence of encapsulated sense organs within the dermis, and by a lack of hair follicles and sebaceous glands. Hair bearing skin, on the other hand, has both hair follicles and sebaceous glands but lacks encapsulated sense organs. There is also wide variation between different body sites. For example, the scalp with its large hair follicles may be contrasted with the forehead, which has only small vellus-producing follicles, albeit associated with large sebaceous glands (McGrath et al., 2004).

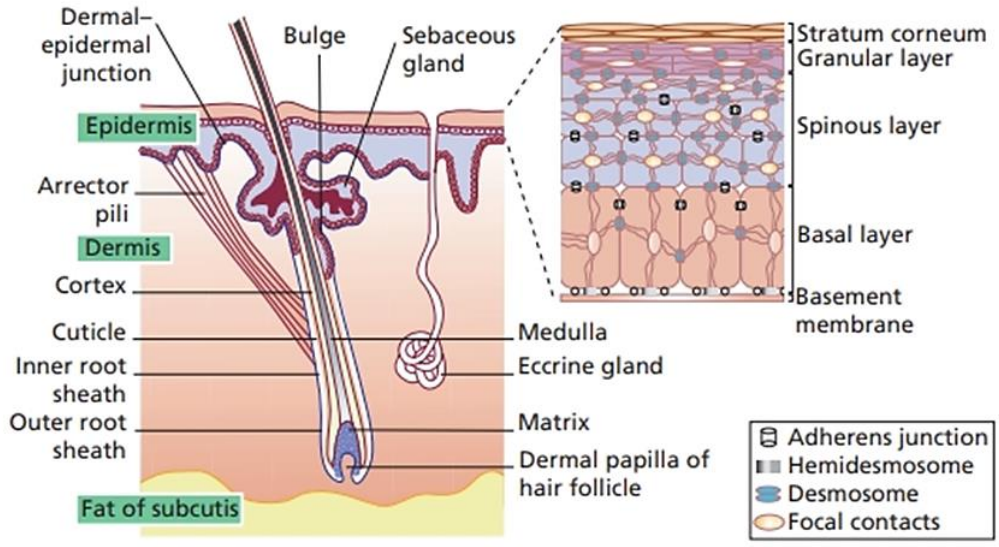



**Figure 13.** Skin and its appendages (McGrath et al., 2004)

The skin, the largest organ in the body, possesses an array of functions, acting as a barrier for protection and prevention of dehydration, as a sensory and thermoregulatory organ, and as an active site of vitamin D synthesis and immune surveillance. The skin is comprised of the epidermis

and the dermis (Cobo et al., 2021; Germann et al., 2021). Morphologically, the epidermis is arranged into distinct layers that reflect the sequential differentiation of keratinocytes as they migrate from the basal layer on the onset of terminal differentiation, having lost the ability to proliferate, to the outermost cornified layers, where they are sloughed off. The epidermis and hair follicle are maintained and regenerated through the existence of stem cells. Epidermal stem cells are relatively quiescent and undifferentiated with a capacity to maintain homeostasis, self-renew tissue, and contribute to wound repair (Ojeh et al., 2015).

### I.3.1. Definition of wounds

A wound is a disruption of normal anatomic structure and function. Wounds result from pathologic processes. Wounds may be classified as those that repair themselves or can be repaired in an orderly and timely process (acute wounds) and those that do not (chronic wounds). Assessment of a wound in the environment in which it occurs is essential for diagnosis, treatment, management, and study. No wound can be assessed in isolation from the patient or his or her environment. Thus, complete wound assessment must include the extent of the wound, associated attributes of the wound, host factors that influence wound status, and environmental factors that impact optimum wound management (Herman et al., 2023).

### I.3.2. Classification of wounds

Wounds can be classified according to several criteria, including their nature, cause, and duration.

**Wound nature criteria**

- **Open wounds**: These involve a disruption in the skin that allows external agents to enter. Common types include:
    - **Abrasions**: Superficial damage to the skin due to friction or scraping.
    - **Lacerations**: Irregular tears in the skin caused by blunt trauma.
    - **Punctures**: Deep, narrow wounds caused by sharp, pointed objects.
    - **Incisions**: Clean cuts made by sharp instruments, such as scalpels.
- **Closed wounds**: These occur when tissue damage is present without a tear in the skin.
    - **Contusions**: Bruises caused by blunt force trauma leading to internal bleeding.
    - **Avulsions**: Partial or complete tearing away of the skin from underlying tissues (Pollock and Schumacher, 2012)

**Cause of injury criteria**

- **Mechanical injuries**: Caused by sharp or blunt physical trauma.
- **Thermal injuries**: Result from exposure to heat or cold (e.g., burns or frostbite).
- **Chemical injuries**: Caused by corrosive substances such as acids or alkalis (Pollock and Schumacher, 2012).

**Wound duration criteria**

- **Acute wounds**:

These heal in an expected time frame with proper treatment (typically within 4–6 weeks). A wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue. A wound may result from precise disruption of tissue by the surgeon's knife (incision) to widespread damage of tissue (e.g., major trauma, burns). A wound may also result from a contusion, hematoma, laceration, or abrasion. The continuity of the skin must be restored expeditiously because it plays a crucial role in maintaining homeostasis (Sharma et al., 2024; Senthil and David, 2008).

- **Chronic wounds**:

Fail to progress through the normal stages of healing, often due to underlying conditions such as diabetes or vascular insufficiency (Sharma et al., 2024). According to the present definition, wounds are considered chronic when healing time does not follow the expected course or fall within the range of what is considered a normal healing trajectory. The entire process is prolonged, and wounds diagnosed or categorized as chronic often resist attempts at treatment. In addition, the quality of tissue may be poor, and a functional closure is not achieved, so the wounds may reoccur. (Maqsood, 2018).

### I.3.3. Process of wound healing via zinc function

Wound healing is an intricate and dynamic process which can be subdivided into a series of phases; including (1) coagulating fibrin clot formation (haemostasis, occurs within seconds to 1 h), (2) inflammatory response (within minutes to days), (3) cell proliferation, re-epithelialization, granulation and angiogenesis (begins 18–24 h after wounding and lasts from days to weeks), and (4) matrix remodeling and scar formation (5–7 days after injury and could persist months to years) (fig. 14). these distinct but overlapping phases involve dynamic coordination of soluble mediators

(reactive oxygen species (ROS), chemokines, cytokines and growth factors), ECM turnover and cellular cross-talk amongst platelets, infiltrating immune cells, resident keratinocytes, endothelial cells, fibroblasts, epithelial cells and stem cells (Lin et al., 2018).

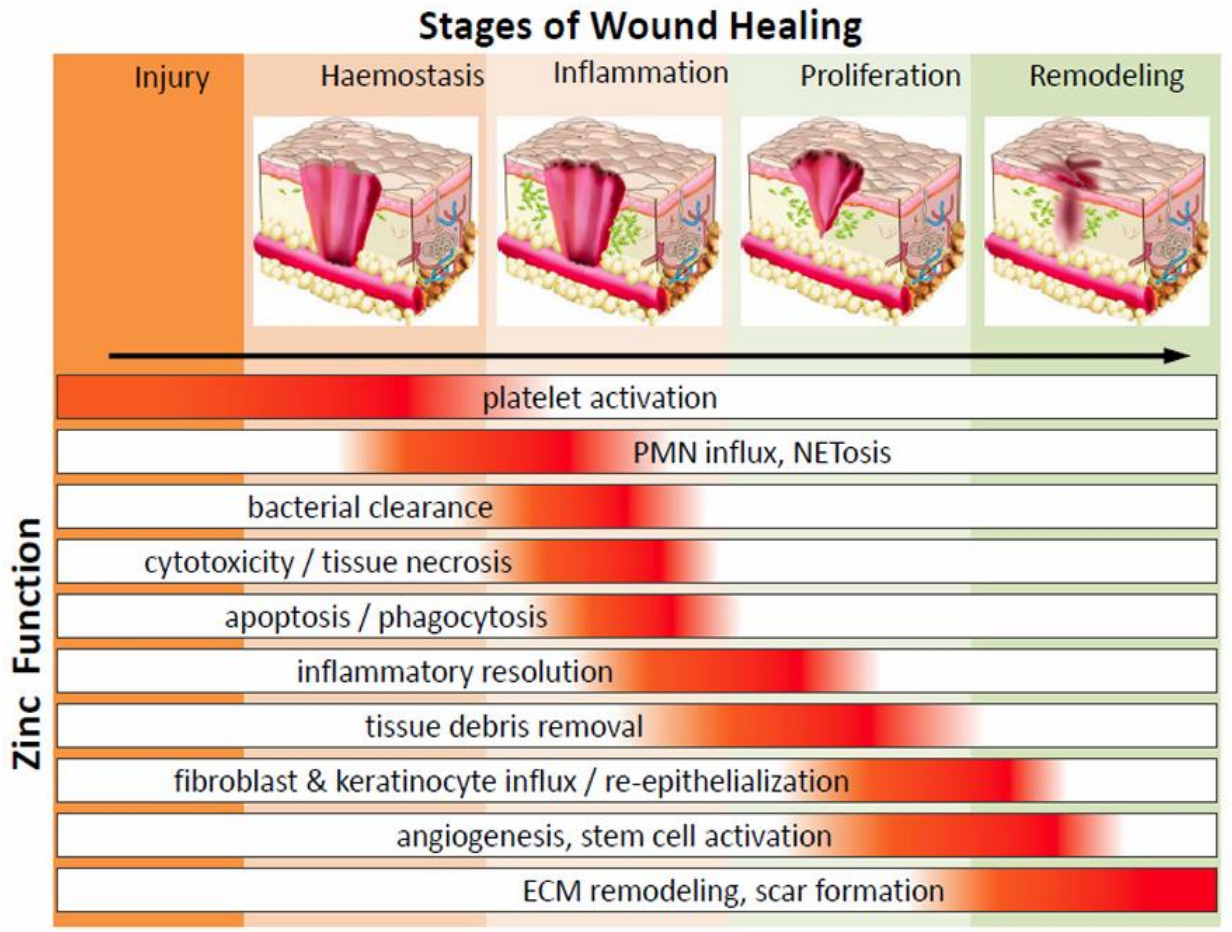



**Figure 14.** Function of zinc throughout the stages of wound healing (Lin et al., 2018)

Zinc is an essential micronutrient, present at less than 50 mg/kg, in the human body. It is important for human health and disease due to its critical roles in growth and development, bone metabolism, central nervous system, immune function and wound healing (Freeland and Sanjeevi, 2015). Zinc deficiency has been linked to delayed wound healing (Lansdown and Mira, 2007). Zinc alters immune responses in a multitude of ways ranging from myeloid-derived cells and inflammatory signaling to lymphocyte differentiation and antibody production (Coger and Million, 2019). The ability of zinc to regulate immune homeostasis is a burgeoning field of study and has been extensively reviewed (Quinton and Allan, 2018).

Zinc contributes to platelet activation by stimulating its entry into the cell, leading to a series of internal changes including increased phosphorylation of certain proteins, which can be inhibited

using protein kinase C (PKC) inhibitors. Studies have also shown that zinc stimulates the secretion of platelet α-granule contents, which contain a wide range of chemokines such as CXCL1, CXCL4, CXCL8, CCL2, and CCL5, among others. These factors attract and activate innate immune cells at the site of injury, suggesting an important role for both zinc and platelets in stimulating the inflammatory phase of wound healing (Pei and Matthew, 2018).

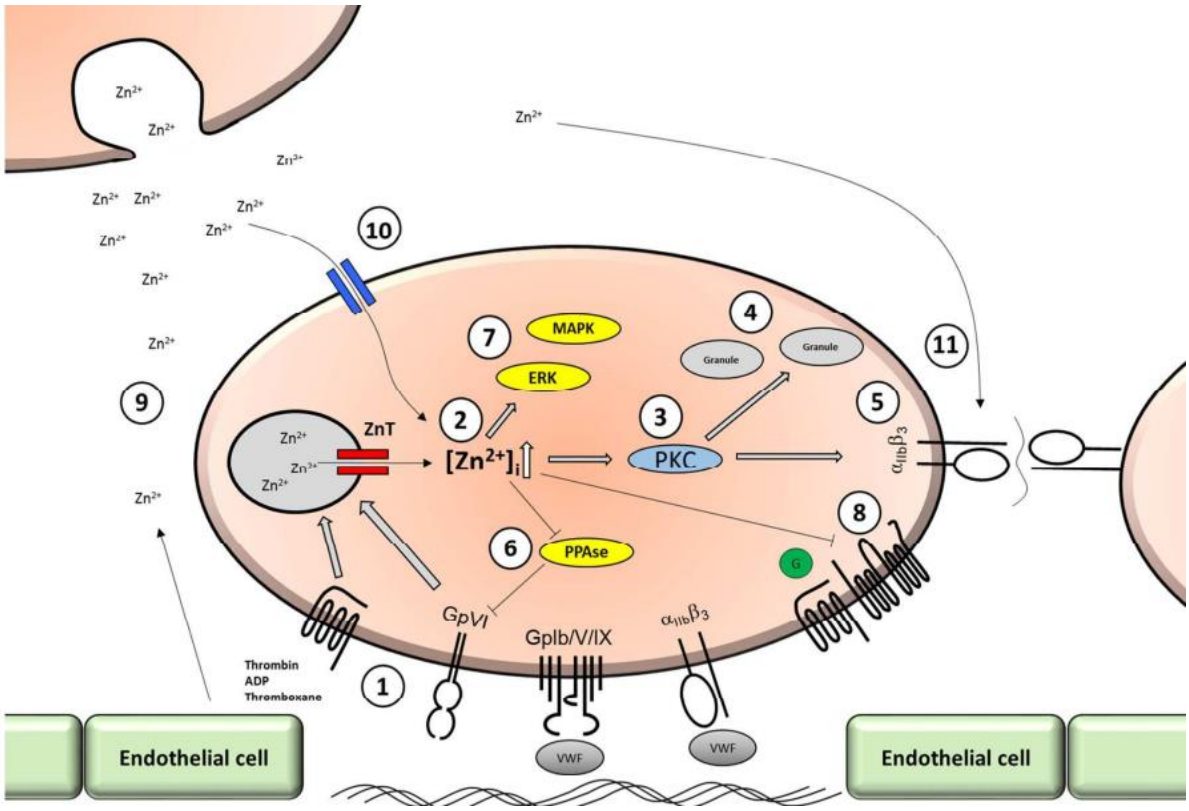



**Figure 15.** Speculative model of the mechanisms and machinery that are influenced by Zn2+ during platelet activation (Taylor and Pugh, 2013)

The mechanisms and machinery that are influenced by Zn2+ during platelet activation, is illustrated in fig 16. (1) *Initial stimulation*: Platelets interact with proteins in the damaged vessel wall (such as collagen and VWF) and are stimulated by substances such as ADP and thrombin. (2) *Intracellular zinc release:* $Zn^{2+}$ is released from internal stores into the cytoplasm via ZnT channels or transporters, increasing its intracellular concentration [$Zn^{2+}$]. (3) *PKC activation:* $Zn^{2+}$ activates the PKC enzyme, promoting granule release and activation of the integrin αIIbβ3 receptor, allowing fibrinogen binding and platelet aggregation. (4) *Enhanced phosphorylation signaling*: $Zn^{2+}$ inhibits phosphatases and increases protein phosphorylation, which activates kinases such as ERK and enhances activating signaling. (5) *Reducing cAMP:* $Zn^{2+}$ inhibits the enzyme adenylate cyclase, which reduces cAMP and increases the platelet response to activation. (6) *Exogenous zinc sources*: $Zn^{2+}$ is released from damaged endothelial cells, subendothelial matrix, and platelet granules. (7)

*Exogenous zinc entry:* $Zn^{2+}$ enters platelets via non-selective channels or transporters. (8) *Direct effect on integrins:* $Zn^{2+}$ may interact directly with integrin αIIbβ3, regulating platelet interaction and contributing to thrombus formation (Niaz, 2020; Taylor and Pugh, 2013; Watson and Nathan, 2015).

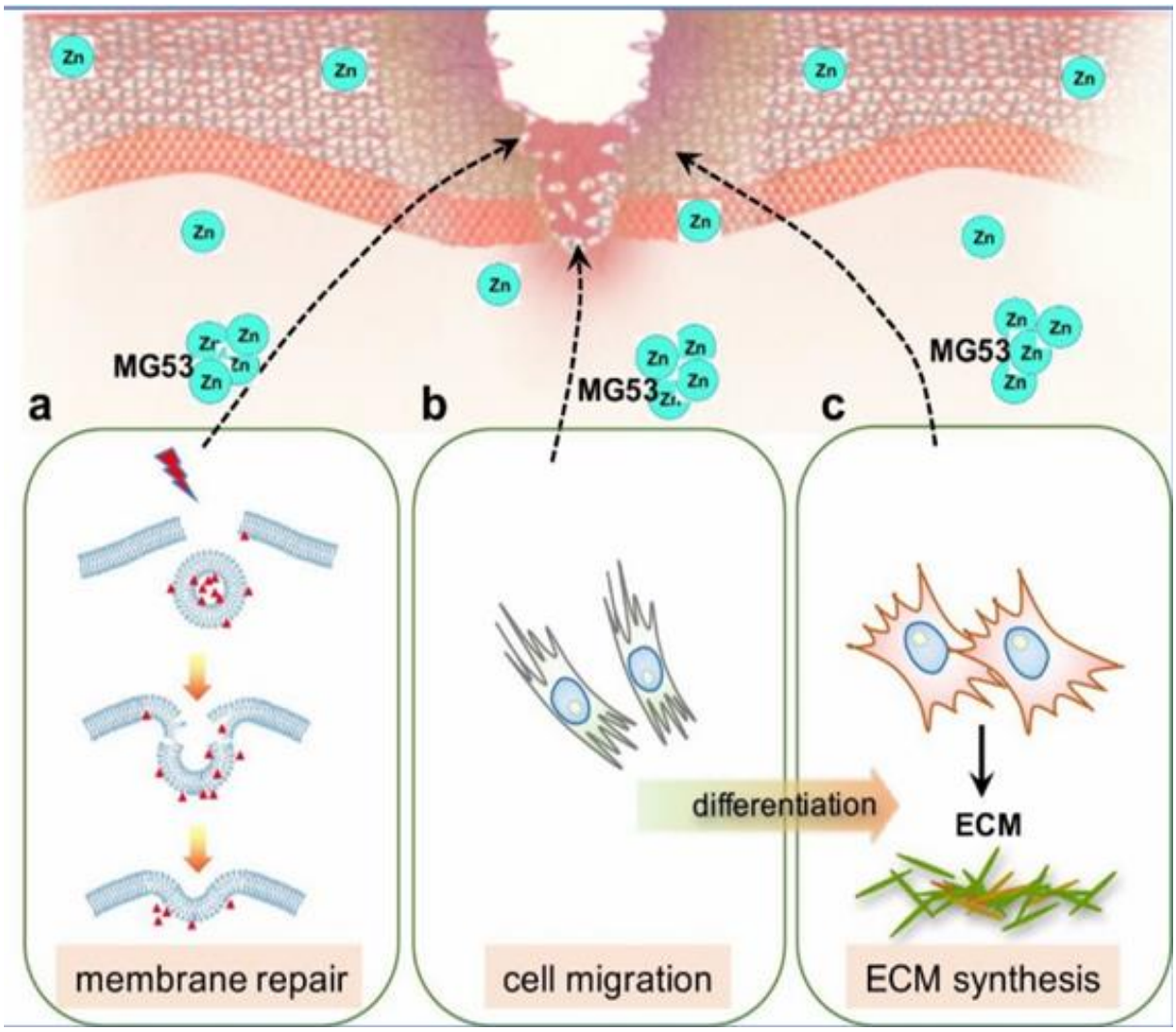



**Figure 16.** Proposed roles of zinc in mitsugumin 53 (MG53) mediated wound healing process (Lin et al., 2018)

In human physiology, zinc plays important roles as cofactor for many metalloenzymes required for cell membrane repair, cell proliferation, growth and immune system function. The pathological effects of zinc deficiency include the occurrence of skin lesions, growth retardation, impaired immune function and compromised would healing. Mitsugumin 53 (MG53) exists as intrinsic intracellular vesicles which could be secreted into blood circulation as a myokine to mediate wound healing. In response to dermal injury, MG53 could exert the following functions. (a) MG53-containing vesicles nucleate the cell membrane repair machinery and trafficking to damaged

cell membrane to protect against acute membrane injury of keratinocytes and fibroblasts. (b) MG53 mediates cytoskeletal stress fibre remodelling to promote fibroblasts migration to the wound sites. (c) MG53 regulates transforming growth factor-β (TGFß) signaling to modulate trans-differentiation of fibroblasts into myofibroblasts and suppresses deposition of extracellular matrix (ECM) proteins during tissue remodeling stage of wound healing. Zinc is proposed to bind to MG53 on its two zinc-binding sites and modulates MG53-mediated wound healing process (Lin et al., 2018).

# CHAPTER II

# Material and methods

This chapter focuses on the study principle, the material and the methods used for the partial characterization and the evaluation of wound healing activity of eggshell membrane and zinc-enriched plant seeds.

## II.1. Study Principle

The eggshell membrane is a natural biomaterial rich in proteins such as collagen (types I, V, and X), glycosaminoglycans (e.g., dermatan sulfate and chondroitin sulfate), and lysozyme, which makes it similar to the extracellular matrix (ECM), supporting cell adhesion, migration, and proliferation; essential steps in wound healing (King'ori, 2011). It also has anti-inflammatory and antioxidant properties that help regulate immune responses and reduce oxidative stress; both key factors in effective wound healing. Additionally, ESM promotes fibroblast proliferation and collagen synthesis, which are necessary for granulation tissue formation and dermal reconstruction (Ruff et al., 2009). Moreover, zinc is a vital trace element involved in various biological functions including enzyme activity, cell proliferation, and immune regulation. It contributes to wound healing by promoting angiogenesis, enhancing the activity of keratinocytes and fibroblasts, and regulating inflammatory cytokines (Freeland and Sanjeevi, 2015). Zinc deficiency has been associated with impaired immune response, delayed epithelialization, and reduced tensile strength in healing tissues (Nazeer et al., 2023).

This study aims to investigate the synergistic effectiveness of zinc and eggshell membrane in promoting wound healing and tissue regeneration. The study hopes to suggest a novel and natural bioactive product to improve tissue-healing processes.

## II.2. Study Materials

Research material include biological and non-biological material presented by lab equipment and devices used in the experimentation. Biological material consist of selected zinc-enriched plant seeds, eggshell membrane, and an animal model.

### II.2.1. Plant material (LNS)

Selected zinc-enriched plant seeds (LNS) were sourced from a local market in El Oued Province, southeastern Algeria. They were thoroughly washed with tap water and dried until use.

### II.2.2. Eggshell Membrane (ESM)

Red eggshell waste was collected from several sweet shops at around 1000 egg. After collecting, the eggshells were thoroughly washed with tap water several times to remove any impurities or egg white. After that, the ESM was manually separated from the outer shell (Tsai et al., 2006) and air-dried than stored until use.

### II.2.3. Animal model

The present study was performed on 24 female rats (*Wistar albino*) weighing between 150 and 200g. All rats were healthy until the experimental endpoint to enable an *in vivo* study to assess wound healing activity. The rats underwent a 10-day adaptation period under ambient laboratory conditions (12-hour light/dark cycle at 25 ± 3°C) (Nga et al., 2020). at the nature and life sciences faculty in the University of Echahid Hamma Lakhder (El-Oued, Algeria) with free access to food and water .

## II.3. Study Methods

### II.3.1. Biochemical Characterization of LNS and ESM

#### II.3.1.1. Dosage of protein

The protein content was determined by the method of Bradford (1976). It is based on interactions between basic amino acid residues; mainly arginine, lysine, and histidine, with the bright dye Coomassie Blue G-250 (fig. 17) (Bianchi et al., 1996). After the binding of the reagent with these hydrophobic amino acids, the absorbance can be obtained using a spectrophotometer at the maximum wavelength of 465-595 nm. Therefore, from this absorbance, the protein concentration is easily deduced by the Beer-Lambert law. A dry glass test tube is filled with 200 µl of the standard or sample. 2 ml of Coomassie reagent is added and mixed for 30 seconds. The absorbance at 595 nm is measured two minutes into the reaction and before an hour has passed. The calibration curve of the concentration of bovine serum albumin help determine the sought protein concentrations (Bradford, 1976).

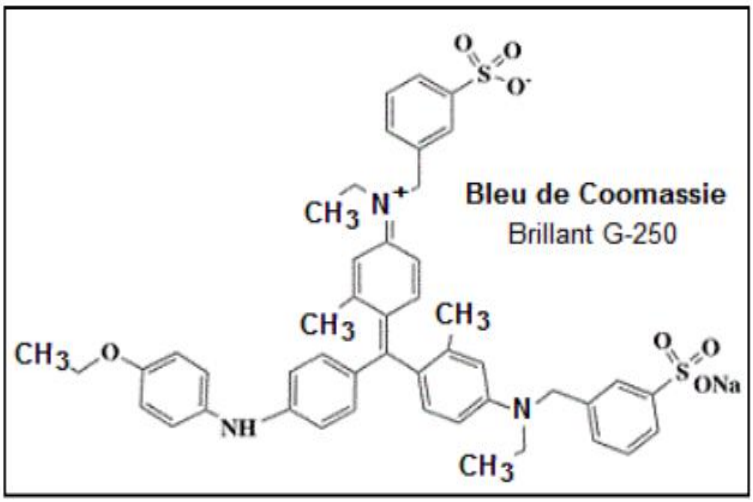



**Figure 17.** Coomassie Blue G-250 brilliant dye (Bradford, 1976).

### II.3.1.2. Dosage of total sugar

The Dubois method (1956) is used to determine total sugar concentrations. Simple sugars, oligosaccharides, polysaccharides, and their derivatives give a yellow-orange color when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and color-stable (Dubois et al., 1956). In the presence of concentrated sulfuric acid and under heat, glycosidic bonds are hydrolyzed. Tetradehydration followed by cyclization of the released monosaccharides gives furfuralic derivatives and 5-formylfuroic acid derivatives. These products condense with phenol to give yellow-orange complexes. The appearance of these complexes is followed by an absorbance measurement at 490 nm, which allows the total saccharide concentration of the analyzed sample to be determined by reference to a calibration curve, for which glucose is used as the standard (Brian-Jaisson, 2014; Brudieux, 2007). In glass tubes, 200 µL of sample or standard are mixed with 200 µL of phenol and 1 ml of concentrated sulfuric acid. The tubes are incubated at 90°C for 5 minutes. They are then left at room temperature for 30 minutes, protected from light. The absorbance is measured at 492 nm (Brudieux, 2007).

### II.3.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared (IR) spectroscopy measures the absorption of infrared radiation by chemical bonds in a material. Chemical structural fragments of molecules, known as functional groups, tend to absorb IR radiation in the same frequency range regardless of the structure of the rest of the molecule that the functional group is in. This correlation between the structure of a molecule and the frequencies at which it absorbs IR radiation allows the structure of unknown molecules to be identified and structural or chemical changes of the molecule to be followed (Simmons, 1999).

### II.3.1.4. X-Ray Diffractometer (XRD)

The X-ray diffraction (XRD) technique is used to analyze the atomic or molecular structure of materials in a non-destructive way. It is particularly effective for crystalline or semi-crystalline substances. The material is often ground into a fine powder, which is why it is also called "X-ray powder diffraction." The technique relies on the principle of diffraction, where X-rays bend when they encounter obstacles or apertures. The extent of diffraction depends on the wavelength of the X-rays compared to the size of the obstacle (Hanafi and Zaki, 2023). Bragg described diffraction and interference of X-rays in a crystal as reflections at the atomic planes of the crystal lattice. The positions of the reflections are calculated using the optical path difference 2s, with s = **d sin θ**, between two reflected rays at neighboring interplanar spacings. As in visible light optics, maxima are produced for integer multiples of **λ** (Ermrich, 2011). It follows **2 d sin θ λ = n** (Bragg's law) d interplanar spacing dhkl (hkl Miller indices):

- **θ** Bragg angle θB
- **2θ** angle between incident and reflected beam
- **n** 'order' of the interference **n** = 1, 2, 3 ..., normally **n** = 1 (called "Reflection of Order)
- **λ** wavelength

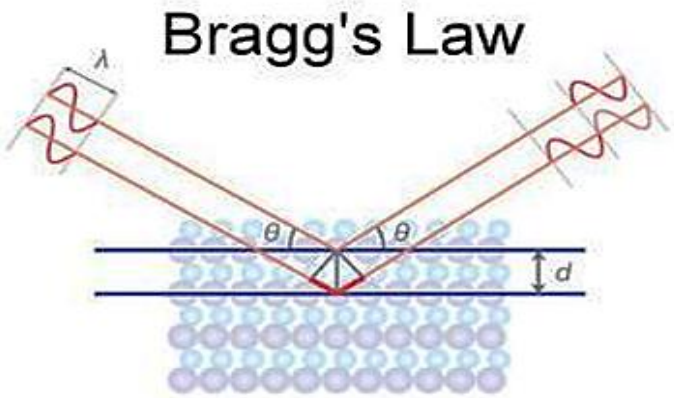



**Figure 18.** Graphical representation of Bragg's law (Hanafi and Zaki, 2023)

### II.3.2. Preparation of ESM/LNS mixture

LNS seeds were dried and ground using an electric grinder into a fine powder. The powder was then sieved at 0.05 mm by vibrational sieving (Tsai et al., 2006). The obtained powder was stored in a tightly sealed container at room temperature (Miganeh et al., 2021). The ESM was thoroughly washed with distilled water several times and dried in an encubator (POL-EKO sp. K) for 24 hours at 37°C. After drying, the ESM was ground using an electric grinder into a fine powder

which was then sieved at 0.05 mm by vibrational sieving (Tsai et al., 2006). After that, the prepared ESM and LNS powders was mixed in equal proportions (50% each). The resulting combination was incorporated into a Vaseline base (Baakdah et al., 2023) by manual stirring. After achieving a homogeneous mixture, the obtained ointment was exposed to ultraviolet light (UV-C) for 20 minutes to ensure decontamination and microbial inactivation (Esmaeili et al., 2024; Kowalski, 2009). The final product was then stored in tightly sealed containers until further use.

### II.3.3. Wound healing activity

Each *Wistar albino* rat were placed in a closed box with a cotton swab containing chloroform drops for temporary immobilization. The hair on the back was removed using a sterile clipper (Ahmed et al., 2019). The area was disinfected with betadine (povidone-iodine), and the rat were anesthetized locally with lidocaine (Mattea et al., 2021). After 3 minutes, a full thickness circular wound measuring 15±3mm in diameter was made in the skin layer at the level of the lumbar spine of each rat (Suneel et al., 2018). The prepared ointments was applied directly to the wound from different groups (5 to 6 rats per group), as follows:

- **Group 1**: served as normal rats presenting healthy skin without any wound incision;
- **Group 2**: served as control rats presenting wounds treated with Vaseline only;
- **Group 3**: served as test rats presenting wounds treated with LNS-Vaseline mix;
- **Group 4**: served as test rats presenting wounds treated with LNS-ESM-Vaseline mix.

The treatment was applied only once per treatment period and then covered with a sterile bandage to prevent wound infection. After that, the rats were placed in individual cages to recover. The wound areas were photographed (adjacent to a ruler) using a digital camera to capture and record images of the wound (i.e., days 1, 3, 4, 5, and 6) (Li et al., 2013). The area of the open wound was estimated using ImageJ software. The area of a given wound, at a given time point, was expressed as a percentage of the area of that wound immediately after injury (i.e., day 1), as follows (Ahmed et al., 2019; Galiano et al., 2004):

$$\text{Wound healing rate (WHR)} = \frac{Ai - A}{Ai} \times 100$$

Where:

- Ai is the initial wound area calculated on day 0
- A is the wound area on day t

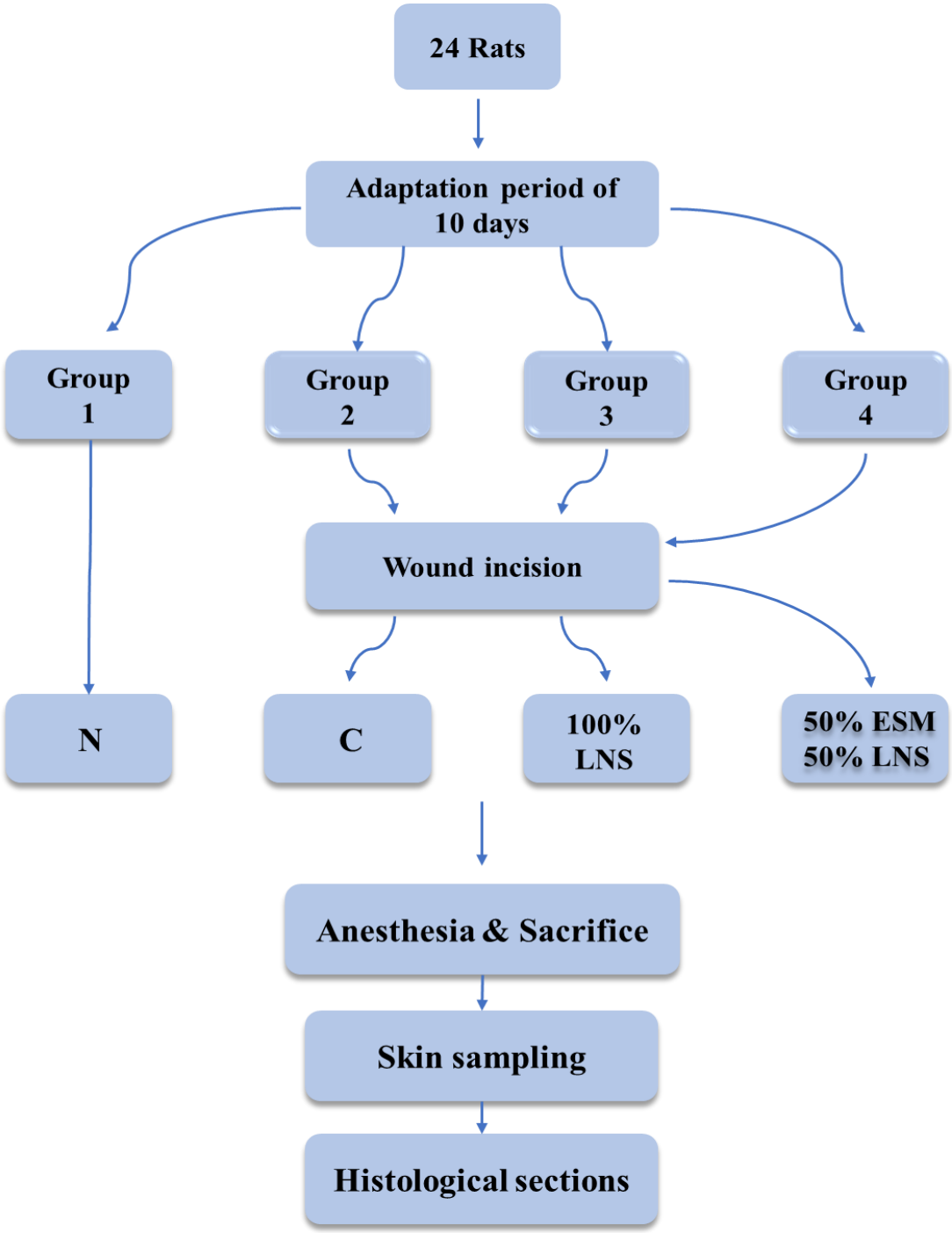



**Figure 19.** Experimental design of wound healing activity

### II.3.3.1. Sacrifice and collection of skin tissue

The rats were anesthetized with chloroform (94%), decapitated, and the wound area was shaved, and skin tissue was excised to complete the experiment (Kamali and Mohri, 2015; Mohri and Rezapoor, 2009). The tissues were immediately preserved in 10% formalin solution for histological analysis (Zeman et al., 2021; Fischer et al., 2008; Kiernan, 2000).

### II.3.3.2. Histological sections

Tissue sections of each specimen are prepared after sacrifice and dissection, following the procedures described below (Andre et al., 2020; Derai, 2016; Bahi, 2015; Djeffal, 2014; Djoudad Kadji et al., 2011):

#### *Fixation*

To preserve structures in a condition as close to the natural status as possible, with halted enzymatic activity, 10% formalin is used as fixative agent.

#### *Dehydration*

In order to create a thin section later without losing the original cellular structure at the time of plasma membrane rupture (sudden water release), this phase aims to eliminate intracellular water. The sample is passed through alcohol baths with progressively higher concentrations (from 50% diluted alcohol to 100% absolute alcohol). Because paraffin is hydrophobic, at this stage the samples become ready for embedding.

#### *Inclusion*

The purpose of embedding is to create histological sections. The most commonly used embedding medium is paraffin (an opaque white resin). Upon removal from the circulation apparatus, the tissue is removed from its cassette using forceps and placed directly into a metal mold containing drops of molten paraffin, ensuring a section of all the tissues is obtained. The mold is then filled with paraffin and placed on a cooling plate to facilitate demolding.

#### *Tissue sectioning*

After embedding, the paraffin blocks are thinly sliced using a microtome. The slices are put on glass slides after being softened in a water bath at 45°C. They are set on metal slide holders and then dried in an oven set to 40°C for about an hour.

#### *Coloring*

The hematoxylin-eosin (or hemalon) technique is used according to Tessier (2012) with minor modifications. The histological sections were immersed in a series of staining baths for a total duration of 67 minutes, following a standardized hematoxylin and eosin (H&E) staining

protocol (fig. 20). Initially, the slides were deparaffinized in two xylene baths, each for 15 minutes. This was followed by rehydration through four ethanol baths of decreasing concentrations (100% to 80%), for a total of 8 minutes. Subsequently, the slides were rinsed in distilled water for 5 minutes, then immersed in a solution containing 1 mL of hydrochloric acid (HCl) and 400 mL of 70% ethanol for 3 minutes, followed by a 1-minute rinse in distilled water. For nuclear staining, the sections were placed in hematoxylin for 30 seconds, rinsed under running tap water for 3 minutes, and then incubated in a hot water bath at 60°C for 10 minutes. A further 3-minute rinse in distilled water was performed before counterstaining with aqueous eosin for 20 seconds. The sections were then rinsed again under tap water for 3 minutes, followed by a final rinse in distilled water. Finally, the sections were dried in an oven at 60°C for 10 minutes to ensure complete drying before microscopic observation (Djoudad-Kadji et al., 2011; André et al., 2008).

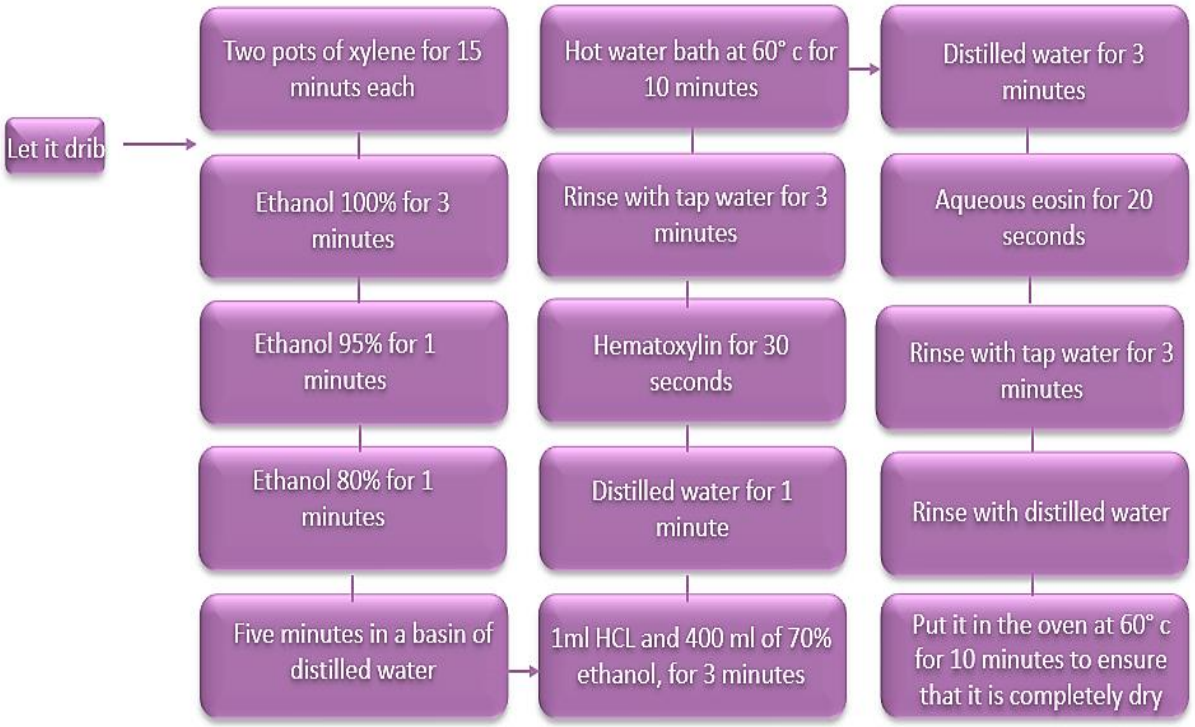



**Figure 20.** Hematoxylin-eosin staining steps

***Microscopic observation***

At this stage, a histological slide is created that is ready for microscopic observation. To ensure clear vision, some synthetic resin drops must be carefully adjusted to the colored lamé by placing them on it. This will prevent air bubbles. Finally, the residues are allowed to dry. After that, it is observed using an optical microscope with magnifications of x40 and x10.

### II.3.4. Statistical analysis

The data analysis was performed using XLSTAT (version 2016.02.28451). Results are presented as means ± standard error of the mean (M ± SEM). The Shapiro-Wilk test was used to assess the normality of the data. The ANOVA test followed by Tukey's HSD test was conducted to identify statistically significant differences. Significance levels were considered as follows: significant (*) if $p \leq 0.05$, highly significant (**) if $p \leq 0.01$, and very highly significant **(***) if $p \leq 0.001$.

# CONCLUSION AND PERSPECTIVES

## Conclusion

Poultry eggs have been extensively consumed for domestic and industrial uses. A large amount of eggshell waste is generated and discarded every year, resulting in a waste of natural resources and a threat to the environment. In this context, the purpose of this study is to evaluate the effects of the eggshell membrane (ESM) and zinc enriched plant seeds (LNS) on wound healing. The study was conducted on eggshell waste collect from sweets shops and nutritional plant seeds obtained from El Oued (Algeria).

For partial characterization, colorimetric assays helped determined the amount of total sugars and proteins in both samples. Moreover, Fourier transformed infrared spectroscopy and X-ray diffraction were used to define some of the structural proprieties of ESM and LNS. The study evaluates wound healing activity *in vivo* by applying an ointment consisting of 50 % of ESM and 50% of LNS mixed with petroleum jelly (Vaseline) as a basis.

Biochemical analysis demonstrated that ESM protein content, reaching 88.94%, compared to only 7.40% in LNS. Total sugar content was also higher in ESM (7.25%) than in LNS (1.50%). These results highlight the fibrous and protein-rich nature of ESM, dominated by collagen and glycoproteins.

FTIR spectroscopy confirmed the presence of protein-related functional groups (e.g., amides) in ESM, whereas LNS spectra showed prominent peaks corresponding to polysaccharides and ester/phosphoryl bonds. Moreover, X-ray diffraction (XRD) revealed a crystalline structure in ESM due to calcium carbonate, while LNS exhibited an amorphous pattern, consistent with its contents of carbohydrates and dietary fibers.

In wound healing activity evaluated in *Wistar albino* rats model, the results showed that the ESM+LNS combination significantly accelerated wound closure compared to LNS alone or to the control group. By day six, the wound healing rate reached approximately 75% in the ESM+LNS group, versus 60% in the LNS group and only 23% in the control group, indicating a clear synergistic effect.

Histological sections confirmed that the ESM+LNS group achieved near-complete skin restoration, with reorganized collagen fibers and reappearance of dermal appendages such as sebaceous glands and hair follicles—features not observed in the other groups. These findings support the hypothesis that LNS contributes antioxidant and pro-angiogenic properties, while ESM provides a structural and bioactive scaffold that accelerates tissue regeneration.

Based on these results, the ESM and LNS combination can be considered a promising natural treatment for enhanced wound healing, uniting the bioactivity of plant-derived compounds with the structural support of an animal-derived matrix.

**Perspectives**

Eggshell membrane powder is a natural byproduct rich in proteins, collagen, and biologically active compounds, thus opening the door to numerous future research directions beyond its application in wound healing.

- One of the most promising avenues involves the use of ESM as a **natural dietary supplement** due to its content of amino acids and collagen, which may support joint, bone, and skin health.
- ESM can also be investigated as a plant growth enhancer when combined with organic fertilizers, thanks to its nitrogenous compounds and trace elements.
- Moreover, emerging studies suggest its potential role in water treatment, where it may act as an adsorbent for removing heavy metals and organic pollutants.
- Another promising application lies in the development of biomedical materials for use **in dentistry or tissue engineering**, such as biofilms or bone graft substitutes, due to its collagen content and tissue-regenerating properties.
- Additionally, it can be processed into nanoparticles for drug delivery systems or as antimicrobial agents.
- In the food industry, eggshell membrane powder may be utilized to **enhance the nutritional value of functional foods**.
- Furthermore, it is being explored as an additive in the production of biodegradable polymers for eco-friendly packaging materials.

Based on these diverse applications, eggshell membrane powder represents a promising biomaterial that warrants further research and development in the fields of health, agriculture, biotechnology, and environmental sustainability.

# REFERENCES

1. Abdul, M. 2022. *Master of Science in Environmental Engineering.* Department of Civil Engineering, Bangladesh University of Engineering and Technology, Dhaka

2. Ahmed, N., Pires, M.L. and Pugh, N., 2020. Zinc: An endogenous and exogenous regulator of platelet function during haemostasis and thrombosis. *Platelets.*: http://mc.manuscriptcentral.com/cpla

3. Ahmed, T.A., Seddik, R.J. and Farid, R.M. (2020). Eggshell membrane: a natural biomaterial with diverse biomedical applications. *Materials Science and Engineering: C*, 109, p.110612.

4. Ahmed, T.A.E., Suso, H.P., Hincke, M.T. and Pedersen, M.E., 2019. Processed eggshell membrane powder regulates cellular functions and increases MMP activity important in early wound healing processes. *PLoS ONE*, 14(8), p.e0201975. https://doi.org/10.1371/journal.pone.0201975

5. Alina, S., K. A. Katarzyna, K Alok , M., Teotia, M., Soni, R.K. and Mittal, J., 2016. Applications of egg shell and egg shell membrane as adsorbents: A review. *Journal of Molecular Liquids*, https://doi.org/10.1016/j.molliq.2016.08.

6. Amirrah, I.N., Lokanathan, Y., Zulkiflee, I., Wee, M.F.M.R., Motta, A. and Fauzi, M.B., 2022. *A comprehensive review on collagen type I development of biomaterials for tissue engineering: From biosynthesis to bioscaffold*. Biomedicines, 10(9), p.2307. https://doi.org/10.3390/biomedicines10092307

7. Anderson, J.W., Baird, P., Davis, R.H., Ferreri, S., Knudtson, M., Koraym, A., Waters, V. and Williams, C.L., 2013. Health benefits of dietary fiber. *Nutrition Reviews*, 67(4), pp.188–205. https://doi.org/10.1111/j.1753-4887.2009.00189.x

8. Andre, J.M., Catala, M., Morere, J.J., Escudier, E., Katsanis, G. and Faye, B., 2020. How many large camelids in the world? A synthetic analysis of the world camel demographic changes. *Pastoralism*, 10, p.25.

9. André, J.M., Catala, M., Morere, J.J., Escudier, E., Katsanis, G. and Poirier, J., 2008. *Histologie: les tissus*. Faculté de Médecine, Université Pierre et Marie Curie. https://mcours.net/cours/pdf/scien/Histologie.

10. Azad, M. and Avin, A., 2018. Scanning electron microscopy (SEM). *International Conference on Hydraulics and Pneumatics – HERVEX*.

11. Baakdah, F., Ashi, A., Almaghrabi, S., Ismail, K.A., Sami, R., Alshehry, G., Aljumayi, H., Algarni, E., Alorabi, J.A., Alshahrani, M.Y., Algehainy, N.A. and Alanazi, M.A., (2023). Healing processes of burn wounds with honey and vaseline as ointment forms: An in-vivo study in Wistar rats. *Materials Express*, 13, pp.2042–2048. https://doi.org/10.1166/mex.2023.2569

12. Bahi, A., 2015. L'effet protecteur des antioxydants naturels dans l'intoxication au mercure chez le rat Albinos Wistar (Aspects biochimiques, immunologiques et histologiques). Thèse de doctorat, Université des Frères Mentouri Constantine, Algérie.

13. Balá, Ž. 2014. "Eggshell Membrane Biomaterial as a Platform for Applications in Materials Science." *Acta Biomaterialia* 10 (9): 3827–3843. https://doi.org/10.1016/j.actbio.2014.03.020

14. Baláž, M., Ficeriová, J. and Briančin, J. (2016). *Influence of milling on the adsorption ability of eggshell waste*. Chemosphere, 146, pp. 458–471. Publisher: Elsevier. DOI: https://doi.org/10.1016/j.chemosphere.2015.12.002

15. Barth, A., 2007. Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1767(9), pp.1073–1101. https://doi.org/10.1016/j.bbabio.2007.06.004

16. Benjamin, J., J. Bielajew, J. C. Hu, and K. A. Athanasiou. 2020. "Collagen Quantification, Biomechanics, and Role of Minor Subtypes in Cartilage." *Nature Reviews Materials*. Department of Biomedical Engineering, University of California, Irvine, Irvine, CA, US. https://doi.org/10.1038/s41578-020-0213-1

17. Bewley, J.D., Bradford, K., Hilhorst, H. and Nonogaki, H., 2013. *Seeds: Physiology of Development, Germination and Dormancy*. 3rd ed. New York: Springer.

18. Bianchi, D.P., Golini, R. and Bortolo, P., 1996. Immobilization of penicillin G acylase on aminoalkylated polyacrylic supports. *Enzyme and Microbial Technology*, 18, pp.592–596

19. Boateng, J.S. and Catanzano, O., 2015. Advanced therapeutic dressings for effective wound healing—A review. *Journal of Pharmaceutical Sciences*, 104(11), pp.3653–3680. https://doi.org/10.1002/jps.24610

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

21. Bonmati, M.B., Ceccanti, P., Nannipieri, J. and Valero, 2009. Characterization of humus–protease complexes extracted from soil. *Soil Biology and Biochemistry*, 41, pp.1199–1209.

22. Boye, J., Zare, F. and Pletch, A., 2010. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, 43(2), pp.414–431. https://doi.org/10.1016/j.foodres.2009.09.003

23. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), pp.248–254. https://doi.org/10.1016/0003-2697(76)90527-3

24. Brem, H. and Tomic-Canic, M., 2007. Cellular and molecular basis of wound healing in diabetes. *Journal of Clinical Investigation*, 117(5), pp.1219–1222. https://doi.org/10.1172/JCI32169

25. Caley, M.P., Martins, V.L.C. and O'Toole, E.A., 2015. Metalloproteinases and wound healing. *Advances in Wound Care*, 4(4), pp.225–234. https://doi.org/10.1089/wound.2014.0581

26. Chouana, T., 2017. Caractérisation structurale et activités biologiques des polysaccharides d'Astragalus gombo Bunge. PhD thesis. Université Clermont Auvergne

27. Chowdhury, S.R., Alam, M.M. and Kabir, A.N.M.H., 2023. Composite biomaterials for skin regeneration: A review. *Materials Science and Engineering: C*, 145, p.114693. https://doi.org/10.1016/j.msec.2023.114693

28. Coates, J., 2000. Interpretation of infrared spectra, a practical approach. In: R.A. Meyers, ed. *Encyclopedia of Analytical Chemistry*. Chichester: John Wiley and Sons Ltd., pp.10815–10837.

29. Cobo, R., García-Piqueras, J., Cobo, J. and Vega, J.A., 2021. The human cutaneous sensory corpuscles: An update. *Journal of Clinical Medicine*, 10(2), p.227. https://doi.org/10.3390/jcm10020227.

30. Coger, V., Million, N., Rehbock, C., Sures, B., Nachev, M., Barcikowski, S., Wistuba, N., Strau, S. and Vogt, P.M., 2019. Tissue concentrations of zinc, iron, copper, and magnesium during the phases of full-thickness wound healing in a rodent model. *Biological Trace Element Research*, 191, pp.167–176. https://doi.org/10.1007/s12011-018-1600-y.

31. Derai, E.H., 2016. *Effet de la combinaison de la vitamine C et la vitamine E sur le métabolisme et la distribution du zinc chez des rats diabétiques sous un régime alimentaire pauvre en zinc*. Thèse de doctorat, Université Badji Mokhtar, Algérie.

32. Djeffal, A., 2014. Évaluation de la toxicité d'un insecticide carbamate "méthomyl" chez le rat Wistar*: stress oxydant*. Thèse de doctorat, Université Badji Mokhtar, Algérie.

33. Djoudad-Kadji, H., Benslimane, S., Chevalier, C., Kadji, B.,Exbrayat, J. M., And Iguer-Ouada, M. (2011). Visualisation des coupes histologiques des follicules ovariens de barbus callensis variation de fixateurs et de colorants. Rev. fr. histotechnol, 24(1), 21-28. https://www.academia.edu/download/33784099/Kadji_et_al.__2011_AFH.pdf

34. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), pp.350–356. https://doi.org/10.1021/ac60111a017

35. Dueñas, M., Hernández, T., Estrella, I., Fernández, D. and Muñoz, R., 2002. Bioactive phenolic compounds of cowpeas (*Vigna sinensis* L). Modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *Journal of the Science of Food and Agriculture*, 82(3), pp.265–272. https://doi.org/10.1002/jsfa.1032

36. Elizabeth, C. N., R. E. B. Watson, and M. J. Sherratt. 2011. "Molecular Aspects of Skin Ageing." *Maturitas* 69: 249–256. https://www.elsevier.com/locate/maturitas

37. Eming, S.A., Krieg, T. and Davidson, J.M., 2007. Inflammation in wound repair: molecular and cellular mechanisms. *Journal of Investigative Dermatology*, 127(3), pp.514–525. https://doi.org/10.1038/sj.jid.5700701

38. Eming, S.A., Martin, P. and Tomic-Canic, M., 2014. Wound repair and regeneration: Mechanisms, signaling, and translation. *Science Translational Medicine*, 6(265), p.265sr6. https://doi.org/10.1126/scitranslmed.3009337

39. Enoch, S., and D. J. Leaper. 2008. "Basic Science of Wound Healing." *Surgery* 26 (6): 31–37. https://doi.org/10.1016/j.mpsur.2007.11.005.

40. Ermrich, M., 2011. XRD for the analyst: Getting acquainted with the principles. *PANalytical*. www.panalytical.com.

41. Esmaeili, S., Rahmati, M., Zamani, S., Arabpour, Z. and Salehi, M., (2024). A comparison of several separation processes for eggshell membrane powder as a natural biomaterial for skin regeneration. *Skin Research and Technology*, https://doi.org/10.1111/srt.70038

42. Fernandez, M. S., C. Escobar, I. Lavelin, M. Pines, and J. L. Arias. 2003. "Localization of Osteopontin in Oviduct Tissue and Eggshell during Different Stages of the Avian Egg Laying Cycle." *Journal of Structural Biology* 143 (2): 171–180.

43. Fischer, A.H., Jacobson, K.A., Rose, J. and Zeller, R., 2008. Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harbor Protocols*, 2008(5), pdb.prot4986. https://doi.org/10.1101/pdb.prot4986.

44. Fladerer, J. P., and S. Grollitsch. 2024. "Eggshell Membrane as a Promising Supplement to Maintain Bone Health: A Systematic Review." *Bone Reports* 21: 101776. https://www.elsevier.com/locate/bonr

45. Freeland, G.J.H., Sanjeevi, N. and Lee, J.J., 2015. Global perspectives on trace element requirements. *Journal of Trace Elements in Medicine and Biology*, 31, pp.135–141

46. Freeland-Graves, J.H. and Sanjeevi, N., 2015. Nutritional role of zinc in wound healing and tissue repair. *Advances in Nutrition*, 6(5), pp.568–570. https://doi.org/10.3945/an.115.009514.

47. Freeland-Graves, J.H., Sanjeevi, N. and Lee, J.J., 2014. *Global perspectives on trace element requirements*. Journal of Trace Elements in Medicine and Biology. https://doi.org/10.1016/j.jtemb.2014.04.006

48. Galiano, R.D., Michaels, J.T., Dobryansky, M., Levine, J.P. and Gurtner, G.C., 2004. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair and Regeneration*, 12(4), pp.485–492. https://doi.org/10.1111/j.1067-1927.2004.12404.x.

49. Ganesan, K. and Xu, B., 2017. Polyphenol-rich lentils and their health-promoting effects. *International Journal of Molecular Sciences*, 18(11), p.2390. https://doi.org/10.3390/ijms18112390

50. Gautron, J., Bain, M. and Hincke, M.T., 2011. Eggshell proteins and their physiological role in avian eggshell formation and protection. *Frontiers in Bioscience*, 16, pp.1339–1361.

51. Gautron, J., Réhault-Godbert, S., Pascal, G. and Nys, Y., 2011. Ovotransferrin is a matrix protein of the eggshell membranes and basal calcified layer of the eggshell. *Matrix Biology*, 30(2), pp.117–124 https://doi.org/10.1016/j.matbio.2010.11.004.

52. Germann, C., Sutter, R. and Nanz, D., 2021. Novel observations of Pacinian corpuscle distribution in the hands and feet based on high-resolution 7-T MRI in healthy volunteers. *Skeletal Radiology*, 50(6), pp.1135–1142. https://doi.org/10.1007/s00256-020-03644-6.

53. Giansanti, F., Massucci, M. T., Giardi, M. F., and Benedetti, P. A. (2021). Eggshell membrane: a biocompatible platform for bioactive compound delivery. International Journal of Molecular Sciences, 22(3), 1350. https://doi.org/10.3390/ijms22031350

54. González-Andrades, M. et al., 2016. Generation of bioengineered human skin with a stratified dermo-epidermal structure. *Journal of Tissue Engineering and Regenerative Medicine*, 10(5), pp.431–441. https://doi.org/10.1002/term.1920

55. Goormaghtigh, E., Cabiaux, V. and Ruysschaert, J.M., 1990. Secondary structure and dosage of soluble and membrane proteins by attenuated total reflection Fourier-transform infrared spectroscopy on hydrated films. *European Journal of Biochemistry*, 193(2), pp.409–420. https://doi.org/10.1111/j.1432-1033.1990.tb19354.x

56. Gupta, A., Kowalczuk, M., Heaselgrave, W., Britland, S., Martin, C., and Radecka, I. (2019). The production and application of hydrogels for wound management: A review. European Polymer Journal, 111, 134–151. https://doi.org/10.1016/j.eurpolymj.2018.12.019

57. Gurtner, G.C., Werner, S., Barrandon, Y. and Longaker, M.T., 2008. Wound repair and regeneration. *Nature*, 453(7193), pp.314–321. https://doi.org/10.1038/nature07039

58. Haddad, B., Mittal, A., Mittal, J., Paolone, A., Villemin, D., Mansour, D., Goussem, M., Asmaa, H., Zineb, H., Boumediene, M., 2021. *Synthesis and characterization of egg shell (ES) and egg shell with membrane (ESM) modified by ionic liquids*. Chemical Data Collections, 33, p.100717. https://doi.org/10.1016/j.cdc.2021.100717

59. Han, C., Chen, Y., Shi, L., Chen, H., Li, L., Ning, Z., Zeng, D. and Wang, D., 2023. *Advances in eggshell membrane separation and solubilization technologies*. Frontiers in Veterinary Science, 10, p.1116126. https://doi.org/10.3389/fvets.2023.1116126

60. Hanafi, A., and M. Zaki. 2023. "Biosorption of Alkali Treated Eggshell Waste for Dye Removal." *Universiti Teknologi MARA* (FEB)

61. Herman, T.F., Popowicz, P. & Bordoni, B. (2023). *Wound Classification*. In: StatPearls Treasure Island (FL): StatPearls Publishing; Updated 17 August 2023.

62. Hincke, M. T., J. Gautron, M. Panheleux, J. M. Garcia-Ruiz, M. D. McKee, and Y. Nys. 2000. "Identification and Localization of Lysozyme as a Component of Eggshell Membranes and Eggshell Matrix." *Matrix Biology* 19 (5): 443–453.

63. Hincke, M. T., Y. Nys, J. Gautron, K. Mann, A. B. Rodriguez-Navarro, and M. D. McKee. 2012. "The Eggshell: Structure, Composition and Mineralization." *Frontiers in Bioscience* 17: 1266–1280. https://doi.org/10.2741/3985.

64. Hincke, T. M., Y. Nys, and J. Gautron. 2010. "The Role of Matrix Proteins in Eggshell Formation." *Journal of Poultry Science Association* 47: 208–219. University of Ottawa, Canada. Department of Cellular and Molecular Medicine. http://www.jstage.jst.go.jp/browse/jpsa

65. Huang, L., Xu, H., Peng, Y. and Wu, D., 2019. Eggshell membrane: A natural biomaterial for tissue engineering. *Bone Research*, 7, p.26. https://doi.org/10.1038/s41413-019-0062-7

66. Hunt, T. K. 1988. "The Physiology of Wound Healing." *Annals of Emergency Medicine* 17 (12): 1265–1273. https://doi.org/10.1016/s0196-0644(88)80351-2.

67. Iqbal, A., Khalil, I.A., Ateeq, N. and Sayyar Khan, M., 2006. Nutritional quality of important food legumes. *Food Chemistry*, 97(2), pp.331–335. https://doi.org/10.1016/j.foodchem.2005.05.011

68. Jackson, M. and Mantsch, H.H., 1995. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Critical Reviews in Biochemistry and Molecular Biology*, 30(2), pp.95–120. https://doi.org/10.3109/10409239509085140

69. Jadach, B., Mielcarek, Z. and Osmałek, T., 2024. Use of collagen in cosmetic products. *Current Issues in Molecular Biology*, 46, pp.2043–2070. https://doi.org/10.3390/cimb46030132.

70. Jiang, T., Liu, W., Lv, Y., Zhang, T. and Li, J., 2019. Eggshell membrane: A natural biomaterial for regenerative medicine. *Frontiers in Materials*, 6, p.175. https://doi.org/10.3389/fmats.2019.00175

71. Jukanti, A.K., Gaur, P.M., Gowda, C.L.L. and Chibbar, R.N., 2012. Nutritional quality and health benefits of pulses. *Crop Journal*, 1(3), pp.117–130.

72. Kähkönen, M.P. *et al.*, 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, 47(10), pp.3954–3962. https://doi.org/10.1021/jf990146l

73. Kamali, H. and Mohri, M., 2015. Effects of heparin, citrate, and EDTA on plasma biochemistry of cat: comparison with serum. *Revue Méd. Vét.*, 166(9–10), pp.275–279. https://www.researchgate.net/publication/282977524.

*74.* Katarzyna M., and Magdalena M. G. 2020. "Collagen-Based Materials in Cosmetic Applications." *Materials* 13: 4217. https://doi.org/10.3390/ma13194217. www.mdpi.com/journal/materials.

75. Kiernan, J.A., 2000. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. *Microscopy Today*, 00-1, pp.8–12. https://doi.org/10.1017/S1551929500057060

76. Kim, K., Jung, S.H. and Lee, J.H., 2021. Bioactive peptides derived from eggshell membrane stimulate skin regeneration. *International Journal of Molecular Sciences*, 22(15), p.7852. https://doi.org/10.3390/ijms22157852

77. King'ori, A.M., 2011. A review of the uses of poultry eggshells and shell membranes. *International Journal of Poultry Science*, 10(11), pp.908–912. https://doi.org/10.3923/ijps.2011.908.912.

78. Kowalski, W.L. (2009). *UV Surface Disinfection*. In: Ultraviolet Germicidal Irradiation Handbook: UVGI for Air and Surface Disinfection (Chapter 11, pp. 233–254). Berlin, Heidelberg: Springer. DOI: https://doi.org/10.1007/978-3-642-01999-9_11

79. Kuehl, L.K. et al., 2021. Skin structure and function: The contributions of the extracellular matrix. *Matrix Biology Plus*, 11, p.100076. https://doi.org/10.1016/j.mbplus.2021.100076

80. Kueper, T., Grune, T., Prahl, S., Linsenmayer, T., Müller, P., Nerlich, A.G. and Elsässer, H.P., 2007. Vimentin is the specific target in skin glycation: AGEs modify vimentin and impair cytoskeletal function. *FASEB Journal*, 21(13), pp.2590–2601.

81. Kumar, M.P.S. and Leaper, D.J. (2008). *Classification and management of acute wounds*. Surgery (Oxford), 26(2), pp. 43–47. Publisher: Elsevier Ltd. DOI: https://doi.org/10.1016/j.mpsur.2007.11.003

82. Kumar, S., Yarmush, M.L., Dash, B.C., Hsia, H.C. and Berthiaume, F., 2018. Impact of complete spinal cord injury on healing of skin ulcers in mouse models. *Journal of Neurotrauma*, 35(6), pp.815–824. https://doi.org/10.1089/neu.2017.5405.

83. Lansdown, A.B.G., Mirastschijski, U., Stubbs, N., Scanlon, E. and Ågren, M.S., (2007). *Zinc in wound healing: Theoretical, experimental, and clinical aspects*. Wound Repair and Regeneration, 15(1), pp.2–16. https://doi.org/10.1111/j.1524-475X.2006.00179.x

84. Lazarus, G. S., D. M. Cooper, D. R. Knighton, D. J. Margolis, R. E. Pecoraro, G. Rodeheaver, and M. C. Robson. 1994. "Definitions and Guidelines for Assessment of Wounds and Evaluation of Healing." *Wound Repair and Regeneration* 2 (3): 165–170

85. Lee, C.H., Singla, A. and Lee, Y., 2020. Biomedical applications of collagen. *International Journal of Pharmaceutics*, 221(1–2), pp.1–22.https://doi.org/10.1016/S0378-5173(01)00691-3

86. Lee, J.Q., Walkey, A.J. and Mizgerd, J.P., 2018. Integrative physiology of pneumonia. *Physiological Reviews*, 98, pp.1417–1464. https://doi.org/10.1152/physrev.00032.2017

87. Li, J., Chen, J. and Kirsner, R., 2013. Pathophysiology of acute wound healing. *Clinics in Dermatology*, 25(1), pp.9–18. https://doi.org/10.1016/j.clindermatol.2006.09.007

88. Li, Z., Zhang, X., Gu, J. and Wang, Y., 2016. Structural and mechanical properties of eggshell membrane as a biomaterial. *Applied Surface Science*, 369, pp.342–351. https://doi.org/10.1016/j.apsusc.2016.02.017

89. Liang, X., Cong, H., Jiang, G., Rao, R.P., He, H. and Ramakrishna, S. (2024). Eggshell membrane: Structure, purification, properties and multifunctional applications. *Food Bioscience*, 60, 104487. https://doi.org/10.1016/j.fbio.2024.104487

90. Lin, P.H., Sermersheim, M., Li, H., Lee, P.H.U., Steinberg, S.M. and Ma, J., 2018. *Zinc in wound healing modulation: A review*. Nutrients, 10, p.16. https://doi.org/10.3390/nu10010016

91. Lopez-Dee, Z., Pidcock, K. and Gutierrez, L.S., 2011. Thrombospondin-1: Multiple paths to inflammation. *Mediators of Inflammation*, 2011, p.296069. https://doi.org/10.1155/2011/296069

92. Maqsood, Irfan. 2018. "Classification of Wounds: Know Before Research and Clinical Practice." *Journal of Genes and Cells* 1–4. https://www.genesandcells.com/journal

93. Marion M.. Bradford. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Reproduction Research Laboratories, Department of Biochemistry, University of Georgia, Athens, Georgia 30602, analytical biochemistry ,72, 248-254)

94. Matej Baláz, Eggshell membrane biomaterial as a platform for applications in materials science , Institute of Geotechnics, Slovak Academy of Sciences, Watsonova 45, 04001 Košice, Slovakia, journal homepage: www.elsevier.com/locate/actabiomat

95. Matej, B. 2014. "Eggshell Membrane Biomaterial as a Platform for Applications in Materials Science." *Acta Biomaterialia* 10 (9): 3827–3843

96. Maxwell, T., K. Hincke, Y. Nys, J. Gautron, K. Mann, A. Bédécarrats, N. Rodriguez, M. Dolfus, and M. McKee. 2012. "The Eggshell Structure, Composition and Mineralization." Frontiers in Bioscience 17: 1266–1280. https://www.researchgate.net/publication/51895246

*97.* McGrath, J. A., R. A. J. Eady, and F. M. Pope. 2004. "Anatomy and Organization of Human Skin." In *Rook's Textbook of Dermatology*, edited by T. Burns, S. Breathnach, N. Cox, and C. Griffiths, 3.1–3.84. Oxford: Blackwell Science Ltd.

98. Melat, C., M. Alef, C. Emin, L. Nalian, P. Stelian, and S. Rodica. 2019. "Collagen Sources and Areas of Use." *European Journal of Interdisciplinary Medicine and Natural Sciences* 2 (1): 122–128. https://doi.org/10.26417/ejis.v2i1.p122-128.

99. Miganeh, B., and A. Dhiren. 2021. "A Comparative Review of Eggshell Powders for Use in Concrete Production." *International Journal of Advanced Research in Engineering and Technology* 12 (5): 280–287. DOI 10.34218/IJARET.12.4.2021.030 :

100. Mittal, A., M. Teotia, R. K. Soni, and J. Mittal. 2016. "Applications of Eggshell and Eggshell Membrane as Adsorbents: A Review." *Desalination* 400: 1–12. https://doi.org/10.1016/j.molliq.2016.08.065

101. Mohri, M. and Rezapoor, H., 2009. Effects of heparin, citrate, and EDTA on plasma biochemistry of sheep: comparison with serum. *Research in Veterinary Science* https://www.sciencedirect.com/science/article/abs/pii/S003452880800115X

102. Monsigny, M., Petit, C. and Roche, A.-C., 1988. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Analytical Biochemistry*, 175(2), pp.525–530.

103. Mori, H.M., Kawanami, H., Kawahata, K., and Inoue, M. (2019). Enhancement of wound healing by plant extracts with antioxidant and anti-inflammatory properties. *Journal of Ethnopharmacology*, 241, p.112013.

104. Nan, Z., Y.-D. Liu, Y. Zhang, T. W. Gu, and L.-H. Peng. 2023. "Pharmacological Functions, Synthesis and Delivery Progress for Collagen as Biodrug and Biomaterial." *Pharmaceutics* 15 (5): 1443. https://doi.org/10.3390/pharmaceutics15051443.

105. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011. *Guide for the care and use of laboratory animals* (8th ed.). National Academies Press. https://doi.org/10.17226/12910

106. Nazeer, M., Saeed, M., Shah, A.M., Zhang, J. and Qamar, W., 2023. The efficiency of zinc sulfate immersion bath on improved wound healing in common carp. *Environmental Science and Pollution Research*, 30, pp.1234–1245. https://doi.org/10.1007/s13201-023-02077-z.

107. Ng, L.M. and Simmons, R., 1999. Infrared spectroscopy. Chemistry Department, Cleveland State University, Cleveland, OH.

108. Nga, E.N., Ngolsou, F., Nyangono Ndongo, M., Soppo Lobe, V., Betote, D.P.H., Benga Mekoulou, C. and Minkande, Z., 2020. Étude toxicologique in vivo de l'extrait aqueux des feuilles de *Psychotria calceata*. *Sciences and Disease*, 21(10)

109. Nimalaratne, C., Lopes-Lutz, D., Schieber, A., and Wu, J. (2015). Free radical scavenging and anti-inflammatory properties of peptide-enriched egg white protein hydrolysate. Food Chemistry, 166, 358–363. https://doi.org/10.1016/j.foodchem.2014.06.017

110. Nunan, R., Harding, K.G. and Martin, P., 2014. Clinical challenges of chronic wounds: Searching for an optimal animal model to recapitulate their complexity. *Disease Models and Mechanisms*, 7(11), pp.1205–1213. https://doi.org/10.1242/dmm.017741

111. Nys, Y., J. Gautron, J. M. Garcia-Ruiz, and M. T. Hincke. 2004. "Avian Eggshell Mineralization: Biochemical and Functional Characterization of Matrix Proteins." *Comptes Rendus Palevol* 3 (6–7): 549–562.

112. O'Brien, F.J., 2011. Biomaterials and scaffolds for tissue engineering. *Materials Today*, 14(3), pp.88–95. https://doi.org/10.1016/S1369-7021(11)70058-X

113. Ojeh, N., Pastar, I., Tomic-Canic, M. and Stojadinovic, O. (2015). *Stem cells in skin regeneration, wound healing, and their clinical applications*. International Journal of Molecular Sciences, 16(10), pp. 25476–25501. Publisher: MDPI (Multidisciplinary Digital Publishing Institute). https://doi.org/10.3390/ijms161025476

114. Park, E., Lee, J.H. and Kim, J.S., 2015. Preparation of egg shell membrane protein hydrolysates and evaluation of their antioxidant activity. *Food Science and Biotechnology*, 24(1), pp.1–7. https://doi.org/10.1007/s10068-015-0001-9

115. Park, J.H., Lee, S.H. and Han, S.H., 2018. Processed eggshell membrane powder regulates cellular functions and increases MMP activity important in early wound healing processes. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 106(4), pp.1524–1532. https://doi.org/10.1002/jbm.b.33951

116. Pastar, I. et al., 2008. Epithelialization in wound healing: A comprehensive review. *Advances in Wound Care*, 3(7), pp.445–464. https://doi.org/10.1089/wound.2013.0473

117. Pei Hui, L., Matthew, S., Haichang, L., Peter, H.U.L., Steinberg, S.M. and Jianjie, M., 2018. Zinc in wound healing modulation. *Nutrients*, 10(1), p.16. https://doi.org/10.3390/nu10010016

118. Pines, M., Knopov, V. and Bar, A., 2020. Eggshell mineralization in the domestic fowl: Cellular, molecular and hormonal regulation. *Poultry Science*, 99(10), pp.4964–4976. https://doi.org/10.1016/j.psj.2020.06.064.

119. Pinto, A.M., Cerqueira, M.T., Pereira, D.M. et al., 2017. Eggshell membrane-derived scaffolds for tissue engineering applications: Structure, properties and biological activity.

*ACS Applied Materials and Interfaces*, 9(36), pp.31606–31618. https://doi.org/10.1021/acsami.7b09580

120. Pollock, P. and Schumacher, J. (2012). *Principles of wound management*. In: *Equine Medicine, Surgery and Reproduction* (pp. 469–487). Elsevier. DOI: https://doi.org/10.1016/B978-0-7020-2801-4.00023-7

121. Proksch, E., Brandner, J.M. and Jensen, J.M., 2008. The skin: An indispensable barrier. *Experimental Dermatology*, 17(12), pp.1063–1072. https://doi.org/10.1111/j.1600-0625.2008.00786.x.

122. Quinton, L.J., Walkey, A.J. and Mizgerd, J.P., 2018. Integrative physiology of pneumonia. *Physiological Reviews*, 98, pp.1417–1464. https://doi.org/10.1152/physrev.00032.2017.

123. Ramshaw, J.A.M., Peng, Y.Y. and Glattauer, V., 2009. Collagens as biomaterials. *Journal of Materials Science: Materials in Medicine*, 20(1), pp.3–8. https://doi.org/10.1007/s10856-008-3541-6

124. Rath, N. C., Huff, G. R., Huff, W. E., and Balog, J. M. (2016). Applications of eggshell membrane in biomedical research and development. Biologicals, 44(4), 188–195. https://doi.org/10.1016/j.biologicals.2016.05.006

125. Rath, N.C., Huff, W.E. and Huff, G.R., 2003. Effects of dietary microencapsulated sodium butyrate on *Salmonella enteritidis* infection in broiler chickens. *Poultry Science*, 82(9), pp.1401–1409.

126. Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine*, 49(11), pp.1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006

127. Rittié, L. and Fisher, G.J., 2015. Natural and sun-induced aging of human skin. *Cold Spring Harbor Perspectives in Medicine*, 5(1), p.a015370. https://doi.org/10.1101/cshperspect.a015370

128. Rodrigues, M., Kosaric, N., Bonham, C.A. and Gurtner, G.C., 2019. Wound healing: A cellular perspective. *Physiological Reviews*, 99(1), pp.665–706. https://doi.org/10.1152/physrev.00067.2017

129. Rose, M. L., and M. T. Hincke. 2009. "Protein Constituents of the Eggshell: Eggshell-Specific Matrix Proteins." *Cellular and Molecular Life Sciences* 66 (16): 2707–2719.

130. Rose-Martel, M., and M. T. Hincke. 2012. "Molecular Markers of Eggshell Mineralization: A Proteomic Approach." *Proteomics* 12 (22): 3084–3095. https://doi.org/10.1002/pmic.201200169

131. Rozario, T. and DeSimone, D.W., 2010. The extracellular matrix in development and morphogenesis: A dynamic view. *Developmental Biology*, 341(1), pp.126–140. https://doi.org/10.1016/j.ydbio.2009.10.026

132. Ruff, K.J., DeVore, D.P., Leu, M.D. and Kopp, K.J., 2009. Eggshell membrane: A possible new natural therapeutic for joint and connective tissue disorders. *Clinical Interventions in Aging*, 4, pp.235–240. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2697621/.

133. Sakai, L.Y., Keene, D.R. and Engvall, E., 2001. Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. *Journal of Cell Biology*, 103(6), pp.2499–2509. https://doi.org/10.1083/jcb.103.6.2499

134. Shahidi, F. and Ambigaipalan, P., 2015. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. *Journal of Functional Foods*, 18, pp.820–897. https://doi.org/10.1016/j.jff.2015.06.018

135. Shahidi, F. and Yeo, J., 2016. Bioactivities of phenolics by focusing on suppression of chronic diseases: A review. *International Journal of Molecular Sciences*, 17(9), p.1374. https://doi.org/10.3390/ijms17091374

136. Sharma, A., Shankar, R., Yadav, A.K., Pratap, A., Ansari, M.A., and Srivastava, V. (2024). *Burden of Chronic Nonhealing Wounds: An Overview of the Worldwide Humanistic and Economic Burden to the Healthcare System*. The International Journal of Lower Extremity Wounds, Advance online publication. Publisher: SAGE Publications. DOI: https://doi.org/10.1177/15347346241246339

137. Shenoy, M., Abdul, N.S., Qamar, Z., Al Bahri, B.M., Al Ghalayini, K.Z.K. and Kakti, A., 2022. Collagen structure, synthesis, and its applications: A systematic review. *Cureus*. https://doi.org/10.7759/cureus.24856.

138. Shewry, P.R. and Halford, N.G., 2002. Seed storage proteins: Structures and biosynthesis. *The Plant Cell*, 14(suppl 1), pp. S43–S56.

139. Shi, Y., Zhou, K., Li, D., Guyonnet, V., Hincke, M.T. and Mine, Y., 2021. *Avian eggshell membrane as a novel biomaterial: A review*. Foods, 10, p.2178. https://doi.org/10.3390/foods10092178

140. Shoulders, M.D. and Raines, R.T. (2009). *Collagen structure and stability*. Annual Review of Biochemistry, 78, pp. 929–958. Publisher: Annual Reviews. https://doi.org/10.1146/annurev.biochem.77.032207.120833

141. Shuster, S., Black, M.M. and McVitie, E., 1975. The influence of age and sex on skin thickness, skin collagen and density. *British Journal of Dermatology*, 93(6), pp.639–643. https://doi.org/10.1111/j.1365-2133.1975.tb05113.x

142. Sibilla, S., and L. Genovese. 2015. "An Overview of the Beneficial Effects of Hydrolysed Collagen as a Nutraceutical on Skin Properties: Scientific Background and Clinical Studies." *The Open Nutraceuticals Journal*. https://www.researchgate.net/publication/276696375

143. Silverstein, R.M., Webster, F.X. and Kiemle, D.J., 2005. *Spectrometric Identification of Organic Compounds*. 7th ed. New York: John Wiley and Sons.

144. Singh, B., Pal, L. and Dey, N., 2020. Biopolymeric composite scaffold for skin tissue engineering: A review. *Chemical Engineering Journal*, 389, p.124469. https://doi.org/10.1016/j.cej.2020.124469

145. Sivakumar, C., Stobbs, J.A., Tu, K., Karunakaran, C. & Paliwal, J. (2023). *Molecular characterization of green lentil flours using synchrotron X-rays and Fourier transform mid-infrared techniques*. Powder Technology, 426, Article 118674. Elsevier BV. DOI: https://doi.org/10.1016/j.powtec.2023.118674

146. Sivakumar, C., Stobbs, J.A., Tu, K., Karunakaran, C. and Paliwal, J. (2024). *Unravelling particle morphology and flour porosity of roller-milled green lentil flour using scanning electron microscopy and synchrotron X-ray micro-computed tomography*. Powder Technology, 436, Article 119470. Publisher: Elsevier BV. DOI: https://doi.org/10.1016/j.powtec.2024.119470

147. Socrates, G., 2001. *Infrared and Raman Characteristic Group Frequencies: Tables and Charts*. 3rd ed. Chichester: Wiley.

148. Stewart, D., Morrison, I.M. and Hsu, T.F., 1997. FT-IR spectroscopy of lignin substructures. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 53(2), pp.227–235. https://doi.org/10.1016/S1386-1425(96)01744-1

149. Surewicz, W.K. and Mantsch, H.H., 1988. New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 952(2), pp.115–130. https://doi.org/10.1016/0167-4838(88)90107-0

150. Taiz, L., Zeiger, E., Møller, I.M. and Murphy, A., 2015. *Plant Physiology and Development*. 6th ed. Sunderland, MA: Sinauer Associates.

151. Taylor, K.A. and Pugh, N., 2016. *The contribution of zinc to platelet behaviour during haemostasis and thrombosis*. Metallomics, 8(2), pp.144–155.

152. Torres-Mansilla, A., Hincke, M., Voltes, A., López-Ruiz, E., Baldión, P.A., Marchal, J.A., Álvarez-Lloret, P. and Gómez-Morales, J., 2023. *Eggshell membrane as a biomaterial for bone regeneration*. Polymers, 15(6), p.1342. https://doi.org/10.3390/polym15061342

153. Tosh, S.M. and Yada, R.Y., 2010. Dietary fibres in pulse seeds and fractions: Characterization, functional attributes, and applications. *Food Research International*, 43(2), pp.450–460. https://doi.org/10.1016/j.foodres.2009.09.005

154. Tsai, W.T., Yang, J.M., Lai, C.W., Cheng, Y.H., Lin, C.C. and Yeh, C.W., 2005. Characterization and adsorption properties of eggshells and eggshell membrane. *Bioresource Technology*, https://doi.org/10.1016/j.biortech.2005.02.017

155. Tvaroška, I., 2024. Glycosylation modulates the structure and functions of collagen: A review. *Molecules*, 29(7), p.1417. https://doi.org/10.3390/molecules29071417.

156. Université d'Oran, 2019. *Fiche technique TP histologie: Préparation d'une lame histologique*. Faculté de Médecine, Service d'Histologie et Embryologie.

157. University of Washington, School of Nursing. 2005. *Biobehavioral Nursing and Health Systems*. Seattle, WA: University of Washington.

158. Van der Rest, M. and Garrone, R., 1991. Collagen family proteins. *The FASEB Journal*, 5(13), pp.2814–2823. https://doi.org/10.1096/fasebj.5.13.1916105.

159. Vandeginste, V (2021). Food waste eggshell valorization through development of new composites: A review. Sustain. Mater. Technol., 29, e00317

160. Vázquez, C., López de Dicastillo, C., López-Carballo, G. and Hernández-Muñoz, P., 2012. Polyphenols and wound healing: A review. *Current Pharmaceutical Design*, 18(4), pp.552–568. https://doi.org/10.2174/138161212799958651

161. Wang, L., Liang, J., Wen, Z. and Guo, T., 2018. Characterization of collagen from eggshell membranes. *Food Chemistry*, 245, pp.1114–1121. https://doi.org/10.1016/j.foodchem.2017.11.06

162. Watson, B.R., White, N.A., Taylor, K.A., Howes, J.M., Malcor, J.D.M., Bihan, D., Sage, S.O., Farndale, R.W. and Pugh, N., 2016. *Zinc is a transmembrane agonist that induces platelet activation in a tyrosine phosphorylation-dependent manner*. Metallomics, 8(1), pp.91–100. https://doi.org/10.1039/c5mt00064e

163. Wong Liong, J. W., J. F. Frank, and S. Bailey. 1997. "Visualization of Eggshell Membranes and Their Interaction with Salmonella enteritidis Using Confocal Scanning Laser Microscopy." Journal of Food Protection 60 (9): 1022–1028.

164. Wong, M., Hendrix, M.J.C. and McGuire, P.G., 2008. Comparative ultrastructural analysis of the eggshell membranes from white leghorn chicken and turkey eggs. *Poultry Science*, 87(1), pp.231–239. https://doi.org/10.3382/ps.2007-00248

165. Xu, B. and Chang, S.K.C., 2008. Total phenolic content and antioxidant properties of eclipse black beans and other common beans as affected by thermal processing. *Journal of Agricultural and Food Chemistry*, 56(18), pp.8959–8967. https://doi.org/10.1021/jf801260g

166. Xue, M. and Jackson, C.J., 2015. Extracellular matrix reorganization during wound healing and its impact on abnormal scarring. *Advances in Wound Care*, 4(3), pp.119–136. https://doi.org/10.1089/wound.2013.0485

167. Yamauchi, K., Katayama, Y. and Maniwa, M., 2001. Molecular properties of eggshell membrane collagen. *Journal of Agricultural and Food Chemistry*, 49(2), pp.595–600. https://doi.org/10.1021/jf000765j

168. Yang, T. and Yan, W., 2025. *Strategies for enhancing the antibacterial efficacy of lysozyme and the resulting outcome*. International Journal of Biological Macromolecules, 267, p.143137. https://doi.org/10.1016/j.ijbiomac.2025.143137

169. Yaning, S., K. Zhou, V. Guyonnet, M. T. Hincke, and Y. Mine. 2021. "Avian Eggshell Membrane as a Novel Biomaterial: A Review." *Foods* 10: 2178. https://doi.org/10.3390/foods10092178UITTO, J. (1997) Understanding Premature Skin Aging. The New England Journal of Medicine, 337, 1463-1465.https://doi.org/10.1056/NEJM199711133372011.

170. Zeman, M.K., Kumar, N.M. and Smith, R.W., 2021. Tissue preparation and fixation for histology. In: Conn's Handbook of Models for Human Aging. Academic Press. https://doi.org/10.1016/B978-0-12-813036-0.00017-1.

171. Zhang, Y., Liu, W., Wang, Y. and Zhao, Y., 2019. Polyphenols and wound healing: A review of their role in promoting angiogenesis and collagen synthesis. *Phytotherapy Research*, 33(12), pp.3141–3151. https://doi.org/10.1002/ptr.6460

172. Zhou, J., S. Wang, F. Nie, L. Feng, G. Zhu, and L. Jiang. 2011. "Elaborate Architecture of the Hierarchical Hen's Eggshell." *Nature Nanotechnology* 4 (2): 171–179. https://doi.org/10.1007/s12274-010-0067-8.

173. Zielinski, H. *et al.*, 2012. Antioxidant activity of lentil (Lens culinaris) and its fractions. *Polish Journal of Food and Nutrition Sciences*, 62(3), pp.165–170. https://doi.org/10.2478/v10222-011-0054-

# ANNEX

## Annex 01: Function and distribution of collagen in the human body

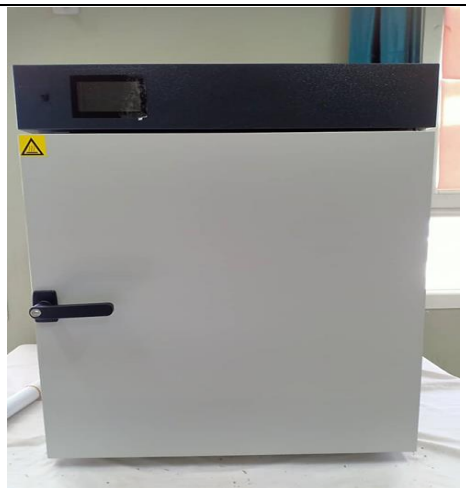
The table below provides examples of selected collagen types, tissue distribution, and uses.

**Table 02: Function and distribution of collagen in the human body (Mingjie et al,.2025).**

| Family | Type | Distribution in tissue | Application/function |
|---|---|---|---|
| Fibril-forming | I<br>II<br>III<br>V<br>XI | Tendon, ligaments, bone, and cornea<br>Lung, cornea, reticular fibers, cartilage, vessel wall, nucleus pulposus, bone, vitreous body ,and skin<br><br>Vitreous body and cartilage | Membranes for guided tissue regeneration<br>Cartilage repair and arthritis treatment.<br>Hemostats and tissue sealants<br>Feedstock for biomaterial in corneal treatments<br>mAbs development for osteoarthritis |
| Basement membrane | IV | Basement membranes | Attachment enhancer of cell culture (mouse neuroblastoma) and diabetic nephropathy indicator |
| Microfibrillar | VI | Dermis, placenta, lungs, cartilage, intervertebral disk, and placenta. | Hemostat |
| Anchoring | VII | Oral mucous, cervix, dermal & epidermal junctions, and skin | Subdermal injection as treatment for dystrophic epidermolysis bullosa (DEB) |
| FACIT | IX<br>XII<br>XIV XIX<br>XX<br><br>XXI | Cornea, cartilage, and vitreous humor<br>Tendon, perichondrium, and ligaments<br>Vessel wall, placenta, liver, dermis, and lungs Human rhabdomyosarcoma<br>Embryonic skin, tendon, corneal epithelium, and sternal cartilage.<br>Blood vessel wall | It co-distributes with type II collagen in cartilage and the vitreous body.<br>Associated to collagen I-containing fibrils.61 XIV collagen is also a fibril diameter regulator in early stages of fibrillogenesis<br>Located in the basement membrane zone and show antiangiogenic and antitumoral properties.<br>Similar to XII and XIV collagen types. The function of this collagen is relatively unknown.<br>Closely related to XII, XIV, and XX collagens. Its expression is stimulated by platelet-derived growth factor, which indicates that this collagen might contribute to matrix assembly of vascular network during blood vessel formation. |
| Transmembrane | XIII<br>XVII | Hair follicle, intestine, liver, dermal & epidermal junctions, epidermis, and lungs<br>dermal & epidermal junctions | Affects bone formation and may have a function in coupling the regulation of bone mass to mechanical use. Involved in inflammation and vasculogenesis.<br>Its putative function is to stabilize adhesion of epithelial cells to the surrounding extracellular matrix. It has important roles in teeth formation |
| Multiplexins | XV<br>XVI XVIII | Kidney, smooth muscle cells, fibroblasts, and pancreas. Keratinocytes and fibroblasts<br>Liver and lungs | It forms a bridge linking large, banded fibrils, likely fibrils containing collagens I and III.<br>As it has been implicated in several diseases, it can be possibly used as a drug target or biomarker.<br>It has major role in determining retinal structure and in closure of the neural tube. |

## Annex 02: Preparation of ESM and LNS powders

The following photos illustrate different steps in preparing ESM and LNS powders.



***Drying of ESM and LNS seeds***

***Grinding of ESM and LNS seeds***


***Sieving of ESM and LNS powders***


***Sterilization of ESM and LNS powders***

**Figure 31: Photos of the preparation steps of ESM and LNS powders**

**Annex 03: Calibration curves of colorimetric assays**

The following figures represent the calibration curves of total sugars and proteins determined by the methods of Dubois et al. (1956) and Bradford (1976) respectively.

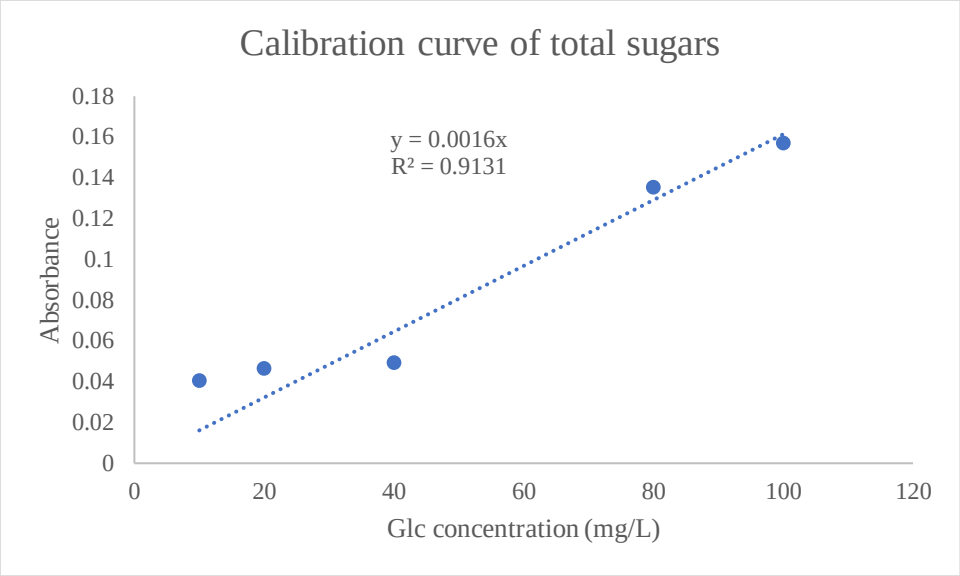



**Figure 32: Calibration curve of total sugars**

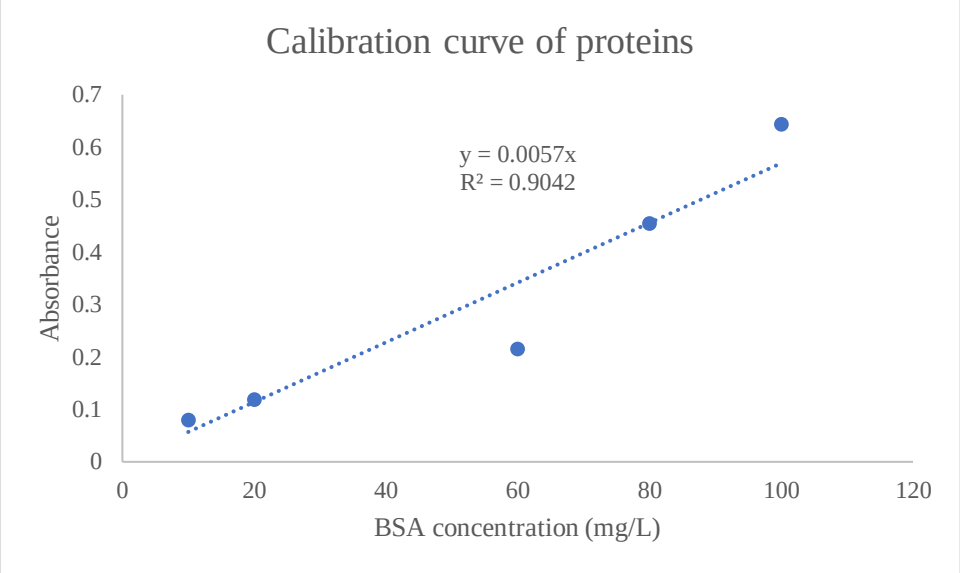



**Figure 33: Calibration curve of proteins**

## Annex 04: Surgical supplies

The figure below represent the surgical supplies used in the experimental step of wound incision.


**Figure 34: Surgical supplies**

## Annex 05: Histological sections' steps

The figure below indicate some steps of histological sectioning of the skin.


**Figure 35: Stages of histological sections**