

**EFFICACY OF *ASPILIA PLURISETA* SCHWEINF IN CUTANEOUS WOUND
HEALING IN A MOUSE MODEL**

**A thesis submitted in partial fulfillment of the requirements for Master of Science degree
(Natural Products and Bio-prospecting) of the University of Nairobi**

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DEDICATION

I dedicate this work to my loving family: Dad, Mom and my siblings, Becky and Ben. I will always treasure you.

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LIST OF ABBREVIATIONS

µm: micrometer

bFGF: basic fibroblast growth factor

BP: British Pharmacopeia

bwt: body weight

CFU: colony forming units

DMSO: dimethyl sulphoxide

ECM: extracellular matrix

EGF: epidermal growth factor

FGF: fibroblast growth factor

H&E: hematoxylin and eosin

HIF: hypoxia inducible growth factor

IL-1: interleukin 1

KARI-TRC: Kenya Agricultural Research Institute- Trypanosomiasis Research Center

kg: kilogram

KGF: keratinocyte growth factor

MBC: minimum bactericidal concentration

MFC: minimum fungicidal concentration

MIC: minimum inhibitory concentration

mg: milligram

ml: milliliter

mmHg: millimeters of mercury

MMPs: matrix metalloproteases

OECD: Organization for Economic Co-operation and Development

PDGF: platelet derived growth factor

TGF- α : transforming growth factor alpha

TGF- β : transforming growth factor beta

TIMP: tissue inhibitor of metalloprotease

TNF- α : tumor necrosis factor alpha

VEGF: vascular endothelial growth factor

WHO: World Health Organization

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ABSTRACT

The skin is more predisposed to wounds because of its direct contact with the environment. The aim of treating wounds is to both hasten their healing and preclude undesirable consequences that may arise in the process. Plant products for wound healing are cheap and locally available; they are also purportedly safer than conventional therapies and so they may provide a viable alternative to expensive conventional wound remedies especially for poor communities in developing countries. *Aspilia pluriseta* has been used in a number of traditional medicine systems to treat lacerations, bruises and burns and it is reputed to aid the healing of such cutaneous lesions. The aim of this study was to evaluate the efficacy of *Aspilia pluriseta* in healing cutaneous wounds and to also test its safety when applied on the skin.

An *Aspilia pluriseta* ointment was formulated for wound healing studies. Full thickness excision wounds were created on male adult Swiss albino mice and treated with the ointments in order to evaluate the influence of the plant material on wound healing. The ointment was applied to two groups of experimental mice. The first group (n=8) and second group (n=8) were treated with 10% and 20% *A. pluriseta* based ointment, respectively. A third and fourth group (n=8 each) were treated with the vehicle ointment alone (Simple Ointment, British Pharmacopeia) and Silver sulfadiazine (Silverex Cream[®]) as the negative and positive controls, respectively. All the mice in the experimental and control groups were treated daily for 21 days. Wound area measurements were taken every three days and biopsies for histology were taken on the 7th, 14th and 21st days post-treatment.

The *A. pluriseta* powder was extracted by cold maceration using methanol to be tested for antimicrobial activity. The methanol-free extract was dissolved in dimethyl sulfoxide (DMSO) and distilled water and tested for activity using five bacteria and one fungus by broth

macrodilution method. The extract was screened qualitatively for major groups of phytochemical compounds.

The 20% *A. pluriseta* based ointment was tested for skin sensitization potential in guinea pigs. It was applied once a week onto the shaved left flank of three guinea pigs for three weeks (induction phase). It was then applied onto the right flank of the guinea pigs (challenge phase) 7 days after the last treatment of the induction phase, and the animals observed 24 hours later for allergic skin reaction. Descriptive statistics were done for quantitative data and one-way analysis of variance was used to compare the means of responses between treatment groups. P-values less than 0.05 were considered statistically significant.

The *A. pluriseta* ointment preparation enhanced wound healing to an extent that was comparable to that of the standard drug (Silverex Cream[®]) in terms of epithelialization time (15.5 ± 1.118 days compared to 16.5 ± 0.616 days for the 20% *A. pluriseta* ointment and standard drug, respectively) and wound contraction (28.17 ± 3.725 and 57.75 ± 6.178 percent wound area reduction on the 6th and 9th days post-wounding, respectively for the 20% *A. pluriseta* powder ointment compared to 14.98 ± 7.194 and 36.37 ± 4.871 for the standard drug on the same days). Histopathology revealed that the plant material accelerated remodeling in the wounded tissue and increased the amount of collagen in the scar compared to the negative control. The extract had marginal antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus agalactiae* with a Minimum Inhibitory Concentration (MIC) of between 50 mg/ml and 200 mg/ml and Minimum Bactericidal Concentration (MBC) ranging from 100 to 800 mg/ml. The extract contained triterpenoids, tannins, glycosides, saponins and phenols. The 20% *A. pluriseta* ointment induced moderate sensitization in guinea pigs.

The demonstrated wound healing activity of *A. pluriseta* offers justification for more in-depth studies into the plant's therapeutic potential. Skin sensitization, however is a possible side effect of topical application of the plant-based ointment. Further study on the efficacy of *A. pluriseta* in the treatment of wounds, its safety and antimicrobial activity is recommended.

1. CHAPTER ONE: INTRODUCTION

1.1. Background information

Abnormalities of wound healing are among the greatest causes of deformity and disability (Ashoka *et al.*, 2011). Wounds also have a significant impact on the quality of life of patients, both human and animal. They also present a heavy economic burden, not least the reduction in the efficiency of the human labor force and productivity of food animals. In addition, wounds act as substrate for infection in injury patients and therefore they prolong the convalescence of such patients. Wound infection is one of the most common diseases in developing countries (Kumar *et al.*, 2006).

Wound healing is the process through which damaged tissue is restored as closely as possible to its pre-injury state. This process is the function of an intricate interaction of blood cells, cytokines and growth factors (Clark, 1991) as well as other connective tissue components. The basic principles of optimal wound healing include minimizing tissue damage, providing adequate tissue perfusion and oxygenation, proper nutrition and provision of a moist wound healing environment. Timely and effective treatment of acute wounds may limit development of chronic wounds, a consequence of non-healing. The goal of studying the pharmacology of wound healing is to measure the influence of various interventions on healing and to screen agents that encourage healing.

1.2. Statement of the problem

While data on the prevalence of wounds or their social impact locally or regionally is lacking, it is clear that therapeutic interventions are needed to manage wounds. Despite big advances in the pharmaceutical drug industry, there is limited availability of agents that stimulate wound healing

(Udupa *et al.*, 1995). Current research has resulted in only few economic and efficient pro-healing agents that can preclude the burden of wounds on patients (Süntar *et al.*, 2011). The high cost of treatment, especially of chronic wounds and unwanted side effects that accompany therapy worsens the problem (Porrás-Reyes *et al.*, 1993; Suh *et al.*, 1998). Plant products for wound healing, on the other hand, are cheap and locally available; they are also purportedly safer than conventional therapies (Raina *et al.*, 2008) and so they may provide a viable alternative to expensive conventional remedies especially for poor communities in developing countries. Many medicinal plants have been reported to possess wound healing activities (Nagori and Solanki, 2011).

Although Africa harbors about 25% of the global pool of genetic resources, its contribution to global commercial natural product based remedies is meager. The continent is also suffering a high rate of loss of its biodiversity with an average annual loss of 1% as opposed to a global level of loss of 0.6% (Iwu, 1993). This is compounded by the accompanying loss of traditional knowledge. The conservation of plant genetic resources, documentation and validation of traditional knowledge are key issues that need to be addressed (Neuwinger, 2000) to counter these losses.

Aspilia pluriseta has been used ethnomedicinally by a number of communities. In Kenya, it has been used ethnomedicinally by the Kikuyu and Luo communities for a variety of conditions. In the East and Southern Africa region, it has been used ethnomedicinally in Tanzania, Rwanda, Burundi and Zimbabwe. Though the plant has been used ethnomedicinally to treat wounds, a review of literature revealed no studies on a scientific platform to validate this wound healing activity (<http://www.prota4u.org/protav8.asp?p=Aspilia+pluriseta>).

1.3. Justification

Understanding the pharmacological and toxicological properties of medicinal plants is important for the optimization of their use in therapy and as adjuncts to conventional medicine (Midiwo *et al.*, 2002). This is in line with the general goal of scientific validation, standardization and safety evaluation of plants of traditional medicine before recommending them for therapy (Gupta and Jain, 2010). This study therefore aimed at validating the ethnomedical use of *A. pluriseta* to support the healing of cutaneous wounds. This was done through evaluation of the plant's efficacy in excision wound healing in experimental mice with a view to understanding the effects on wound healing parameters and to unravel the potential for its further development.

1.4. Null hypothesis

Aspilia pluriseta does not have wound healing properties.

1.5. Objectives

1.5.1. General objective

The general objective of this study was to determine the efficacy of *Aspilia pluriseta* in wound healing using morphometric, antimicrobial, safety and phytochemical parameters.

1.5.2. Specific objectives

The specific objectives were:

- 1) To determine the wound healing activity of *Aspilia pluriseta* powder ointment on excision wounds in mice using morphometric parameters.
- 2) To determine the antifungal and antibacterial activity of methanolic extract of *Aspilia pluriseta*.
- 3) To identify the major classes of phytochemicals in the methanolic extract of *Aspilia pluriseta*.

- 4) To determine the skin sensitization potential of *Aspilia pluriseta*-powder-based ointment after repeated topical administration in guinea pigs.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. WOUND HEALING

2.1.1. Definition, causes and occurrence of wounds

A wound is an injury that results from the disruption of the normal tissue anatomy and function. It may result in a break of the epithelium with or without loss of underlying connective tissue. Wounds may result from physical, chemical, microbial agents or immunological mechanisms (Raina *et al.*, 2008). Acute wounds heal normally in an orderly and efficient manner, and within an expected timeframe. Chronic wounds on the other hand occur when there is an interruption in the healing process and consequently, healing time is protracted and the healing outcome is distorted. It is estimated that currently, 6 million people have chronic wounds around the world (Kumar *et al.*, 2007). Wounds impart a heavy burden on patients and healthcare provision agencies. The overall cost of wound management has not been studied in many countries, including Kenya. In the United States of America (USA), the cost of institutional care on patients with chronic wounds is estimated to be US\$ 1000 per day. The estimated expenditure for the provision of wound care to human patients worldwide tops US\$ 7 billion (Shefali and Bhaduri, 1999).

Wounds and other dermatologic conditions are one of the five most common reasons why people seek medical care in developing countries. Wounds are common especially in rural populations where manual labor in the fields and other activities that expose people to trauma, combined with delayed wound treatment, predispose to wound complications (Ryan, 1992).

2.1.2. Tissue response to injury

When a wound results, the possible outcomes are normal healing, excessive healing or deficient healing. Normal wound healing is the typical repair response, while excessive and deficient healing are considered pathologic responses (Diegelmann and Evans, 2004).

Tissue response to injury may entail any one of a number of events. Regeneration is the replacement of the exact tissue morphology and function after the loss or compromise of that tissue after injury. This occurs in lower life forms such as some species in the Order *Caudata* e.g. salamanders. Some mammalian tissues such as hepatic tissue and the epidermis possess some regenerative capacity. Also, fetal tissue is reported to heal in pattern that closely mimics regeneration (Krummel *et al.*, 1987). Moreover, studies in the African spiny mouse (*Acomys* species) have shown evidence of regeneration of the skin and its adnexa after skin autotomy (Seifert *et al.*, 2012).

Normal repair is another form of tissue response to injury and it is the process resulting in the establishment of a balance between scar tissue formation and resolution (Diegelmann and Evans, 2004). Excessive healing in turn is characterized by excessive lay-down of collagen and other connective tissue components. This results in altered morphology and compromised function of the tissue. Examples include fibrosis (keloids and hypertrophic scars), strictures, adhesions and contractures. Deficient healing occurs when there is insufficient connective tissue lay-down. Consequently, the scar tissue is weak and can easily dehisce, as is the case with chronic ulcers (van Zuijlen *et al.*, 2002; Diegelmann and Evans, 2004). The latter two responses are recognized as pathological wound healing (Diegelmann and Evans, 2004).

Dermal wound healing is primarily accomplished by deposition of a connective tissue matrix, contraction and epithelialization. Wounds with regular margins, for instance surgically created wounds, whose margins are then brought in close apposition using staples or sutures for instance, heal by primary intention. This involves the deposition of collagen, proteoglycans (connective tissue ground substance) and attachment proteins (connective tissue matrix deposition). Wounds that remain open (those whose margins are not apposed) heal by contraction. This is the centripetal motion of cells and connective tissue matrix from the wound edge towards the center of the wound. Tomasek *et al.* (2002) suggest this motion to be the result of contractile fibroblasts (myo-fibroblasts) activity.

2.1.3. The healing cascade

Wound healing is the process that results in the restoration of normal anatomy and function after injury, and regaining of tissue tensile strength. It is a process attended by integrated cellular and biochemical events and characterized by four phases: hemostasis, inflammation, proliferation and remodeling. These phases though distinct, overlap in time. The success of this complex process is owed largely to intricate cell signaling that is effected by *cytokines*. These chemical signals direct and control each stage of the process (Greenhalgh, 1996).

Injury causes the exposure of tissue collagen to blood platelets (Diegelmann and Evans, 2004). The platelets aggregate at the site and degranulate, releasing chemical factors aimed at hemostasis and at the end of this process a fibrin clot is formed. As well as stopping bleeding, this fibrin clot provides a provisional matrix for cell migration (Clark, 2001). In addition to haemostatic factors, platelets produce cytokines that initiate wound healing. These include the Platelet Derived Growth Factor (PDGF) and the Transforming Growth Factor beta (TGF- β).

Platelet Derived Growth Factor (PDGF) attracts neutrophils, macrophages, smooth muscle cells and fibroblasts to the site and also stimulates mitogenesis of fibroblasts and smooth muscle cells. Transforming Growth Factor beta (TGF- β) on the other hand attracts macrophages and stimulates their expression of more cytokines including FGF (Fibroblast Growth Factor), PDGF, TNF α (Tumor Necrosis Factor α) and IL-1 (Interleukin 1). TGF- β also enhances fibroblast and smooth muscle cell chemotaxis and modulates collagen and collagenase production (Diegelmann and Evans, 2004). Coagulation and activated-complement pathways also lead to the expression of vasoactive and chemotactic factors; injured or activated parenchymal cells also produce these, and they serve to recruit inflammatory leukocytes to the site of injury (Clark, 1996).

Neutrophils migrate to the site within minutes of injury. They remove foreign material while debriding the site of non-functional host cells and damaged matrix components. They are important in controlling microbial wound invasion and they do so through phagocytosis and oxidative killing of microbes through the *respiratory burst* phenomenon. They are the main cellular players, and an important marker of the inflammatory phase of healing. They are then either shed as eschar or are phagocytosed by macrophages (Singer and Clark, 1999).

Mast cells are also involved in wound healing. They release granules packed with enzymes and active amines, including Histamine. These amines account for the inflammation around the site, characterized by its classic signs: *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain). The amines also mediate the passage of mononuclear cells into the site from blood vessels by increasing the permeability of the blood vessel walls (Diegelmann and Evans, 2004).

Monocytes from blood are activated to become wound macrophages within about 48 hours after injury. Chemotaxis of monocytes from blood to the injury site is thought to be induced by

fragments of extracellular matrix protein, monocyte chemo-attractant protein 1 and transforming growth factor β (TGF β) (Singer and Clark, 1999). Activated macrophages express growth factors such as PDGF and vascular endothelial growth factor (VEGF), which initiate granulation. Further, using integrin receptors, macrophages and monocytes adhere to specific extracellular matrix proteins. This adherence is important for firstly, activation of macrophages for phagocytosis, secondly, transformation of monocytes to reparative or inflammatory macrophages, and thirdly, stimulation of expression of colony stimulating factor, tumor necrosis factor α (TNF α) and PDGF. Macrophages are arguably the most essential inflammatory cells in normal healing. Inhibition of wound macrophages results in a delay in wound healing. Their presence in the wound heralds the end of the inflammatory phase and the ushering in of the proliferative phase (Diegelmann and Evans, 2004). Overall, the inflammatory phase last for approximately four days.

The proliferative phase lasts a few weeks and comprises of neovascularization, granulation and re-epithelialization (Schreml *et al.*, 2010). It is characterized by a predominance of fibroblasts. The fibroblasts are responsible for the synthesis, lay-down and remodeling of the new matrix. Other important cells are macrophages and endothelial cells. Macrophages provide a source of growth factors necessary to promote fibroplasias and angiogenesis. Endothelial cells form new blood vessels necessary for the delivery of oxygen and nutrients to support active cell metabolism at the wound site (Singer and Clark, 1999).

Fibroblast activity is presumably spurred on by cytokines especially PDGF and TGF β as well as ECM molecules (Gray *et al.*, 1993; Roberts and Sporn, 1996; Xu and Clark, 1996). The fibroblasts therefore start to multiply and express appropriate integrin receptors and move into

the wound. The new ECM provides a scaffold and conduit for attachment and migration of the fibroblasts. The matrix is composed of fibrin, fibronectin and hyaluronic acid. Once attached to the matrix, fibroblasts commence the production of collagen, primarily collagen Type I (Prockop and Kirivikko, 1995). The collagen is produced at the endoplasmic reticulum of the fibroblasts. A vital step in this process is the hydroxylation of proline and lysine residues of the amino acid chains forming procollagen. This is important for the stability of the eventual triple helical structure of collagen (Peterkofsky, 1991). The down-regulation of this hydroxylation (for instance in anaerobic wounds or in cases of vitamin C deficiency) results in collagen of poorer quality which has lower melting temperatures than normal collagen (Diegelmann and Evans, 2004). After hydroxylation, the collagen undergoes more processing in and out of the fibroblasts.

As the proliferative phase progresses, macrophages become a key source of cell signaling factors, notably TGF β , a cytokine very vital in the regulation of fibroblast function. Transforming growth factor β (TGF β) is also produced by platelets and T-lymphocytes (Diegelmann and Evans, 2004). The cytokine has a threefold activity on extracellular matrix (ECM) lay-down. Firstly, it increases the expression of genes for collagen, proteoglycans and fibronectin, with consequential increased production of matrix proteins. Secondly, TGF β retards the production ECM degrading proteases, and finally, it stimulates the production of Tissue Inhibitor of Metalloprotease (TIMP) (Hall *et al.*, 2003).

Collagen synthesis and cross-linking ensures that the capillary bed that forms subsequently is stable and well anchored. Improper collagen cross-linking has been implicated in cases of non-specific post-surgical hemorrhage in patients with otherwise normal hemostatic parameters (MacKay and Miller, 2003).

2.1.3.1. *Epithelialization*

Epithelialization is the process through which epithelial cells migrate into the wound and finally cover it. Macrophages, platelets and keratinocytes produce Epidermal Growth Factor (EGF) and Transforming Growth Factor α (TGF α) and it is these growth factors that stimulate the process. The process begins within hours of injury, and the advancing epidermal cells form a stratum delimiting the wound from clotted blood and damaged stroma (eschar) (Singer and Clark, 1999). This process of centripetal migration of epidermal cells from the wound margin and residual skin appendages for example hair follicles, into the wound is called *epiboly* (Stenn, 1981). Epidermal cells undergo a morphologic alteration in order to achieve this. Their intracellular tonofilaments are retracted (Paladini *et al.*, 1996), the intercellular desmosomes are dissolved and they form peripheral cytoplasmic actin filaments (Goliger and Paul, 1995). The net effect is the loss of physical attachment between cells of epithelial sheets that discourage cell movement, and the expression of intracellular cytoskeletal features that encourage individual cell migration. Lateral movement is further enabled by dissolution of hemidesmosomes, the cytoskeletal plaques that anchor epidermal cells onto the basement membrane (Singer and Clark, 1999).

Further, there is increased expression of integrin receptors on the epidermal cells' surfaces. These receptors enhance cell-ECM interaction. Using these receptors, the migrating epidermal cells chart a path dissecting the non-viable eschar from viable tissue as earlier mentioned. To further enable this migration, there is the production of ECM digesting *collagenase* and *plasminogen activator* by the epidermal cells. The latter has a two-pronged effect: activation of plasmin, and further activation of collagenase. Epidermal cell migration is thus enabled by enzymatic degradation of ECM ahead of the advancing cells (Singer and Clark, 1999). Other epidermal cells begin proliferating behind the forward migrating cells a day or two after injury.

This migration and proliferation is thought to be stimulated by the absence of neighboring cells at the wound margin, the so-called *free edge effect* (Singer and Clark, 1999) as well as paracrine, endocrine, autocrine and intracrine stimulation (Diegelmann and Evans, 2004; Singer and Clark, 1999). Basement membrane proteins then follow the epidermal cells towards the center of the wound. The epidermal cells revert to their usual phenotype, re-establishing intercellular connections and connections with the basement membrane (Singer and Clark, 1999). After epithelial cells have fully bridged and covered the wound, enzymes are produced that dissolve the scab attachment, so that it falls off (Diegelmann and Evans, 2003).

2.1.3.2. Neovascularisation

Neovascularization is the process through which new blood vessels are formed and laid down in the new stroma. The process depends on the newly formed ECM for a scaffold and is also supported by the migration and mitogenic stimulation of endothelial cells (Madri *et al.*, 1996). This angiogenic activity is owed to a number of cytokines: VEGF, TGF β , angiogenin, angiotropin, angiopoietin 1, thrombospondin and acidic and basic fibroblast growth factors. These cytokines are produced by fibroblasts, macrophages and endothelial cells (Singer and Clark, 1999). Low oxygen tension as well as elevated lactic acid levels in the wound tissue also stimulates the process. Oxygen tension in the wound tissue operates a signaling pathway through the Hypoxia Inducible Factor (HIF) that is responsible for turning VEGF production on or off. Many of the cytokines apparently induce angiogenesis by stimulating production of Basic Fibroblast Growth Factor (BFGF) and VEGF by macrophages and endothelial cells. Activated epidermal cells also produce VEGF. The BFGF exerts its role in the early stages of the healing process (within three days of injury) while VEGF is more active from the fourth to the seventh day after injury (Nissen *et al.*, 1998).

Wound proteases digest the basement membrane allowing the movement of endothelial cells and their formation of new blood vessels under the influence of the aforementioned angiogenesis factors. The appropriate ECM, the expression of provisional matrix receptors on the endothelial cells and the production and activity of protease are also vital for angiogenesis (Singer and Clark, 1999). Once granulation is complete, angiogenesis ceases and a lot of the new blood vessels disintegrate through apoptosis (Ilan *et al.*, 1998).

2.1.3.3. Contraction

Contraction commences around the second week of healing. Fibroblasts assume myofibroblast morphology. The fibroblast cytoskeleton is altered with an increase in the amount of actin-containing microfilaments and a change in the microfilaments' intracellular orientation to line up along the cytoplasmic phase of the plasma membrane. There is also intercellular and cell-matrix linkages. This is postulated to occur under the influence of PDGF, TGF β , fibroblast-matrix connection *via* integrin receptors and cross-linking between collagen bundles (Singer and Clark, 1999).

2.1.3.4. Extracellular matrix reorganization and collagen dynamics

Procollagen undergoes processing in the extracellular space after being secreted by fibroblasts. One of the major processes is the formation of intermolecular and intramolecular cross-links between the fibrils. This occurs under the influence of lysyl oxidase enzyme (Hornstra *et al.*, 2003). The collagen matures and gains more strength and stability as more cross-links are formed.

The synthesis and secretion of collagen by the fibroblasts occurs concurrently with collagen breakdown. The collagen breakdown is effected by proteolytic enzymes called matrix

metalloproteases (MMPs). These enzymes are produced by macrophages, fibroblasts, epidermal cells and endothelial cells. Wound repair is a balance between the MMPs' destruction of collagen and the activity of tissue inhibitors of metalloproteases. For the first three weeks post-wounding, wounds gain only about 20% of their final strength. This is followed by a slow gain in tensile strength that is the result of collagen remodeling. Nevertheless, the eventual scar tissue never attains equal tensile strength to that of normal dermal tissue. The possible maximum tensile strength of scar tissue compared to normal skin is reported to be 70% (Singer and Clark, 1999).

2.1.4. Factors affecting the rate of wound healing

The process of wound healing is made of four overlapping phases: hemostasis, inflammation, proliferation and tissue remodeling or resolution (Gosain and DiPietro, 2004). Factors interfering with any one or more of these phases affect the rate of wound healing. These factors are generally divided into two categories, local and systemic factors. Local factors are those that directly influence the characteristics of the wound itself and include oxygenation, infection, foreign matter in the wound and blood supply to the wound. Systemic factors are those that relate to the overall health or disease state of the patient, which affect the ability to heal. The systemic factors mainly include age and gender, ischemia, concurrent diseases, obesity, medications, drug and substance abuse, immune suppression and nutrition (Guo and DiPietro, 2010; Nagori and Solanki, 2011). In addition, the diameter and depth of the wound affects the rate of healing.

2.1.4.1. Infection

The absence of an intact epithelium allows easy access of tissue by infective agents, first and foremost those bacteria normally residing on the skin surface. Bacterial invasion results in a state

of infection and microbial replication. The extent of this state has been categorized into four groups namely, contamination, colonization, local infection/critical colonization and invasive infection. Contamination refers to the presence of bacteria in the wound without replication while colonization is the presence of bacteria with replication. Local infection or critical colonization is a state of bacterial replication in the wound with a start of tissue response. Invasive infection is the state of tissue damage as a result of bacterial replication (Edwards and Harding, 2004; Dow *et al.*, 1999).

The mere presence of bacterial wound contamination does not necessarily impede healing. In fact, sub-infective levels of contamination seem to have a prohealing effect associated with enhanced inflammation and granulation (Laato *et al.*, 1998). The occurrence of pathologic wound infection is dependent on bacterial load, bacterial virulence and the host immune response (Wysocki, 2002). The infective doses of different bacterial agents vary according to the species of bacteria involved and also the interaction among a number of species. Traditionally, bacterial counts of above 1×10^5 have been regarded as above the threshold of infection. However, it has been felt that the infective dose for a certain organism that triggers a host immune response (as opposed to a certain fixed figure), should be the benchmark for classifying wounds as infected (Kingsley, 2001).

The interaction of many bacterial species is of great significance in affecting wound healing. Higher numbers of interacting species have been found to correlate positively with delayed healing (Edwards and Harding, 2004). Initial bacterial wound invaders are resident flora on the skin surface followed by other Gram-positive bacteria in the early chronic phase. Later chronic wounds usually have multi-specific bacterial invasion with anaerobic organisms and Gram-

negative rods for example *Pseudomonas aeruginosa* present in them. Wounds on different body locations will however display different specific attendance depending on what species of commensals occur in these sites.

Frequently isolated bacterial species are: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and β -hemolytic streptococci. Others include *Escherichia coli* and *Corynebacterium* sp. (Kumar *et al.*, 2006). However, the use of routine cultures to identify bacterial species present in wounds has been reported as not being conclusive. This has been because of the identification of bacteria that cannot be cultured routinely by the use of molecular tools and enhanced culturing methods. Such tools and methods have demonstrated the presence of a wider spectrum of bacterial species (Hill *et al.*, 2003).

Bacteria in wounds tend not to live singly, but in *biofilms*. These are complex communities of microorganisms in a self-secreted polysaccharide matrix (Edwards and Harding, 2004). While biofilms may possibly comprise a single species, they are more commonly the aggregations of many bacterial species, aerobic and anaerobic (Hansen *et al.*, 2007).

The efficiency of bacterial elimination from wounds by the host immune system is markedly decreased between free living microorganisms and those living within biofilms. For instance, 1000 times more leukocytes are needed to kill the same amount of *Staphylococcus aureus* in a biofilm compared to free living *S. aureus* (Westgate *et al.*, 2010). This resistance is also seen against chemotherapeutic agents. This resistance to chemotherapeutic agents is exclusive of standard antimicrobial resistance mechanisms (DeKievit *et al.*, 2001). It is probably caused by reduced penetration of the biofilm by antimicrobial agents and phagocytic cells because of the self secreted matrix. However, it has been found that even in the event that antimicrobial agents

do penetrate the matrix, their activity is below the expected level. This could be due to phenotypic alteration of the bacterial cells in the biofilm in response to intercellular signaling, as well as their more sessile growth pattern and a lowered metabolic rate (Edwards and Harding, 2004). In addition to existence within biofilms, *S. aureus* and *E. coli* have been reported to act via specific adherence proteins to hamper inflammatory response and enhance attachment onto the host cells (Westgate *et al.*, 2010).

A low-grade presence of bacterial colonization serves in the interest of healing, rather than against it. However, higher numbers and persistence of bacteria in wound tissue is obviously detrimental. The specific effects of bacterial colonization are summarized in Table 1.

Table 1: Effects of microbial colonization on the healing of wounds (Edwards and Harding, 2004)

Phase of Wound Healing	Effect of Bacterial Colonization
Inflammatory Phase	Depletion and exhaustion of platelets and chemotactic factors. Reduced leukocyte activity by bacterial products. Retaliatory increased production of cytotoxic enzymes and ROS (respiratory burst) with possible bystander damage on host tissue. Production of vasoactive amines by host cells with subsequent vasoconstriction, tissue hypoxia.
Proliferative phase	Deposition of friable granulation as a result of increased matrix metalloproteases production and reduced production of tissue inhibitor of metalloproteinase.
Epithelialization	Inhibited migration of epidermocytes, digestion of dermal proteins. Epithelial damage by proteases.
Extracellular matrix reorganization	Reduced numbers and activity of fibroblasts; production of disorganized collagen. High collagen turnover resulting in a weak scar. Reduced collagen cross-linking, weak scar and dehiscence.

2.1.4.2. *Oxygenation*

Oxygen is a prerequisite at every stage of healing. Even though its complete role in wound healing is not fully understood, its importance in cell metabolism, especially aerobic metabolism seems to be primary (Schreml *et al.*, 2010). It is vital in oxidative killing of pathogens to control infection, induction of angiogenesis, enhancement of keratinocytes differentiation, cell migration, epithelialization, fibroplasia, collagen synthesis and wound contraction (Rodriguez *et al.*, 2008).

Wound healing, a reparative process with massively increased cellular activity needs a lot of biochemical energy, and oxygen is important for the production of the cellular energy currency through aerobic glycolysis, citric acid cycle and in oxidation of fatty acids (Tandara and Mustoe, 2004). Oxygen is also vital in the activity of NADPH-linked oxygenase. This is a strictly oxygen-dependant enzyme which catalyses the production of reactive oxygen species (ROS), important in oxidative killing of microorganisms (Allen *et al.*, 1997).

Wound tissue oxygenation is the product of the oxygen supply, and the rate of consumption of the oxygen in the wound tissue. Oxygen supply is in turn dependent on gaseous exchange at the lungs, blood oxygen carrying capacity and tissue perfusion at the site and its surroundings (Schreml *et al.*, 2010). Vascular disruption in the immediate post-injury wound and increased cellular metabolism render the wound tissue hypoxic relative to normal tissue. This is not necessarily detrimental. It serves as a signal for the induction of many processes in wound healing, most notably, cytokine production from the various cellular sources (Rodriguez *et al.*, 2008). Prolonged hypoxia however causes delayed wound healing. Chronic wounds are conspicuously hypoxic with Tandara and Mustoe (2004) reporting tissue oxygen tensions in

chronic wounds at between 5 and 20mmHg in contrast to between 30 and 50mmHg in normal tissue.

2.1.4.3. Nutrition

This is a systemic factor that has profound effect on wound healing. Total malnutrition or some specific nutrient deficiencies are negatively significant in surgical or traumatic wound healing. Specific nutritional supplementation is indicated in patients with less than optimum nutritional status afflicted with non-healing wounds. Important dietary entities include carbohydrates, fats, proteins, vitamins and minerals (Guo and DiPietro, 2010).

Carbohydrates and fatty acids are important in meeting cellular energy demands that are increased in the face of active repair. When they are deficient, alternative energy sources, usually amino acids are catabolised, and this may deplete them resulting in a secondary deficiency (Arnold and Barbul, 2006). In addition to energy metabolism, fatty acids have a role in the anabolism of new repair components during healing. Essential fatty acids (omega-3 and omega-6) are important in cytokine production, cell metabolism, gene expression and angiogenesis on site, and overall, they serve to promote host immune function (McDaniel *et al.*, 2008; Shingel *et al.*, 2008; Arnold and Barbul, 2006).

Generally, protein deficiency negatively affects immunity. Locally, its effects include impairment of neovascularization, fibroplasias, ECM synthesis and wound remodeling. Proteins are some of the most important nutritional factors to wound healing (Gogia, 1995; Guo and DiPietro, 2010). In this group, the amino acids Arginine and Glutamine are very vital. Physiologic demands for arginine are increased during times of stress. Arginine also has an effect on the immune, endocrine and circulatory (vascular tone and endothelial cell activity)

systems, as well as on wound healing. It is also a precursor to proline, one of the major amino acid constituents of collagen (the other being glycine). As such high levels of these amino acids are needed especially during the proliferative phase of healing (Shepherd, 2003; Campos *et al.*, 2008). It has been found that the supplementation of arginine as an adjuvant to therapy during wound healing enhances the healing process (Campos *et al.*, 2008). Glutamine is a non-essential amino acid, but some circumstances like tissue injury make it essential. It is an important energy source for fibroblasts, lymphocytes, epithelial cells and macrophages (Arnold and Barbul, 2006; Campos *et al.*, 2008). Glutamine is also important in protein and nucleic acids synthesis in fibroblasts (MacKay and Miller, 2003). It stimulates the early inflammatory phase of healing (Campos *et al.*, 2008).

Vitamins have their general role as antioxidants and anti-inflammatory agents. They also are often co-factors in various enzyme-catalyzed biochemical reactions. Vitamin A (retinol) and vitamin C (L-ascorbic acid) bear great significance to wound healing. Vitamin E (tocopherol) is also important but to a less understood extent of its mode of activity than the former two.

Generally, vitamin A has its role in epithelial and bone tissue development, cellular differentiation and immune function modulation (MacKay and Miller, 2003). Locally in healing wounds, it serves as an anti-oxidant, promotes fibroplasias, modulates cellular differentiation and multiplication, promotes collagen and ECM ground substance synthesis and reduces ECM degradation by MMPs (Burgess, 2008). In addition, it has been reported to reverse corticosteroid-induced delayed healing as well as increase collagen cross-linking and hence tensile strength of scars in animal models (MacKay and Miller, 2003).

Vitamin C deficiency results in delayed wound healing, underlining its importance to the process. The deficiency has been correlated with decreased collagen synthesis, fibroplasias and angiogenesis as well as increased capillary fragility resulting in hemorrhage (vitamin C deficiency is considered a differential diagnosis in cases of non-specific post-surgical hemorrhage in patients with normal coagulation parameters)(MacKay and Miller, 2003). Vitamin C is also important in the hydroxylation of proline and lysine residues in procollagen prior to its secretion, a step vital to the stability of the collagen finally produced (Gross, 2000). Furthermore, it is a powerful anti-oxidant, modulates immune function (promotes neutrophils response to chemotaxis and also enhances lymphocyte transformation) and stimulates angiogenesis (MacKay and Miller, 2003).

Vitamin E is regarded as helpful in achieving minimal scarring and is popular in the cosmetic industry. Being a lipophilic anti-oxidant, it plays a major role in protecting lipid biological membranes from oxidative damage. However, animal model studies are conflicting and its exact role in pro-healing is vaguely understood (MacKay and Miller, 2003).

A number of micronutrients and trace elements have been found to be cofactors to a large number of enzymes attending the wound healing cascade. Deficiencies therefore retard healing and conversely, supplementation has been found to accelerate healing. Magnesium is a cofactor to enzymes involved in collagen synthesis. Copper is a cofactor for cytochrome oxidase and the cytosolic antioxidant, superoxide dismutase. It is also important in ensuring proper collagen cross linking. Zinc is a cofactor for RNA (ribonucleic acid) and DNA (deoxyribo-nucleic acid) polymerase, while iron is important in the hydroxylation of proline and lysine residues of

procollagen hence the overall stability of collagen (Shepherd, 2003; Arnold and Barbul, 2006; Campos *et al.*, 2008).

2.1.4.4. Age

Increased age is a major factor impacting negatively on wound healing. This factor is gaining significance especially because the elderly human population is expanding faster than any other age group according to WHO estimates (<http://www.who.int/topics/ageing/en>). Age associated inhibition of wound healing stems from interference with the inflammatory response. This includes delaying immune cell migration into the wound area, interference with cytokine production and hampered phagocytic activity of macrophages (Swift, 2001). Swift (1999) also reports delayed epithelialization, impaired collagen synthesis and neovascularization in animal models.

Androgen hormones in ageing males (5- α -dihydrotestosterone and testosterone) are also reported to be detrimental to wound healing. Estrogens, though, accelerate healing (Gilliver *et al.*, 2007).

2.1.4.5. Diabetes

Diabetes is a growing health concern worldwide. Patients are plagued by a dual wound threat. Firstly, acute wounds healing impairment has been documented in diabetes (Guo and DiPietro, 2010). Secondly, diabetic patients stand a high risk of developing non-healing diabetic foot ulcers. These ulcers are estimated to occur in 15% of all diabetes cases and precede 84% of all diabetes related lower leg amputations (Brem and Tomic-Canic, 2007).

Wounds in patients of diabetes are characterized by sustained hypoxia, hyperglycemia and high levels of MMPs, all detrimental to healing (Tandara and Mustoe, 2004). At the cellular level,

there is impairment of T-cell immunity, leukocyte chemotaxis, phagocytosis and anti-infective activity. There is also concurrent fibroblast and epidermal cell dysfunction (Guo and DiPietro, 2010).

2.2. MANAGEMENT AND CARE OF WOUNDS

The aim of treating wounds is to either shorten the time required for healing or to minimize undesirable consequences that may attend the process (Raina, 2008). The envisaged clinical endpoint in management and care of wounds is wound closure and total epithelialization (MacKay and Miller, 2003). Optimizing wound healing involves minimizing host tissue damage, debriding off necrotic tissue, enhancing tissue perfusion and oxygenation, optimizing nutrition and maintaining a moist environment (Pierce and Mustoe, 1995). The ideal wound healant therefore should facilitate granulation, enhance immunity, aid in debridement, control infection, reduce pain and discomfort and promote angiogenesis and perfusion. It should, in addition be affordable, easy to both apply and remove, and cause minimal patient discomfort. In fact, the Food and Drug Administration of the USA requires that potential wound healants not only support but also augment healing (Pierce and Mustoe, 1995).

2.2.1. Conventional methods of wound management

These methods are based on an understanding of the pathogenesis of wounds, and involve the correction of the underlying cause and the treatment of all the undesired occurrences subsequent to wounding. The efforts are directed both locally on the wound itself, and systemically and may consist of: infection control, debridement, immobilization, nutritional and glycemc control, and circulation control.

The interventions include medical care for example the use of antibiotics, wound debridement, use of topical agents for example the use of growth factors, wound dressing, wound closure and wound coverage, for instance the use of skin grafts. A variety of adjunctive therapies, such as hyperbaric oxygen, therapeutic ultrasound, electrical stimulation and electromagnetic therapy are currently under investigation and have been recommended for use in pressure ulcers.

2.2.2. Traditional methods of wound management

Traditional systems of medicine are based on empirical knowledge and experience gained through millennia (Kumar *et al.*, 2007). Drugs that enhance the healing of wounds have traditionally been developed mainly from plants, animal and mineral products. Ethnopharmacological studies from around the world report hundreds of plants reputed to aid wound healing. They are mostly used as pastes from fresh plant parts applied directly onto wounds (Kumar *et al.*, 2007).

2.2.3. Herbal products in conventional wound management

Many plants have demonstrated wound healing potential using various extractive forms and animal/wound models. A few of these have been formulated into different dispensable dosage forms for example creams, powders, impregnated gauze pads and gel matrices into which plant extracts have been incorporated. This is evidenced by vast numbers of potential value added herbal wound healants reported in review papers (Kumar *et al.*, 2007; Gupta and Jain, 2010) and numerous patent applications for new herbal-based wound healing products (Nagori and Solanki, 2011).

While the exact biochemical basis for the action of the botanicals is only beginning to be understood, the products with pro-wound healing effects are thought to work by enhancing blood

clotting, fighting infection and accelerating the rate of wound healing. Some, especially poly-herbal formulations have been reported to promote gains in tensile strength in incision wounds and epithelialization and contraction in excision wounds (Raina *et al.*, 2008).

The chemical compounds in plants that are responsible for healing are mainly secondary metabolites. Their diversity is therefore almost as wide as the species producing them, this is due to the fact that these metabolites are characteristically produced by only a limited range of plant species. Furthermore, a single plant species may be the source of more than one compound credited with aiding healing. For example, *Centella asiatica*, a plant with good wound healing properties has three chemical entities that are reported to enhance healing: *asiatic acid*, *asiaticoside* and *madecassic acid* (Gupta and Jain, 2010).

The solvents mainly used in the extraction of wound healing compounds are water and alcohols. For instance, in a review of ethno-pharmacologic approaches to wound healing in India, of the 64 plants reported to have wound healing activity, 54 had been extracted using water alone or together with another solvent. Alcohols (methanol and ethanol) were also common solvents (Kumar *et al.*, 2007). It is unclear whether it is an absolute fact that wound healing compounds are the more polar constituents of the spectrum of chemicals found in any plant (hence the choice of more polar solvents), or if the predominance of water and alcohol as solvents for extraction is arbitrary.

2.3. TOXICITY OF HERBAL REMEDIES

Although the general perception is that herbal remedies are safe and of low toxicity, they are, in theory, just as likely as their synthetic counterparts to cause adverse reactions. Long use and experience boasted of by herbalists does not by any means qualify as the standard for the

measure of safety (Ernst *et al.*, 1998). As such, safety tests are an indispensable prerequisite before the recommendation of any herbal products for use as wound healants.

Some members of the family *Asteraceae* contain potential allergens: *sesquiterpene lactones*, *polyacytelenes* and *thiophenes* (Aronson, 2009). These chemicals mainly manifest their toxicity in the form of contact dermatitis and allergy, effects that have been reported by many investigators as reviewed by Aronson (2009).

Mice exposed to 450mg/kgbw daily of *Aspilia pluriseta* root bark aqueous extract via the intraperitoneal route for one month developed moderate lymphoid depopulation of the spleen (Piero *et al.*, 2011).

2.4. ASPILIA PLURISETA SCHWEINF (ASTERACEAE)

2.4.1. Description of *Aspilia pluriseta*

Aspilia pluriseta, commonly known as *dwarf aspilia* is a perennial, woody herb with multiple branches from a woody rootstock (Figure 1). It is usually trailing with the distal part erect though it is sometimes erect, up to 1.5m high. The leaves are very rough and bear three prominent veins from the base. The flower heads are yellow in color. It is a plant of open woodland and grassland, with a preference for areas with black cotton soils (Gachathi, 2007). Its altitude range is between 1360 and 1560 meters above sea level. Its worldwide distribution is limited to East and Southern Africa (Flora of Zimbabwe, www.zimbabweflora.co.zw). The plant belongs to the genus *Aspilia* and the family *Asteraceae*.



Figure 1: *Aspilia pluriseta* (center of picture, yellow flowers)

2.4.2. Ethnomedical utilization of *Aspilia pluriseta*

The plant is locally (in Kenya) referred to as *muuti* (Kikuyu), *wuti* (Kamba), *ol-oiyabase* (Maasai) and *Shilambila* (Luhya). Various communities in Kenya as well as some in the rest of the Eastern and Southern Africa use the plant ethnomedically (http://www.africamuseum.be/collections/external/prelude/view_plant?pi=01500) as outlined in Table 2. The use of plant pulp on fresh wounds is common among almost all the communities that use the plant ethnomedically.

2.4.3. Chemistry and bioactivity of *Aspilia pluriseta*

Rodriguez *et al.* (1985) isolated Thiorubrine A from *Aspilia* species including *A. pluriseta*. Thiorubrine A is a potent photosensitizer with bioactivity against a wide array of pathogenic organisms. It belongs to a family of secondary metabolites that have a wide distribution in *Asteraceae*. However, Page (1997) failed to corroborate Rodriguez's findings, reporting no significant amounts of thiorubrines in plants of *Aspilia* (*Wedelia*) genus sourced from Kenya and Uganda.

Sebisubi *et al.* (2010) isolated a number of diterpenes from *A. pluriseta* aerial parts. Four of these diterpenes demonstrated moderate activity against chloroquine sensitive (D6) and chloroquine resistant (W2) *Plasmodium falciparum*. Cos *et al.* (2002) reported that the aqueous extract of the plant exhibited antiviral activity against HIV-1 virus with a good selectivity index.

Piero *et al.* (2011) did a phytochemical screen on the aqueous root bark extract of *A. pluriseta* and observed the presence of flavonols, flavones, flavonoids, chalcones, tannins, bound anthraquinones and sterols. The aqueous extract of the plant was reported by the same author to exhibit hypoglycemic properties in alloxanised mice.

Table 2: Ethnomedical uses of *Aspilia pluriseta* (adapted from <http://www.metafro.be>)

Country	Ailments ethnomedically treated by <i>Aspilia pluriseta</i>
Burundi	Injuries, cuts or wounds; constipation; diarrhea; otalgia; bacterial/fungal skin conditions; headache; gynecologic conditions; heart and other chest conditions; dizziness, vertigo and syncope; helminthoses and other endoparasitoses; stomatitis; emesis; dyspnoea and asthma; anemia and cachexia; toothaches and dental caries; dyspepsia, stomach pain and related complaints; fever and malaria; psychosis.
Kenya	Conjunctivitis and other eye disorders; dyspepsia, stomach pain and related ailments; injuries cuts or wounds; bacterial and fungal skin conditions, abscesses, boils, pruritus, skin ulcer and other skin conditions; diarrhea; cough, pneumonia, chest pain and related complaints; mumps otitis, otalgia and related complaints; post-partum disorders; helminthoses and other endoparasitoses; bleeding (Kokwaro, 2009; Gachathi, 2007; Johns <i>et al.</i> , 1995; Njoroge <i>et al.</i> , 2004; Njoroge and Bussmann, 2006, 2007; Njoroge and Kibunga, 2007).
Rwanda	Mastitis in cattle
Zimbabwe	Hygroma, edema or dropsy; constipation; diarrhea; obstetric disorders; anorexia; dyspnea and asthma; anemia and cachexia; nervous disorders; dyspepsia, stomach pain and related complaints; amenorrhea; amnesia; coma; magic (Gelfand <i>et al.</i> , 1985).

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. EXPERIMENTAL ANIMALS

Male Swiss Albino mice, 3 months old were purchased from KARI-TRC. They were housed in the University of Nairobi, Department of Public Health, Pharmacology and Toxicology animal house under room conditions and natural light. They were caged in groups of 6 in standard solid-bottom cages bedded with wood shavings. Cage bedding was changed once every week. Guinea pigs were purchased from the Central Veterinary Laboratories, Kabete. Albino guinea pigs and mice were preferentially selected to facilitate the observation of skin lesions. They were kept in a wood shavings bedded enclosure. All the animals were fed standard pelleted diet, Unga Mice Pencils for the mice and Unga Rabbit Pellets for the guinea pigs, and allowed *ad libitum* access to water. In addition, the guinea pigs were fed fresh vegetables every two days.

3.2. COLLECTION AND PREPARATION OF THE PLANT MATERIAL

Aspilia pluriseta aerial parts (leaves stems and flower heads) were collected in Ruiru (1° 9' 0" South, 36° 58' 0" East) in the month of January, 2012. It was identified by a taxonomist from the East Africa Herbarium (National Museums of Kenya) and a voucher specimen deposited. The plant parts were washed with tap water and then dried in the shade for ten days. They were then ground to a fine powder using an electric mill and then packed in dry zip-locked plastic bags and stored at room temperature awaiting extraction. The packaged plant powder was labeled with the name of the investigator, botanical name of the plant, collection site and packing date and stored away from direct sunlight.

3.3. PREPARATION OF PLANT EXTRACTS

Extraction of plant material was carried out with methanol (analytical grade) using cold maceration method. The plant powder was soaked in the solvent for 72 hours and agitated regularly. A liter (1000 ml) of solvent was used for every 100 grams of the plant powder, a solvent to sample ratio of 10:1 (Das *et al.*, 2010). At the end of the 72 hrs, filtration was done, first using cotton wool and then using filter paper (Whatman, № 1). The filtrate was then centrifuged for 5 minutes at 3000 revolutions per minute. The solvent was removed *in vacuo* using a rotary evaporator (Büchi, Switzerland) with the water-bath maintained at 50°C until the extract was near dry. The extract was dispensed into previously weighed and labeled screw-cap amber colored jars. The jars were left uncapped on a sand bath at 50°C, until a constant weight was achieved, signifying that all the solvent had evaporated off. The extract per-cent yield was determined as the percentage of extract weight to the weight of the plant powder. The jars were then capped and stored at 4°C until use.

3.4. DETERMINATION OF THE WOUND HEALING ACTIVITY OF *ASPILIA*

PLURISETA

3.4.1. Wound ointment preparation

The dry plant powder was separated into different particle sizes, +180, +150, +125, +106 and -106 µm using a series of different pore size sieves and a mechanical sieve shaker. Simple Ointment (British Pharmacopeia) base was prepared as follows: white soft paraffin, cetostearyl alcohol, hard paraffin and lanolin were weighed in a crucible in 17:1:1:1 ratio. The crucible was placed in a water bath at 85°C to melt the contents, after which these were stirred constantly until the mixture solidified.

The 10% and the 20% *A. pluriseta* ointments were prepared by triturating 10 grams and 20 grams respectively of the +125 μ m powder (particle size of between 125 and 150 μ m) into 90 grams and 80 grams respectively of the earlier formulated simple ointment. The trituration was done on a glass slab using a stainless steel spatula until a uniformly mixed plant material ointment was achieved. The respective ointments were dispensed into separate labeled containers and stored at room temperature awaiting use.

3.4.2. Excision wound healing assay

The backs of the mice were depilated using an electric clipper (Wahl, USA). The depilated area was disinfected by swabbing using 5% povidone iodine. A 100 mm² square area was marked using a stencil. Excision wounds through the full thickness of the skin (including *panniculus carnosus* muscle) were created using a pair of thumb forceps and Mayo's scissors along the edges of the marked squares. This was done with the animals under halothane anesthesia. Each wound was traced onto translucent paper for area calculations. Tracing was done with the animals under light halothane anesthesia. The animals were then randomly assigned one of four groups of eight members each. These groups were representative of the various treatments and were designated numbers 1, 2, 3 and 4. Group 1 animals were treated using the 10% *A. pluriseta* powder ointment; group 2 with 20% *A. pluriseta* powder ointment; group 3 with 100% Simple Ointment (formulated as explained under 3.4.1). Group 4 animals were treated with Silverex Cream[®] (Ranbaxy) containing 1% silver sulfadiazine and 0.2% chlorhexidine gluconate. All the treatments were administered topically onto the wounds. Members of respective groups were marked appropriately with two sets of marks on each animal's tail, one depicting the treatment group the animal belonged to and the other designating the individual animal number within the group, as shown in Figure 2.



Figure 2: Identification marks used for the animals in the wound healing assay

The three blue marks on the proximal part of the tail depict the treatment group while the two marks on the distal part of the tail depict the animal number in the group.

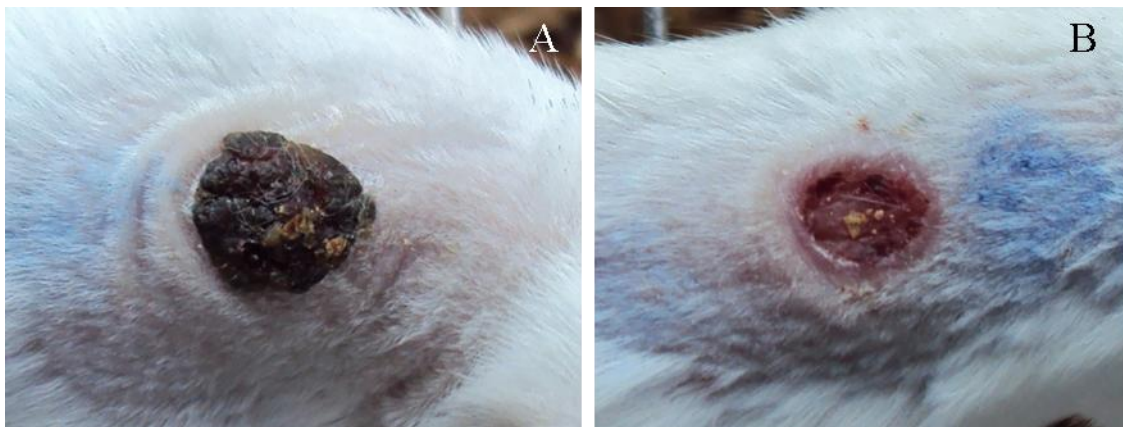


Figure 3: The gross appearance of different animals' wounds on day 9 after wound creation

Photograph A shows a wound with the scab intact (dark raised area at the center of the photograph), while the scab of the wound in photograph B has fallen off (pink area at the center of the photograph).

The wounds were left open and treated daily (Süntar *et al.*, 2009) upto the 21st day (Nisbet *et al.*, 2010). They were observed daily for extent of healing (Figure 3) and they were traced every three days (as earlier described) for determination of the extent of wound area reduction. The wound area was determined using 1mm² graph paper and the measurement was computed thus: the difference between the current and the initial wound area expressed as a percentage of the initial wound area as shown next.

$$\text{Percent Wound Area Reduction} = \frac{\text{Initial Wound Area} - \text{Specific Day Wound Area}}{\text{Initial Wound Area}} \times 100$$

On the 7th and 14th day post wounding, an animal was randomly selected from each group and euthanized using halothane in a gas chamber. The wound and scar tissue was harvested for histology. Any hair growing over the wound area from the wound edges was trimmed off. The day that the wound was fully epithelialized (when no raw area was left after the scab fell off) was recorded for each animal. The rest of the animals were all euthanized on the 21st day and their scar tissue harvested. All the harvested tissues were fixed in 10% formalin for histology.

3.4.3. Histopathology

Euthanasia of the animals for collection of tissue specimens was carried out using an overdose of halothane anesthetic in a gas chamber. A biopsy incorporating the wound and part of the surrounding tissue was collected using a thumb forceps and Mayo's scissors. The samples were fixed in 10% neutral buffered formalin and then routinely processed, blocked with paraffin wax and 5µm microtome-cut sections stained with Hematoxylin and Eosin (H&E), and Masson's Trichrome stains (Nisbet *et al.*, 2010). The tissue sections were observed under a light microscope (Leica DM500) with a ×40 and oil immersion (×100) objective lens, and micrographs taken using a digital camera (Olympus E-330) coupled to a microscope. Different types of cells were identified by morphology and staining characteristics.

The histological parameters that were taken were the population of neutrophils, macrophages and fibroblasts. The extent of neovascularization, collagen lay-down and epithelialization was also evaluated. The qualitative scoring system of histopathological lesions as shown in Table 3 which was adapted from Nisbet *et al.* (2010) and Süntar *et al.* (2009) with modification on macrophages enumeration was used. Three high power (×400 magnification) fields were randomly selected and examined for the determination of the number of blood vessels as well as the population of neutrophils, macrophages, fibroblasts and extent of collagen deposition (qualitative determination of the density of each parameter).

Table 3: Qualitative scoring system used for histologic evaluation of the wounds

PARAMETER	SCORE			
	–	+	++	+++
Collagen	None	Scant	Moderate	Abundant
Epithelialization	None	Partial	Complete but immature/thin	Complete and mature with keratinization
Neovascularization	None	Upto 5 vessels/high power field	6-10 vessels/ high power field	More than 10 vessels/ high power field
Fibroblasts	None-minimal	Few fibroblasts	More fibroblasts	Fibroblast predominance
Macrophages	None	Few macrophages	More macrophages	Macrophage predominance
Neutrophils	None	Few neutrophils	Moderate neutrophils population	Neutrophils predominance

3.5. ANTIMICROBIAL ASSAY

The broth macrodilution method was used for the antimicrobial activity assay. The methanol extract was tested for activity against five bacterial and one fungal species. The bacteria tested were *Staphylococcus aureus* (ATCC-25923), *Bacillus cereus* (ATCC-11778), *Pseudomonas aeruginosa* (ATCC-27853), *Streptococcus agalactiae* and *Escherichia coli* (ATCC-25922). *Candida albicans* was also tested as a representative fungal species.

A stock solution of the *A. pluriseta* methanol extract having a concentration of 1600 mg/ml was prepared using distilled water. Mueller Hinton broth was prepared routinely and dispensed into screw capped culture tubes, 2 ml per tube. Nine concentrations of the plant extract were tested. The concentrations were achieved by serial double dilution. The extract concentrations ranged between 3.125 mg/ml and 800 mg/ml. Distilled water was used as the negative control while Gentamicin sulphate (39.0625 µg/ml) and Amphotericin B (16 µg/ml) dissolved in distilled water were used as the positive controls for the antibacterial and the antifungal tests, respectively. After thorough mixing of the media and the extract on a vortex mixer, 1 ml of a 24 hour bacterial or fungal suspension was dispensed into the tubes. The suspensions were adjusted to 0.5 McFarland Standard, which is equivalent to approximately 1×10^8 colony forming units per ml (CFU/ml). The bacteria were incubated at 37°C for 24 hours and the fungus for 48 hours at room temperature.

After incubation, the tubes that did not exhibit grossly visible growth in form of turbidity were selected. One ml was pipetted from each tube and dispensed onto sterile culture plates then subcultured in Mueller Hinton agar using the pour-plate method and further incubated for 24

hours. This subculturing was aimed at evaluating the extent of killing or inhibitory activity of the extract against the test microorganisms.

The parameters of interest were the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the bacteria and minimum fungicidal concentration (MFC) for *Candida albicans*. The MIC was the lowest concentration of the extract or the standard drug that did not permit visible growth of the microorganism after the initial 24 or 48 hour incubation. This was determined by the visual examination of the culture tubes for turbidity. The MBC or MFC was the lowest concentration of the test substance that killed all the organisms. This was determined from plate cultures subcultured from the initial broth cultures. The plates were examined for the growth of colonies of the microorganisms. The lowest concentration of the test substance at which there was no growth of any colonies of the respective microorganism was taken as the MBC or MFC.

3.6. PHYTOCHEMISTRY

The methanol extract was screened for major phytochemicals compounds. The chemical classes screened for include diterpenes, glycosides, phenols, tannins and proteins (Tiwari *et al.*, 2011).

3.6.1. Copper Acetate Test

This test was set up to test for the presence of diterpenes. A few drops of copper acetate solution were added to the extract. Of interest was the appearance of an emerald green color which is indicative of the presence of diterpenes.

3.6.2. Salkowski's Test

This was done to screen for the presence of triterpenes. Chloroform was added to the extract and mixed. The mixture was filtered and a few drops of concentrated sulphuric acid added to the filtrate. Development of a yellow color confirms presence of triterpenes in a mixture.

3.6.3. Test for Saponins

The foam test was employed. Two ml of distilled water was added to about 0.5 gm of the extract and the mixture was shaken. The appearance of foam that persists for 10 or more minutes indicates the presence of saponins.

3.6.4. Gelatin Test

This test was done to screen for the presence of tannins. A 1% gelatin solution was added to the extract and mixed on a vortex mixer. Appearance of a white precipitate indicated the presence of tannins in the extract.

3.6.5. Ferric chloride test

This test was done to test for the presence of phenols. Four drops of ferric chloride were added to the extract. Development of a blue-black color indicated the presence of phenols in the extract.

3.6.6. Modified Borntrager's Test

The purpose of this test was to screen the extract for anthranol glycosides. A few drops of ferric chloride were added to the extract. The set-up was placed in a boiling water bath for 5 minutes and then cooled. An equal volume of benzene was added and mixed. The benzene layer was pipetted out and to it, a few drops of ammonia solution were added, and the mixture was observed for a color change. Appearance of a pink color is positive for anthranol glycosides.

3.6.7. Legal's Test

This was employed to screen for the presence of cardiac glycosides in the extract. The extract was treated with sodium nitropruside in pyridine and sodium hydroxide. The mixture was observed for the development of a pink or red color which is confirmatory for the presence of cardiac glycosides.

3.6.8. Xanthoproteic Test

This was set up to test the extract for presence of proteins. About 5 drops of concentrated nitric acid were added and the mixture observed for development of yellow color which confirms the presence of proteins.

3.7. SKIN SENSITIZATION ASSAY

This was carried out along OECD Test Guideline 406 (OECD, 1992) with modifications on the number of animals and the use of an occlusive patch. The Buehler Non-adjuvant test with modifications was applied on 3 female adult (3 months old) guinea pigs. Albino guinea pigs were used to facilitate the observation of skin reactions. In order to reduce the number of animals used for this test, only one ointment was tested for skin sensitization. The 20% *A. pluriseta* ointment was used since it demonstrated higher activity than 10% *A. pluriseta* ointment in the wound healing assay. The reduction in the number of animals used was desired because of animal welfare.

The ramp area of the guinea pigs was depilated using an electric clipper. A 4 cm² square area was marked on the right posterior flank, about 1cm from the dorsal midline and a film of the 20% *A. pluriseta* powder ointment was applied on it. This application was repeated on the 7th and 14th days and constituted the induction phase of the trial.

On the 28th day, the side opposite to the one used for the induction was cleared of hair and marked as earlier mentioned. This new site was used for the challenge phase of the assay. The ointment was equally applied on the marked area. 6 hours after the application of the ointment, the residual ointment was wiped off using moist cotton wool. 24 hours later, the site was observed for a skin reaction, and this was scored as explained in the test guideline. Observation of the site was repeated 24 hours later. A second challenge application was undertaken on the 35th day to confirm the results observed on the 28th day.

3.8. DATA ANALYSIS

The data was entered into Microsoft Excel spreadsheets. Descriptive statistics was done on wound contraction and epithelialization time values. The means (\pm standard error of means) of responses from the *A. pluriseta* ointment treated groups were compared with each other and with the means of responses from the control groups using one way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significant.

4. CHAPTER FOUR: RESULTS

4.1. WOUND HEALING ASSAY

4.1.1. Time taken to achieve total gross epithelialization

The time taken to achieve total gross epithelialization of the wounds for the various groups is shown in Table 4. Wound epithelialization in the group treated with the 20% *A. pluriseta* powder ointment took approximately a day less than the standard/reference drug treated group and almost three days lower than the time taken by the negative control group. However, the wound epithelialization time was not statistically different for any of the test or control groups ($p \geq 0.05$) when analyzed by ANOVA.

Table 4: Summary of mean (\pm SEM) total gross wound epithelialization time

Treatment Group	Epithelialization Time in Days (Mean\pmSEM)
10% <i>Aspilia pluriseta</i> ointment	17.2 \pm 1.398
20% <i>Aspilia pluriseta</i> ointment	15.5 \pm 1.118
Simple Ointment (negative control)	18.2 \pm 0.735
Silverex[®] Cream (positive control)	16.5 \pm 0.616

4.1.2. Wound contraction

The rate of wound area reduction for the various treatment groups is shown in Table 5 and Figure 4. In the first two weeks of the wounds, the 20% *A. pluriseta* ointment treated group showed more wound contraction, especially in the 6th and 9th day post wounding with 28% and 58% area reduction compared to 15% and 36% for Silverex[®] Cream in those days.

Table 5: Percent wound area reduction values after treatment of mice with *A. pluriseta* ointment on various days of treatment (Mean±SEM)

Treatment Days

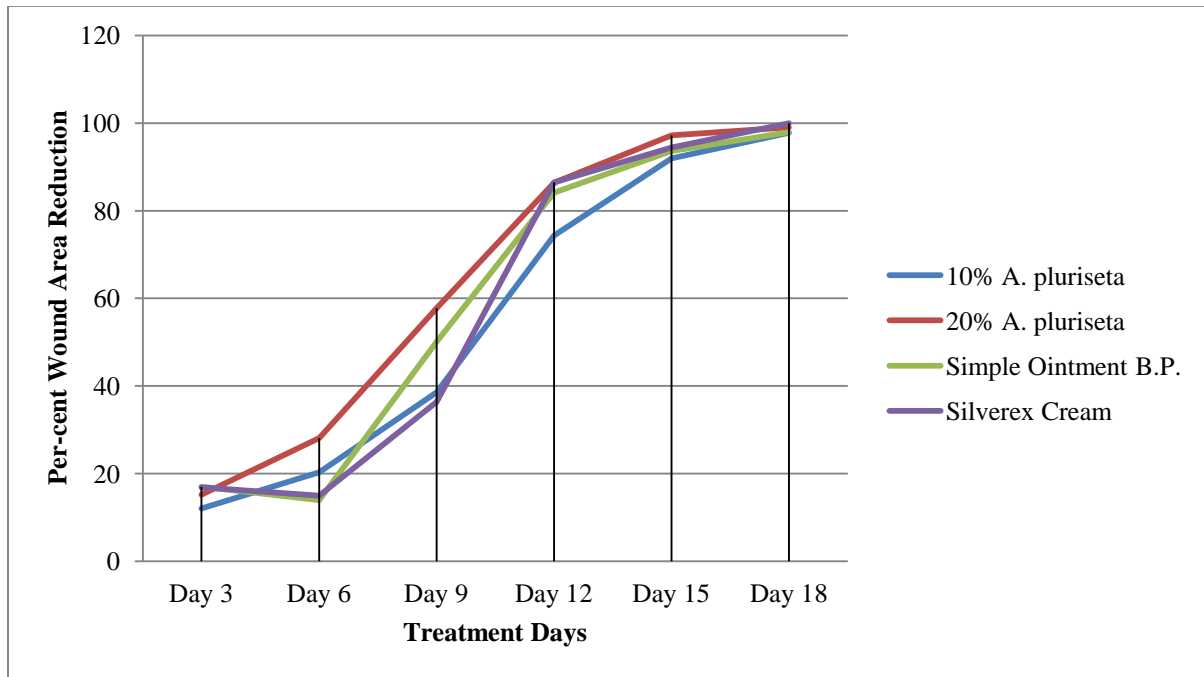
Treatment	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
10% <i>A. pluriseta</i> Ointment	12.05±2.894	20.29±6.733	38.56±9.577	74.31± 8.583	91.95±4.211	97.78±1.456	100
20% <i>A. pluriseta</i> Ointment	15.24±2.343	28.17±3.725	57.75±6.178	86.37± 5.807	97.19±1.851	99.05± 0.606	100
Simple Ointment (B.P.)	17.04±2.086	13.94±3.581	50.09±8.124	84.13±4.405	93.61±2.566	97.90±1.002	100
Silverex® Cream	16.9±1.481	14.98±7.194	36.37±4.871	86.46±2.185	94.44±2.272	100	100

Key:

10% *A. pluriseta* Ointment: 10% *Aspilia pluriseta* ointment

20% *A. pluriseta* Ointment: 20% *Aspilia pluriseta* ointment

Figure 4: Percent wound area reduction at various days of treatment of mice with *A. pluriseta* ointments



Key:

10% *A. pluriseta*: 10% *Aspilia pluriseta* ointment

20% *A. pluriseta*: 20% *Aspilia pluriseta* ointment

4.1.3. Histopathology

Histopathology findings are summarized in Table 6. Healing was characterized by various histological changes representing the different phases of healing.

On the 7th day post wounding, there were numerous neutrophils and intense neo-vascularization with numerous endothelial cells in all the groups. In addition, there were few fibroblasts and macrophages, scanty collagen and a thin and incomplete epidermis at this time. Granulation tissue in wounds treated with Simple Ointment (B.P.) was loosely packed and interspersed with empty spaces which indicated the presence of edema; the few present fibroblasts had large elliptical nuclei and light chromatin density, showing that they were not yet fully mature. The granulation tissue in the 20% *A. pluriseta* ointment group was more densely packed than that in the Simple Ointment (B.P.) (negative control) group. The cells in the Silverex[®] cream and the 20% *A. pluriseta* ointment treated groups were more mature with packed chromatin and intensely basophilic nuclei. There was hemorrhage in sections from the 20% *A. pluriseta* ointment treated animal (Figure 5).

On the 14th day of the wounds, there was a general reduction in the intensity of neo-vascularization and an increase in macrophage and fibroblast populations as well as collagen lay-down. All the animals except those treated with the 20% *A. pluriseta* ointment had hemorrhage and hemosiderin-laden macrophages. Collagen deposition appeared to be more in the Silverex[®] cream and in the 20% *A. pluriseta* ointment treated wounds. There was focal edema and congestion in sections from the Silverex[®] cream treated wounds.

On day 21, wounds from all the groups were fully epithelialized. Vascularization and the number of neutrophils were diminished. There were numerous fibroblasts and more collagen but few

macrophages. In the Simple Ointment (B.P.) group, the vascular response similar to that seen in the 14th day with numerous endothelial cells and capillaries was sustained and there was some edema in the granulation tissue. The granulation tissue also appeared to be more loosely deposited than that in the other groups (Figure 6). The 10% and 20% *A. pluriseta* ointment treated wounds had more collagen and a more compacted stroma (Figure 5, Figure 7). Moreover, the epidermis in the *A. pluriseta* ointments treated groups was denser than in the other groups with little spaces between individual cells as shown in Figure 7.

Table 6: Histological scores for the wounds at various days of treatment of mice with *A. pluriseta* ointments

Day	Treatment Group	Neutrophils	Macrophages	Fibroblasts	Collagen	Vascularization	Epithelialization
7	10% <i>A. pluriseta</i> Ointment	+++	+	+	+	+++	+
	20% <i>A. pluriseta</i> Ointment	+++	+	+	+	+++	+
	Simple Ointment (B.P.)	+++	+	+	+	++	+
	Silverex® Cream	++	++	+	+	+++	+
14	10% <i>A. pluriseta</i> Ointment	+++	++	+	+	++	+
	20% <i>A. pluriseta</i> Ointment	++	++	++	++	++	+
	Simple Ointment (B.P.)	+++	++	+	+	++	+
	Silverex® Cream	+++	++	++	++	+++	+
21	10% <i>A. pluriseta</i> Ointment	+	++	+++	++	++	++
	20% <i>A. pluriseta</i> Ointment	+	+	+++	+++	+	+++
	Simple Ointment (B.P.)	+	++	+	++	+++	++
	Silverex® Cream	+	++	+++	+++	++	+++

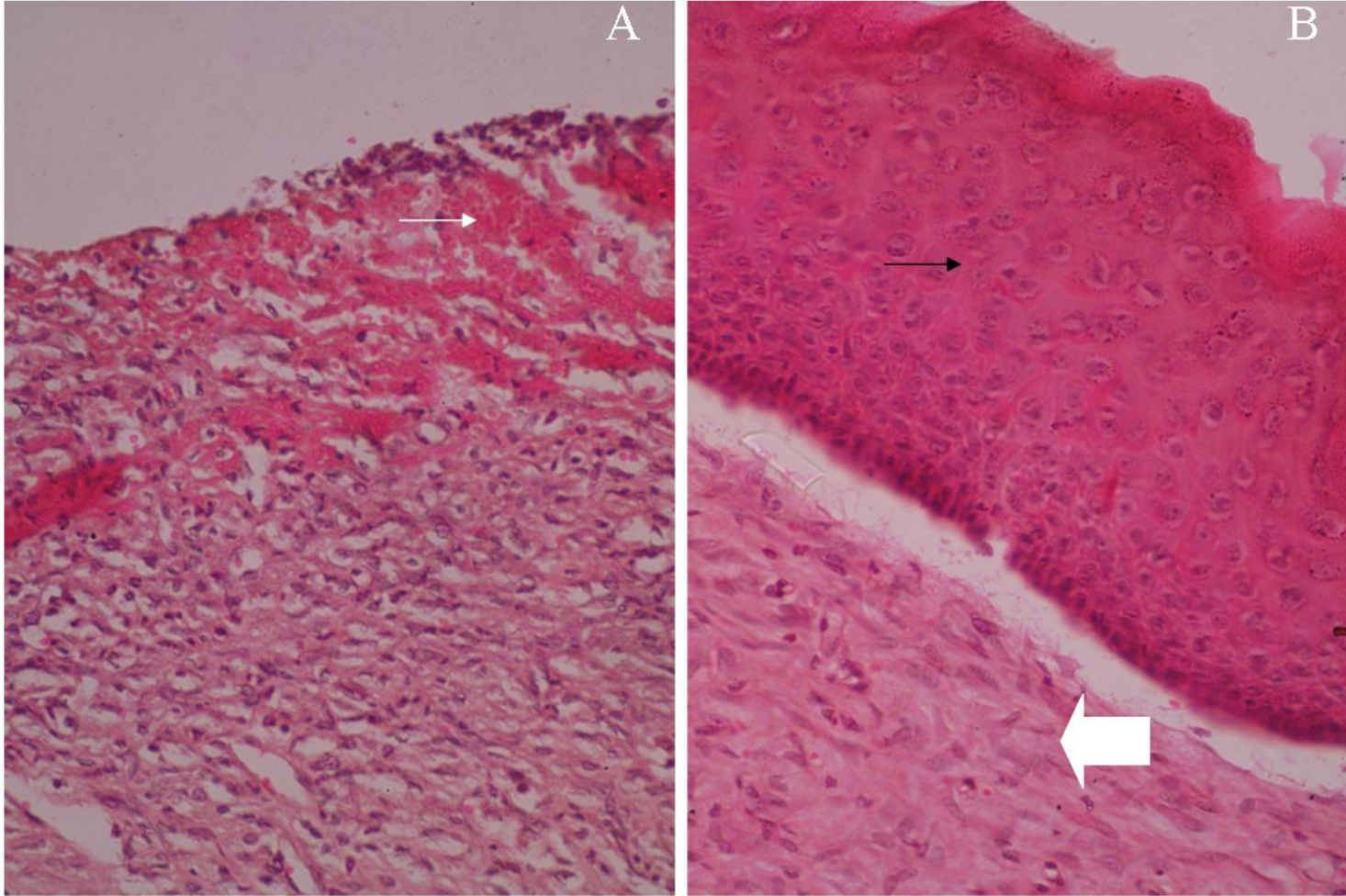


Figure 5: Photomicrographs of sections from the 20% *A. pluriseta* ointment group; H&E, $\times 400$.

The image on the left (A) is from Day 7 while that on the right is from Day 21 day post-wounding. The white arrow shows hemorrhage in the 7th day section; the black arrow shows a thick and dense epidermis, white block arrow shows compacted collagen in the dermis in Day 21.

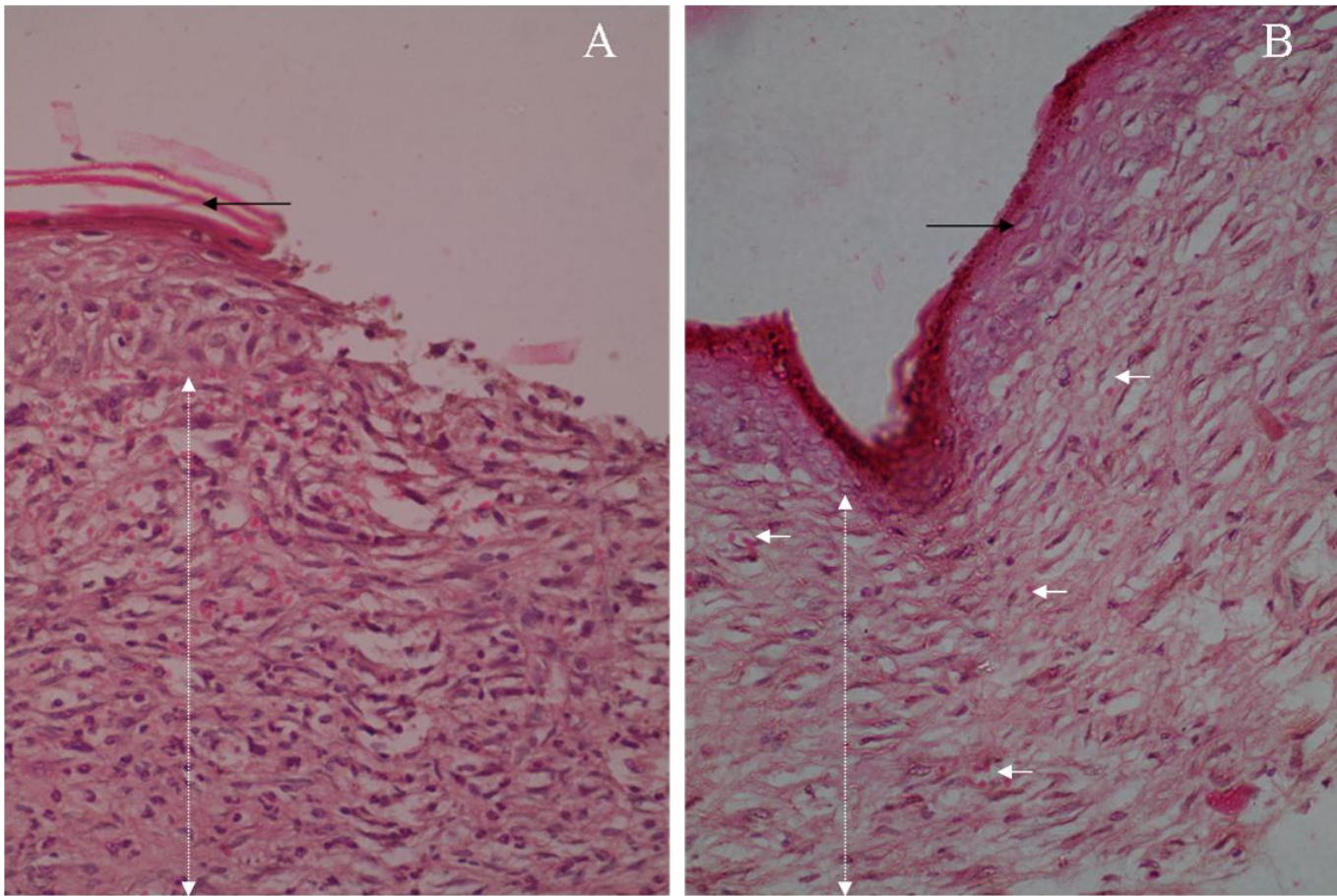


Figure 6: Photomicrographs of sections from wounds treated with Simple Ointment B.P. (negative control group) (H&E Stain, ×400).

Image A is from Day 7 while image B is from Day 21. The black arrows show the epidermis, which is thin and incomplete in Day 7 section and complete in Day 21 section. Note the loose stroma (white dashed arrows) in the Day 21 section, a thin epidermis and numerous blood vessels (white arrows).

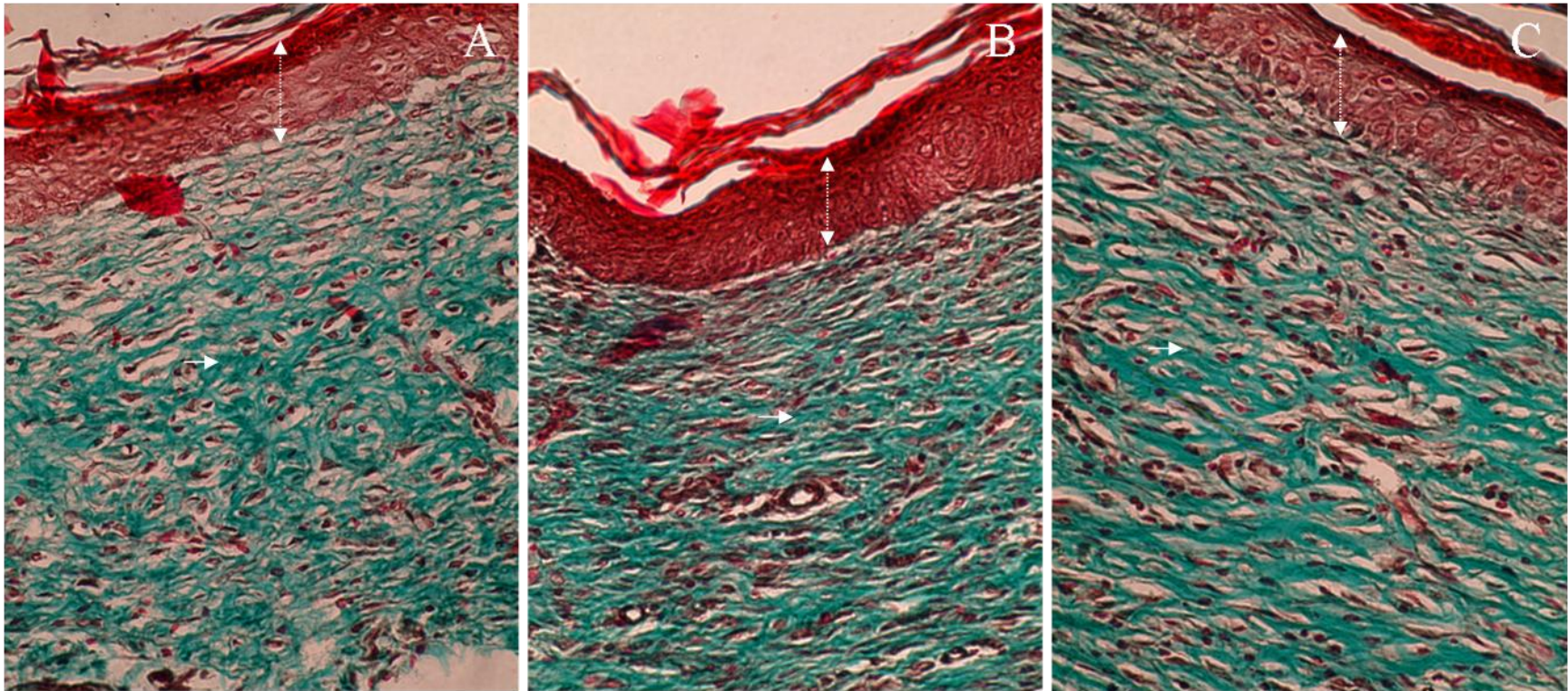


Figure 7: Comparison of the collagen lay-down and epidermal reconstruction between three treatment groups on Day 21 (Masson's Trichrome Stain, $\times 400$).

Photomicrograph A is from the negative control group, B is from the 10% *A. pluriseta* ointment group and C is from the reference drug (Silverex Cream[®]) group. The collagen tissue (green, white arrows) in A is not organized in any specific orientation as opposed to that in B and C. The collagen is more densely laid down in B than in C with less intervening space between bundles of collagen fibers in B. The epidermis (white dashed arrows) in B is more compact with little space between individual cells than in either A or C.

4.2. ANTIMICROBIAL ACTIVITY

The results of the antimicrobial activity assay are summarized in Table 7 and Table 8. The methanol extracts showed marginal activity against the organisms tested. There was a concentration related inhibition of growth after 24 hour incubation of the organisms in presence of the extract. Visible growth, indicated by turbidity of the test tube contents was inhibited by 50 mg/ml *A. pluriseta* methanol extract for *Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus agalactiae*. The same was achieved at 100 and 200 mg/ml, respectively for *Escherichia coli* and *Pseudomonas aeruginosa*. *Candida albicans* was inhibited by 100 mg/ml after 48 hour incubation at room temperature.

After subculturing onto Mueller Hinton agar plates and further incubation, the extract concentration of 800 mg/ml completely eliminated all the tested organisms. *Streptococcus agalactiae* was eliminated by a concentration eightfold lower, 100 mg/ml as shown in Table 8. The MFC for *Candida albicans* was 800 mg/ml.

Both Amphotericin B and gentamicin (positive controls) used completely eliminated all the organisms at the concentrations tested, while the negative controls allowed exuberant growth of the organisms.

Table 7: Minimum Inhibitory Concentration of *A. pluriseta* methanol extract against selected microorganisms

Organism	Minimum Inhibitory Concentration (MIC)
<i>Bacillus cereus</i> ATCC- 11778	50mg/ml
<i>Staphylococcus aureus</i> ATCC- 25923	50mg/ml
<i>Pseudomonas aeruginosa</i> ATCC-27853	200mg/ml
<i>Escherichia coli</i> ATCC- 25922	100mg/ml
<i>Streptococcus agalactiae</i>	50mg/ml
<i>Candida albicans</i>	100mg/ml

Table 8: Minimum Bactericidal Concentration of *A. pluriseta* methanol extract against selected bacteria

Organism	Minimum Bactericidal Concentration (MBC)
<i>Bacillus cereus</i> ATCC- 11778	800mg/ml
<i>Staphylococcus aureus</i> ATCC- 25923	800mg/ml
<i>Pseudomonas aeruginosa</i> ATCC-27853	800mg/ml
<i>Escherichia coli</i> ATCC- 25922	800mg/ml
<i>Streptococcus agalactiae</i>	100mg/ml

4.3. PHYTOCHEMICAL COMPOSITION

The methanol extract of *A. pluriseta* contained various major classes of phytochemicals. The results of the qualitative phytochemical screening are outlined in Table 9.

Table 9: Phytochemical composition of the methanol extract of *A. pluriseta*

Phytochemical Compound Class	Presence in the Methanol Extract
Triterpenes	Positive
Tannins	Positive
Proteins	Negative
Anthranol Glycosides	Positive
Cardiac Glycosides	Negative
Saponins	Positive
Phenols	Positive
Diterpenes	Negative

4.4. SKIN SENSITIZATION ASSAY

The 20% *A. pluriseta* ointment did not elicit any irritation during the initial application or at any time in the course of the induction period. The ointment induced moderate sensitization after the challenge exposure on the 28th day. This was indicated by a confluent erythema on the skin area used for challenge exposure (Figure 8). The erythema persisted for approximately 48 hours and then resolved on its own. This exposure was repeated 7 days later (35 days after the first application) to confirm the results obtained, and similar observations were made.



Figure 8: Picture showing the challenge site for skin sensitization assay (black outline) on the guinea pig rump after application of 20% *A. pluriseta* ointment

This photograph was taken about 30hrs after challenge application of the ointment to test for skin sensitization. Note the diffuse reddening of the area marked with an ellipse. This erythema was associated with an allergic reaction to the 20% *A. pluriseta* ointment.

5. CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. DISCUSSION

The *A. pluriseta* based ointments tested had an appreciable effect on excision wounds but induced skin allergy in guinea pigs. In addition, the methanol extract of the powdered *A. pluriseta* aerial parts had marginal activity against various Gram positive and Gram negative microorganisms and were found to contain a number of major classes of phytochemicals. These findings are generally in agreement with like studies on other medicinal plants as discussed below.

A. pluriseta-based ointments supported the healing of the excision wounds in an *in vivo* mouse model. The target of wound healing research is mainly the development of wound healing agents that are as close as possible to an ideal agent. Such an agent should augment healing (Pierce and Mustoe, 1995) and also be affordable, comfortable and convenient. Plant derived wound healing agents approximate that ideal (Gupta and Jain, 2010). This is the first time that the wound healing activity of *A. pluriseta* has been evaluated on an *in vitro* or *in vivo* model. The wound healing enhancement that was observed in this study is similar to that seen in other experiments on *in vivo* support of wound healing by plant extracts (Kumar *et al.*, 2007). The wound healing activity closely corroborates the work of Okoli *et al.* (2007) who reported enhancement of epithelialization by the methanol extract of *Aspilia africana*, a member of the same genus as the test plant, *A. pluriseta*.

The methanol extract exhibited a marginal killing activity (Rios and Recio, 2005) on the microorganisms tested but an appreciable inhibitory effect on some of the microbes. This is in contrast with the observation of Rodriguez *et al.* (1985) who reported the presence of an

antibiotic compound, Thiorubrine A, in tissues of plants from *Asteraceae* family, and especially members of *Aspilia* genus, including *A. pluriseta*. The outcome of antimicrobial activity tests using plant extracts is affected by a number of factors (Ncube *et al.*, 2008), main among them being ecologic factors, mode of extraction, choice of the antimicrobial activity test method and the choice of the test microorganisms (Hammer *et al.*, 1999; Nostro *et al.*, 2000). Non-uniformity between this experiment and that done by Rodriguez *et al* (1985) may explain the differences in the results obtained. Rios and Recio (2005) proposed 1 mg/ml concentration of crude extracts as the cut-off value for antibiotic activity by the extracts. Concentrations higher than this cut-off value kill the microorganisms by non-specific toxicity that would most likely harm normal cells as well. Since the antimicrobial activity observed was in very high concentrations, far above the 1mg/ml cut-off, the inference is that support of *Aspilia pluriseta* on wound healing is exclusive of infection control. The killing effect on the test microorganisms was likely due to toxicity of the extract.

The *A. pluriseta* methanol extract was found to contain triterpenes, saponins, tannins, anthranol glycosides and phenols. These compounds are typical constituents of plant tissue. Piero *et al.* (2011) found flavonols, flavones, flavonoids, chalcones, tannins, anthraquinones and sterols in aqueous extract of *A. pluriseta* root bark, which is similar, at least in part, to what was found in the aerial parts methanol extract tested in this project.

The compounds found in the plant may account for the exhibited biological activity. Tannins, triterpenoids and saponins have been reported to support the healing process of cutaneous wounds. Tannins and triterpenoids are thought to support wound healing due to their astringent properties which are postulated to be responsible for the compounds' support of wound

contraction and epithelialization (Manjunatha *et al.*, 2005). Tannins have been shown to improve contraction, granulation formation, collagen organization and expression of VEGF (Li *et al.*, 2011). Saponins, in turn, support healing by stimulation of fibronectin production by fibroblasts. Fibronectin facilitates the interaction of fibroblasts with the ECM and also fibroblast migration, and in this way promotes healing (Kanzaki *et al.*, 1998). However, these investigators reported much higher activity than what has been observed in this study. This difference in the degree of activity may be accounted for by the fact that whole plant material was used in this project as opposed to extracts and purified compounds used in the other mentioned reports. The tannins, triterpenes and saponins are the most likely compounds to have caused the enhancement of healing observed.

The plant powder ointment caused moderate skin sensitization in guinea pigs. Histologic indicators of sensitization however were not observed in the mice used for the wound healing assay. Absence of allergic reaction in mice does not preclude the results of the definitive test in guinea pigs. The *Asteraceae* family (whose many members are ornamental plants) has been singled out as the largest cause of allergic dermatitis in florists (<http://en.wikipedia.org/wiki/Asteraceae>). This allergenic property is postulated to be due to the presence of *sesquiterpene lactones*, *polyacetylenes* and *thiophenes* (Aronson, 2009) in *Asteraceae* plant tissue.

The findings of this project point to a multi-pronged enhancement of healing by *A. pluriseta* ointments. The plant material supported early polymorphonuclear inflammation, while at the same time allowing the recruitment of wound macrophages. It may also have induced an early intensive granulation. This inference is supported by the more intense granulation seen in the 7th

day sections of the *A. pluriseta* ointment treated groups. In addition to the presence of more fibroblasts in these sections, these fibroblasts appeared to be more mature (they had more basophilic nuclei and were more spindle shaped) than those of the negative control group. Moreover, the plant-based ointments seemed to enhance collagen synthesis and deposition- sections from these groups were more fibrotic than those from the control groups (Figure 5 and Figure 7). Also, the *A. pluriseta* ointments promoted more epidermal remodeling. The 21st day neo-epidermis from the animals treated with the ointments was denser with little space between cells (Figure 5).

The formulation of the ointments might have had an influence on the activity of the *A. pluriseta* ointments and the negative control group (Simple Ointment, B.P.). The oleaginous, hydrophobic and non-polar nature of Simple Ointment could have inhibited activity from polar constituents of the plant material, (the release potential for polar active constituents by an oleaginous vehicle would be low). Due to this, some activity from the plant powder could have been hampered, producing an apparent inactivity by the plant powder. The ointment base, on the other hand, has occlusive, emollient and protectant properties which may have favored healing by maintaining a moist wound healing environment, supporting new cells and providing a physical barrier protecting the wound from external insult. This may have favored healing in all the animals to which the ointments were applied, including the negative control group.

5.2. CONCLUSIONS

The following conclusions were made from the results obtained in the study.

- ✓ *Aspilia pluriseta* possesses wound healing activity. This validates the ethnomedical use reported by various communities locally and in the region.
- ✓ The contribution of the antimicrobial activity of *A. pluriseta* to the wound healing process is minimal.
- ✓ The wound healing activity of *A. pluriseta* is attributable to the phytochemicals in the plant tissue.
- ✓ Allergy induction occurs with repeated topical application of the *A. pluriseta* ointment. This may hinder exploitation of the plant for its wound healing potential.

5.3. RECOMMENDATIONS

The following points are the recommendations from this study and scope for further work.

- ✓ The demonstrated wound healing activity should be further studied. Different extracts of the plant parts and different dosage forms should be tested for their influence on healing of wounds. The plant should also be tested for its effect on other wound types for example peptic ulcers, corneal ulcers and diabetic wounds.
- ✓ In order to conclusively elucidate the antimicrobial activity of the plant extracts, it would be prudent to test a range of extracts of different polarities and to also bear in mind activity differences that may be due to other factors such as ecology, season of harvesting, plant part being tested and the plant lifecycle.
- ✓ The effect of long term application of the plant onto normal as well as wounded skin should be further studied to rule out any delayed adverse effects before the plant can be recommended for therapy. The observed increased fibrosis should also be studied further to determine its reversibility and the effect on function of tissue after healing.
- ✓ The skin sensitization effect of *A. pluriseta* ointment should be studied further. Different extraction methods may be explored in bid to remove the allergens from the plant tissue.
- ✓ The demonstrated activity suggests that the plant may have potential in the development of commercially viable drugs for wound healing and skin revitalization. This potential should be explored in pharmacologic, pharmaceutical and clinical trials.

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