Preclinical Screening of Wound Healing efficacy of *Sonneratia apetala* Buch. - Ham. extract on Excision Wound Model in Wistar rats

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CERTIFICATE

This is to certify that Ms. Ishani Mukherjee has carried out the research on the project entitled "Preclinical Screening of Wound Healing efficacy of *Sonneratia apetala* Buch. - Ham. extract on Excision Wound Model in wistar rats" under my supervision, in the division of Pharmacology and Toxicology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.

He has incorporated his findings in to this thesis of the same title being submitted by his in partial fulfillment of the requirement for the award of Degree of Master of Pharmaceutical Technology, Jadavpur University. I am satisfied that he has carried out his thesis with proper care and confidence to my entire satisfaction

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DECLARATION

I declare that **"Preclinical Screening of Wound Healing Efficacy of *Sonneratia apetala* Buch. -Ham. extract on Excision Wound Model in Wister rat"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Ishani Mukherjee
Signature of the student

Full Name- Ishani Mukherjee

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Dedicated to My

Family & My Mentor

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DATE: 29/08/24

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PREFACE

The present study entitled "Preclinical Screening of Wound Healing Efficacy of *Sonneratia apetala* Buch. -Ham. extract on Excisional Wound Model in wistar rats" covers original research work conducted by the author for the award of Master of Pharmacy in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata.

The enormous diversity of the plant kingdom has become a target for drug companies looking for novel medications and lead compounds. Their widespread availability, low toxicity, and little or non-existent adverse effects have motivated us to use medicinal herbs in the treatment of a variety of ailments. Traditional applications necessitate background in order to be of proper value, and as a result, today they are a significant component of study. As a result, the thesis covered the above-mentioned study in a coherent way in respect to the other research-related aspects.

In conclusion, the detailed study has been put together in a way that justifies the work's relationship to establishing pharmacological activities, notably wound healing activity.

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CHAPTER 1: INTRODUCTION

1.1. Process of Wound Healing

The skin is the largest organ by surface area in the human body, serving as a critical barrier that protects internal tissues from mechanical damage, microbial infection, ultraviolet radiation, and extreme temperatures. A wound is a common injury caused by internal and/or external factors, often associated with various immunological events, such as necrosis and inflammation. Significant tissue damage and infection are key characteristics of wounds, frequently accompanied by other co-morbidities (Flanagan, M. 2013). Wound healing is a complex process involving immunohistochemistry, tissue regeneration, and remodeling. Traditional procedures for treating various types of wounds have been used since early human history. The wound healing process is a complex physiological event in the human body that involves the sequential activation of multiple cell types and signaling pathways in a coordinated manner. Consequently, it is highly vulnerable to injuries, which have significant implications for both individual patients and the healthcare economy. In the United States, non-healing wounds result in approximately $50 billion in healthcare costs annually, surgical and trauma scars account for nearly $12 billion, and burns contribute $7.5 billion each year (Rodrigues et al., 2019). Individuals with diabetes, the elderly, and those with genetic disorders such as sickle cell disease are particularly prone to abnormal wound healing, leading to long-term complications. Despite the availability of various wound healing therapies, they have only been moderately effective, highlighting the need for more effective treatments. Skin repair involves the intricate coordination of various cell types through sequential steps. The epidermis, the outer impermeable layer, endures harsh environmental conditions and houses sebaceous glands, sweat glands, and hair follicles. The dermis, rich in extracellular matrix, vasculature, and mechanoreceptors, provides the skin with strength, nutrients, and immunity. Beneath the dermis, subcutaneous adipose tissue functions as an energy reserve and a constant source of growth factors for the dermis. Each layer also contains resident immune cells that continuously monitor for damage. During wound healing, multiple cell types within these three layers must coordinate precisely through stages such as hemostasis, inflammation, angiogenesis, growth, re-epithelialization, and remodelling. These stages, although sequential, often overlap, making skin repair one of the most complex processes in the human body (Sorg et al., 2017; Cañedo-Dorantes et al., 2019).

Despite the body's considerable innate reparative ability, various cellular aspects of an individual's injury response can become diminished, compromising wound closure. This diminishment is often due to pathological systemic changes, such as those associated with advanced age or uncontrolled diabetes. Indeed, age and diabetes are primary risk factors for developing chronic wounds, which are wounds that take longer than 12 weeks to heal. Unfortunately, these chronic wounds, including venous ulcers, pressure sores, and diabetic foot ulcers, represent a significant unmet clinical need and are increasing globally. This discussion explores the current understanding of skin repair and highlights impaired cellular behaviors that underpin chronic wound healing pathology. The application of emerging research technologies will be crucial in further elucidating the cellular and molecular basis of both acute and pathological repair (Takeo et al., 2015).

The initial response to a wound involves the constriction of injured blood vessels and activation of platelets to form a fibrin clot, which stops blood flow and provides a scaffold for incoming inflammatory cells. Neutrophils are quickly recruited to the clot as the first line of defense against bacteria. Within 48-96 hours post-injury, monocytes are recruited and transform into tissue-activated macrophages at the wound site. The adaptive immune system, including Langerhans cells, dermal dendritic cells, and T cells, is also activated to address both self and foreign antigens. There is increasing interest in understanding the heterogeneity within these immune cell populations and the roles specific subsets play in clearing cellular debris versus resolving infection. Following the inflammatory phase, angiogenesis occurs, involving the proliferation, migration, and branching of endothelial cells to form new blood vessels. Concurrently, pericytes within the basal lamina are activated to scaffold and provide structural integrity to the endothelial cells. Some research suggests that these activated pericytes are mesenchymal stromal cells with increased plasticity (Chouhan et al., 2019). Additionally, circulating progenitor cells from the bone marrow support new blood vessel formation during wound healing, with most cellular diversity occurring within the perivascular space. As new blood vessels form, resident fibroblasts proliferate and invade the clot to form contractile granulation tissue. Some fibroblasts differentiate into myofibroblasts, which draw the wound margins together. The dividing fibroblasts deposit extracellular matrix and transition the wound microenvironment from an inflammatory to a growth state. Re-epithelialization occurs simultaneously, involving the proliferation of unipotent epidermal stem cells from the basement

membrane and the de-differentiation of terminally differentiated epidermal cells (Zhang et al., 2017; Senoo, 2013). Repair of the epidermal layer also involves reconstructing skin appendages, with tissue-resident stem cells for sebaceous glands, sweat glands, and hair follicles activating local appendage repair. This defense extends to a sophisticated immune barrier response that protects against pathogenic infection while supporting commensal microorganisms through an elegantly adapted host–microbiota axis. The skin has also evolved efficient and rapid mechanisms to close breaches in its barrier, collectively known as the wound healing response. Wound repair is classically simplified into four main phases: hemostasis, inflammation, proliferation, and dermal remodeling, which together result in architectural and physiological restoration following damage.

While these epidermal stem cells are mostly unipotent during homeostasis, they become highly plastic in response to injury and can differentiate into other cell types to rapidly repair the epidermis during wound healing. Within the subcutaneous adipose tissue, stromal vascular cells and their subsets are well characterized. These cells release growth factors and cytokines that are critical for neovascularization and wound repair. Inflammatory cells within the subcutaneous tissue have also garnered attention, especially in conditions of obesity and type 2 diabetes, as increased inflammation can alter the outcome of wound repair (Martin, P. 1997; Lévesque et al., 2010).

1.2. Factors affecting Wound Healing

Wound healing is a complex, multifaceted process influenced by various factors that can either facilitate or hinder the restoration of tissue integrity. Understanding these factors is crucial for developing effective treatments and improving patient outcomes. The key factors affecting wound healing can be broadly categorized into local and systemic factors (Hess, C. T. 2011).

1.2.1. Local Factors

Infection: The presence of bacteria in a wound can significantly delay healing by prolonging the inflammatory phase, causing additional tissue damage, and increasing the risk of complications. Effective wound management, including proper cleaning and the use of antibiotics, is essential to prevent and treat infections.

Oxygenation: Adequate oxygen supply is vital for wound healing as it supports cellular functions, collagen synthesis, and the formation of new blood vessels (angiogenesis). Poor oxygenation, often seen in conditions such as chronic obstructive pulmonary disease (COPD) or peripheral artery disease, can impede the healing process.

Moisture: Maintaining an optimal moisture balance in the wound environment is crucial. A moist environment promotes cell migration and re-epithelialization, whereas excessive dryness can lead to the formation of a scab that hinders healing. Conversely, excessive moisture can cause maceration and damage surrounding tissues.

Pressure: Continuous pressure on a wound, particularly in bed-bound or immobile patients, can impair blood flow and oxygen delivery, leading to pressure ulcers. Regular repositioning and the use of pressure-relieving devices are important preventive measures (Bereznicki, L. 2012).

1.2.2. Systemic Factors

Age: Aging is associated with a slower wound healing process due to reduced cellular proliferation, decreased collagen synthesis, and impaired immune response. Older adults are also more likely to have comorbidities that can further complicate healing.

Nutrition: Adequate nutrition is fundamental for wound healing. Protein, vitamins (especially A and C), and minerals (such as zinc) are critical for cell proliferation, collagen formation, and immune function. Malnutrition or specific nutrient deficiencies can significantly delay healing.

Chronic Diseases: Conditions such as diabetes mellitus, cardiovascular disease, and chronic kidney disease can impair wound healing. For example, diabetes is associated with reduced blood flow, neuropathy, and a heightened inflammatory response, all of which contribute for delayed healing and an increased risk of infection.

Medications: Certain medications can affect wound healing. Corticosteroids, for instance, can reduce inflammation and collagen synthesis, delaying the healing process. Chemotherapy and radiation therapy can also impair cell proliferation and tissue repair.

Lifestyle Factors: Smoking and excessive alcohol consumption can negatively impact wound healing. Smoking reduces blood flow and oxygen delivery, while alcohol can impair immune function and delay the inflammatory response.

Wound healing is a dynamic and intricate process influenced by a hybrid of local and systemic factors. Effective management of wounds requires a comprehensive understanding of these factors to implement appropriate interventions. By addressing underlying conditions, optimizing the wound environment, and ensuring adequate nutrition and care, healthcare providers can significantly enhance the healing process and improve patient outcomes (Anderson et al., 2012).

1.3. Types of Wound

1.3.1. Acute (healing) wound

Acute wounds are injuries that typically heal through an orderly and timely reparative process within weeks to months, provided that no complications arise. They are often the result of sudden trauma or surgical procedures. Acute wound healing, as observed in primary healing, is a carefully regulated, systemic cascade of overlapping processes that necessitate the timely completion of several cellular processes, such as phagocytosis, chemotaxis, mitogenesis, and the synthesis of extracellular matrix components. These actions occur in a sequence corresponding to the emergence of distinct cell types in the wound at different points in the healing process (Enoch et al., 2008).

1.3.1.1. Causes of Acute wound

The major causes for acute wound are mainly physical like traumatic injury, surgical wounds, and environmental conditions (Hurlow et al., 2022). The primary causes of acute wounds include:

Traumatic Injuries

- **Cuts and Lacerations**: Sharp objects such as knives, glass, or metal can cause cuts or lacerations that vary in depth and severity.
- **Abrasions**: Scraping or rubbing of the skin against a rough surface can cause abrasions, which are usually superficial.
- **Puncture Wounds**: Sharp, pointed objects like nails or needles can cause puncture wounds, which are typically deep and narrow.

- **Burns**: Thermal injuries from hot objects, flames, steam, or scalding liquids can cause varying degrees of burns (first, second, or third degree).
- **Contusions**: Blunt force trauma, such as a blow or impact, can cause contusions (bruises), leading to bleeding under the skin without breaking it.
- **Avulsions**: A tearing away of tissue, often resulting in a flap or complete loss of skin and underlying tissue.
- **Bite Wounds**: Animal or human bites can cause puncture, laceration, or crushing injuries, often complicated by infection (Hurlow et al., 2022).

Surgical Wounds

- **Incisions**: Surgical cuts made intentionally during procedures to access internal organs or structures.
- **Excisional Wounds**: Removal of tissue, such as tumors or cysts, leaving a surgical wound that needs to heal.

Environmental and Occupational Injuries

- **Chemical Burns**: Exposure to corrosive chemicals, such as acids or alkalis, can cause chemical burns and tissue damage.
- **Frostbite**: Exposure to extreme cold can cause frostbite, damaging the skin and underlying tissues.
- **Radiation Burns**: Exposure to high levels of radiation, such as during radiation therapy, can cause radiation burns.

Acute Medical Conditions

- **Acute Infections**: Rapidly spreading infections, such as necrotizing fasciitis, can cause tissue damage and acute wounds.
- **Acute Inflammatory Conditions**: Conditions such as acute dermatitis can cause skin breakdown and ulceration (Ding et al., 2022).

1.3.1.2. Risk Factors for Acute wound

- **Age**: Younger individuals tend to heal faster, whereas older adults may have delayed healing due to reduced skin elasticity and slower cellular regeneration.
- **General Health**: Individuals with underlying health conditions, such as diabetes or immune deficiencies, may experience slower wound healing.
- **Lifestyle Factors**: Smoking, poor nutrition, and lack of physical activity can impair the body's ability to heal acute wounds effectively.
- **Environmental Factors**: Working in high-risk environments, such as construction or manufacturing, increases the likelihood of sustaining acute injuries (Reichert, W. M. 2007).

1.3.1.3. Healing Process

Acute wound healing produces a very dynamic cascade of cellular signaling and behavioral events that guarantee the skin barrier closes quickly. Little changes to this response almost never interfere with the healing of wounds because of high levels of redundancy and compensation mechanisms (Pieper et al., 1999).

1.3.2. Chronic (Non-healing) wound

A chronic wound is one where the normal healing process is disrupted at one or more stages—hemostasis, inflammation, proliferation, or remodeling resulting in a delay in healing beyond the expected time. Various factors, including growth factors, cytokines, proteases, and cellular and extracellular elements, play crucial roles in different stages of the healing process. Alterations in one or more of these elements can impede wound healing (Wolcott et al., 2010).

Causes:

Changes in one or more of these components are necessary because growth factors, cytokines, proteases, and cellular and extracellular components are crucial at various phases of the healing process. Factors both systemic and local that prevent wound healing insufficient blood supply in the area Impaired healing could be caused by systemic factors. Non-healing can also be caused by condition-specific variables (e.g., neuropathy in diabetes; ischaemia in peripheral vascular disease). The following factors have been linked to an increased risk of developing chronic

wounds: modifications to the extracellular matrix's composition, changes in the cell profile and activity, failure to undergo epithelialization, and the presence of microorganisms and free radicals (Monaco et al., 2003).

Local Factors

- **Inadequate Blood Supply**: Poor blood circulation limits the delivery of oxygen and nutrients essential for tissue repair.
- **Infection**: The presence of bacteria and other pathogens can cause prolonged inflammation and tissue damage, preventing proper healing.
- **Mechanical Stress**: Continuous pressure or friction on the wound area can impede healing, as seen in pressure ulcers.
- **Foreign Bodies**: Objects or debris in the wound can cause persistent inflammation and delay the healing process.
- **Moisture Imbalance**: Too much moisture can lead to maceration, while too little can cause the wound to dry out and form scabs that hinder healing (Eggleston, R. B. 2018).

Systemic Factors

- **Diabetes**: Neuropathy and reduced immune function in diabetic patients can lead to non-healing wounds, particularly in the lower extremities.
- **Peripheral Vascular Disease**: Ischemia resulting from narrowed blood vessels restricts blood flow to the wound, impairing healing.
- **Advanced Age**: Aging is associated with a decline in cellular function and immune response, making wound healing less efficient.
- **Nutritional Deficiencies**: Lack of essential nutrients, such as proteins, vitamins (especially vitamin C and vitamin A), and minerals (such as zinc), can impair the body’s ability to repair tissue.
- **Chronic Inflammatory Diseases**: Conditions like rheumatoid arthritis or lupus can cause prolonged inflammation that disrupts the healing process.

- **Medications**: Certain drugs, such as corticosteroids and immunosuppressants, can inhibit wound healing by reducing inflammation and immune response.

Cellular and Molecular Factor

- **Growth Factors and Cytokines**: Disruptions in the production or activity of growth factors and cytokines, which are crucial for cell signaling during healing, can lead to chronic wounds.
- **Protease Imbalance**: Overproduction of proteases, such as metalloproteinases, and reduced activity of their inhibitors can degrade essential proteins in the extracellular matrix, preventing proper tissue formation.
- **Altered Cellular Activity**: Changes in the behavior and function of key cells involved in wound healing, such as fibroblasts and keratinocytes, can impair the repair process.
- **Extracellular Matrix (ECM) Composition**: Alterations in the ECM, which provides structural support to cells, can hinder cell migration and tissue regeneration.
- **Presence of Free Radicals**: Excessive free radicals can cause oxidative stress and damage to cellular components, disrupting the healing process.
- **Impaired Epithelialization**: Failure of the wound to re-epithelialize (formation of new skin) can leave it open and vulnerable to further damage and infection (Eaglstein et al., 1997; Mustoe, T. 2004).

Role of Proteases and Inflammatory Response

In chronic wounds, the production and activity of proteases, which are tightly regulated in the healing of acute wounds, are disrupted. Several types of metalloproteinases, including type-2 and type-9, have been identified within the fluid of chronic wounds but are absent in the fluid of acute wounds. Other proteases, such as neutrophil elastase, are significantly higher in chronic wounds, while the activity of tissue inhibitors of metalloproteinases is decreased. The normal inflammatory response seen in the healing of acute wounds is significantly altered in chronic wounds. Wound fluid from chronic venous ulcers is rich in pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and transforming growth factor-β1. As the chronic

wound begins to heal, the levels of these cytokines decrease, indicating a correlation between non-healing wounds and increased levels of pro-inflammatory cytokines (Zhao et al., 2016).

Impact on Quality of Life

Chronic wounds and burns significantly decrease the quality of life for patients due to increased physical pain and socio-economic complications. Unlike burns, the incidence and prevalence of chronic wounds have been increasing, mainly due to population aging, leading to higher costs for national health systems. Therefore, the development of new and more cost-effective technologies and therapies is essential to improve the long-term sustainability of national health systems.

Healing Process

In most cases, healing restores barrier function and the skin's tensile strength to near-normal levels. However, unlike prenatal wound healing, which is a regenerative process that recreates the original skin architecture, adult wound healing results in a fibrotic scar that serves as a rapid patch for the wound. Excessive scarring can lead to hypertrophic scarring and keloid formation, driven by differential cellular responses to mechanical stress within the healing skin. Impairments in the wound healing response can also result in chronic wounds, which are common in conditions such as diabetes, vascular disease, aging, and hemoglobinopathies. Improper care of these wounds can lead to recurrence, limb amputations, and mortality (Bryant et al., 2015; Järbrink et al., 2016).

1.4. Types of Wound Healing

1.4.1. Primary Healing (First Intention)

Primary healing occurs when a wound is closed within 12–24 hours of its creation, such as with a clean surgical incision or a clean laceration. The wound edges are brought together directly using sutures, tissue glue, tapes, or mechanical devices. This type of healing involves only minimal disruption of the epithelial basement membrane and limited cell death of epithelial and connective tissue. Consequently, epithelial regeneration is more prominent than fibrosis. With a proper balance between all healing phases—including cellular proliferation, collagen metabolism, and extracellular matrix degradation—wounds heal efficiently and quickly reach complete closure (Southwick et al., 2018).

1.4.2. Delayed Primary Healing

Delayed primary healing is used for contaminated or poorly delineated wounds that are initially left open to prevent infection, such as bites or abdominal wounds after peritoneal soiling. The skin and subcutaneous tissues are not immediately brought together (sutures might be placed but not tied). After a few days, once the host's defenses have debrided the wound, closure can be performed. Phagocytic cells are recruited into the wound within 3–4 days, and inflammatory cells eliminate contaminating bacteria. Even after a delay of several days, wound edges can be approximated, with collagen metabolism remaining unaffected and the wound retaining its tensile strength as if closure had been immediate (Young et al., 2011).

1.4.3. Secondary Healing (Second Intention)

Secondary healing is required for wounds with extensive soft tissue loss, such as those from major trauma, severe burns, or certain surgical procedures (e.g., laparostomy). In these cases, epithelial cell regeneration alone cannot restore the original architecture. Instead, granulation tissue grows from the wound margin, followed by extracellular matrix accumulation and collagen deposition. These open, full-thickness wounds close through wound contraction and epithelialization. For instance, large skin defects can reduce to 5–10% of their original size within six weeks, primarily through contraction. Myofibroblasts, which have characteristics of both fibroblasts and smooth muscle cells, play a crucial role in this type of healing. They appear in the wound around three days post-injury, reaching their peak between the tenth and twenty-first days. Healing by secondary intention is slower and may lead to contractures, especially over joints, potentially resulting in functional limitations (Prasetyono, T. O. 2009).

1.4.4. Superficial (Partial-Thickness) Wounds

Healing of superficial wounds, such as superficial burns, split-thickness donor graft sites, and abrasions, involves injuries that affect the epithelium and the superficial part of the dermis. The basal layer of cells remains intact, and epithelial cells from dermal appendages, hair follicles, and sebaceous glands replicate to cover the exposed dermis. These cells migrate towards each other from the basal layer to encircle the wound. Healing occurs solely through epithelialization, leading to nearly complete anatomical and physiological restoration (Prasetyono, T. O. 2009).

1.5. Phases of Wound Healing

Healing of acute wounds, as seen in primary healing, is a well-regulated, systemic cascade of overlapping processes that require the coordinated completion of various cellular activities, including phagocytosis, chemotaxis, mitogenesis, and synthesis of extracellular matrix components. These activities occur in a sequence that correlates with the appearance of different cell types in the wound at various stages of the healing process. The process involves four overlapping but well-defined phases: hemostasis, inflammation, proliferation, and remodeling/scar maturation. The regulation of these events is multifactorial, involving various important cells and growth factors (cytokines) at different stages of the healing process (Singh et al., 2017).

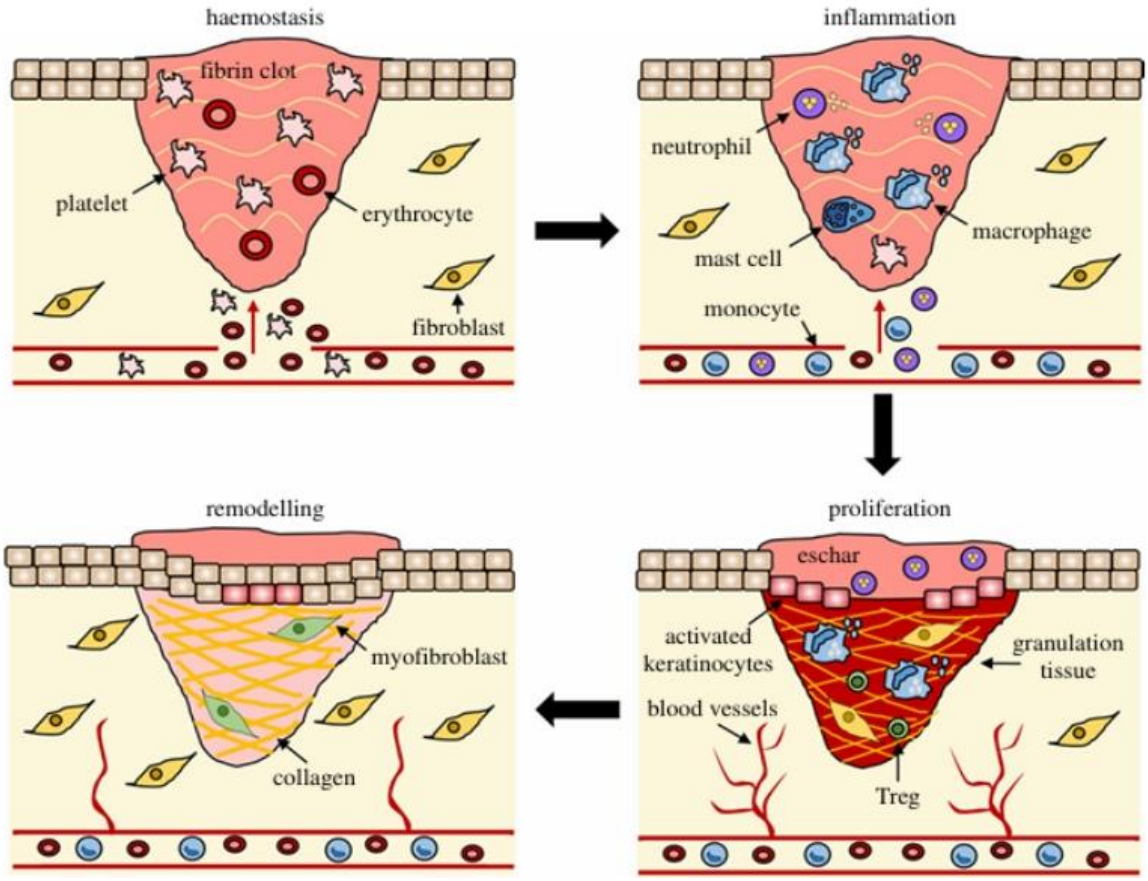

Figure 1: Phases of wound healing (Wilkinson et al., 2020).

1.5.1. Haemostasis (immediate)

Vascular Contraction and Clot Formation

Right after an injury, blood vessels rapidly contract, and a clot forms to prevent excessive blood loss. Platelets, which are key players in hemostasis and coagulation, become activated upon encountering the subendothelial matrix of the damaged blood vessels. Platelet receptors, such as glycoprotein VI, interact with extracellular matrix (ECM) proteins like fibronectin, collagen, and von Willebrand factor, promoting adhesion to the vessel wall (Velnar et al., 2009).

Platelet Activation and Degranulation

Thrombin activates platelets, causing them to change shape and release alpha and dense granules that contain bioactive molecules, reinforcing the coagulation process. An insoluble clot, or eschar, made up of fibrin, fibronectin, vitronectin, and thrombospondin forms to plug the wound and stop the bleeding. Besides stopping the bleeding, the eschar also protects against bacterial invasion, provides a scaffold for incoming immune cells, and holds a reservoir of cytokines and growth factors that guide early wound repair (Golebiewska et al., 2015).

Immune Cell Recruitment and Early Infection Control

Platelets are essential in recruiting immune cells to the injury site, either by capturing them directly in the eschar or by releasing chemokine attractants upon degranulation. The platelet secretome also contains growth factors that stimulate resident skin cells, including fibroblasts and keratinocytes. As the most abundant cell type during the early repair phase, platelets play a significant role in preventing bacterial infection. They express toll-like receptors (TLRs) that regulate the production of antimicrobial peptides (Golebiewska et al., 2015).

Switching Off Coagulation

Once an adequate clot has formed, the coagulation process is switched off to prevent excessive thrombosis. Platelet aggregation is inhibited by prostacyclin, thrombin is inhibited by antithrombin III, and coagulation factors V and VII are degraded by activated protein C. Meanwhile, the injured vessel wall is repaired by smooth muscle cells and endothelial cells that proliferate in response to the platelet-derived growth factor (PDGF). Endothelial progenitors are

also recruited to aid in this process, as mature endothelial cells have limited capacity for proliferation (Golebiewska et al., 2015).

Table 1: Cells responsible for wound healing.

S. No.	Cell type	Function in wound healing	References
1.	**Platelets**	➢ involved in thrombus formation ➢ α granules are a rich source of inflammatory mediators including cytokines (e.g. TGF-β, PDGF, β-thromboglobulin, platelet factor-4) ➢ Major initial stimulus for inflammation	(Nurden et al., 2008).
2.	**Neutrophils**	➢ First cells to infiltrate site of injury. ➢ Phagocytosis and intracellular killing of invading bacteria	(Wilgus et al., 2013).
3.	**Monocytes (macrophages)**	➢ Contains phagocytose and destroy invading bacteria ➢ clean up necrotic tissue and detritus; ➢ abundant in inflammatory mediators, such as cytokines; ➢ promote angiogenesis, collagen synthesis, and fibroblast division	(Nurden et al., 2008).
4.	**Lymphocyte**	➢ Not clearly defined ➢ May produce cytokines in certain types of wound	(Kim et al., 2019).
5.	**Fibroblasts**	➢ Produce various components of the ECM, ➢ including collagen, fibronectin, hyaluronic acid,	(Raziyeva et al., 2021).

		proteoglycans ➢ synthesize granulation tissue ➢ Help to reorganize the 'provisional' ECM	

1.5.2. Inflammation (Day 1-3)

Innate inflammation evolved as a primary defense against pathogenic invasion at wound sites. This immune response is triggered by injury-induced signals, such as damage-associated molecular patterns (DAMPs) from necrotic cells and tissue damage, and pathogen-associated molecular patterns (PAMPs) from bacterial components. These PAMPs and DAMPs activate resident immune cells, including mast cells, Langerhans cells, T cells, and macrophages, by binding to pattern recognition receptors, which then initiate downstream inflammatory pathways. The release of pro-inflammatory cytokines and chemokines subsequently attracts circulating leukocytes to the injury site. Pro-inflammatory molecules also promote vasodilation and the expression of endothelial cell adhesion molecules, such as selectins, which aid in neutrophil and monocyte adhesion and migration (Segel et al., 2011). Neutrophils, arriving early after injury, are recruited to the wound from damaged vessels, attracted by chemoattractants like interleukin 1 (IL-1), tumor necrosis factor-alpha (TNF-α), and bacterial endotoxins such as lipopolysaccharide (LPS). In response to pro-inflammatory signals and activation of inflammatory pathways like NF-κB, neutrophils and other wound cells release their own cytokines. Neutrophils eliminate necrotic tissue and pathogens through phagocytosis and the release of reactive oxygen species (ROS), antimicrobial peptides, eicosanoids, and proteolytic enzymes. They also form extracellular traps, which are webs of DNA coated with antimicrobial peptides and cytotoxic histones, to trap and kill pathogens (Kim et al., 2008).

Circulating monocytes enter the wound tissue and differentiate into macrophages in response to the local environment. Although macrophages are generally thought to be recruited after neutrophils, an initial wave of monocytes can enter the wound simultaneously with neutrophils. Macrophages are key effector cells in tissue repair, known for their versatility and high plasticity. They peak in wound infiltration 72 hours after injury in mice and 7 days post-injury in humans. Like neutrophils, macrophages engulf necrotic debris and pathogens through evolutionarily

conserved receptors, but they also exhibit different behaviors and morphological changes in response to cytokines (Rodero et al., 2014). Classically activated macrophages, induced by pro-inflammatory stimuli such as LPS and interferon-gamma (IFN-γ), promote inflammation by releasing ROS, inflammatory cytokines (like IL-1, IL-6, and TNF-α), and growth factors (such as vascular endothelial growth factor, VEGF, and PDGF). These macrophages phagocytose apoptotic neutrophils, replacing them as the main inflammatory mediators. Later stages of inflammation are marked by a transition to an alternative activation state, either through the differentiation of newly recruited monocytes or the switching of existing macrophages to an anti-inflammatory phenotype. This switch can be stimulated by changes in cytokines, efferocytosis, miRNAs, transcription factors, and the modulation of inflammatory receptors. The significant presence of neutrophils and macrophages in wounds may have overshadowed the roles of other myeloid cells in wound repair. Recent studies have shown that resident T cells are crucial for the early injury response, while circulating T cells are important for resolving inflammation. Aged and diabetic mice exhibit reduced resident dendritic epidermal T cells and delayed healing, but subcutaneous administration of these T cells can restore healing. Additionally, the removal of anti-inflammatory regulatory T cells delays tissue repair. Mast cells also contribute to wound healing by releasing histamine, which aids in neutrophil recruitment during early inflammation (Das et al., 2015).

1.5.3. Proliferation (day 3 to week 2)

The proliferative phase begins around day 3 and lasts for 2-4 weeks after wounding. It is marked by fibroblast migration, deposition of the extracellular matrix, and formation of granulation tissue. This phase involves extensive activation of keratinocytes, fibroblasts, macrophages, and endothelial cells to coordinate wound closure, matrix deposition, and angiogenesis. Keratinocytes are activated by changes in mechanical tension, electrical gradients, hydrogen peroxide, pathogens, growth factors, and cytokines as early as 12 hours post-injury. This activation causes keratinocytes at the wound edge to undergo a partial epithelial-mesenchymal transition. As the proliferative phase progresses, the provisional fibrin/fibronectin matrix is replaced by newly formed granulation tissue. Epithelialization of the wound represents the final stage of this phase. Fibroblasts appear in the wound 2-4 days after injury, with endothelial cells following about one day later. Attracted by factors like platelet-derived growth factor and

transforming growth factor-β, fibroblasts proliferate and produce matrix proteins such as fibronectin, hyaluronan, collagen, and proteoglycans. These components help construct the new extracellular matrix, which supports further cell ingrowth and is essential for repair. The extracellular matrix provides turgor to soft tissues, rigidity to bone, a substratum for cell adhesion, and regulates the growth, movement, and differentiation of cells. It consists of fibrous structural proteins (collagens, elastin) and an interstitial matrix of adhesive glycoproteins embedded in a proteoglycan and glycosaminoglycan gel. This matrix becomes highly organized around epithelial cells, endothelial cells, and smooth muscle cells, forming the specialized basement membrane (Shaw et al., 2016).

Collagens, synthesized by fibroblasts, are the most abundant proteins in the body and play a vital role in wound repair by providing strength and integrity to tissues. Various growth factors and cytokines induce collagen synthesis during the proliferative and remodeling phases. Collagen types (e.g., I, III, V) form fibrils and constitute most of the connective tissue in healing wounds, while other types (e.g., IV) are non-fibrillar and part of the basement membrane. Adhesive glycoproteins, such as fibronectin and laminin, link extracellular matrix components and cells. Fibronectin mediates cell attachment, spreading, and migration, and enhances the sensitivity of certain cells to growth factors. Integrins are glycoproteins that mediate cell-cell and cell-matrix adhesion, integrating extracellular matrix functions with the cytoskeleton. Proteoglycans, consisting of glycosaminoglycans linked to a protein backbone, regulate the structure and permeability of the extracellular matrix, and modulate cell growth and differentiation. By 3-5 days post-injury, granulation tissue, indicative of optimal healing, is well established. It appears pink, soft, and granular, and is characterized by proliferating fibroblasts and capillary loops in a loose extracellular matrix. Angiogenesis, or the formation of new blood vessels, occurs during this phase through four steps: proteolytic degradation of the parent vessel basement membrane, endothelial cell migration towards the angiogenic stimulus, endothelial cell proliferation, and maturation into capillary tubes. New vessels are initially edematous due to incomplete junctions and increased transcytosis. Angiogenic factors induce capillary sprout invasion into the fibrin/fibronectin-rich wound clot, forming a microvascular network in the granulation tissue. The density of blood vessels decreases as collagen accumulates, forming a scars (Nunan et al., 2015).

Granulation tissue bleeds easily if traumatized, and its appearance indicates wound status. Healing wounds are moist, shiny, hyperemic, and reddish, while excessive, friable, beefy-red wounds suggest poor healing. Epithelialization begins within a few hours of wounding as a single layer of epidermal cells migrates from the wound edges, forming a delicate cover over the exposed area. By about 12 hours, mitotic activity increases in basal epithelial cells at the wound edges. These cells migrate as a sheet, extending lamellipodia along the advancement edge and loosening their firm attachments to the dermis to migrate across the provisional matrix. When advancing epithelial cells meet, contact inhibition halts further movement, and a new basement membrane regenerates. Epithelialization requires a moist environment, adequate nutrition, bacteriological control, and is modulated by growth factors like keratinocyte growth factor, epidermal growth factor, and basic fibroblast growth factor (Honnegowda et al., 2015).

1.5.4. Remodeling and scar maturation (week 1 to several weeks)

The synthesis and remodeling of the extracellular matrix begins concurrently with the development of granulation tissue and continues over extended periods. There is continuous synthesis and breakdown of collagen as the extracellular matrix is constantly remodeled, reaching a steady state about 21 days after wounding. Wound contraction occurs through interactions between fibroblasts and the surrounding extracellular matrix and is influenced by various cytokines, including transforming growth factor-β, platelet-derived growth factor, and basic fibroblast growth factor. Collagen degradation is achieved by specific metalloproteinases produced by fibroblasts, neutrophils, and macrophages at the wound site. The synthesis and secretion of metalloproteinases are regulated by growth factors, cytokines, and phagocytic stimuli. Metalloproteinases include interstitial collagenases, which cleave fibrillar collagen types I, II, and III; gelatinases (or type-IV collagenases), which degrade amorphous collagen and fibronectin; and stromelysins, which catabolize various constituents of the extracellular matrix, including proteoglycans, laminin, fibronectin, and amorphous collagen (Darby et al., 2014). Metalloproteinases depend on zinc ions for their activity and should be distinguished from other proteases like neutrophil elastase, cathepsin G, and plasmin, which can also degrade the extracellular matrix but are not metalloenzymes. The activity of metalloproteinases is tightly regulated to prevent the degradation of essential collagen and impaired healing. They are typically produced as inactive precursors (zymogens) that must be activated by certain proteases

(e.g., plasmin) likely present only at injury sites. Activated collagenases can be rapidly inhibited by specific tissue inhibitors of metalloproteinases, produced by most mesenchymal cells. Further remodeling of the wound causes a decrease in the activity of metalloproteinases and an increase in the activity of tissue inhibitors of metalloproteinases. This process also reduces the density of macrophages and fibroblasts, halts the outgrowth of capillaries, reduces blood flow and metabolic activity, and decreases the size of the underlying contractile connective tissue, bringing the wound margins closer together (Amadeu et al., 2004). Ultimately, the granulation tissue scaffolding evolves into an avascular scar composed of largely inactive fibroblasts, dense collagen, fragments of elastic tissue, and other components of the extracellular matrix. As the scar matures, fibronectin and hyaluronan are broken down, and collagen bundles increase in diameter, which corresponds with increasing tensile strength of the wound. However, these collagen fibers never regain the original strength of unwounded skin, achieving a maximum of 80% of the strength of unwounded skin (Larouche et al., 2018).

1.6. Current scenario for the treatment of wound healing

Despite ongoing efforts, there remains no effective method to treat or prevent the under healing or over healing of wounds. The surge of new products and drugs in the wound closure market underscores the significant need for an ideal wound healing therapy, which is currently nonexistent. Chronic wounds present a substantial and growing biomedical challenge, and the global market for anti-fibrotic therapies exceeds $10 billion. Many existing therapies are not only unpredictable and often ineffective but also painful to apply and difficult to use (Las Heras et al., 2020). There are several reports on topical adjuvant therapies for wound healing, particularly strategies for improving healing in challenging wounds such as chronic wounds and burns. The primary treatment strategy for chronic wounds is to correct the wound's dysregulated physiological state to promote healing. This includes ensuring the patient's nutritional and hydration status is adequate and managing any comorbidities contributing to ulcer development. Infection control, proper wound oxygenation, and debridement are essential, as is minimizing excess pressure or other mechanical stress on the wound. Treatment varies depending on the type of chronic wound. Arterial ulcers often require surgical revascularization combined with debridement. Diabetic ulcers involve debridement and basic wound care, including cleansing and using dressings that maintain moisture while minimizing contact with wound exudates (Jeffcoate

et al., 2004). Venous and pressure ulcers are treated with standard wound care practices, with surgical options like primary closure or skin grafting available if necessary. Additional strategies to aid wound healing include negative pressure wound therapy (NPWT) and hyperbaric oxygen therapy (HBO). NPWT is thought to clear exudate and harmful bacterial products from the wound, stabilize it mechanically, increase tissue perfusion, and promote granulation tissue formation at the cellular level. However, evidence supporting NPWT in chronic wounds is mixed. HBO aims to increase tissue oxygenation and reduce inflammation, but its efficacy reports are inconsistent. Becaplermin (REGRANEX Gel) is a topical recombinant human PDGF-BB used as an adjuvant therapy for diabetic neuropathic ulcers of the lower extremity (Han, S. K. 2023). However, when compared to drugs like the angiotensin analogue NorLeu3-A(1-7), becaplermin showed inferior wound healing capacity in terms of accelerating the closure of full-thickness wounds. Additionally, becaplermin should be used cautiously in patients with malignancies and is contraindicated in those with malignancies at the application site, as using three or more tubes is associated with increased mortality due to malignancy.

1.6.2. Recent therapeutic strategies- Nanomedicine for Wound Healing

1.6.2.1. Nanomedicine strategies to address bacterial infection

Chronic wounds are often prone to infection. While the body's defense system can initially control the growth and spread of microorganisms, pathogens such as bacteria can quickly evolve, adapt, or develop biofilms, leading to infections that impair wound healing. Nanomedicine has shown promise in eliminating these infections. Nanomaterials are being used as direct antimicrobial agents due to their "nano" characteristics and intrinsic antimicrobial properties, as well as carriers of antibiotics or other antimicrobial agents. The exact mechanisms by which nanoparticles (NPs) exert toxicity against bacteria are not fully understood, but several theories have been proposed. NPs may have a bactericidal effect through direct interaction with the bacterial cell wall, releasing toxic ions, or generating reactive oxygen species (ROS) (Wang et al., 2020). The physical interaction between NPs and bacterial cell walls involves van der Waals forces, hydrophobic or electrostatic forces, and receptor-ligand interactions, which can affect the permeability and integrity of the membrane. NPs can also cross the cell membrane and interact adversely with subcellular components such as proteins and DNA. This interaction can induce oxidative or nitrosative stress, deactivate proteins, and alter gene expression and protein

synthesis, ultimately inhibiting bacterial growth or causing bacterial death. The antibacterial effects of ions released by NPs vary depending on their composition and the bacterial species. For instance, metallic and ionic forms of copper and silver NPs produce hydroxyl radicals that damage essential proteins and DNA, while iron ions from superparamagnetic iron oxide NPs (SPIONs) can serve as a critical nutrient for bacterial growth and survival. Additionally, NPs can disrupt the function of efflux pumps by binding to their active sites or altering their kinetics, impairing homeostasis, or causing detrimental changes to the membrane's surface charge (Xiu et al., 2021).

1.6.2.2. Nanomedicine improving Angiogenesis

During angiogenesis, new blood vessels form from existing vasculature to deliver oxygen, nutrients, and inflammatory response components essential for wound healing. This process is crucial throughout life, playing a significant role in various health and disease conditions, including tumor growth, metastasis, retinopathy, and coronary artery disease. In wound healing, angiogenesis is vital for the proliferative phase, where new capillaries grow into the wound and form granulation tissue. Without angiogenesis, only superficial wounds would heal effectively (Mukherjee et al., 2020). While acute wounds produce normal granulation tissue, impaired angiogenesis in chronic wounds leads to defective granulation tissue, delaying the progression to the proliferation phase.

Nanomaterials have been reported to promote angiogenesis in various tissue regeneration scenarios, such as bone and nerve tissue repair, post-ischemia reperfusion repair, and wound healing. The use of nanomaterials for promoting angiogenesis may be a promising alternative to traditional growth factors like VEGF-A or PDGF, which can sometimes promote pathological angiogenesis, thrombosis, and fibrosis. Biological angiogenic agents, such as growth factors, are often costly, require complex processing, and may have short half-lives or become unstable in the chronic wound environment. For instance, fibroblast growth factor 7 showed initial success in treating venous ulcers in a phase 2 clinical trial but did not significantly increase the percentage of full wound healing within 20 weeks, primarily due to insufficient retention of the growth factors in the wound. Nanomaterials as delivery vehicles may address this issue (Kargozar et al., 2020).

Gold nanoparticles (Au NPs) have demonstrated significant angiogenic value in wound repair. These versatile nanomaterials exhibit biocompatibility and unique physico-optical characteristics. Studies have shown that Au NPs coated onto hydrocolloid membranes (HCM) significantly increase the expression of angiogenesis biomarkers like VEGF, angiopoietin 1, and angiopoietin 2 in cutaneous wounds in rat models, leading to faster wound closure. Another study found that Au NPs combined with photo biomodulation therapy (PBMT) accelerated wound contraction and increased angiogenesis in rats, with Au NPs alone also showing better results than controls. Additionally, combining Au NPs with antibacterial agents has yielded both antibacterial and angiogenic effects (Sharifi et al., 2020).

Hypoxia-mimicking materials such as silicate ions also support angiogenesis in wounds. Silicate ions released from biomaterials can trigger the angiogenesis cascade by promoting the expression of proangiogenic factors like VEGF and FGF and stimulating nitric oxide (NO) production by endothelial cells, resulting in new blood vessel formation. Loading wound dressings with hypoxia-mimicking materials has shown promise in treating chronic wounds. For example, SiO2 nanoparticles in a freeze-dried polyvinylpyrrolidone (PVP) and PVP-gel matrix accelerated the healing of full-thickness dermal wounds in a murine model, showing higher vascularization levels compared to controls (Kargozar et al., 2020).

Interestingly, nanoparticles can also suppress angiogenesis by reducing the vascular lumen and inhibiting new blood vessel formation. Some NPs exhibit antiangiogenic properties depending on their type, size, and other physicochemical properties. For instance, smaller Au and Ag NPs (20 nm) have been found to be more potent antiangiogenic materials than larger counterparts (100 nm), effectively suppressing VEGF-induced activation of VEGF receptor-2 (Hoseinzadeh et al., 2022).

1.6.2.3. Nanomedicine for the treatment of chronic inflammation

Using nanodevices to manage inflammation is highly effective in aiding the healing of chronic wounds. Nanoparticles (NPs) are promising candidates for targeted delivery and sustained release of anti-inflammatory agents. For instance, gold nanoparticles (Au NPs) have been used to deliver carbon monoxide-releasing molecules incorporated into bovine serum albumin (BSA). While low concentrations of carbon monoxide (CO) have anti-inflammatory effects, high concentrations can be harmful. Traditional metal and nonmetal CO-releasing molecules face

issues like low water solubility and rapid CO release. These issues were initially addressed by using proteins as carriers of CO, known as CO-releasing molecules (CORMs), which are soluble, stable, and safe. However, protein-CORMs have short circulation times and non-targeted distribution once inside the body. To overcome these limitations (Chen et al., 2021), CORMs were conjugated to gold NPs, extending their circulatory half-life and improving targeted delivery and sustained release of CO. This nano formulated CORM demonstrated high cellular uptake efficacy, strong anti-inflammatory effects, and quick tissue regeneration in wound areas.

Fullerene, with its highly unsaturated structure and high electron-receptor properties, is considered an excellent radical scavenger. Fullerene can neutralize benzyl and methyl radicals, forming stable radical or nonradical adducts. Studies have tested various water-soluble fullerene derivatives for their ability to accelerate wound healing in ex vivo human skin models and in vitro mouse models of skin irritation. Several derivatives, including ALM-liposome, C70-tetrainositol, C70-ethanolamine, and ALP-P, showed accelerated wound healing without cytotoxicity. Although this study did not include chronic wounds with stalled inflammation, the accelerated healing might be due to fullerene's modulatory effect on keeping reactive oxygen species (ROS) levels low, providing a constant stimulatory effect during wound healing. Fullerenes have also been shown to regulate immune reactions in chronic inflammatory skin diseases like atopic dermatitis by significantly reducing IL-4 and IL-5 concentrations (Spesia et al., 2017).

Nanoparticle systems can be beneficial in all stages of wound healing. For example, cerium oxide (CeO2) nanoparticles loaded with L-arginine can accelerate hemostasis through a nano bridging effect, where NPs adsorb onto polymer or protein chains and act as cross-linkers. CeO2 NPs can eradicate bacterial infection through ROS production upon sunlight irradiation. These NPs can also capture excess ROS and release loaded arginine under the catalysis of inducible nitric oxide synthase (iNOS), producing nitric oxide (NO). NO, in combination with L-arginine, promotes the proliferation stage of wound healing (Ye et al.,2016).

1.6.3. Advanced therapeutic options for wound healing

Advanced therapeutic options include stem cell therapy, laser therapy and newer chemotherapy which are listed below.

1.6.2.1. Stem Cell Therapies

Wound care is a significant medical challenge, and this section reviews some of the most innovative proposed therapies. The presence of epidermal stem cells (ESCs) in the skin is advantageous for therapy adoption, as these cells influence the proliferation and migration of fibroblasts and keratinocytes and promote angiogenesis.

Mesenchymal stem cell (MSC) therapy involves techniques using scaffolds seeded with MSCs and biomaterials like collagen or cellulose. MSCs stimulate granulation tissue formation, angiogenesis, and future vascularization. They recruit endothelial cells by releasing factors such as FGF and ANG1. MSCs modify TNF alpha production and reduce the function of natural killer (NK) cells during the inflammatory phase, altering IFN gamma activity. Using MSCs during the final healing phase, particularly scar formation, lowers the TGFbeta1/TGFbeta3 ratio, increases IL10, and reduces IL6 and IL8 levels (Rodrigues et al., 2019).

1.6.2.2. Chemotherapy for Wound Healing

Various drugs impact wound healing, including anticoagulants, antimicrobials (such as antibiotics), anti-angiogenesis agents (e.g., bevacizumab, aflibercept), antineoplastic drugs, antirheumatoid drugs, nicotine, steroids, and vasoconstrictors. Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are particularly noteworthy. Among new drugs, Exendin 4 (Exe4) is a significant candidate. Exe4 is a naturally occurring peptide with a 53% similarity to Glucagon-Like Peptide-1 (GLP1), an intestinal insulinotropic peptide part of the incretin hormone family. GLP1 plays a crucial role in postprandial insulin secretion, and experimental evidence suggests this may promote tissue regeneration (Zhao et la.,2024).

The long-term outcomes of intralesional triamcinolone acetonide (TA) injections can vary significantly among patients. Since their introduction in 1961, TA injections have been found to either improve the appearance of scars or be similar in effect to a placebo. Other topical treatments for scarring include immune modulators and chemotherapeutic agents like 5-fluorouracil, which is a well-established anti-scar therapy, and more recently, imiquimod 5% cream. Imiquimod works by stimulating the production of proinflammatory cytokines, leading to increased collagen breakdown within the scar. However, its use has been linked to additional disfigurement due to hyperpigmentation and hypopigmentation of the treated skin. Intralesional

injections of bleomycin, an antineoplastic agent, have been found to reduce the surface area of large keloids and hypertrophic scars (Zhuang et al., 2021). When combined with electroporation, which involves short, intense electrical pulses to increase membrane permeability to drugs, bleomycin significantly reduced scar size and erythema. Despite promising initial results, this combination therapy requires multiple sessions and is not yet considered an established treatment for keloids. CO2 lasers have shown efficacy in recontouring keloids by activating fibroblasts and rearranging collagen fibers, though multiple treatments are necessary, and higher treatment frequencies can lead to increased local erythema. A novel therapy developed by Waibel and colleagues utilized fractional laser treatments to enhance the delivery of topical triamcinolone, resulting in improved scar hypertrophy and texture, though the improvement in scar color was less notable. Topical application of basic fibroblast growth factor (FGF-2) has shown clinical success in improving scar color and texture in various types of wounds, including burns, chronic wounds, and split-thickness skin-grafted wounds. While this treatment has shown promise in Japan, it is not yet available for clinical use in Europe or the USA (Zhuang et al., 2021).

1.6.2.3. Laser Therapies for Wound Healing

The use of phototherapy for wound healing is gaining importance. Lasers, especially fractional ablative lasers like CO2 or erbium, are critical in assisted healing treatments. Non-ablative fractional lasers or vascular lasers are also commonly used. Photodynamic therapy (PDT) combines harmless light in the protoporphyrin absorption spectrum with non-toxic photosensitizing dyes. PDT has been explored for chronic wounds due to its ability to reduce microorganisms by inducing reactive oxygen species (ROS) without causing antibiotic resistance. PDT also promotes tissue regeneration, decreases metalloproteinase activity, and regenerates collagen. However, its clinical use for wound healing is not yet widespread due to limited studies and the need for multiple sessions with current lights and photosensitizers (Chaves et al., 2014).

Low-level laser light therapy (LLLT) induces cellular modifications (photobiomodulation) that lead to beneficial clinical effects. LLLT can be applied with low fluences or laser light, but light-emitting diodes (LEDs) are now promoted to simplify the technique. LLLT works by cytochrome c absorbing photons, which increases ATP production and boosts ROS and transcription factors. However, LLLT's role remains controversial. LEDs are revolutionizing

lighting due to their ease of use, though they differ technically from LLLTs. LEDs tend to decrease the inflammatory state of the lesion and allow targeted biofilm modulation (Chaves et al., 2014).

1.7. Medicinal plants used in wound healing

India is home to a diverse and abundant flora, which has been integral to traditional medicinal practices like Ayurveda, Unani, and Siddha. Herbal medicines, derived from plants, have been used for centuries to treat various diseases and physiological conditions. These plant-based drugs play a significant role in both traditional and modern medicine. Historically, plants have been a crucial part of human healthcare, especially in India and China. Today, many drugs are developed from plant compounds, which are isolated and modified to enhance their efficacy against numerous diseases. The World Health Organization (WHO) reports that over 80% of the global population relies on traditional medicine for various ailments. In developed nations, 25% of medical drugs are derived from plants and their components, with medicinal plant use being prevalent among rural populations in developing countries. Medicinal plants, which can be used directly or indirectly to treat different conditions, are a cornerstone of traditional medicine and are widely recognized for maintaining good health in developing regions (Jamshidi-Kia et al., 2017).

Researchers worldwide are exploring the potential of medicinal plants to aid humanity, with more than 30% of pharmaceutical products based on plant sources. The use of plant-based medicines dates back to 4000-5000 B.C., with the medicinal properties of these plants attributed to small doses of active compounds that produce physiological effects in humans and animals. Key bioactive compounds in medicinal plants include alkaloids, glycosides, resins, gums, and mucilages. Developed countries often import raw medicinal plant materials from developing countries, where they are processed into high-priced medicines that are then sold back to the developing world. The relationship between humans and plants has deep historical roots, with medicinal preparations historically derived from plant parts or as crude extracts. Today, a significant number of effective drugs for treating life-threatening diseases are plant-derived. Many therapeutic compounds are isolated directly from medicinal plants or are modifications of these compounds. In many developing countries, around 25% of therapeutic drugs are plant-

based or derived from plants, with their use being well-known among rural indigenous populations (Ghosh et al., 2013).

The discovery of plants' healing powers by ancient civilizations was a result of trial and error, yet the effectiveness of medicinal plant therapies based on empirical findings over millennia is remarkable, despite some erroneous attributions of therapeutic properties. Infectious diseases remain a leading cause of premature death globally, causing significant morbidity and mortality, particularly due to bacterial infections like those caused by Escherichia coli, Salmonella, and Staphylococcus aureus. The widespread and excessive use of antibiotics has led to drug resistance in human pathogens, presenting a global challenge in clinical settings. This situation underscores the need for alternative antimicrobial therapies. Screening local medicinal plants for antimicrobial properties is a promising approach, as plant materials are a vital resource for combating serious diseases. According to WHO, nearly 80% of the world's population relies on traditional medicine and therapies involving active constituents from various plant extract. Research in this area is an emerging field that combines microbiology and ethnobotany aiming to identify new antimicrobial agents from plants (Ahmed et al., 2024).

Table 2: Medicinal plants used for wound healing.

SL. No.	Name of medicinal plant	Active compound	Mechanism of action /Therapeutic activity	References
1.	*Aloevera*	Acemannan	Immunomodulatory, Antiviral, Anticancer, Antidiabetic	(Liang et al., 2021)
2.	*Andrographis paniculata*	Andrographolide, Kalmeghin	Antimalarial, Antimicrobial Antioxidant and Antiviral.	(Al-Bayaty et al., 2012)
3.	*Angelica sinensis*	Ferulic acid and Butylidene phthalide	Anticancer, Anti-inflammatory,	(Bi et al., 2021)

			Antioxidant, and Immunomodulatory	
4.	*Blumea balsamifera*	L-Borneol	Antifungal, Antiobesity, Antiplasmodial, Antitumour	(Masyudi et al., 2022)
5.	*Camellia sinensi*	Epicatechin-3-gallate, Epicatechin, Epigallocatechin, Epigallocatechin-3 gallate	Anti-inflammatory, Antimicrobial Antiobesity, Antioxidant, Cardioprotective Neuroprotective	(Kouhihabibidehkordi et al., 2021)
6.	*Curcuma longa*	Curcuminoids	Antibacterial, Anti-inflammatory and Antioxidant	(Bouchama et al., 2023)
7.	*Cinnamomum cassia*	Cinnamaldehyde	Anticancer, Antidiabetic, Anti-inflammatory, Antimicrobial, and Antioxidant	(Khaled et al., 2024)
8.	*Entada phaseoloides*	Tannin	Antibacterial, Antioxidant	(Sharanya et al., 2022)
9.	*Lonicera japonica*	Biflavonoids, Dicaffeoylquinicacid	Anti-inflammatory, Antimicrobial and Antioxidant	(Li et al., 2020)
10.	*Panax ginseng*	GinsenosidesRb1, Rb2,Rc,and Rd	Antiaging, Antiallergic, Anticancer, Anti-inflammatory, Antimicrobial,	(Yang et al., 2023)

			Antioxidant, Immunomodulating	
11.	*Rheum officinale*	Emodin	Antiaging, Antiallergic, Anticancer, Anti-inflammatory, Antimicrobial, Antioxidant, Immunomodulating, Antibacterial, Antioxidative, Haemostatic	(Akbar et al., 2020)
12.	*Salvia miltiorrhiza*	Cryptotanshinone, Danshensu, Salvianolic acid B	Anticancer, Anti-inflammatory, Antimicrobial, Antioxidant, Antiplatelet aggregation, Proangiogenic	(Jung et al., 2020)
13.	*Stemona tuberosa*	Tuberostemonine N	Antibacterial, Anti-inflammatory, Antioxidant, Antitussive, and Neuroprotective	(Nguyen et al., 2024)
14.	*Wedelia trilobata*	Kaurenoicacid and Luteolin	Antimicrobial, Antioxidant, and Antitumour	(Azmee et al., 2023)
15.	*Zanthoxylum bungeanum*	Afzelin, Hyperoside, Quercitrin and Rutin	Anaesthetic, Antiasthma, Anti-inflammatory,	(Okagy et al., 2021)

			Antioxidant and Antitumour	

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CHAPTER 2: AIM, OBJECTIVES & PLAN OF WORK

2. AIM AND OBJECTIVES

2.1. AIM

To perform preclinical screening of wound healing activity of *Sonneratia apetala* Buch. -Ham on wounded rat.

2.2. OBJECTIVES OF WORK

- Antioxidant assay of the selected mangrove plant.
- Total phenolic content (TPC) and total flavonoid content (TFC) determination.
- Acute skin irritation assay
- Ointment formulation
- Evaluation of the in-vivo wound healing activity of the *Sonneratia apetala* on wounded male albino wistar rats.

2.3. RATIONALE OF WORK

The process of wound healing involves a multifaceted and dynamic sequence of events that demands the coordinated actions of diverse cells, tissues, and biochemical processes to facilitate tissue repair. Presently, a limited number of pharmaceuticals are accessible that have the capacity to enhance the speed of wound healing significantly. Throughout history, wounds have been managed using herbal remedies. *Sonneratia apetala* (*S. apetala*) is predominantly found in regions including Southeast Asia, the Indian subcontinent, and coastal areas of Africa and Australia. *S. apetala* has a significant historical background in traditional medicine, where it has been used for the treatment of various conditions including respiratory ailments, inflammation, and pain. Due to its historical usage and preliminary research on its bioactivities, there exists a compelling necessity to scientifically validate and investigate the wound healing properties of *S. apetala*. Despite its widespread presence, the medicinal attributes and chemical components of *S. apetala* have not been extensively examined. Initial studies have suggested that *S. apetala* demonstrates notable antioxidant and anti-inflammatory properties, which play a vital role in the process of wound healing. The pharmacological effects of this plant demand in-depth validation through vivid preclinical investigation, and the bioactive constituents accountable for such therapeutic effects requires thorough identification. This preclinical study seeks to bridge these

research gaps by systematically examining the wound healing potential of *S. apetala* in a preclinical settings using wound excision model in rats.

2.4. PLAN OF WORK

- Collection of *Sonneratia apetala* Buch. -Ham plant's fruit.
- Hydro-alcoholic extraction of the selected plant by soxhlet assisted extraction.
- Preliminary phytochemical study of the extract.
- DPPH radical scavenging assay.
- Total phenolic content (TPC) and Total flavonoid content (TFC) determination.
- Ointment formulation
- Acute skin irritation assay
- Evaluation of In-vivo wound healing potential
- Wound size Measurement
- Tissue collagen marker determination
- Histopathology

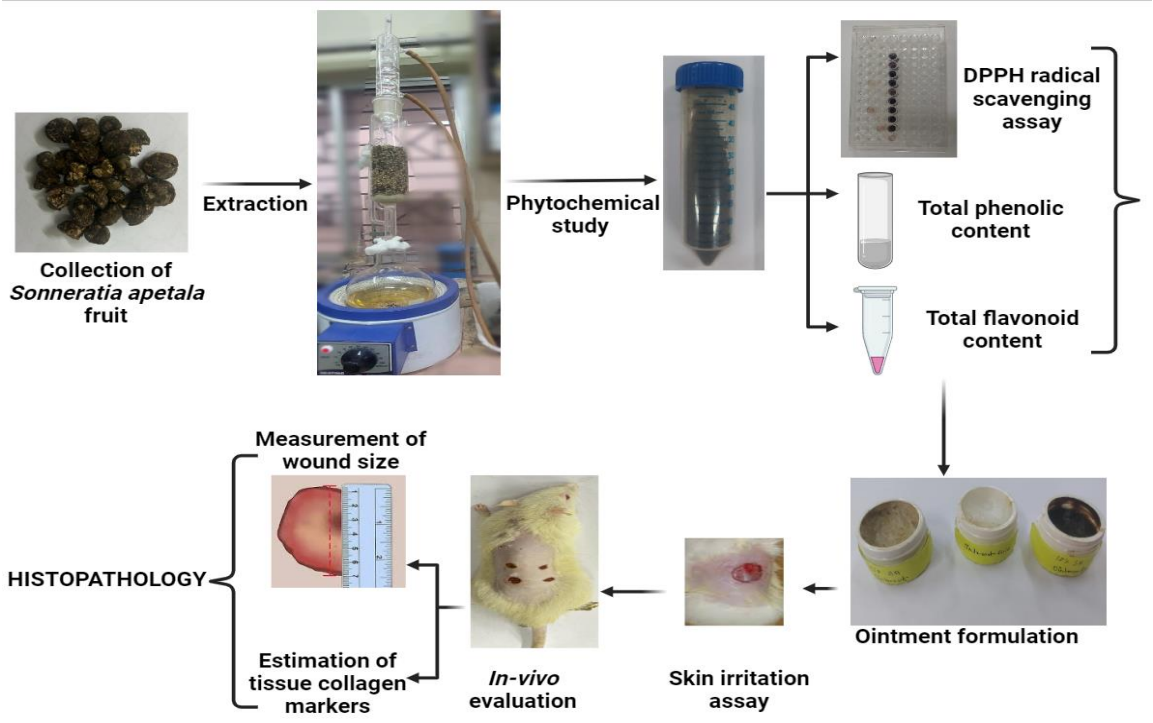


Figure 2.1: Graphical representation for the Plan of work.

CHAPTER 3: PLANT PROFILE AND LITERATURE REVIEW

3.1. Plant Taxonomy of *Sonneratia apetala* Buch. -Ham

Domain: Eukaryote

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Eudicots

Order: Myrtales

Family: Lythraceae

Genus: *Sonneratia*

Species: *Sonneratia apetala* (Zhong et al., 2020)

3.2. BOTANY OF *SONNERATIA APETALA*

Sonneratia apetala, commonly known as mangrove apple or mangrove crabapple, is a species of plant in the family Lythraceae. It is one of the key mangrove species found in coastal and estuarine environments, particularly in the Indo-West Pacific region. Here are some key aspects of its botany:

Morphological Characteristics

- **Tree Structure**: *Sonneratia apetala* is a medium-sized tree that can grow up to 15 meters in height. The trunk is often buttressed, and the bark is greyish-brown, rough, and fissured.
- **Leaves**: The leaves are simple, opposite, and ovate to elliptical in shape. They are leathery, dark green on the upper surface, and paler beneath. The leaves have a smooth margin and a blunt or slightly notched apex.
- **Flowers**: The flowers are large, white, and fragrant, typically blooming at night. They are solitary or in small clusters, with numerous long, white stamens that give the flower a brush-like appearance. The flowers are adapted for pollination by nocturnal animals such as bats and moths.

- **Fruit**: The fruit is a fleshy, globose berry that is green when immature and turns brown when ripe. It contains several seeds and is buoyant, aiding in dispersal by water.

Figure 3.1: Fruits of *Sonneratia apetala.*

3.3. CHEMISTRY OF *SONNERATIA APETALA*

The chemistry of ***Sonneratia apetala*** involves various bioactive compounds that contribute to its medicinal properties and ecological functions. These compounds are distributed in different parts of the plant, such as leaves, bark, and fruits. The primary classes of compounds include flavonoids, tannins, saponins, alkaloids, and phenolic acids (Islam et al., 2020).

3.3.1. Major Bioactive Compounds present in *Sonneratia apetala*

3.3.1.1. Flavonoids: Flavonoids are a diverse group of phytonutrients known for their antioxidant properties. In *Sonneratia apetala*, compounds such as quercetin, kaempferol, and their glycosides have been identified. These compounds are involved in protecting the plant against UV radiation, pathogens, and herbivores.

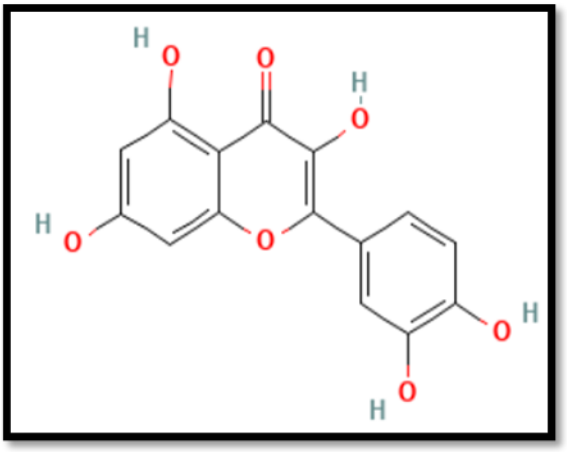
3.3.1.2. Tannins: Tannins are polyphenolic compounds with astringent properties. They play a role in defense against herbivores and pathogens and contribute to the plant's overall resilience. Ellagitannins and Gallo tannins are common in mangrove species, including *Sonneratia apetala*.

3.3.1.3. Alkaloids: Alkaloids are nitrogen-containing compounds that often have significant pharmacological effects. Specific alkaloids present in *Sonneratia apetala* contribute to its medicinal properties, such as analgesic and anti-inflammatory effects.

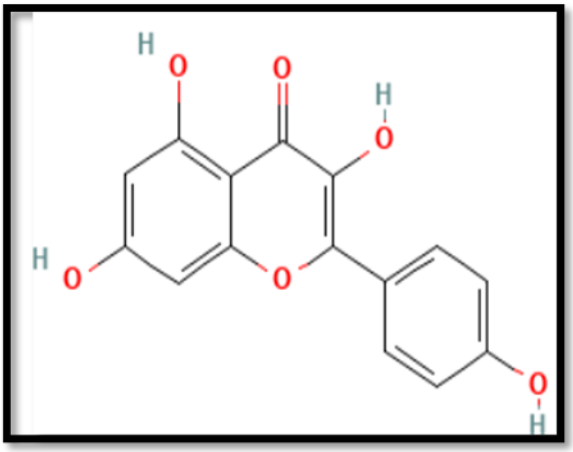
3.3.1.4. Phenolic Acids: Phenolic acids such as gallic acid and ellagic acid, are known for their antioxidant and anti-inflammatory properties. These compounds help in protecting the plant from oxidative stress and contribute to its medicinal benefits (Liu et al., 2023; Uddin et al., 2024).

3.3.2. Chemical Structures of major bioactive compounds in *Sonneratia apetala* (pubchem.nih.gov)

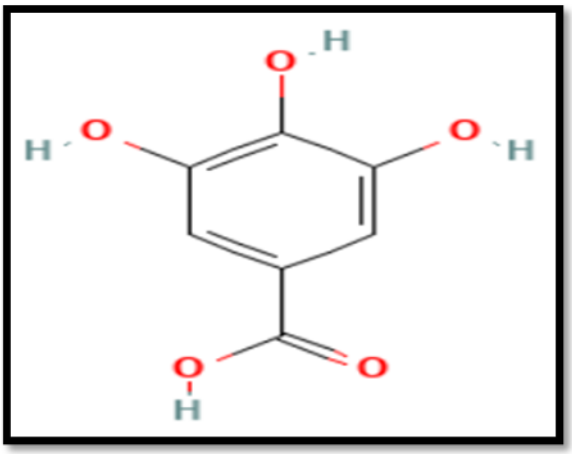
Here are the chemical structures of some key compounds found in *Sonneratia apetala*:



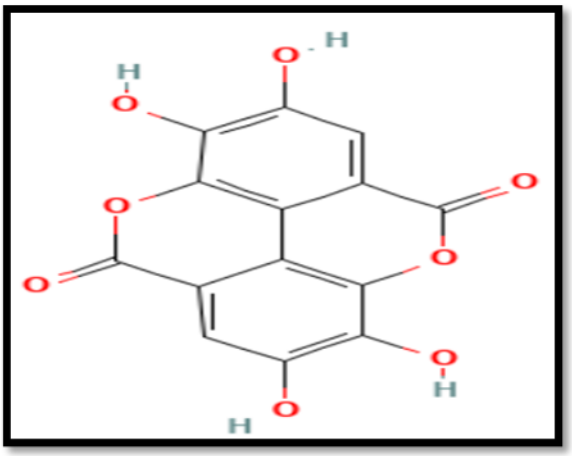
Quercetin



Kaempferol



Gallic acid



Ellagic acid

3.4. ETHNOMEDICINAL EVIDENCES OF *Sonneratia apetala*

3.4.1. Antimicrobial activity of *Sonneratia apetala* (Tauhidur et al., 2020)

In this study the authors isolated fifty-six endophytes from the leaves, roots, bark, and fruits of *Sonneratia apetala*, a pioneer mangrove plant in the Sundarbans, Bangladesh. A total of 56 isolates were obtained, and 12 different species within 8 genera were identified using morphological and molecular characteristics. The antimicrobial activity of ethyl acetate (EtOAc) and methanolic (MeOH) extracts of these 12 different species was analyzed by resazurin assay, and the minimum inhibitory concentrations (MICs) were determined. The fungal extracts exhibited antimicrobial activities against more than one tested bacterium or fungus among 5 human pathogenic microbes, namely Escherichia coli NCTC 12241, *Staphylococcus aureus* NCTC 12981, Micrococcus luteus NCTC 7508, *Pseudomonas aeruginosa* NCTC 7508, and Candida albicans ATCC 90028. Overall, methanolic extracts demonstrated greater activity than ethyl acetate extracts. *Aspergillus niger* and *Fusarium equiseti* were the most active isolates and showed activity against the microorganisms under investigation. Methanolic extracts of Colletotrichum gloeosporioides and A. niger exhibited the lowest MIC (0.0024 mg/mL) against P. aeruginosa. This study indicated that endophytic fungi isolated from S. apetala species possessed potential antimicrobial properties, which could be further investigated.

3.4.2. Anti-oxidant activity of *Sonneratia apetala* (Hossain et al., 2016)

The study evaluated nutrient compositions in the pericarp and seed of the fruit. Each pericarp and seed was successively fractionated into n-hexane, diethyl ether, chloroform, ethyl acetate, and methanol. Polyphenol contents and antioxidant activities of different pericarp and seed fractions were measured using various in vitro methods, and phenolic compounds were determined by HPLC. Carbohydrates, proteins, lipids, and ash contents were found to be 29.6%, 8.8%, 2.8%, and 25.5% of dry weight in the pericarp, whereas in the seed, these values were 28.3%, 11.5%, 4.2%, and 22.7%, respectively. Among the mineral macro-elements, potassium content was the highest, followed by sodium, calcium, magnesium, phosphorus, and sulfur, while in micro-elements, iron was the largest, followed by manganese, zinc, and copper. The methanol fraction of the seed exhibited the highest polyphenol content (221.9 mg gallic acid equivalent/g fraction), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (IC50 = 2.1 μg/mL), and nitric oxide (NO) (IC50 = 490.8 μg/mL) free radical scavenging activities. Similarly, the methanol fraction of the seed also

demonstrated very strong reducing power (OD = 1.67 at 100 μg/mL), Fe2+ chelating activity, and total antioxidant capacity.

3.4.3. Hepatoprotective and anti-inflammatory activity of *Sonneratia apetala* (Liu et al., 2019)

The aim of this study was to evaluate the antioxidant activity and hepatoprotective effect of a mangrove plant Sonneratia apetala fruit extract (SAFE) on APAP-induced liver injury in mice. Mice were orally pretreated with SAFE (100, 200, and 400 mg/kg) daily for one week. The control and APAP groups were intragastrically administered distilled water, and the NAC group was treated with N-Acetyl-L-cysteine (NAC) before APAP exposure. The results indicated that SAFE significantly improved survival rates, attenuated hepatic histological damage, and decreased the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum in APAP-exposed mice. SAFE treatment also increased glutathione (GSH) level and glutathione peroxidase (GSH-Px) activity, enhanced catalase (CAT), and total antioxidant capacity (T-AOC), as well as reducing malondialdehyde (MDA) levels in the liver. Additionally, the formation of tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and elevation of myeloperoxidase (MPO) in APAP-exposed mice were inhibited after SAFE treatment. SAFE also demonstrated high DPPH radical scavenging activity and reducing power in vitro. The main bioactive components of SAFE, such as total phenol, flavonoid, condensed tannin, and carbohydrate, were determined. The study concluded that SAFE exerted a potential protective effect against APAP-induced acute liver injury, which might be associated with the antioxidant and anti-inflammatory activities of SAFE.

3.4.4. Antidiabetic and antibacterial activity of *Sonneratia apetala* (Hossain et al., 2013)

Seeds of *Sonneratia apetala* were found to contain very high levels of polyphenols (300 ± 8.2 mg GAE/g extract), flavonoids (30.6 ± 0.7 CE/g extract), anthocyanins (2.3 ± 0.03 μmol/g extract), and vitamin C (4.0 ± 0.08 mg/g extract). The IC50 values for DPPH and NO free radical scavenging were 4.3 and 49.4 μg/mL for the seed extract, and 59.8 and 751.6 μg/mL for the pericarp extract, respectively. The seed extract also exhibited very high reducing power (O.D. 1.14 at 50 μg/mL extract) and total antioxidant capacity (280.8 GAE or 310.24 AAE/g extract). In streptozotocin (STZ)-induced type 2 diabetic rats, the group treated with seed extract showed a decrease in serum glucose levels from 13.75 ± 2.21 mmol/L (at 30 min) to 10.3 ± 1.75 mmol/L

(at 135 min), while in the pericarp-treated group, levels decreased from 14.36 ± 2.16 to 11.32 ± 1.74 mmol/L. The area under the glucose curve was more significantly decreased in the seed-treated group compared to the pericarp-treated group. Susceptibility tests revealed that the seed extract inhibited the growth of both gram-positive and gram-negative pathogenic bacteria. Therefore, fruits of *S. apetala*, especially its seeds, were found to be rich in phenolics, flavonoids, and compounds with antioxidant, antidiabetic, and antibacterial properties.

3.4.5. Cytotoxic activities of *Sonneratia apetala* (Uddin et al., 2024)

Extracts from the plant components of *Sonneratia apetala* were found to exhibit significant antioxidant and cytotoxic effects. The edible mangrove fruit of *S. apetala* was identified as a natural herb, rich in bioactive properties and functional phytochemicals. Both the methanol seed (MS) and methanol pericarp (MP) extracts of *S. apetala* demonstrated remarkable antioxidant activity, attributed to the exceptionally high levels of polyphenolic compounds they contained. The various biological properties of the fruit were partially attributed to the synergistic effects of identified polyphenols such as (−)-epicatechin (ET), catechin hydrate (CH), rutin hydrate (RH), trans-ferulic acid (TFA), trans-cinnamic acid (TCA), myricetin (MT), and kaempferol (KF). Notably, the anti-proliferative effect demonstrated statistical significance at a concentration of 300 μg/mL for both extracts. Furthermore, the seeds of the *S. apetala* plant were recognized as a potential source of both hydrophilic and lipophilic functional food components, aiming to improve human physiological health.

3.5. LITERATURE REVIEW

3.5.1. Review on Wound Healing and their Pathogenesis

(Wilkinson et al 2020) suggested wound healing as a complex, dynamic process supported by a myriad of cellular events that must be tightly coordinated to efficiently repair damaged tissue. Chronic, non-healing wounds can develop as a result of alteration in wound-linked cellular behaviors, which is seen in diabetes and aging. Because these wounds are so common and frequently repeat, they pose a substantial socioeconomic burden. Therefore, a better molecular and clinical knowledge of the mechanisms underlying wound repair is desperately needed. Here, we go over the biological underpinnings of tissue restoration and talk about how our growing

knowledge of wound pathology could assist in establishing more effective wound treatments in the future.

(Mills et al., 2020) provided an insight on the pathogenesis of wound healing as a complex process comprising four distinct but overlapping stages including haemostasis, inflammation, proliferation and remodelling. In skin, the overall aim of the wound healing process is to reform the barrier to the outside environment, in as short a time as possible, to prevent infection and fluid loss. Wound healing involves numerous growth factor and cytokine signalling cascades which help to remove infection from the wound and reform the damaged skin. The process of wound healing was described along with the various triggers that can contribute to the formation of diabetic ulcers, pressure injuries and arterial and venous ulcers. Finally, wound infection is introduced as it is becoming increasingly prevalent and is creating a significant challenge for wound management due to the emerging prevalence of antimicrobial resistance. Overall, these challenges are leading to a pressing need for improved treatments and clinical approaches for the treatment of wounds.

(Gonzalez et al., 2016) discussed about the regeneration and tissue repair processes that consist of a sequence of molecular and cellular events which occur after the onset of a tissue lesion in order to restore the damaged tissue. The exsudative, proliferative, and extracellular matrix remodeling phases are sequential events that occurs through the integration of dynamic processes involving soluble mediators, blood cells, and parenchymal cells. Exsudative phenomena that take place after injury contribute to the development of tissue edema. The proliferative stage seeks to reduce the area of tissue injury by contracting myofibroblasts and fibroplasia. At this stage, angiogenesis and reepithelialization processes can still be observed. Endothelial cells are able to differentiate into mesenchymal components, and this difference appears to be finely orchestrated by a set of signaling proteins that have been studied in the literature. This pathway is known as Hedgehog. The purpose of this review is to describe the various cellular and molecular aspects involved in the skin healing process.

(Enoch & Leaper, 2007) suggested the various pathological pathways involved during the progression of wound. Changes in any one of these components may be accountable for the delayed healing observed in chronic wounds, as growth factors, cytokines, proteases, and intracellular and extracellular factors are all critical during different phases of the healing process.

Additionally, as demonstrated by hypertrophic scars and keloids, deregulation at specific phases of the healing process can result in excessive accumulation of collagen and the production of erroneous scars. This review addressed all kinds of wound healing as well as the cellular and molecular processes that were associated with acute wound healing and the mechanisms leading to abnormal healing.

3.5.2. Animal Models of Wound Healing

(Bruno et al., 2024) in their study evaluated the effect of hydromethanolic extract of *Dioscorea bulbifera* (HEDB) on wound size, percentage of wound contraction and tissue hydroxyproline in male wistar rat excision wound showing enhanced wound closure, contraction, and tissue hydroxyproline levels compared to controls. The dose of 200 mg/kg produced the best wound closure and percentage wound contraction, whereas 800 mg/kg produced the greatest amounts of tissue hydroxyproline. These results provide a solid scientific basis for the traditional medicinal use of *HEDB* in wound healing procedures. This study provides enough evidence that wound excision animal model is one of the finest model to establish the use of medicinal plants for wound healing.

(Saeed & Martins-Green, 2024) suggested that different research has explored various methods to treat chronic wounds, yet no effective treatment has been found so far. Despite the existence of thousands of cancer drugs, there is only one drug approved for treating chronic wounds. Moreover, there is a lack of models that accurately replicate human chronic wounds. This review evaluates different models used to study the biology of impaired healing and chronic non-healing wounds. Notably, it highlights a promising model involving aged and obese db/db-/- mice. The chronic wounds in these mice exhibit characteristics similar to human chronic wounds, such as increased oxidative stress, chronic inflammation, damaged microvasculature, abnormal collagen matrix deposition, lack of re-epithelialization, and the spontaneous formation of multi-bacterial biofilms. The paper emphasizes the importance of developing chronic wound models that closely mimic human conditions to test potential treatments for healing chronic wounds.

(Pandey & Gupta, 2023) reviewed about the wounds that can be categorized based on a number of factors, including the underlying cause, the location of the damage, the mechanism causing the symptoms, the depth and tissue loss of the wound, or the clinical presentation. Acute wound

healing occurs in four phases, based on research using animal models. It is inevitable that comparable underlying processes will be involved in chronic wounds. Adequate phases of wound healing include hemostasis, inflammation, proliferation or granulation, and remodeling or maturation. The way we comprehend and use information has undergone a significant shift. This study examined all facets of wound healing, encompassing all pathways and model for wound healing.

(Grada et al., 2018) suggested about the animal models that provide invaluable information and can be correlated with human wound healing. When it comes to interpretation and implementation, one must not fail to recognize differences in each animal model. The investigator must assess the merits and limitations of each model according to the experimental objectives. Creating an animal model that reflects the complexity and heterogeneity of chronic wounds in humans may be an unattainable goal because they are an outcome of multifactorial process that is influenced by both intrinsic and extrinsic factors such as impaired circulation, infection, chronic inflammation, poor nutrition, aging, limited physical activity, and chronic disease, among others. Useful models are designed such that these impairments are comparable, thus permitting a higher degree of validity. Given the ongoing advances in genetic manipulation of mice and other animal species, new, more useful models of the wound repair will eventually emerge.

3.5.3. Current Scenarios for the treatment of Wound Healing

(Chauhan et al., 2023) provided the insight on current therapy for wound healing which is retarded by steroidal drugs strictly when taken in medium to high dose levels. Corticosteroids and dexamethasone show their effect in all phases of the wound healing process. Medicinal plants are found to be very advantageous in the healing of the wound, elevating the rate of wound healing with lesser risk to the patient. Antioxidants activate the healing process by progressing a new wound cover in the antioxidant activity, which is the main aspect of wound healing and leads to the anti-inflammatory effect at an early phase and activates rejuvenation processes in later phase of wound healing. The assessment of the wound healing parameters, including antioxidant, fibroblast proliferation, and collagen production, was employed for scientific support.

(Kolimi et al., 2022) revived about the various conventional approaches such as cell therapy, gene therapy, growth factor delivery, wound dressings, and skin grafts etc., that are being utilized for promoting wound healing in different types of wounds. However, all these abovementioned therapies are not satisfactory for all wound types, therefore, there is an urgent demand for the development of competitive therapies. The benefits and drawbacks of a number of cutting-edge and novel wound healing techniques, including extracellular matrix-based approaches, cold plasma treatment therapy, stem cell therapy, nanotherapeutics, 3D bioprinted skin, and extracellular matrix-based approaches, were discussed. Lastly, the difficulties encountered by these novel approaches were also examined along with a brief outlook.

(Mirhaj et al., 2022) in their review reported that wound dressing involves the use of a scaffold, usually using biomaterials for the delivery of medication, autologous stem cells, or growth factors from the blood. Antibacterial and anti-inflammatory drugs are also used to stop the infection as well as accelerate wound healing. With an increase in the ageing population leading to diabetes and associated cutaneous wounds, there is a great need to improve the current treatment strategies. This research critically reviews the current advancement in the therapeutic and clinical approaches for wound healing and tissue regeneration. The results of recent clinical trials suggest that the use of modern dressings and skin substitutes is the easiest, most accessible, and most cost-effective way to treat chronic wounds with advances in materials science such as graphene as 3D scaffold and biomolecules hold significant promise.

(Schilrreff & Alexiev, 2022) in their review, they discussed the role of the immune system, the involvement of inflammatory mediators and reactive oxygen species, the complication of bacterial infections in chronic wound healing, and the still-underexplored potential of natural bioactive compounds in wound treatment. We focus on natural compounds with antioxidant, anti-inflammatory, and antibacterial activities and their mechanisms of action, as well as on recent wound treatments and therapeutic advancements capitalizing on nanotechnology or new biomaterial platforms. Plants and microorganisms such as bacteria, fungi, microalgae, cyanobacteria, and archaea have proven to be an excellent source of bioactive compounds. In particular, plant-derived compounds have been used worldwide for thousands of years as traditional treatments for numerous diseases. Natural bioactive compounds with high levels of antioxidant, anti-inflammatory, and antimicrobial properties could be of great benefit for chronic

wound healing. Anti-inflammatory drugs can quickly regulate the levels of various inflammatory factors and normalize the inflammatory response of chronic wounds with severe inflammation. Several studies have documented the use of extracts from natural origin for the development of bioactive wound treatments, which provide opportunities for eliminating the inflammatory response and accelerating wound healing.

3.5.4. Natural Products for Wound Healing

(Bilal et al., 2024) study aimed to evaluate the in vivo wound healing potential of *Psidium guajava* L. extract (PGLE) by wound excision model owing to its ethnomedicinal use as a wound healer since ancient times. PGLE were shown to have in vitro antioxidant potential along with antibacterial activities against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeroginosa, and* Methicillin-resistant *Staphylococcus aureus* with the minimum inhibitory concentration of 250, 400, 400, and 500 µg/ml respectively was shown by PGLE and found biocompatible with $1.1 \pm 0.16\%$ hemolysis. PGLE also shown to have promoting epithelialization and wound contraction and it also elevated hydroxyproline content which makes this potential candidate for wound healing. PGLE was reported anti-oxidant by elevating catalase, peroxidase, and glutathione content while depleting lipid peroxidation.

(Bajpai et al., 2024) reported an in-depth evaluation of natural chemicals derived from plants that aid in wound healing. They reported that it covers the chemical sources of these compounds as well as the biological mechanisms that underlie their healing benefits. There are several steps involved in wound healing, and any disruption to this process can lead to incorrect wound healing. The complex process of restoring the structure and function of injured tissues is controlled by the release of many cytokines and growth factors at the site of injury. Numerous plants or compounds derived from plants with high concentrations of antioxidants together with anti-inflammatory, immunomodulatory and antibacterial activities were shown to be very beneficial for wound healing. They provide a list of natural compounds that were found effective in wound healing and some of the include astaxanthin, myricetin, apigenin, resveratrol, curcumin, asiatic acid and the others.

(Ibrahim et al., 2018) in their study reported that wound healing process can be facilitated by natural products with medicinal properties. Several research has been conducted regarding the ability of natural compounds with anti-inflammatory, antioxidant, antibacterial, and pro-collagen synthesis capabilities to heal wounds. The bioactive phytochemical components of the several chemical families, including tannins, terpenoids, alkaloids, essential oils, flavonoids, saponins, and phenolic compounds, may have contributed to their therapeutic qualities. Every bioactive substance may have a unique role in the qualities of wound healing. For example, tannins and flavonoids have antiseptic and antibacterial properties, and saponins can increase the synthesis of pro-collagen. These phytochemicals have the ability to alter one or more stages of the healing process for wounds. The purpose of this review is to highlight the functions and applications of a few natural products, such as sea cucumber, vitamin E, honey, and Curcuma longa, in aiding in the healing of wounds. With great results, these natural products have been utilized widely in the management of wound care. In this study, the pre-clinical and clinical evidence investigations of these natural compounds are also covered.

(Dai et al., 2017) in their research the curcumin was successfully formulated as an amorphous nanosolid dispersion and favorably released from gelatin-based biomimetic NMs that could be easily applied topically to experimental wounds. They showed synergistic signaling by the released curcumin during the healing process that included the mobilization of wound site fibroblasts by activating the Wnt signaling pathway, partly mediated through Dickkopf-related protein-1, and persistent inhibition of the inflammatory response through decreased expression of monocyte chemoattractant protein-1 by fibroblasts. With a combination of these effects, the curcumin/gelatin-blended NMs enhanced the regenerative process in a rat model of acute wounds, providing a method for translating this ancient medicine for use in modern wound therapy.

3.5.5. Pharmacologic al activities of *Sonneratia apetala*

(Bose et al., 2023) reported about *Sonneratia apetala* (SA) as a tree which is an evergreen species that is known for its rapid growth and natural occurrence. The main phyto-constituents reported in SA are betulinic acid, lupeone, lupeol, stigmast-5-ene 3beta, β-amyrin hexadecaneate, 5β-cholestane-3α, 7α-diol, and physcoion. Some chemical constituents present in SA are gibberellin, quercetin, caffeic acid, (-) catechin, and epicatechin. The fruits and bark have

antioxidant, antidiabetic activity, antibacterial, hepatoprotective effect and astringent activity, anticancer activity, hypouricemic activity, and gastroprotective effects. The constituents of bark and leaf include flavonoids, alkaloids, tannins, glycosides (anthraquinone and cardiac), terpenoids, saponins, steroids, protein and amino acids, steroid and gums, carbohydrates, vitamins (thiamine, riboflavin) and certain minerals. This review also reported its ecological, salt regulatory and reproductive features as well.

(Mithila et al., 2023) performed a study on iron overload that results in oxidative damage to various biomolecules including DNA, proteins and lipids which ultimately leads to cell death. The SA fruit, known for its high antioxidant content and various bioactive properties, had its powder fractionated using n-hexane (Hex), chloroform (Chl), and methanol (Met) to assess their effectiveness in mitigating iron overload. Additionally, their in vivo efficacy in alleviating iron overload and iron-induced oxidative stress was tested in mice injected intraperitoneally with ferric carboxymaltose at a dosage of 100 mg/kg body weight (bw). The in vivo results indicated that the Met and Hex treated mice had significantly ($P<0.05$) reduced iron levels (including serum and liver iron and ferritin concentrations, and serum total iron-binding capacity) compared to the iron-overloaded group. Moreover, at this concentration, the Met fraction prevented iron-induced oxidative stress in the liver tissues of iron-overloaded mice by restoring reducing power, total antioxidant capacity, and total protein levels. Therefore, the SA fruit, particularly its Met fraction, shows potential for treating iron overload and related toxicity.

(Nagababu & Rao, 2017) reported in their investigation that involved the screening and evaluation of SA leaf crude extracts for the phytochemicals, antibacterial activity and antioxidant activity. Green synthesis of silver nanoparticles using aqueous leaf extract. The results of every experiment performed indicated the detection of most phytochemicals in leaves and their substantial biological actions. In isolation, purification, and determination of the precise active principle in extract responsible for the biological activity, more research is required. This was the first report of green synthesis for this plant of silver nanoparticles. Because phytochemical-based nanoparticles have more antibacterial action, they might be a useful tool in the development of medications to combat bacteria resistant to antibiotics.

(Hossain et al., 2017) demonstrated in their study about the seeds powder of SA was successively fractionated using n-hexane, diethyl ether, chloroform, ethyl acetate, and methanol.

The fractions were tested for their antibacterial, anti-diarrheal, analgesic, and cytotoxic properties. The methanol fraction of seeds (MeS) exhibited strong inhibition against *Escherichia coli strains, Salmonella Paratyphi A, Salmonella Typhi, Shigella dysenteriae, and Staphylococcus aureus*, but not *Vibrio cholerae*, at a concentration of 500 μg/disc. All fractions significantly inhibited castor oil-induced diarrhea and delayed its onset in mice at a dose of 500 mg extract/kg body weight. Among these, MeS demonstrated the highest effectiveness in reducing diarrheal episodes, while the n-hexane fraction (HS) significantly ($P<0.05$) prolonged the onset time of diarrhea compared to the positive control. Additionally, HS ($P<0.005$) was the most effective in reducing acetic acid-induced writhing in mice at the same concentration. Both the HS and diethyl ether fractions notably increased the reaction time of mice in the hot plate test at 500 mg extract/kg. Furthermore, all fractions exhibited strong cytotoxic effects in brine shrimp lethality assays. These findings indicate that S. apetala seeds could be highly beneficial as nutraceuticals.

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CHAPTER 4: EXTRACTION AND PHYTOCHEMICAL SCREENING

4.1. Collection of *Sonneratia apetala* Buch. -Ham

The fruit of plant *Sonneratia apetala* Buch. -Ham (SA) was collected from Sundarbans Mangrove Forest in month of August- September 2023 and identified using a reference sample housed at Jadavpur University's Department of Pharmaceutical technology. The fruit was thoroughly cleaned and sanitized using tap water. The outer skin was then peeled off and course grinded using mixer grinder. After that the fruit part was kept in leaf dryer for period of 3-4 days at 50^{o}C temperature. After drying the fruit sample was kept in zipper pouch for storage.

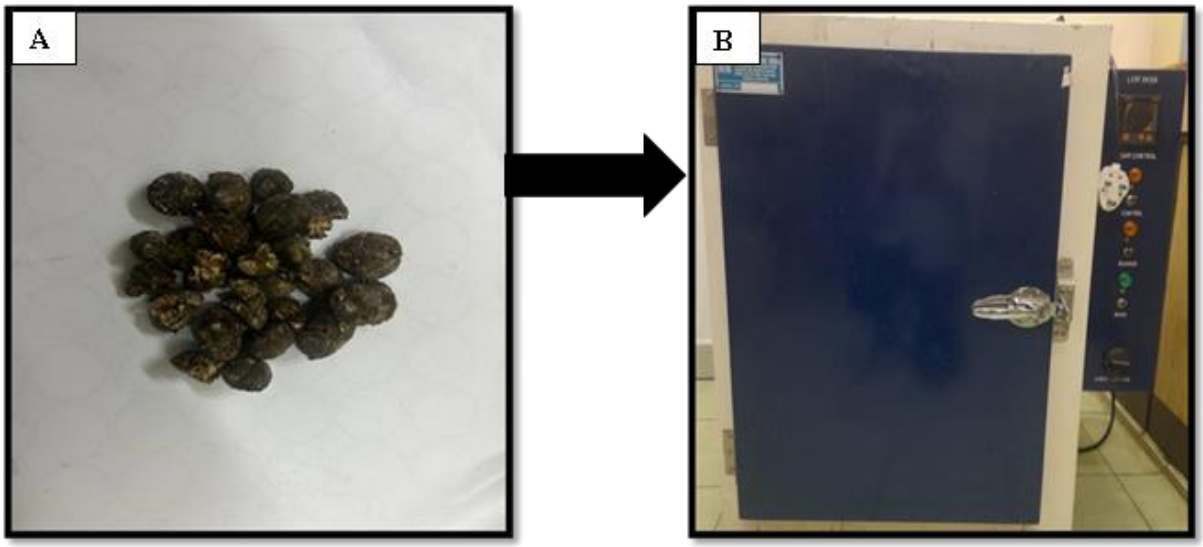

Figure 4.1: SA fruits dried using Leaf Dryer**.**

(A: Collected Fruits of SA**; B:** Leaf dryer for the drying of SA Fruit).

4.2. Extraction using Soxhlet assisted Apparatus

The SA fruit was extracted in a Soxhlet assisted apparatus using hydroalcoholic extract iof SA methanol: water (70:30) solvant. The previous step was repeated twice after filtering the extract. The filtrate extract was evaporated using rotary evaporator and it was lyophilized using lyophilizer at -40 degree celcius. The w/w yield of hydroalcoholic extract of SA (HASA) was found to be 16 %.

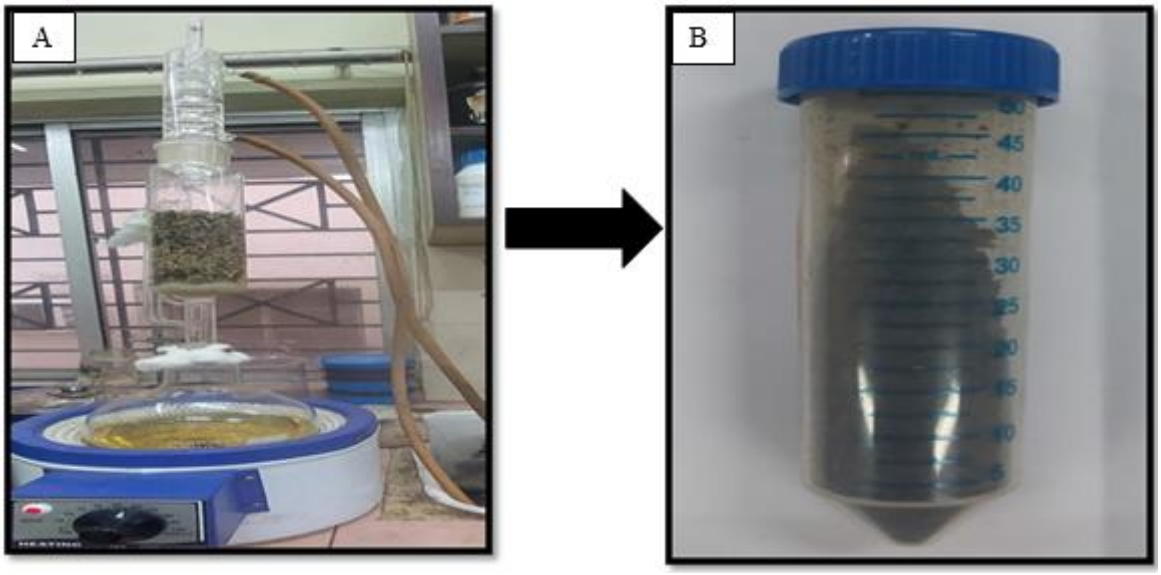

Figure 4.2: Extraction process of SA fruits via Soxhlet apparatus using (Hydro-alcoholic 70:30 solvent).

(A: Set up of Soxhlet apparatus for hydroalcoholic extract of SA; **B**: Isolated Hydroalcoholic extract of *Sonneratia apetala* (HASA).

4.3. Phytochemical Screening

Methodology

Phytochemicals are natural compounds produced by plants that have bioactive properties such as alkaloids, flavonoids, saponins, tannins, steroids, protein, and other metabolites (Graceclin et al., 2013; Ugochukwa et al., 2013). The qualitative analysis was conducted to identify the presence of various phytochemicals in the Hydroalcoholic extract *Sonneratia apetala* Buch. -Ham (HASA). It is therefore essential to determine the phytoconstituents present in a plant extract in order to claim their pharmacological importance.

4.3.1. Test for Steroid

4.3.1.1. Liebermann-Burchard Test

10 mg of extract was dissolved in 1 ml of chloroform. 1 ml of Acetic Anhydride was added following the addition of 2 ml of concentrated sulphuric acid. Formation of reddish violet or pinkish colour indicated the presence of steroids (Zhou et al., 2004).

4.3.1.2. Salkowski Test

1 ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by acid layer indicated the presence of steroid (Bosila et al., 2005).

4.3.2. Test for Alkaloids

4.3.2.1. Mayer's test

1.2 ml of extract was taken in a test tube. 0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff coloured precipitate gives positive test for alkaloids (Saha et al., 2021).

4.3.2.2. Dragendorff's test

0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendorff's reagent were added in 2 ml solution of extract in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloids (Saha et al., 2021).

4.3.2.3. Wagner's test

2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml Wagner's reagent. Formation of reddish brown indicated the positive response for alkaloids (Saha et al., 2021).

4.3.3. Test for Flavonoids

4.3.3.1. Alkaline reagent test

2 ml of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoids (Ugochukwu et al., 2013).

4.3.3.2. Shinoda's test (Palanisamy et al., 2012)

Small quantity of the extract was dissolved in alcohol. Two to three pieces of magnesium followed by concentrated hydrochloric acid was added and heated. Appearance of magenta color demonstrates presence of flavonoids.

4.3.4. Test for Saponins

1 ml solution of the extract was diluted with distilled water to 20 ml and shake in a graduated cylinder for 15 minutes. Development of stable foam suggested the presence of saponins. 1 ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins (Sinha et al., 1985)**.**

4.3.5. Test for Tannins

5 ml of extract solution was allowed to react with 1 ml 5% ferric chloride solution. Greenish black coloration indicated the presence of tannins. 5 ml of extract was treated with 1 ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins (Scegelman et al., 1969).

4.3.6. Test for Glycoside

4.3.6.1. Legal's test

The extract was dissolved in pyridine and sodium nitroprusside solution added to make it alkaline. The formation of pink red to red color shows the presence of glycosides (Salwaan et al., 2012).

4.3.6.2. Bontrager's test

A few ml of dilute sulphuric acid added to 1 ml of the extract solution. Boiled, filtered and extracted the filtrate with chloroform. The chloroform layer was treated with 1 ml of ammonia. The formation of red color shows the presence of anthraquinone glycosides (Salwaan et al., 2012).

4.3.7. Test for Protein

4.3.7.1. Biuret Test

The extract was treated with 1 ml 10% sodium hydroxide solution and heated. A drop of 0.7% copper sulphate solution to the above mixture was added. The formation of purplish violet color indicates the presence of proteins (Kumar et al., 2012).

4.3.7.2. Millon Test

3 ml test solutions were mixed with 5 ml Million's reagent separately. White precipitate was formed which on heating turned to brick red. It indicates the presence of proteins (Kumar et al., 2012).

4.3.8. Test for Carbohydrate

4.3.8.1. Benedict's test

Test solution was mixed with few drops of Benedict's reagent i.e. alkaline solution containing cupric citrate complex boiled in water bath, observed for the formation of reddish brown precipitate to show a positive results for the formation of carbohydrate (Bhandary et al., 2012).

4.3.8.2. Molisch test

In 2 ml of the extract, 1 ml of α-naphthol solution along with concentrated sulphuric acid via the sides of test tubes were added. Purple or reddish violet colour at the junction of the two liquid was observed that signified the presence of carbohydrates in the plant extract (Salwaan et al., 2012).

4.3.9. Test for Triterpenoid

4.3.9.1. Salkowski test

The test extract was treated with few drops of concentrated sulphuric acid. Formation of yellow colour at the lower layer suggested the presence of triterpenoid (Nayak et al., 2011).

Results

Figure 4.3: Various Phytochemical test performed with HASA.

Table 4.1: Presence or absence of different phytoconstituents in HASA.

S. NO.	PHYTOCONSTITUENT	PRESENCE/ ABSENCE
1.	STEROIDS	-ve
2.	ALKALOIDS	+ve
3.	FLAVONOIDS	+ve
4.	SAPONINS	-ve
5.	TANNINS	+ve
6.	GLYCOSIDES	+ve
7.	PROTEINS	-ve
8.	CARBOHYDRATES	+ve
9.	TRITERPENOIDS	+ve

'+ve' indicates the presence of that particular phytoconstituents while '-ve' indicated the absence of that phytoconstituents.

Discussions

The presence of alkaloids, flavonoids, tannins, carbohydrate, glycoside and triterpenoids was observed during the preliminary phytochemical screening of HASA. Flavanoids are known to possess great antioxidant activities due to the presence of compounds like quercetin and kaempferol and even the presence of carbohydrates improves the antioxidant property of the extract. The presence of tannins provide the astringent properties to the plant extract whereas the alkaloids and triterpenoids are responsible for the analgesic and anti-inflammatory activities of the SA plant extract (Uddin et al., 2024; Liu et al., 2019). This different pharmacological relevance of the SA plant extract declares them to be a potential candidate for the management of wound healing.

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CHAPTER 5: TOTAL PHENOLIC, FLAVONOID CONTENT AND DPPH RADICAL SCAVENGING ACTIVITY

5.1. Introduction

Wound healing, Diabetes mellitus, cardiovascular disease, neurological illnesses, chronic kidney disease, tissue injuries, and other diseases are all highly impacted by oxidative stress through lipid peroxidation, DNA, and protein damage. Wound healing is linked to an oxidative stress due to an imbalance of free radicals and antioxidants in the body results in overproduction of reactive oxygen species which lead to cell, tissue damage, and delayed wound healing. The antioxidant potential of HASA was determined using the DPPH radical scavenging technique in this study. Antioxidants derived from fragrant, spicy, medicinal, and other plants were investigated in order to create natural antioxidant compositions for food, cosmetic, and other purposes. Terpenoids, phenolic metabolites, and alkaloids are the three principal types of plant compounds. Among these three classes, phenolic chemicals are the most essential for nutritional applications and have received the greatest attention. Phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolysable and condensed tannins), and flavonoids are examples of phenolic chemicals. These chemicals protect plants from oxidative damage and have been employed by humans as antioxidants. It is of tremendous interest to discover new and safe antioxidants from natural sources for use in natural antioxidants, functional foods, and nutraceuticals.

5.2. Drugs and Chemicals

1, 1- Diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), naphthyl ethylene diamine dihydrochloride, sodium nitroprusside, ascorbic acid, trichloroacetic acid (TCA), diamine tetra acetic acid (EDTA), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), Thiobarbituric acid (TBA) and Quercetin. All reagent used were of high analytical grade.

5.3. Total Phenolic Content (TPC)

The Folin-Ciocalteu (FC) reagent was used to measure the total phenolic content, slightly changing the reference technique of Jana et al., 2024. In a conical flask, 250 µl (1 mg) of extract, 200 µl of distilled water, and 300 µl FC were mixed together and agitated for 30 minutes. After

that, the mixture was agitated for two hours at room temperature with 300 µl of Na2CO3 (2%) added. At 760 nm, the absorbance was measured using distilled water as a blank. The following formula was used to get the total phenolic content. 760 nm absorbance equals 0.001 x Pyrocatechol (Mg) + 0.0033 (Jana et al., 2024)

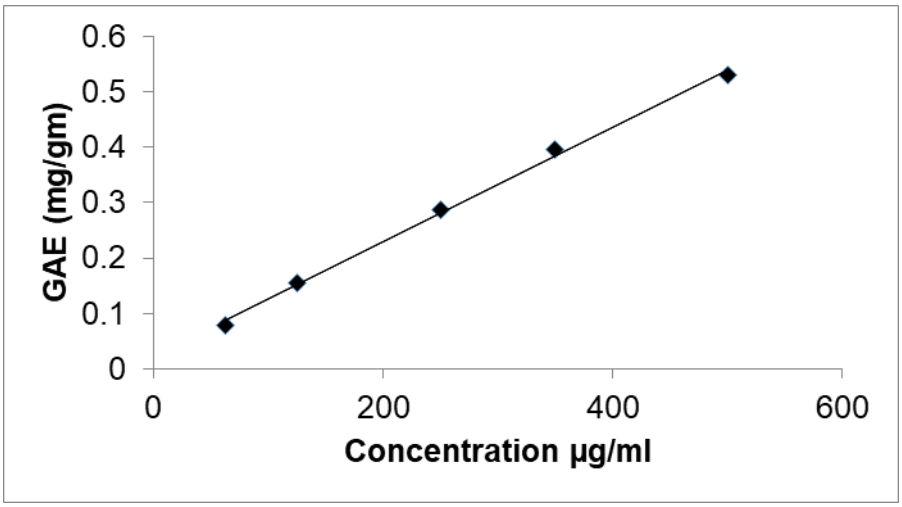

Figure 5.2: Total phenolic content estimation.

5.3.1. Result - Total phenolic content for the extract as determined from the amount of pyrocatechol formed during the assay method was found to be 62.5 GAE.mg/gm.

5.4. Total Flavonoid Content (TFC)

5.4.1. Methods

The Dowd technique was used to calculate the total flavonoid content. The extract solution (0.1 mg/mL) was combined with 200 µL of 2% aluminium trichloride (AlCl3) in methanol. Spectrophotometer measurements for absorption at 415 nm were taken. Next 10 minutes in comparison to a blank sample made up of 250 µL of extract solution with 200 µL of methanol and no AlCl3. Equipped with a standard curve and quercetin (25–200 µg/200 µL methanol) as the standard, the total flavonoid concentration was calculated. The expression for the total flavonoid content is µg of quercetin equivalents (QE)/mg of extract (Singha et al., 2024).

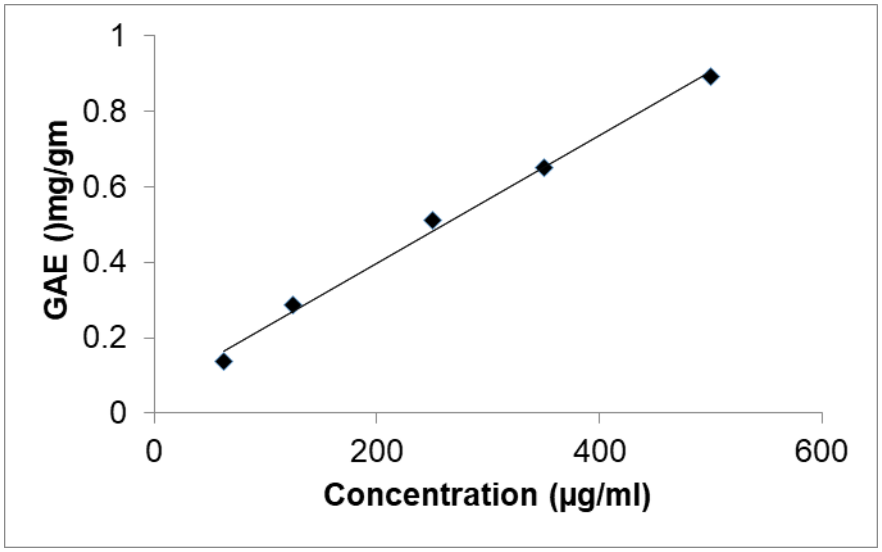

Figure 5.2: Total Flavonoid Content estimation.

5.4.2. Results

Flavonoids are large class of benzo-pyrone derivatives, ubiquitous in plants exhibit antioxidant activity. The flavonoid content of HASA is shown in Figure 6.2. Total flavonoid content of HASA was calculated to be 102 mg/gm of quercetin equivalents (QE)/mg of extract.

5.5. DPPH Radical Scavenging Activity

5.5.1. Methods for DPPH radical scavenging activity

The antioxidant properties of *Sonneratia apetala* extract were evaluated using DPPH free radical scavenging assay performed in our lab. In the test, 100µl of both the sample and the DPPH solutions (200µg/ml) were taken in 96 well microplate and left to stand in dark for 10 minutes at room temperature. Spectrophotometer was used to quantify the reduction in absorbance of the sample solution at 517 nm. Ascorbic acid was used as control. The IC50 value (g/ml) was used to express the free radical scavenging activity (lab reference). The inhibition % was calculated using the following formula (Banerjee et al., 2023).

Inhibition $\%=A_c-A_s/A_c\times100$

Where, A_s is the 'absorbance of test substance' and A_c is the 'Absorbance of Control'

5.5.2. Statistical Analysis

All the values are given as mean ± SEM. The IC50 (50% inhibitory concentration) values were calculated for the graphs plotted between concentrations versus percentage inhibition using GraphPad Prism software 9.0.

5.5.3. Results

The observations of the in vitro antioxidant activities demonstrated that the HASA exhibited excellent antioxidant properties. The sample showed a gradual decrease in DPPH activity with the increasing concentration, as indicated by the DPPH test. The IC50 values for ascorbic acid and HASA were found to 66.66 ±0.53 and 101.309± 0.78 respectively, based on the DPPH graph

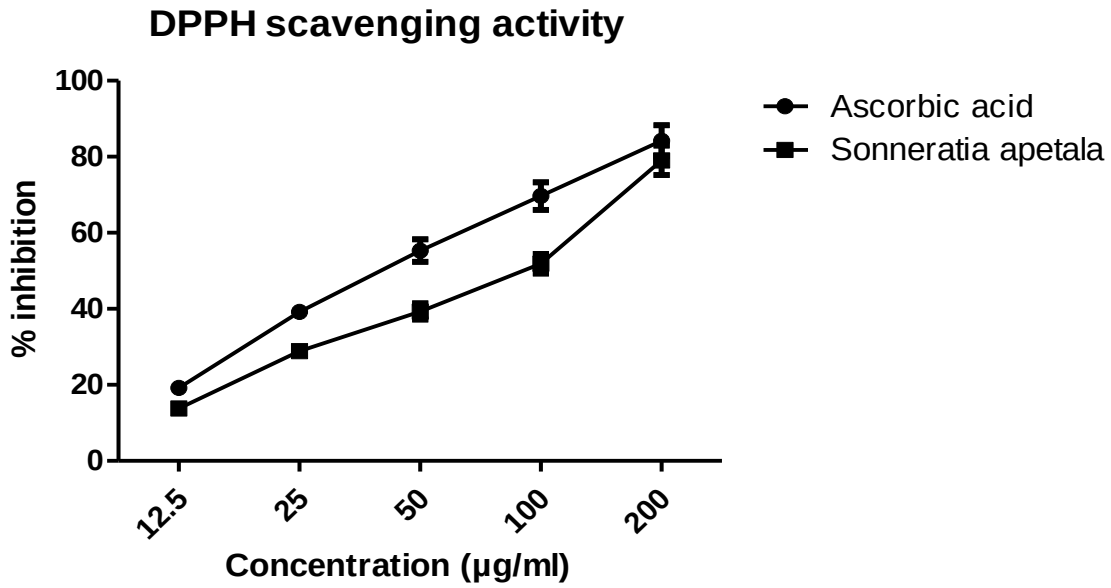

Figure 5.3: 1, 1- Diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity of extract and ascorbic acid. The data represents the % of DPPH inhibition. Each point in the graph represents the values obtained during the experiments (mean ± SEM).

5.6. Discussions

The use of DPPH provides easy and rapid way to evaluate antioxidant activity. The mechanism involved is a stable nitrogen centered free radicals, the colour of which change from violet to yellow upon reduction by either the process of hydrogen-or-electron donation. Substance which are able to perform the reaction. The DPPH scavenging of plant was compared with standard and was prominent that showed the in vitro antioxidant activity of the plant extract as shown in Figure 6.1. Approximately 6% of the oxygen we breathe is converted into harmful oxygen-derived free radicals through our body's normal processes. These radicals have the potential to cause damage to various cellular systems and substances in our body. To counteract these free radicals, antioxidants are necessary. Antioxidants help to neutralize free radicals by accepting an electron or hydrogen atom, thereby preventing cellular damage. In order to test the antioxidant capacity of a component, molecule, or extract, it is important to ensure its effectiveness. One solution to this problem is to use dietary supplements containing natural antioxidants. These supplements can serve as an alternative to traditional medications, providing a means to boost the body's antioxidant defenses. DPPH is often used to assess the antioxidant capabilities of herbal extracts or compounds measuring the decrease in absorbance at 517 mm, indicating a decrease in free radicals. The purpose of the study was to evaluate the in vitro antioxidant activity of HASA via estimation DPPH scavenging potential of the extract. The presence of phenolic and flavonoid content ensured the HASA’s anti-oxidant activity as shown in Figure 5.1 and 5.2. It was also confirmed that the HASA had a high potential of possessing antioxidant characteristics as per the results of DPPH scavenging test as depicted in Figure 5.3.

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CHAPTER 6: DEVELOPMENT OF TOPICAL OINTMENT AND ACUTE SKIN IRRITATION ASSAY

6.1. Development of Ointment Formulation

Ointment of Hydro-alcoholic extract of *Sonnaratia apetala* Buch. -Ham (HASA) was prepared of following doses i.e. 5% and 10% as mentioned in the table below (Demilew et al., 2018).

Table 6.1: Ointment Formulation

Formula	Ingredients	Amount (%w/w)
5% w/w ointment preparation	Hydro-alcoholic extract of *Sonneratia apetala* Buch. -Ham	5
	Soft paraffin	80
	Hard paraffin	5
	Bees wax	5
	Cetosteryl alcohol	5
10% w/w ointment preparation	Hydro-alcoholic extract of *Sonneratia apetala* Buch. -Ham	10
	Soft paraffin	75
	Hard paraffin	5
	Bees wax	5
	Cetosteryl alcohol	5
Ointment Base	Soft paraffin	80
	Hard paraffin	10
	Bees wax	5
	Cetosteryl alcohol	5

Procedure:

Simple ointment was prepared using hard paraffin, cetostearyl alcohol, white soft paraffin, and wool fat 100 g of simple ointment base was prepared by placing hard paraffin (10 g) in a beaker and melted over water bath. The other ingredients such as cetostearyl alcohol (5 g), white soft paraffin (80 g), and bees wax (5 g) were added in descending order of melting point, respectively, after removing from melting. All the ingredients were melted over a water bath

with constant stirring until they became homogeneous. The mixture was removed from the heat and stirred until cold. To prepare hydroalcoholic extract ointment, 10 g and 20 of the powdered extract were incorporated into portion of simple ointment base to prepare 5% and 10% (w/w) ointment, respectively, by trituration. The remainder of simple ointment base was gradually added and mixed thoroughly. Finally, the extract ointment was transferred to a clean container for topical application during the animal experiment (Demilew et al., 2018).

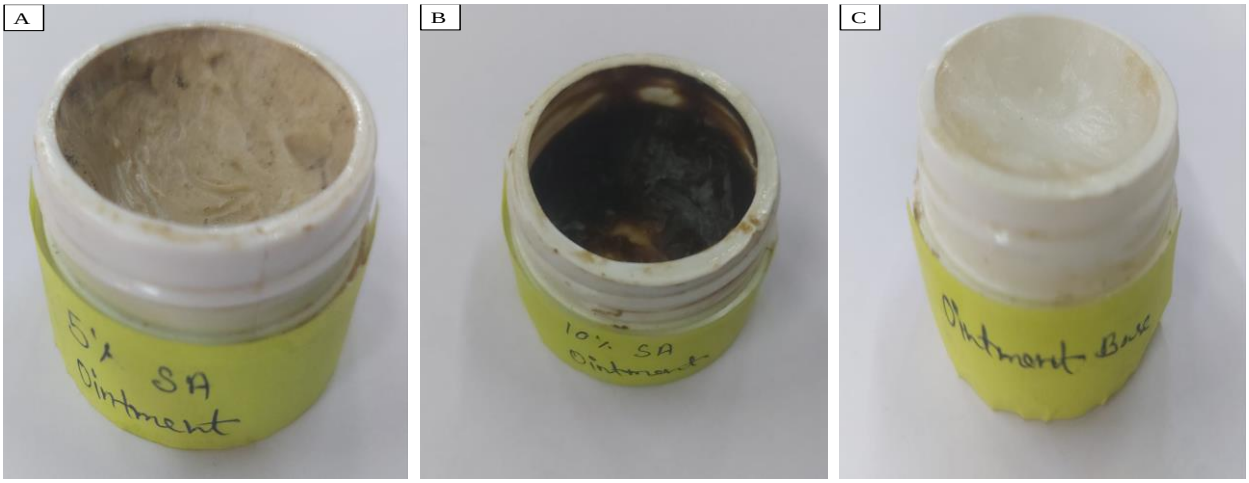

Figure 7.1: Showing different Ointment formulations of Hydroalcoholic extract of *Sonnaratia apetala*.

6.2 Storage

All the three formulations were stored in a close container and kept at Room temperature.

6.3. Acute skin irritation assay of ointment

As per OECD 402, method provides information on health hazard likely to arise from short term exposure to a test chemical by dermal route. Test chemicals should not be administered at doses that are known to cause marked pain and distress due to potential corrosive or severely irritant actions (OECD, 2017).To test the wound healing properties of HASA ointment on laboratory animals, their dorsal hair were emoved properly using hair removal cream then they were anaesthetized with Ketamine to create a wound. The experimental animals were then divided into 3 groups, each group's exposed area received applications of 5%, 10% ointment, and 0.8% formalin as a standard irritant respectively (Ankomah et al., 2022).

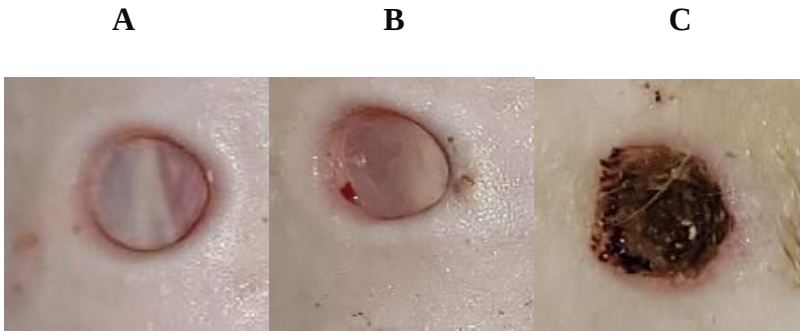

Figure 6.2: Depiction of wound creation in three different group of animals in which each group's exposed area received applications of 5%, 10% ointment, and 0.8% formalin as a standard irritant respectively. Grp A = 5% w/w hydro-alcoholic extract of *S. apetala*, No lesion occur; Grp B= 10% w/w of hydro alcoholic extract of *S. apetala* ointment, no lesion occur and Grp C = Aqueous solution of 0.8% formalin, erythema and oedema, skin rash and inflammation was observed

6.4. Discussions

Two formulations were prepared of different concentration i.e. 5% and 10% of HASA to form ointment for topical application for wound healing. Further, the skin irritation assay of the formulated ointment was performed to check the skin compatibility of the formulation. The animals were observed for seven days to search for any signs of swelling or inflammation, such as oedema and erythema, in the region being investigated. When all the ointment formulations were applied to the skin for around a week, it did not cause skin irritation, such as erythema and oedema. We conclude that the formulated ointment of different concentration are safer for topical use in animal studies.

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CHAPTER 7: *IN-VIVO* EXICISION WOUND HEALING MODEL

7.1. Introduction

Wound healing is a significant challenge in clinical practice, given its complexity and long-standing importance throughout human history. The skin acts as the primary barrier against infections, making effective wound healing essential. The process of healing a skin wound is intricate and dynamic, involving three overlapping phases: inflammation, proliferation, and maturation, often referred to as remodeling. This well-coordinated process begins with the influx of cytokines and growth factors at the wound site immediately after injury, aiming to eliminate pathogens and clear cellular debris. During the proliferation phase, granulation tissue forms through angiogenesis, with keratinocytes and fibroblasts migrating to the wound site. The maturation phase focuses on restoring the skin barrier, repairing granulation tissue within the scar, and regressing blood vessels. Over time, the newly formed tissue strengthens, with collagen production peaking and then decreasing after three weeks, while cross-linking and reorganization continue for months. The primary goal of wound treatment is to accelerate healing and minimize the risk of complications. Any existing therapies are not only unpredictable and often ineffective but also painful to apply and difficult to use (Las Heras et al., 2020). There are several reports on topical adjuvant therapies for wound healing, particularly strategies for improving healing in challenging wounds such as chronic wounds and burns. The primary treatment strategy for chronic wounds is to correct the wound's dysregulated physiological state to promote healing. This includes ensuring the patient's nutritional and hydration status is adequate and managing any comorbidities contributing to ulcer development. Infection control, proper wound oxygenation, and debridement are essential, as is minimizing excess pressure or other mechanical stress on the wound. Treatment varies depending on the type of chronic wound. A significant portion of the global population relies on plants and plant extracts for healthcare, with over 400 plant species used in remedies for wound healing. *Sonneratia apetala*, part of the Lythraceae family, has been studied for the isolation of secondary metabolites and the evaluation of their biological activities. In previous reported studies *Sonneratia apetala* has been reported to have anti-bacterial, anti-oxidant along with anti-inflammatory activities. These pharmacological properties are likely due to the combined effects of phenols, tannins, and flavonoids. Plants have played a significant role in traditional medicine, contributing to the treatment or prevention of various diseases. They are known to reduce the risk of chronic conditions such as diabetes,

cancer, cardiovascular disorders, and other illnesses (Lalitha et al., 2019). These pharmacological properties of this plant shows the potential of *Sonneratia apetala* in the wound healing process.

7.2. Materials and Methods

7.2.1. Drug and Chemicals

A pharmacy provided the Mupirocin(15g) ointment, and Hi- Media Laboratories Pvt. Ltd. (Mumbai, Maharashtra, India) provided all other chemicals.

7.2.2. Animals

The State Centre for Laboratory Animal Breeding (SCLAB), Buddha Park, B 14, Block B, Kalyani, West Bengal 741235, provided male Wister rats (180-220 grams), aged between 6-8 weeks. Following that animals were taken care properly in the Departmental Animal House both prior and throughout the experiment. Temperature was maintained between 20-26 ˚C, Relative Humidity was 44-56%, and 12hour cycles of light and dark was maintained with the right food and bedding. Prior to the primary animal experiment, the Institutional Animal Ethics Committee (JU/IAEC-24/77) approved the experimental work.

7.3. In-vivo Wound Healing Studies

7.3.1 Excision Wound Model

Excision wound healing models were used to evaluate the wound healing activity of Hydroalcoholic extract of fruit of *Sonneratia apetala* (HASA). Firstly, the experimental rats were given Ketamine hydrochloride (25mg/kg) to anaesthetized them (Rhea and Dunnwald, 2020). Animal's dorsal surface hair was removed through depilator (hair removing cream). The dorsum of all rats was rinsed with double distilled water. A biopsy punch device (diameter, 6mm) was used to induce the 4 full-thickness circular wound two on either side of dorsal line in each rat. The skin flap was cut out with the help of scissors and removed using angular forceps. After recovering from anaesthesia, rats were placed individually in a cage for further study. The wounded rats were treated continuously twice daily for 16 days with 5% extract (low dose) and 10% extract (High dose) and ointment base in respective group of animals. Wound closure rate was measured each alternative days by placing a tracing paper over the wound and tracing it out,

area of this impression was wound gap. The area of wound at the time of wounding was considered as 100 %.

Percentage of wound closure rate = (Wound area on Day 0 - Wound on Day n)/Wound area on day zero)*100.

Figure 7.1: Depiction of Wound excision model on experimental rats.

7.3.2. Dose Selection and Treatment Protocol

All 12 animals were divided into 6 groups as following:

Group 1: Normal Control (Distilled water p.o), Group II: Wound Control, Group III: excision wound + 5g Mupirocin ointment (standard topical control), Group IV: Excision wound + ointment base, Group V: Excision wound +5% ointment of SA extract (topical), Group VI: Excision wound + 10% ointment of SA extract(topical).

Table 7.1: Animal Grouping and Treatment Protocol

Sr.no	**No. of groups**	**Treatment**	**Species/strains**	**Total no. of animals**
1	Normal control	Distilled water p.o	Male Albino Wistar rats	2

2	Wound Control	Excision wound	Male Albino Wistar rats	2
3	Standard Topical Control	Excision wound + 5g Mupirocin ointment	Male Albino Wistar rats	2
4	Ointment Base Control	Excision wound + ointment base	Male Albino Wistar rats	2
5	5 % HASA Ointment	Excision wound + 5% ointment of SA extract (topical)	Male Albino Wistar rats	2
6	10 % HASA Ointment	Excision wound + 10% ointment of SA extract (topical)	Male Albino Wistar rats	2 **Total= 12**

7.3.3. Estimation of Connective tissue parameters

These studies were done in 96 well plate following the procedure as described in with slight modification. For this study, 250 mg of wet, granulated tissues were dried at 50˚C for roughly 24 hours before being weighed and put into glass test tubes with stoppers. Each test tube contained 1ml of 6 N HCL after dissolving 40 mg of dry tissue in it. The test tubes were then placed in a pot of boiling water for 24 hours, with 12 hours per day, to allow for hydrolysis. The acid was then neutralised using 10N NaOH after cooling the hydrolysate, and phenolphthalein was used as an indicator. The neutral hydrolysates were diluted to a concentration of 20 mg/ml with distilled water. The standard curve method and the appropriate substrate were then used determine whether hydroxyproline and hexuronic acid were present in these hydrolysates (Habash-Bseiso et al., 2005).

7.3.4. Tissue Hydroxyproline content (HPR) Estimation

Each tube received an addition of 0.3 ml of hydrolysate, 0.01 M CuSO4, 2.5 N NaOH, and 6% H202. After giving the tubes a good shake, they were placed in a water bath that was 70˚C. After

10 minutes, tubes were taken out of the water bath and allowed to cool for 5 minutes by submerging them in cold water. After that, 0.7 ml of a 5% solution of para dimethyl amino-benzaldehyde and 1.4 ml of 3 N H2SO4 were added to the test tube. The test tubes were once more submerged in 75˚C water bath for 15 minutes, after which they were allowed to cool for 10 minutes under a regular water stream-Each tube's tissue content's colour intensity was measured using UV spectroscopy at 540 nm in comparison to a blank. The standard curve method, which was composed of standard 4-hydroxy L-proline from 100 to 1000 ug/mL using 3 mg/mL of working solution, was used to determine the tissue's Hydroxyproline content (Sona et al., 2020).

7.3.5. Tissue Hexuronic Acid (HUA) content Estimation

Distilled water was used to dilute 0.150 mL of hydrolysate to 0.5 P 2.5 mL of 0.025 M Borax in concentrated sulphuric acid was placed in stoppered tubes, and these tubes were placed o test tube rack to cool to 4˚C.Then add 0.5 mL of diluted hydrolysate while maintaining 4˚C in the temperature, then sealed the glass tube with a stopper and, in order to maintain the temperature, shook it forcefully after a gradual initial shake. After that, the tubes were heated for 15 minutes in a hot water bath to return the temperature to normal. After each tube had received 0.1 mL of 0.125% carbazole in absolute alcohol, been thoroughly shaken, and spent 15 minutes in a hot water bath, the tissue's colour intensity was evaluated against 2blank.The amount of hexuronic acid in the samples was determined using a standard curve constructed with D (+) Glucurono-6 3-lactone (HI Media Laboratories Pvt. Ltd., Kolkata, India), from 4 to 40µg/ml working solution (Sanwal and Chawdhary, 2011).

7.4. Histology

After 16 days, the skin tissues were excised for histological analysis Each experimental group of rats is given sample of tissue (5 m thick) from the healed wound, which is then fixed in 10% buffer formalin for histopathological analysis (Masson- Meyers et al., 2020). MT stain were applied for formation of collagen fibres and arrangements in granulation tissue and H&E stain for epidermal layer, keratosis layer, hair follicles and fibroblast.

7.5. Statistical analysis

Results from statistical analysis are presented as mean ± SEM. Following a one-way ANOVA, the statistical significance between the groups was examined using Tuckey's multiple comparison test. Statistics were judged significant at $P<0.05$ using GraphPad Prism Software Version 10.1.2.

7.6. Results

7.6.1. Wound contraction Rate

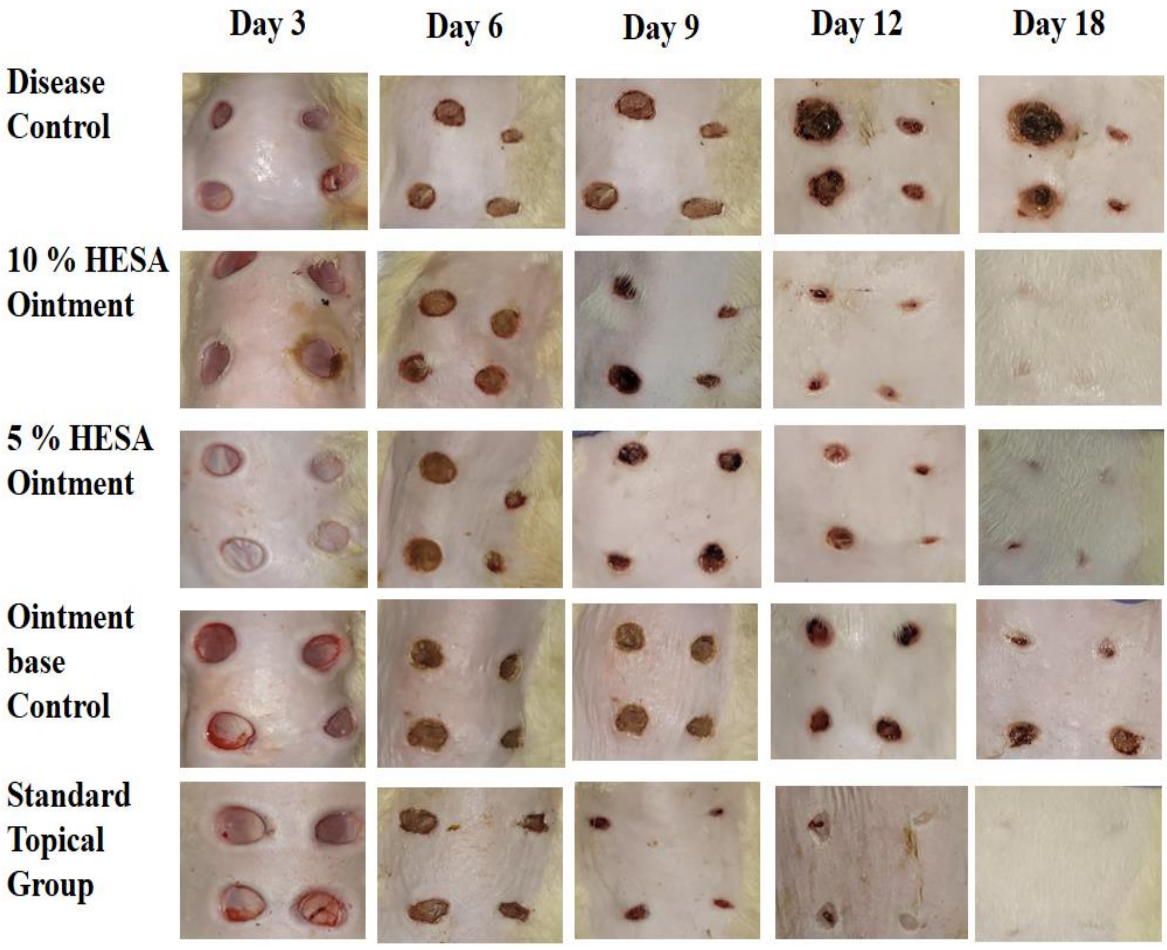

Figure 7.2: Wound contraction on various days of protocol.

Table 7.2: Effect of HASA Ointment on percentage of wound contraction.

Group	**Day 0 (mm2)**	**Day 3 (mm2)**	**DAY 6 (mm2)**	**Day 9 (mm2)**	**Day 12 (mm2)**	**Day 15 (mm2)**	**DAY 18 (mm2)**	**% Heal**	**SD**	**SEM**
5 % HASA Oinntment	113.04	91.5624	84.9056	52.78	40.69	36.30	19.36	82.64	1.001735	0.7083
	113.04	84.9056	75.3914	50.24	40.99	34.19	21.23	81.22		
10 % HASA Oinntment	113.04	98.47	88.20	60.79	47.75	40.49	18.08	84	0.923167	0.6527
	113.04	88.20	84.90	58.05	40.69	34.19	16.61	85.30		
Standard Topical Control	113.04	69.36	60.79	42.98	32.15	28.26	12.56	88.88	0.805316	0.5694
	113.04	75.39	63.58	45.34	34.19	30.17	13.84	87.75		
Ointment base	113.04	91.56	84.90	69.36	55.38	40.69	30.17	75	1.198153	0.8472
	113.04	98.47	81.67	66.44	52.78	38.46	28.26	73.30		
Disease	113.04	102.01	88.20	78.50	63.58	55.38	40.69	64.00	1.394	0.9886

Control									572	
	113.04	98.47	84.90	75.39	60.79	52.78	38.46	65.97		

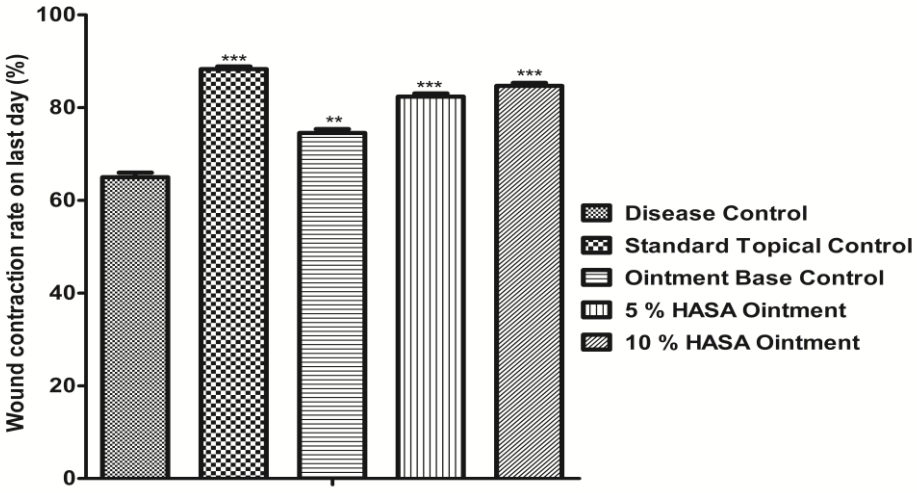

Figure 7.2: Wound contraction rate on last day of study

Normal control: Distilled water p.o; **Wound Control:** Excision wound; **Standard Topical Control:** Excision wound + 5g Mupirocin ointment (Topical); **Ointment Base Control**: Excision wound + ointment base (Topical); **5 % HASA Ointment**: Excision wound + 5% ointment of SA extract (topical); **10% HASA Ointment**: Excision wound + 10% ointment of SA extract (topical); ***: p ≤0.001 w.r.t Wound Control; **: p ≤0.01 w.r.t Wound Control. Results are expressed in terms of Mean ± SEM; n=2

7.6.2. Hydroxyproline content Estimation

Table 7.3: Effects of HASA Ointment on Hydroxyproline content Estimation in form of Mean ± SEM.

Sr.no	No. of groups	Mean± SEM
1	Normal control	0.049 ± 0.0019
2	Wound Control	0.018 ± 0.0003
3	Standard Topical Control	0.046 ± 0.0017
4	Ointment Base Control	0.032 ± 0.0013
5	5 % HASA Ointment	0.0 39 ± 0.0015
6	10 % HASA Ointment	0.044 ± 0.0018

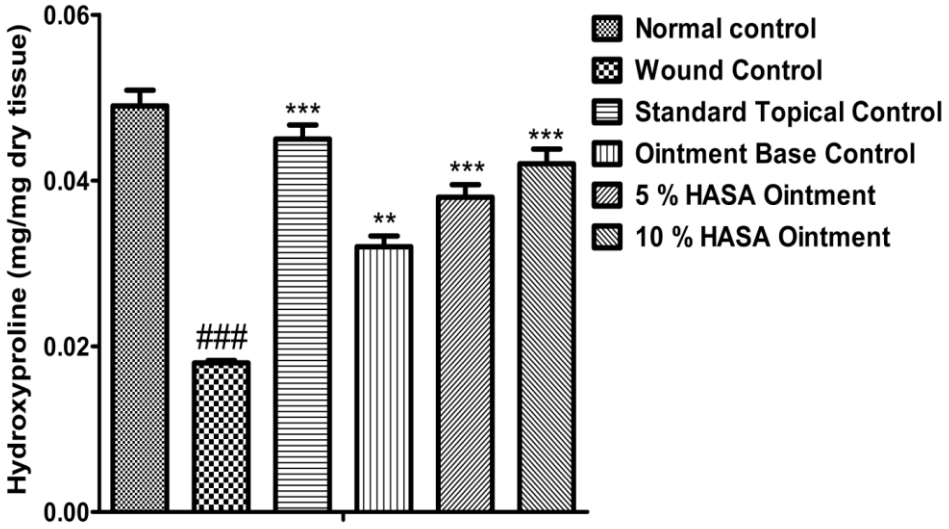

Figure 7.3: Effect of HASA Ointment on Hydroxyproline content estimation.

Normal control: Distilled water p.o; **Wound Control:** Excision wound; **Standard Topical Control:** Excision wound + 5g Mupirocin ointment (Topical); **Ointment Base Control**: Excision wound + ointment base (Topical); **5 % HASA Ointment**: Excision wound + 5% ointment of SA extract (topical); **10% HASA Ointment**: Excision wound + 10% ointment of SA extract (topical); ###: p ≤0.001 w.r.t Normal Control; ***: p ≤0.001 w.r.t Wound Control. Results are expressed in terms of Mean ± SEM; n=2

8.6.3. Hexuronic acid content Estimation

Table 8.4: Showing Hexuronic acid estimation values in form of Mean ± SEM.

Sr.no	No. of groups	Mean± SEM
1	Normal control	0.049 ± 0.0019
2	Wound Control	0.018 ± 0.0003
3	Standard Topical Control	0.046 ± 0.0017
4	Ointment Base Control	0.032 ± 0.0013
5	5 % HASA Ointment	0.0 39 ± 0.0015
6	10 % HASA Ointment	0.044 ± 0.0018

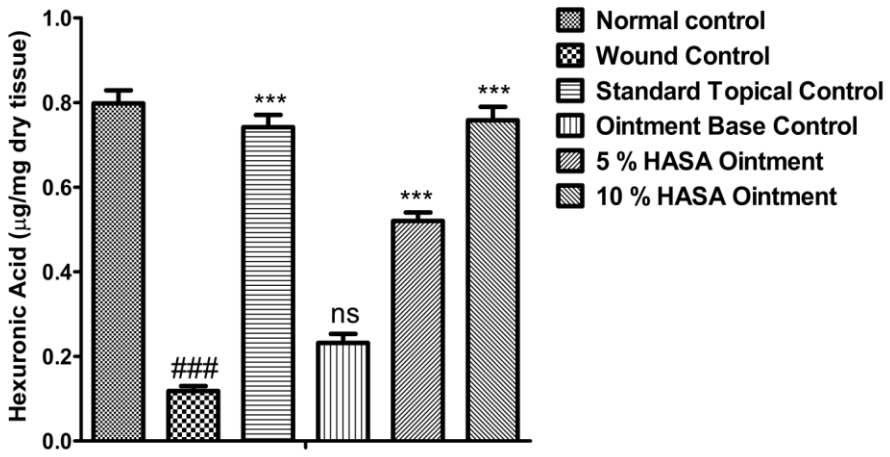

Figure 7.4: Effect of HASA on Hexuronic acid content estimation.

Normal control: Distilled water p.o; **Wound Control:** Excision wound; **Standard Topical Control:** Excision wound + 5g Mupirocin ointment (Topical); **Ointment Base Control**: Excision wound + ointment base (Topical); **5 % HASA Ointment**: Excision wound + 5% ointment of SA extract (topical); **10% HASA Ointment**: Excision wound + 10% ointment of SA extract (topical). ###: p ≤0.001 w.r.t Normal Control; ***: p ≤0.001 w.r.t Wound Control; ns: non significant. Results are expressed in terms of Mean ± SEM; n=2

Discussion:

The graph shows the significant reduction in the hexuronic acid level in treatment groups when compared to diseases control that determines the collagen formation is higher in treatment groups that resulted in quicker healing process in treatment groups as compared to that in diseases control

8.6.4. Histopathological Examination

8.6.4.1. Results:

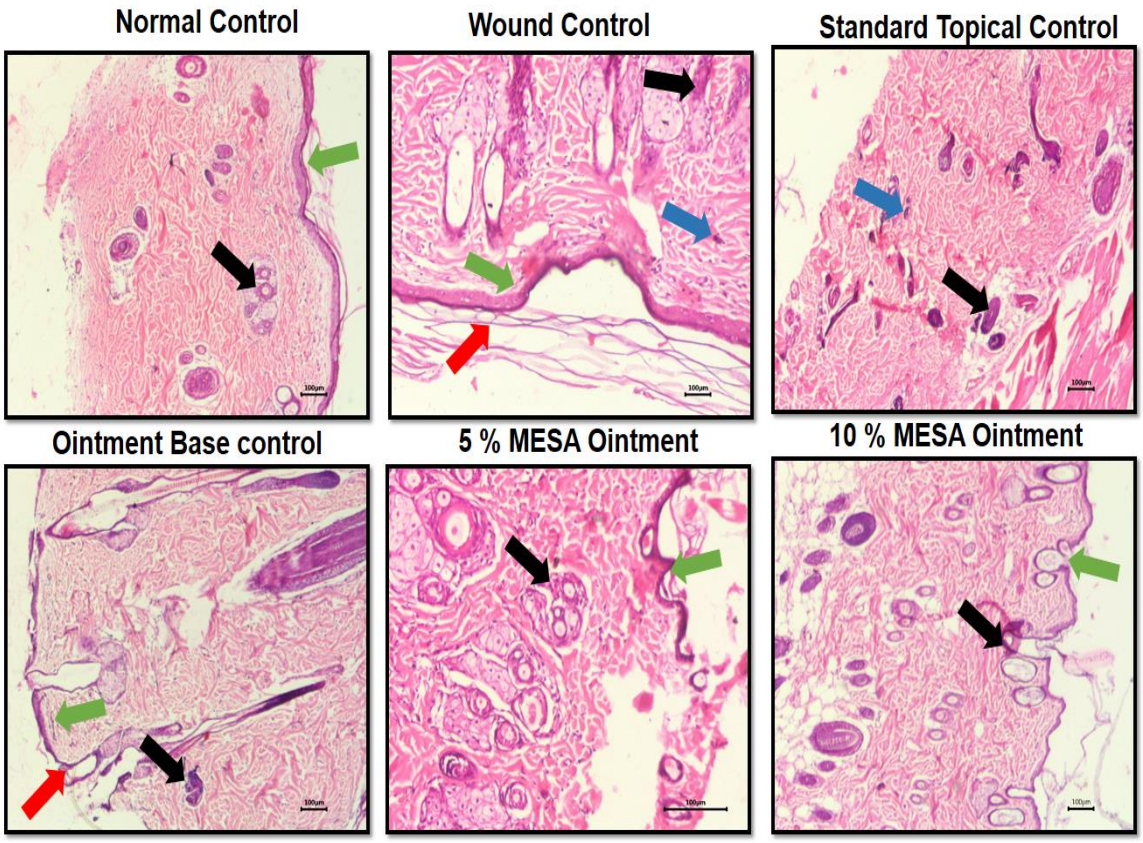

Figure 7.5: Effect of HASA on histopathological examination using H & E staining.

(Green arrow represents the epidermal layer, the red arrow represents the keratosis layer, the black arrow represents hair follicles and the blue arrow represents fibroblast).

Normal control: Distilled water p.o; **Wound Control:** Excision wound; **Standard Topical Control:** Excision wound + 5g Mupirocin ointment (Topical); **Ointment Base Control**: Excision wound + ointment base (Topical); **5 % HASA Ointment**: Excision wound + 5%

ointment of SA extract (topical); **10% HASA Ointment**: Excision wound + 10% ointment of SA extract (topical)

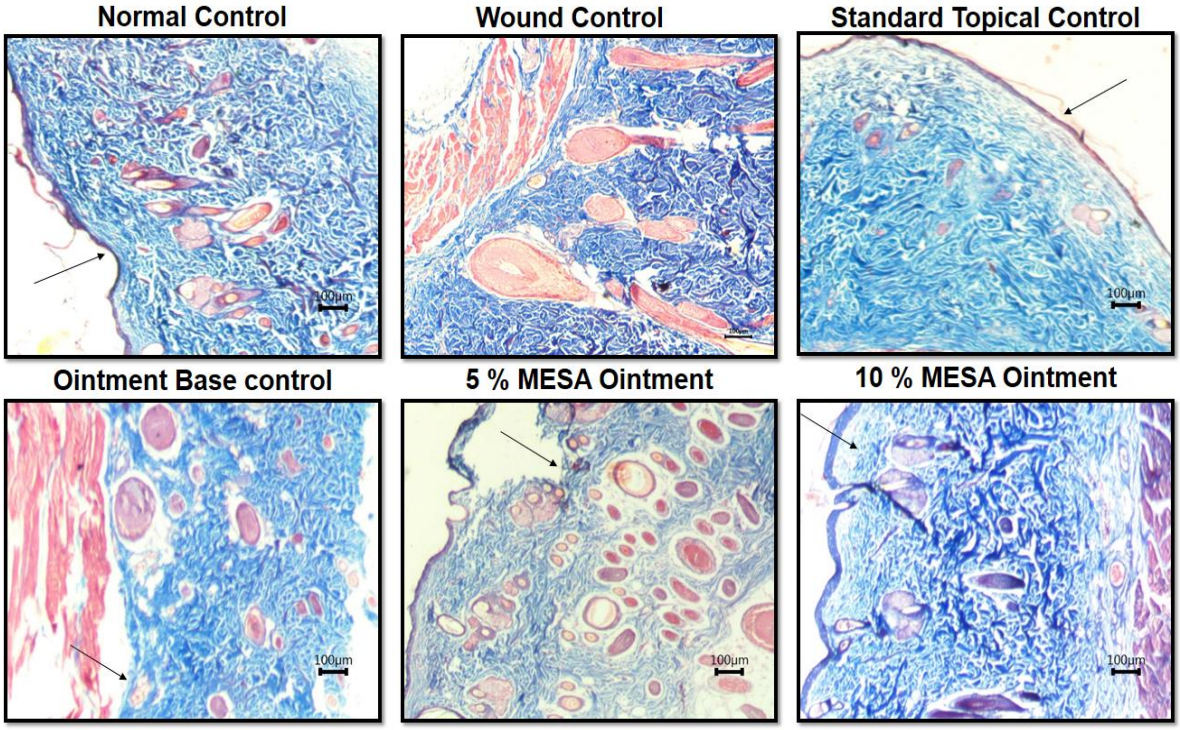

Figure 7.6: Effect of HASA on histopathological examination using M & T staining.

(Arrow shown collagen and fibrotic tissues).

Normal control: Distilled water p.o; **Wound Control:** Excision wound; **Standard Topical Control:** Excision wound + 5g Mupirocin ointment (Topical); **Ointment Base Control**: Excision wound + ointment base (Topical); **5 % HASA Ointment**: Excision wound + 5% ointment of SA extract (topical); **10% HASA Ointment**: Excision wound + 10% ointment of SA extract (topical)

7.6.4.1. Discussions

In haematoxylin and eosin staining the normal control group showed normal architecture of the skin and epidermal cells whereas the disease control group showed the disrupted epidermal layer, keratosis was not visible, tissue were not granulated, and fibro blast were absent, showing clearly that the wounds of the disease control group were not healed as depicted in Figure 7.5 However, in treatment groups of low dose and high dose treated groups, thick and intact epidermal layer,

and a significant keratosis layer were observed above the epidermal layer, the tissues were granulated, and hair follicles and fibroblast regeneration were observed, as shown in Figure 7.5 This confirms that the tissues of the HASA treated groups were wholly reconstructed and healed. In the OB treated tissue section indicates mild wound healing with regenerated epidermal layer, tissue granulation, and hair follicle glands. The tissue section of the Standard, also showed a moderately healed wound with a regenerated epidermal layer, tissue granulation, mild fibroblast, and hair follicles.

In Masson's trichrome staining Collagen synthesis plays a significant role in wound healing. To determine the effect of HASA ointment on wound healing, the skin tissues of all the experimental rats on day 18 were stained with Masson's Trichrome. Masson's Trichrome stained collagen with blue, where the cytoplasm and red blood cells were stained red. The disease control group showed numerous inflammatory cells and irregularly arranged lightly stained collagen fibers, as shown in Fig. 7.6 The HASA treated groups showed no inflammatory cells and distinct well-organized collagen fibers stained with deep blue arranged in a mesh like pattern showing an increase in collagen in the treated groups compared to disease control as shown in Figure 7.6. A few inflammatory cells can be observed in OB treated group with denser collagen fibers compared to the control group.

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CHAPTER 8: SUMMARY AND CONCLUSION

8.Summary and Conclusions

Phytochemical analysis of Hydroalcoholic extract of *Sonnerratia apetala* Buch. -Ham. (HASA) showed the presence of alkaloids, phenols, steroids, and flavonoids. According to a several reports, the antioxidant characteristics of certain steroids and phenolic compounds (coumarins and flavonoids) have pharmacological effects. These active components help the body's natural ability to repair wounds by boosting collagen fibre viability and strength, either by strengthening circulation, reducing cell damage, or encouraging DNA synthesis. Although the principal components of HASA, such as flavonoids, triterpenoids, and alkaloids, may be important in the process of wound healing, more phytochemical research is required to identify the active compound(s) that promote wound healing (Bruck de Souza et al., 2020).

The phytochemical analysis also revealed the presence of alkaloids, phenols, steroids, and flavonoids. According to a number of scientific research, the antioxidant characteristics of certain steroids and phenolic compounds have beneficial effects. These active components help the body's natural ability to repair wounds by boosting collagen fibre viability and strength, either by strengthening circulation, reducing cell damage, or encouraging DNA synthesis. Although the principal components of HASA, such as flavonoids, triterpenoids, and alkaloids, may be important in the process of wound healing, more phytochemical research is required to identify the active compound(s) that promote wound healing.

Wound contraction rate helps us to identify the effectiveness of the treatment on the speedy recovery of wound and as per the findings in our study the treament groups showed higher wound contraction rate as compared to disease control which signifies the effective wound healing activity of both 5% and 10 % HASA ointment formulation which depicts the finding of previous research (Thangavel et al., 2020).

Epithelization, contraction, and connective tissue implantation are the mechanisms that contribute to wound healing. The controlled production, deposition, and maturity of new collagens are key components of the healing process (Eming et al., 2007). Collagen is the main extracellular protein in the granulation tissue of a healing wound, and collagen synthesis in the injured area increases quickly after an injury. Collagen disintegration releases free hydroxyproline and associated peptides

As a result, measuring this hydroxyproline has been utilised as a measure of collagen turnover. The higher concentration of hydroxyproline in the excision wound suggests quicker collagen turnover, which promotes quick healing and increases the breaking strength of the treated wounds. The matrix molecules hexuronic acids serve as the building blocks for the creation of new extracellular matrix. By strengthening electrostatic and ionic interactions with collagen fibres, glycosaminoglycans are known to stabilise them. The observed data supported this fact as the HASA ointment treatment groups showed higher levels of Hydroxyproline and Hexuronic acid levels as depicted in graphs which showcases the faster collagen turnover which supported quicker healing in treatment groups which was not the case in disease control group (Preet et al., 2022).

The histopathological examination also supported the effectiveness of HASA ointment on wounded rats by H & E staining for epidermal layer, keratosis layer, hair follicles and fibroblast along with M & T staining was done for the formation of collagen fibres and arrangements in granulation tissue In treatment groups of low dose and high dose treated groups, thick and intact epidermal layer, and a significant keratosis layer were observed above the epidermal layer, the tissues were granulated, and hair follicles and fibroblast regeneration were observed, as shown in histopathological findings of H & E staining. This confirms that the tissues of the HASA treated groups were wholly reconstructed and healed. In the OB treated tissue section indicates mild wound healing with regenerated epidermal layer, tissue granulation, and hair follicle glands. The tissue section of the Standard, also showed a moderately healed wound with a regenerated epidermal layer, tissue granulation, mild fibroblast, and hair follicles. The disease control group showed numerous inflammatory cells and irregularly arranged lightly stained collagen fibers, as shown in histopathological findings. These findings were also resembling the reports of the previous study (Assar et al., 2021).The HASA treated groups showed no inflammatory cells and distinct well-organized collagen fibers stained with deep blue arranged in a mesh like pattern showing an increase in collagen in the treated groups compared to disease control as shown in histopathological findings. A few inflammatory cells can be observed in OB treated group with denser collagen fibers compared to the control group.

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