ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

WOUND HEALING ACTIVITY GUIDED ISOLATION OF ACTIVE CONSTITUENT (S) FROM THE LEAVES OF ALLOPHYLUS ABYSSINICUS (HOCHST.) RADLK.

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By:
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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with my approval as a University advisor.

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1 INTRODUCTION

1.1. Wound as a health problem

Every year, millions of people experience burns, suffer from nonhealing wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring. Effective wound treatment requires carefully considered interventions often requiring multiple clinic or hospital visits. The resulting costs of wound care are staggering. The global advanced wound management market valued at $3.6 billion in 2008 is forecast to grow by 4.7% annually for the next seven years to reach $5 billion by 2015 (Karen and Lillian, 2006). Chronic wounds and their treatment are an enormous burden on the healthcare system, both in terms of their cost and the intensity of care required. The overall cost to society in lost productivity and decreased quality of life is not measurable but is certainly considerable (Stadelmann *et al*., 1998). The financial growth will primarily be driven by an increase in the incidence of chronic wounds such as venous, diabetic and pressure ulcers (Karen and Lillian, 2006).

The prevalence of chronic ulcerated wounds has been estimated to range from 120 per 100,000 persons 45 to 64 years of age to more than 800 per 100,000 persons 75 years and older (Pieper *et al*., 1999). These chronic wounds include pressure, venous, arterial, and diabetic neuropathic ulcers. The prevalence of many of these wounds increases with age, with 50% to 70% of pressure ulcers occurring in individuals over the age of 70 years. The incidence of pressure ulcers may be as high as 11% in hospitalized patients and as high as 24% in nursing home residents. In addition, the incidence of ulcer recurrence after complete healing approaches 30% within 12 months. Approximately 50,000 amputations per year are directly due to infected, non healed ulcers in diabetic patients. In addition to the numbers of chronic wounds in the population, there are a large number of acute wounds, adding to society's wound burden (Robson *et al*., 2001).

The costs to society for caring patients with wounds are difficult to determine. The market for wound care products exceeds $7 billion. Home health care services provided to 7,000,000 patients, one third of whom are being seen for the treatment of wounds, cost more than $42 billion (Meehan *et al*., 1999; Pieper *et al*., 1999). The treatment of wounds that heal is approximately one half the costs of those that remain unhealed. Total hospital charges for patients with pressure ulcers were reported to be 5.3 times the charges for all other hospitalized patients; the mean length of stay was 4.5 times greater. The direct and indirect medical costs associated with wound treatment are enormous. Added to this must be the tangible and intangible costs that are incurred as the result of patient disability that results in lost wages, decreased productivity, and a diminished quality of life. Realizing the present scope of the problems associated with wound healing and their complexity as recorded from the beginning of man's existence underscores the importance of reviewing the known biologic features of wound healing and the approaches to maximize its efficiency. To make the problems worse, in spite of tremendous advances in the pharmaceutical industry, the availability of drugs capable of stimulating the process of wound repair is still limited (Robson *et al*., 2001).

1.2 Wound healing and the healing cascade

In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) exist in steady-state equilibrium, forming a protective barrier against the external environment. Once the protective barrier is broken, a wound will be inflicted (Shai and Maibach, 2005; Nagori and Solanki, 2011). Wound is defined simply as the disruption of the cellular and anatomic continuity of a tissue that may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue (Robson *et al*., 2001; Raina *et al*., 2008).

Based on the nature of the repair process, wounds can be of either acute or chronic type. Acute wounds are typically tissue injuries caused by cuts or surgical incisions that complete the wound healing process within the expected time frame (Strodtbeck, 2001). In contrast, chronic wounds are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation. As a result, the healing process is delayed, incomplete, and does not proceed in a coordinated manner, subsequently resulting in poor anatomical and functional outcome (Menke *et al*., 2007). It could be due to repeated insults to the tissues and/or other underlying pathophysiology that interferes the normal trajectory of healing (Strodtbeck, 2001). Chronic wounds cause a major disability and are characterized by chronicity and frequent relapse (Menke *et al*., 2007).

Wound healing is a natural and spontaneous phenomenon (Franklin and Dawson, 2008), but not all wounds heal in precisely the same manner due to differences in the etiology of the wound, presence or absence of infection, and medical or surgical interventions. Which of these components predominates depends on whether the wound is closed immediately (first intention), allowed to granulate (secondary intention), or has delayed primary closure (third intention). The process of wound healing may be best understood by dividing it into phases. These phases are somewhat arbitrary, as they overlap in time, physiology, and cell type, with each phase not entirely completed before the next begins (Molnar, 2007). Hence it could be classically divided into four stages: hemostasis, inflammation, proliferation and remodeling (Janis *et al*., 2010).

1.2.1 Hemostasis

Healing begins the instant the wound is made (Molnar, 2007). Tissue injury provokes immediate activation of the extrinsic and intrinsic coagulation pathways. When the skin is cut, the body responds with a complex mechanism that protects from exsanguination. Vasoconstriction is almost immediate to decrease blood loss, but enough blood is released in the wound to stimulate Hageman Factor XII to initiate the clotting cascade (Williamson and Harding, 2004).

Collagen, present in all tissues of the body and ironically the major protein of wound healing, is exposed in the wound, resulting in stimulation of the alternate complement pathway as well as platelet adherence and degranulation. Along with complement and Hageman pathway stimulation, numerous additional vasoactive and chemotactic components are released. Blood fibrinogen quickly converts to fibrin, which, along with platelets, helps to form what is commonly called a scab. The scab ultimately provides a temporary protective barrier. The fibrin forms a pathway to aid cell migration, especially for the fibroblast. One of the most active components of the hemostatic phase of wound healing is the platelet. Platelets are present in the blood; they rapidly aggregate and degranulate in the wound. With degranulation, numerous cytokines, such as platelet derived growth factors (PDGF) are released. PDGFs are potent cytokines with numerous functions, including being a chemo attractant for neutrophils, one of the dominant cells of the inflammatory phase. The stage of hemostasis does more than just stop exsanguination. It also initiates the process of healing by creating a protective layer to minimize the infection risk while providing both a biochemical milieu and physical framework for the stages that follow. Thus, the hemostatic stage prepares for and influences the onset of the next stage of healing (Molnar, 2007).

1.2.2 Inflammation

The inflammatory phase begins as early as two hours after injury. Activated neutrophils leave the circulation to begin the debridement of devitalized tissue and phagocytosis of any infectious agents or foreign bodies. Neutrophils also secrete cytokines and other specific chemical signals needed to attract specialized cells that are important in repairing the injured tissue (Menke and Diegelmann, 2006). In addition, neutrophils release a battery of proteolytic enzymes, such as elastase and matrix metalloproteinase-8 (MMP-8), to assist in their movement through the tissue and needed for the removal of damaged extra cellular matrix (ECM). Mast cell granules are filled with enzymes, such as chymase and tryptase, histamine, and other active amines, which when released cause the classic signs of inflammation: rubor (redness), calor (heat), tumor (swelling), and dolor (pain) (Diegelmann and Evans, 2004). The crucial inflammatory cells are neutrophils and activated monocytes (macrophages). Neutrophils and macrophages are responsible for bound wound bed preparation and the initial milieu of the healing wound. In order to expedite healing, cellular debris and bacteria are cleared (Menke and Diegelmann, 2006). The inflammatory cells also release cytokines [including interleukins (IL-1, IL-6, IL-8) and tumor necrosis factor alpha (TNF-∝)] and growth factors [PDGF, transforming Growth factor (TGF-β, TGF-∝), insulin like growth factor-1(IGF-1), and fibroblast growth factor (FGF)] that not only serve to continue the inflammatory phase but also serve to initiate the proliferative phase by recruiting fibroblasts and epithelial cells (Diegelmann and Evans, 2004).

1.2.3 Proliferative phase

The proliferative phase includes: neoangiogenesis, formation of granulation tissue and ECM, and re-epithelialization (Schreml *et al.*, 2009). Key cells for these processes are the fibroblasts and keratinocytes. The warm, moist microenvironment of the wound also facilitates proliferation and repair (Eaglstein, 2001). The leaked fluid or exudate is rich in cytokines that stimulate new tissue growth (Rodriguez *et al*., 2008).

The process of restoring the vascular network is called neovascularization or angiogenesis. Neovascularization is stimulated by growth factors and tissue hypoxia. At the start of the wound healing process, the most viable parts of the wound are those with access to a vascular supply (Chung *et al*., 2010). This creates a hypoxic gradient between the avascular wound center and the vascularized wound borders (Barrientos *et al*., 2008). Increased endothelial permeability allows nutrients to escape from the intact vascular bed into the interstitium and injury site. This creates a nutrient-rich microenvironment that sustains the developing repair process until neovascularization occurs. Closure of the wound surface is necessary to create a hypoxic wound environment. The fibrin clot formed during hemostasis provides a temporary cover that is eventually replaced by new epidermal cells. This creates a closed system in which angiogenesis can proceed (Van der Veera *et al*., 2009). Hypoxia is thought to induce macrophages into secreting angiogenic growth factors. Lactic acidosis also stimulates angiogenesis (Kanczler and Oreffo, 2008). Platelet and macrophage-derived growth factors, such as FGF and vascular endothelial growth factor, are released by endothelial cells and stimulate angiogenesis along the wound edges (Hoeben *et al*., 2004). New blood vessels bud or sprout from intact vessels in the underlying dermis. The new capillary buds join to form capillary loops thereby establishing blood flow within the wound (Satish and Kathju, 2010). New sprouts or buds extend from the capillary loops further into the wound environment. The process of arborization is thus stimulated by hypoxia within the wound environment and results in the growth of new vessels throughout the wound. Another essential requirement of neovascularization is the formation of an appropriate ECM with an adequate supply of oxygen. Collagen deposition is critical for aortic endothelial cell migration (McDougall *et al*., 2006). Several key enzymes that are oxygen dependent control collagen synthesis (Mole, 2008).

Re-epithelization of a wound occurs when keratinocytes completely cover the surface of the skin defect. As the largest group of epithelial cells within the epidermis, keratinocytes along the wound edges undergo intense mitotic activity. Keratinocytes, stimulated by locally released growth factors, proliferate and begin their migration across the wound bed within 12 to 24 hours after injury (Roho vsky and D'Amore, 1997; Strodtbeck, 2001). Because the site of proliferation is usually proximal to the injury, the new keratinocytes must migrate to the repair site. Migration requires a fluid environment and involves a complex series of steps controlled by a chemotactic gradient generated by various growth factors. In the absence of a fluid surface, the keratinocyte secretes proteolytic enzymes that enable it to burrow downward to find the necessary moisture for migration (Schultz *et al*., 2003). The first step of migration involves separation of the keratinocytes from each other and their anchors to the cell basement membrane. The migrating keratinocyte then undergoes transformation by elongating itself in the direction growth is needed. The leading edge of the elongated keratinocyte attaches to a new spot in the wound bed. The cell then contracts, pulling itself forward across the wound surface. This process is repeated until the migrating cells from opposing sides of the wound touch each other. At the point of contact, migration ceases in a process known as contact inhibition (Garrett, 1998). Another process used for keratinocyte migration is leapfrogging. Leapfrogging occurs when a single cell moves only 2 or 3 cell lengths and then stops, allowing consecutive cells to climb over (Strodtbeck, 2001). As keratinocytes proliferate and migrate across the wound, they also participate in shaping the ECM by expressing surface markers that enhance migration across the matrix. Supporting structures are selectively degraded and resynthesized to provide temporary anchors during the migratory phase. Once migration is complete, the keratinocytes stabilize themselves by forming firm attachments to each other and the new basement membrane. Migration proceeds from the wound edges towards the center in a centripetal manner (Gurtner *et al*., 2008). When the skin surface is completely covered with new epidermal cells, the wound is considered closed. Early closure of an open wound with a viable epidermis is essential because it induces remodeling of the underlying tissue (Strodtbeck, 2001).

The third and final mechanism of proliferation and repair is the development of granulation tissue. Granulation tissue, a transitional substance that replaces the fibrin/fibronectin matrix, begins to appear about 4 days after injury (Gurtner *et al*., 2008). Granulation occurs as the fibrin clot scaffold is replaced with new tissue rich in hyaluronan (hyaluronic acid), fibronectin, and other ECM compounds. Because granulation tissue is very active metabolically and supports the proliferation of a variety of cells and proteins, it is also highly vascular. This accounts for its classic pinkish-red appearance. The predominant cell type found in granulation tissue is the fibroblast (Strodtbeck, 2001). Fibroblasts are dermal cells that produce collagen and numerous other substances that comprise the ECM. ECM is composed of substances that promote adhesion and migration (fibronectin); glycoaminoglycans that promote tissue hydration (hyaluronan); proteoglycans or matrix proteins that are involved in the regulation, migration, storage, and expression of a variety of substances including growth factors, enzymes, and coagulation proteins (chondroitin sulfate); and glycoproteins that provide tissue strength and resiliency (collagens, elastin) (Gurtner *et al.*, 2008). Fibroblast migration and proliferation are triggered by signals from PDGF, TGF, FGF, and complement 5a (C5a) released by activated cells of the inflammatory response (Strodtbeck, 2001). The influx of fibroblasts causes the provisional matrix of fibrin/fibronectin to be degraded and replaced with a new matrix. After migration to the wound, fibroblasts begin to synthesize the proteins hyaluronan and fibronectin (Ghosh *et al.*, 2006). This new matrix is composed of fibronectin and collagen that provides a scaffold for cell migration and organization (Satish and Kathju, 2010). Normal dermal fibroblasts and the fibroblasts involved in the formation of granulation tissue differ in both structure and function. Wound fibroblasts tend to be involved in collagen synthesis rather than proliferation (Mariggio *et al.*, 2009). These fibroblasts produce and release proteoglycans, glycosaminoglycans, and collagen (Lee *et al.*, 2004). They also participate in the process of wound contraction after differentiation into myofibroblasts (Junkera *et al.*, 2008). Differentiated fibroblasts contain contractile proteins such as actin (Hinz and Gabbiani, 2003). Myofibroblasts are arranged in densely packed groups. This arrangement, in conjunction with their special contractile properties, allows the myofibroblast to pull wound edges together through the process of contraction (Strodtbeck, 2001). Contraction decreases healing time because it decreases the size of the wound and reduces the amount of ECM needed to repair the defect (Lorenz and Longaker, 2008). Contraction also facilitates re-epithelization by shortening the distance migrating keratinocytes must travel (Satish and Kathju, 2010). The structure and composition of granulation tissue undergoes constant change as it matures. Although collagen becomes the predominant protein, there are at least 19 different types of collagen (Palpandi *et al.*, 2010). The type of collagen present in a tissue varies with the tissue. For example, skin collagen is 80% type I and 20% type III. (Strodtbeck, 2001). The new granulation tissue contains type I, III, and V collagen fibers (Eckes *et al*., 1996). Thirty percent of the collagen is type III collagen, which does not contribute to restoring tensile strength in the wound. At 3 weeks after injury, the healing wound has approximately 20% of its final strength (Strodtbeck, 2001).

1.2.4 Remodeling

The tissue remodeling phase represents the late processes of healing, taking place up to two years following injury in normal healing conditions (Shai and Maibach, 2005). The wound develops its final strength during this stage of wound healing. The key cells for remodeling are macrophages and fibroblasts. ECM reshaping by cross-linking collagens, cell maturation, and programme cell death or apoptosis are the mechanisms used in wound remodeling (Strodtbeck, 2001). Increased collagen production and breakdown continue for 6 months to 1 year after injury. The initial type III collagen is replaced by type I collagen until a type I: type II ratio of 4:1 is reached, which is equal to normal skin (Ferguson and O'Kane, 2004). Although wound strength increases, it never achieves more than 80% of the preinjury strength. ECM-bound growth factors and MMPs are activated by macrophages and fibroblasts, resulting in degradation of the matrix and differentiation of cells (Diegelmann and Evans, 2004; Ethridge *et al*., 2007). Collagen is first released in precursor form as a triple helix protein called procollagen. Procollagen is then formed into fibers that are arranged in parallel fashion and cross-linked to form thicker and stronger strands. This process converts the loose granulation tissue matrix into a stable ECM (Kjær, 2004). There are substantial differences between the repaired tissue and non injured skin. The new connective tissue is not as well anchored to the underlying connective tissue matrix and is thicker than normal skin. During this time, fibronectin and hyaluronan are replaced, collagen bundles grow in size and strength, neovascularization ceases, and metabolic activity within the ECM declines. The density of cells, such as macrophages, keratinocytes, fibroblasts, and myofibroblasts, is reduced by apoptosis (Strodtbeck, 2001). Keratinocytes are the first cells to undergo programmed cell death; myofibroblasts are the second. Remodeling is thus a balance between the synthesis of new collagen and the degradation of old (Robnson *et al*., 2001). Remodeling is regulated by fibroblasts through the synthesis of ECM components and MMPs that control cell differentiation. As the wound healing process is switched off, the new connective tissue matures and changes from pinkish-red to a white color (Davis and Senger, 2005).

1.3 Impaired wound healing

Successful wound healing requires the timely and optimized function of many different cell types, structural elements, molecular mediators, and processes. Disturbances of any of these functions result in impaired wound healing (Guo and Dipietro, 2010). Impaired healing can be manifested in different ways. These include hypertrophic scars, keloids and contractures (Dealey, 2005).

Hypertrophic scars occur when there is an excessive fibrous tissue response during the healing process resulting in excessive deposition of collagen and a thick wound scar. The ratio of type I to type III collagen is lower than in normal skin. Hypertrophic scars are more common after traumatic injury, especially large burns. They occur shortly after the injury or surgery and remain limited to the area of the injury. They will generally flatten out with time, about one to two years (Dealey, 2005). Histologically, hypertrophic scars are characterized by collagen bundles that are fine, well organized, and parallel to the epidermis. Unlike keloids, myofibroblasts are present, and alpha smooth muscle actin is expressed in a nodular pattern. Mucin is absent, and hyaluronic acid is a major component of the papillary dermis (Kose and Waseem, 2008).

A keloid is a benign hyperproliferative growth of dense fibrous tissue developing from an abnormal healing response to a cutaneous injury (Robles and Berg, 2007). Unlike hypertrophic scars, keloids do not gradually flatten out (Dealey, 2005). Individuals with darkly pigmented skin are genetically prone to keloid formation (Kose and Waseem, 2008). Histologically, keloids are characterized by thick, large, closely packed bundles of disorganized collagen. Mucin is deposited focally in the dermis, and hyaluronic acid expression is confined to the thickened, granular/spinous layer of the epidermis (Kose and Waseem, 2008).

Wound contraction is part of the normal healing process but occasionally contraction will continue after re-epithelialisation has occurred, resulting in scar contraction. This type of scar contracture can lead to joint contracture with subsequent loss of mobility, functional loss, delay in return to work and a poor cosmetic result, any of which may necessitate surgery (Dealey, 2005).

1.4 Factors affecting wound healing

Numerous metabolic and pathophysiologic factors can contribute to poor healing that include local causes such as wound infection, tissue hypoxia, necrosis and systemic causes that include diabetes mellitus, malnutrition, and steroid use, among others (Guo and Dipietro, 2010).

1.4.1 Wound infection

Wound infection is probably the most common reason for impaired wound healing (Robson *et al*., 2001). All open wounds may be considered contaminated but not infected. For a wound to be infected, the bacterial colony count should exceed 10^5 organisms per gram of tissue. Common bacterial skin infections include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeroginosa*, *Streptococcus pneumoniae*, *Klebsiella pneumoni (*Pattanayaka and Sunitab, 2008), *Staphylococcus faecalis, Clostridium perfringens, Clostridium tetani, Coliform bacilli* and enterococcus (Odimegwu *et al*., 2008). There are several mechanisms by which wound infections impair tissue repair. Soft tissue cellulitis or fasciitis prolongs the inflammatory phase of normal wound healing, induces the increased expression of tissue proteases leading to the destruction of surrounding tissue, delays epithelialization and collagen deposition, and disrupts the normal progression of the inflammatory, proliferative, and remodeling phases of acute tissue repair. Inappropriately elevated tissue proteases may also digest wound growth factors and membrane receptor sites necessary for the normal stimulation of cells involved in tissue (Robson *et al*., 2001).

1.4.2 Underlying diseases

Underlying disease in the injured host is another common cause for impaired wound healing. Delayed tissue repair is a widely recognized complication in patients with diabetes mellitus.

Tissue inflammation is abnormal, and fibroblast and endothelial cell proliferation is impaired. Collagen and glycosaminoglycan deposition is decreased in diabetic patients, and wound remodeling is impeded in the setting of chronic hyperglycemia and insulin deficiency (Guo and Dipietro, 2010). Diabetic patients are also more susceptible to wound infection as a result of impaired neutrophil chemotaxis and phagocytosis. The clean wound infection rate is higher in diabetic patients (11%) than in the general patient population (Franz, 2006). The peripheral sensory neuropathy that often develops in diabetic patients results in a loss of sensory protection. This is especially important in the foot, where combined with frequent structural atrophy, the foot is subjected to increased pressure, shear forces, and repeated traumatic injury that may result in dermal ulceration. In long-standing diabetes, occlusive peripheral vascular disease and microangiopathy may be accelerated, which leads to reduced tissue perfusion; as a result, healing processes are impeded further. A situation of prolonged hypoxia, which may be derived from both insufficient perfusion and insufficient angiogenesis, is detrimental for wound healing. Hypoxia can amplify the early inflammatory response, thereby prolonging injury by increasing the levels of oxygen radicals (Mathieu *et al.*, 2006; Woo *et al.*, 2007).

1.4.3 Inflammation

Although inflammation is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain and aggravate many disorders (Anthony *et al.*, 2009). Indeed, in experimental models of repair, inflammation has shown to delay healing and to result in increased scarring. Prolonged inflammation known as chronic inflammation, a hallmark of the non-healing wound, delays healing and predisposes tissue cancer development and can lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. But, in normal situation inflammation is normally closely regulated by the body (Robert *et al.*, 2004; Eming *et al.*, 2007).

1.4.4 Age

Increased age is a major risk factor for impaired wound healing. It is commonly recognized that, in healthy older adults, the effect of aging causes a temporal delay in wound healing, but not an actual impairment in terms of the quality of healing (Gosain and DiPietro, 2004;

Keylock *et al*., 2008). Delayed wound healing in the aged is associated with an altered inflammatory response, such as delayed T-cell infiltration into the wound area with alterations in chemokine production and reduced macrophage phagocytic capacity (Guo and Dipietro, 2010). Increased secretion of inflammatory mediators, delayed infiltration of macrophages and lymphocytes, impaired macrophage function, decreased secretion of growth factors, delayed re-epithelialization, delayed angiogenesis and collagen deposition, reduced collagen turnover and remodeling, and decreased wound strength have been observed in aged individuals (Gosain and DiPietro, 2004).

1.4.5 Malnutrition

Nutrition has been recognized as a very important factor that affects wound healing. Patients with chronic or non-healing wounds and experiencing nutrition deficiency often require special nutrients. Energy, carbohydrate, protein, fat, vitamin, and mineral metabolism all can affect the healing process (Arnold and Barbul, 2006).

Together with fats, carbohydrates are the primary source of energy in the wound-healing process. Glucose is the major source of fuel used to create the cellular ATP that provides energy for angiogenesis and deposition of the new tissues (Shepherd, 2003). The use of glucose as a source for ATP synthesis is essential in preventing the depletion of other amino acid and protein substrates (Arnold and Barbul, 2006). Collagen synthesis requires hydroxylation of lysine and proline, and co-factors such as ferrous iron and vitamin C. Impaired wound healing results from deficiencies in any of these co-factors (Campos *et al*., 2008).

Protein is one of the most important nutrient factors affecting wound healing. A deficiency of protein can impair capillary formation, fibroblast proliferation, proteoglycan synthesis, collagen synthesis, and wound remodeling (Campos *et al*., 2008).

Vitamins C (L-ascorbic acid), A (retinol), and E (tocopherol) show potent antioxidant and anti-inflammatory effects. Vitamin C has many roles in wound healing, and a deficiency in this vitamin has multiple effects on tissue repair. Vitamin C deficiencies result in impaired healing, and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, and increased capillary fragility. Also, vitamin C deficiency leads to an impaired immune response and increased susceptibility to wound infection (Arnold and Barbul, 2006; Campos *et al.*, 2008). Vitamin E, an antioxidant, maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. Vitamin E also has antiinflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds (Arnold and Barbul, 2006; Burgess, 2008).

Several micronutrients have been shown to be important for optimal repair. Magnesium functions as a co-factor for many enzymes involved in protein and collagen synthesis, while copper is a required co-factor for cytochrome oxidase, for cytosolic antioxidant superoxide dismutase and for the optimal cross-linking of collagen. Zinc is a co-factor for both ribo nucleic acid (RNA) and DNA polymerase, and zinc deficiency causes a significant impairment in wound healing. Iron is required for the hydroxylation of proline and lysine, and, as a result, severe iron deficiency can result in impaired collagen production (Shepherd, 2003; Arnold and Barbul, 2006; Campos *et al.*, 2008).

1.4.6 Oxidative stress

During the inflammatory phase, neutrophils produce free radicals. The presence of these radicals will result in oxidative stress leading to lipid peroxidation, DNA breakage, and enzyme inactivation at the wound site (Kumar *et al.*, 2007). This suggests that antioxidants may be of therapeutic use. Compounds with free-radical-scavenging properties have shown to improve significantly wound healing and protect tissues from oxidative damage (Thiem and Grosslinka, 2003).

1.4.7 Drugs

Many allopathic drugs are known to inhibit wound healing. Topically applied steroids inhibit fibroblast proliferation and collagen synthesis and may cause peripheral vasoconstriction at the wound interface (Sussman, 2007). They may also cause hypopigmentation, dermal atrophy, and scar widening (Manuskiatti and Fitzpatrick, 2002). Antiplatelet drugs like aspirin and other non steroidal antiinflammatory drugs (NSAIDs) impair wound healing by inhibiting prostacyclin synthesis, inflammatory mediators derived from arachidonic acid metabolism and platelet aggregation. Colchicine reduces granulocyte migration and cytokine release. It reduces fibroblast synthesis, interrupts extracellular transport of procollagen, while collagenase synthesis increases collagenolysis and inhibits wound contraction. Anti-coagulants like warfarin and heparin inhibit proper coagulation and can adversely affect wounds by increasing the risk of haematomas and seroma formation. They may also cause tissue necrosis such as ‘purple toe syndrome’ (Sussman, 2007).

1.5 Models used to study wound healing

Wound healing activity can be performed by using either *in vivo* or *in vitro* models.

1.5.1 *In vivo* models

1.5.1.1 Incision wound model

Wound breaking strength is the most important parameter to be studied in incision wound model (Ghasemi *et al*., 2010). Two longitudinal paravertebral incisions can be made through the skin and cutaneous muscles at a distance 1 cm from the midline on either side of the vertebral column of anaesthetized rat/mice (Annan and Dickson, 2008). The parted skin is sutured and the skin breaking strength of the wound is measured after ten days of wound induction (Barua *et al*., 2009).

1.5.1.2 Excision wound model

Excision wound is made by excising the full thickness of circular skin from the animal under anaesthesia (Karodi *et al*., 2009). Then wound contraction is assessed by tracing the wound area first on transparent paper and subsequently transferring to a graph paper (Barua *et al*., 2009). In excised wound, since the edges are not in contact with each other, contraction and epithelialization are necessary for the repairing process (Ghasemi *et al*., 2010). Hence, epithelialization and wound contraction are the two parameters to be studied in case of excision wound (Nalwaya *et al*., 2009; Malviya *et al*., 2009).

1.5.1.3 Dead space analysis

Dead space wound can be induced by making a pouch through a small cut in the skin of the animal (Paschapur *et al*., 2009). A polypropylene tube (Azeez *et al*., 2007) or a sterile cotton pellet (Nayak *et al*., 2010) is to be implanted subcutaneously beneath the skin. The day of the wound creation is considered as day zero (Azeez *et al*., 2007). On day 10, the animals are sacrificed by overdose of anaesthesia, the polypropylene tubes or cotton pellet are carefully removed and dried in an oven at 60°C to a constant weight, and the weight is recorded. The level of increase (%) in the weight of granuloma tissue formed is calculated relative to the control (Okoli *et al.,* 2009). The samples are kept at -70°C for biochemical analysis until assayed. Dead space wound is important to study the physical and mechanical changes in the granuloma tissue (Paschapur *et al*., 2009). In dead space wound; granulation tissue dry weight, breaking strength and hydroxyproline content are important parameters to be studied (Malviya *et al*., 2009).

1.5.1.4 Burn wound model

Burn wound is an injury, especially to the skin by fire, heat, radiation, electricity or caustic agents. Burn wound cause disruption of the skin's mechanical integrity and allows environmental microbes to cause infection and delay wound healing (Ahmad *et al*., 2005). Partial thickness burn wound is inflicted upon animals starved overnight under anaesthesia by pouring hot molten wax at 80^{o}C into a metal cylinder with circular opening, placed on the back of the animal (Srivastava and Durgaaprasad, 2008). It can also be inflicted by applying round aluminum templates heated to 75ºC to the moistened, clipped and depilated dorsal skin for 5 seconds (DiPietro and Burns, 2003). Wound contraction, period of epithelialization and scar formation are evaluation parameters in this model (DiPietro and Burns, 2003; Mrityunjoy *et al*., 2007)

1.5.2 *In vitro* models

In vitro tests are now widely employed in ethnopharmacological research because of ethical reasons and their usefulness in bioactive-guided fractionation and determination of active compounds (Houghton *et al*., 2005). Among many some are discussed below.

1.5.2.1 *In vitro* test for fibroblast growth stimulation

Fibroblasts are trypsinized, centrifuged and resuspended in MEM/15%FBS/1% L-glutamine. The cells will be seeded at 37°C in a humidified incubator of 5% $C0_2$. The fibroblast cells will be incubated and assayed after five days using the neutral red assay method to assess the effect of the extracts on the growth of the cells (Annan and Dickson, 2008).

1.5.2.2 Chorioallantoic membrane (CAM) model

In this model, embryonated chicken eggs (9 days old) are selected and a small window will be made in the shell (Barua *et al*., 2009). Albumin is removed on the 4th day after fertilization to drop the embryo away from the shell and to allow the CAM to develop in a way that was accessible to treatment (Melkonian *et al*., 2000). Through the window, a sterile disc treated with the extract of interest is placed inside the egg at the junction of two blood vessels. The window is resealed and the egg will be incubated at 37^0c for three days. The window will then be opened and the growth of new capillary will be observed (Barua *et al*., 2009).

1.5.2.3 Antioxidant activity

It is believed that reactive oxygen species are deleterious to wound healing due to their harmful effects on cells and tissues (Annan and Dickson, 2008). It can be performed by lipid peroxidation assay or free radical scavenging assay.

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems of plant and animal origin. The mechanism involves a process whereby unsaturated lipids are oxidized to form additional radical species as well as toxic by-products that can be harmful to the host system. Lipid peroxidation assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA-TBA_2 adduct that absorbs strongly at 532 nm. This reaction is the most popular method for estimating MDA in biological samples. However, interference can be a significant problem in some biological samples if not dealt with appropriately (Sanchez-Moreno, 2002).

The main methods in free radical scavenging assay comprise superoxide radical scavenging, hydrogen peroxide scavenging, hypochlorous acid scavenging, hydroxyl radical scavenging, the scavenging of radical cation 2,2-azinobis-[3-ethylbenzothiazoline-6-sulphonate] (ABTS) or the trolox equivalent antioxidant capacity (TEAC) method, the scavenging of stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and the scavenging of radical cation N, N-dimethyl-*p*-phenylenediamine (DMPD) method (Sanchez-Moreno, 2002).

DPPH radical scavenging activity is the easiest method to assess the antioxidant activity of natural products. The DPPH scavenging activity of the plant of interest is measured from the bleaching of a purple coloured methanol solution of DPPH which is used as a reagent in a spectrophotometric assay (Annan and Dickson, 2008).

1.5.2.4 Antimicrobial activity

Open wounds are particularly prone to infection, especially by bacteria, and provide an entry point for systemic infections. Infected wounds heal less rapidly and often result in the formation of unpleasant exudates and toxins with concomitant killing of regenerating cells. Antibacterial and antifungal compounds in a traditional remedy may prevent this occurring and may underlie its use in treating wounds (Houghton *et al*., 2005).

1.6 Existing therapy aimed for wound healing

Most of the currently used wound healing agents are topical antimicrobial agents or antiseptics. Topical antimicrobial therapy is one of the most important methods of wound care (Esimone *et al*., 2009). As an example neomycin-bacitracin powder (Cicatrin®), gentamycin ointment, tetracycline ointment, nitrofurazone ointment are among the standard antibiotics used in wound healing (Esimone *et al*., 2005; Annan and Dickson, 2008; Esimone *et al*., 2009; Nalwaya *et al*., 2009). Topical iodine in the form of Lugol's solution regenerates human scar tissue back to normal (David, 2008). Povidone-iodine (5% Betadine) cream is also used for wound healing purpose (Kumar *et al*., 2009). But some of these agents have the following limitations. Antiseptics may increase the intensity and duration of inflammation (Mehibub and Chowdhury, 2009). Bacitracin when used for prolonged time causes hypersensitivity reactions (itching, swelling and anaphylaxis). Polymyxin B sulphate, also causes hypersensitivity reactions; neomycine causes ototoxicity and hypersensitivity reactions, povidone-iodine causes delayed healing, contact dermatitis and metabolic acidosis; silver sulfadiazine 1% cream causes superinfection, rash and toxic to human keratinocytes and fibroblasts. Nitrofurazone (0.2%) causes abnormal accumulation of fluid in the body tissue or cavities causing swelling or distention of the affected parts. Gentamycin causes skin hypersensitivity, ototoxicity and nephrotoxicity when the drug is used in large volume for extended period. Sodium hypochlorite (Dakin's solution), dissolves blood clots and may also delay clotting, causes bleeding, acidosis may also result following continuous use (Thornton *et al.*, 2003).

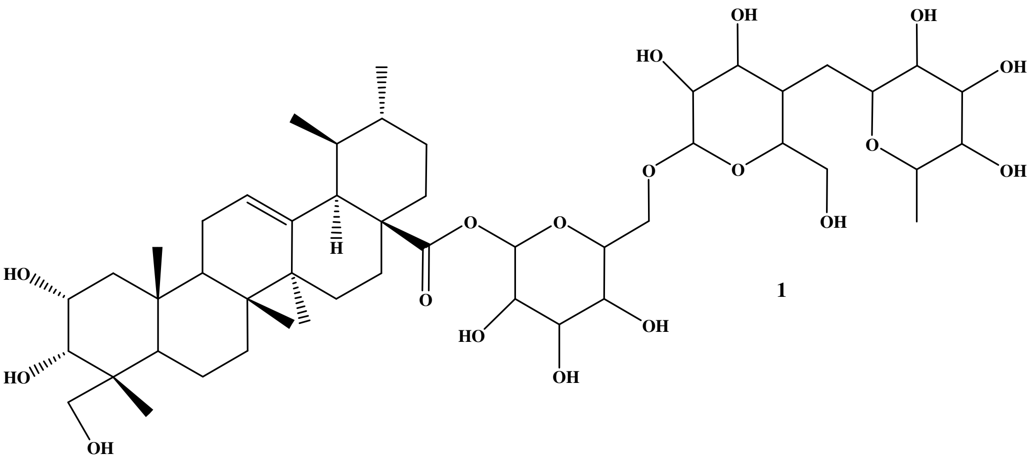
1.7 Plants and phytochemicals with reported wound healing activity

About 60% of the world population and 60–90% of the population of developing countries rely on traditional medicine for their primary health care (Kunwar and Bussmann, 2008). In addition one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of synthetic modern drugs (Mantle *et al.*, 2001). These present a clear indication for the crucial role of medicinal plants as therapeutic alternatives to conventional medicine (Abu-Al-Basal, 2010). Since time immemorial man has used various parts of plants in the treatment and prevention of many ailments (Chah *et al.*, 2006). In developed countries 25% of the medical drugs are based on plants and their derivatives (Principe, 2005) and the use of medicinal plants is well known among the indigenous people in rural areas of many developing countries. Research on wound healing agents is one of the developing areas in modern biomedical sciences and many traditional practitioners across the world have valuable information of many lesser-known or unknown wild plants for treating wounds and burns (Kumar *et al.*, 2007). The phytomedicines for wound healing are not only cheap and affordable but are also purportedly safe as hyper sensitive reactions are rarely encountered with the use of these agents. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. However, there is a need for scientific validation, standardization and safety evaluation of plants of the traditional medicine before these could be recommended for healing of the wounds. Various herbal products have been used in management and treatment of wounds over the years (Raina *et al.*, 2008). A few plants/plant products with promising activities are discussed as follows.

The process of wound healing is promoted by several natural products which are composed of active principles like triterpenes, alkaloids, flavonoids etc (Sumitra *et al.*, 2005). Asiaticoside (**1**) from *Centella asiatica* (Shukla *et al.*, 1999), β-sitosterol (**2**) (Krishnan, 2006) and a glycoprotein (Choi *et al.*, 2001) from the gel of Aloe vera, oleanolic acid (**3**) from *Anredra diffusa* (Letts *et. al.*, 2006), quercetin (**4**), isorhamnetin (**5**) and kaempferol (**6**) from *Hippophae rhamnoides* (Fu *et al.*, 2005), curcumin (**7**) from *Curcuma longa* (Jagetia and Rajanikant, 2004), proanthocyanidins and reseveratrol (**8**) from *Vitis vinifera* (Khanna *et* *al*., 2002), acylated iridoid glycosides from *Scrophularia nodosa* (Stevenson *et al*., 2002), phenolic acids from *Chromolaena odorata* (Phan *et al*., 2001), (+)-epi-bisabolol (**9**) from *Peperomia galioides* (Villegas *et al*., 2001), fukinolic acid (**10**) (Figure 1.1) and cimicifugic acids from *Cimicifuga* species (Kusano *et al*., 2001), xyloglucan from *Tamarindus indicus* (Burgalassi *et al*., 2000) are some of the important plant derived wound healing compounds which were tested in animal models (Diallo *et al*., 2002). Diallo *et al*. (2002) stated that polysaccharides are also partly responsible for the process of wound healing; for example, arabinogalactans from the root of *Angelica acutiloba*, acidic heteroglycans from the leaves of *Panax ginseng*, acemannan from the gel of Aloe vera and general polysaccharides from the leaves of *Plantago major* are reported to have wound healing activity. Besides aromatic plants have a long history of use for treating wounds; especially essential oils obtained from the various parts of plants are very effective in treating small to medium wounds, skin abrasions, excoriations, skin infections and other topical health problems provided an appropriate concentration of essential oil is used (Kerr, 2002).

1.8 *Allophylus abyssinicus* Hochst. Radlk.

Several medicinal plants are used in folklore for wound treatment. One of such plants is *A. abyssinicus* Hochst. Radlk. (Sapindaceae). It is a medium-sized to large tree with grayish-green bark. The leaves are spirally arranged with irregularly toothed marigins. (Vollsen, 1989). In wound care using herbal medicine, the dried and powdered leaves of *A. abyssinicus* are topically applied with subsequent healing. In addition, it is also used for the treatment of boils, sexually transmitted diseases, hunchback and crooked limbs. It is also used during child birth. The ethnomedicinal uses of this plant suggest its usefulness in wound treatment and stimulated our interest to study the leaf extracts for potential application in wound care.



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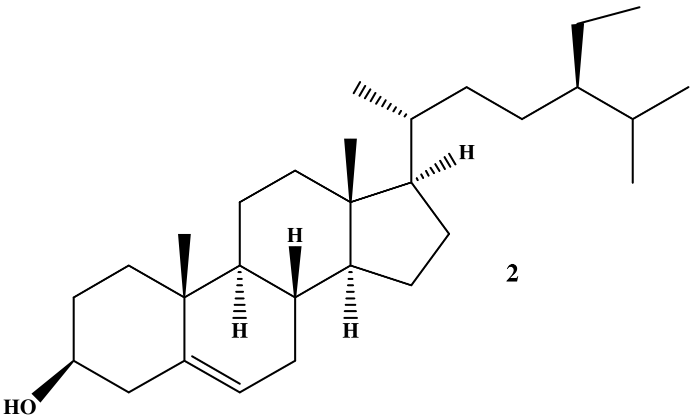

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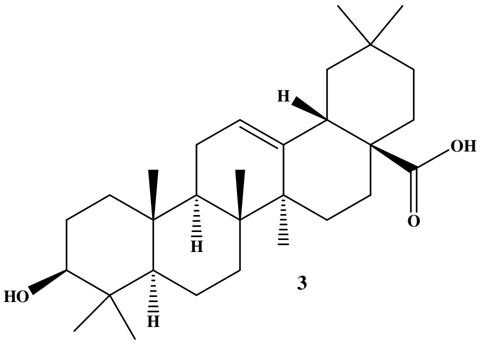

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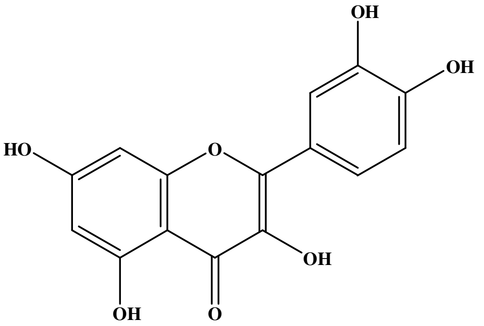

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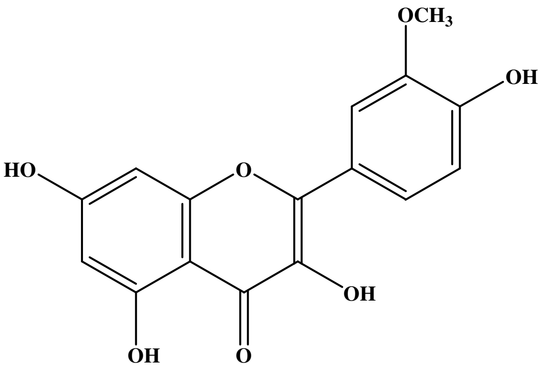

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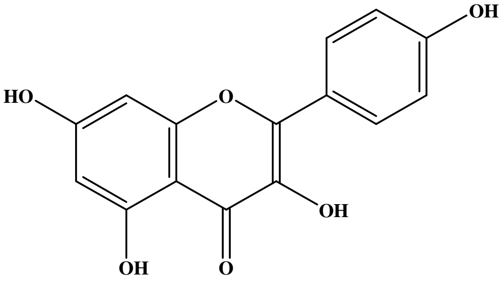
Figure 1.1: Promising phytochemicals for wound healing.



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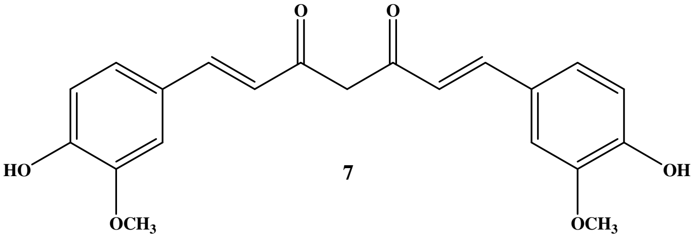

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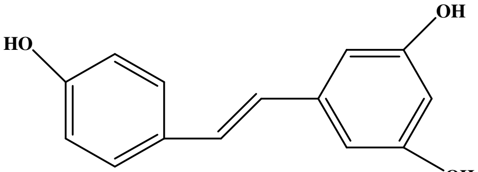

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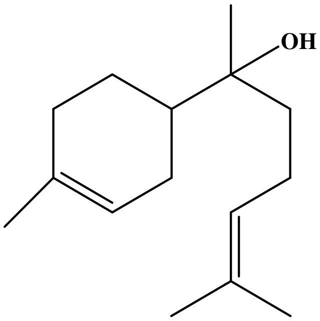

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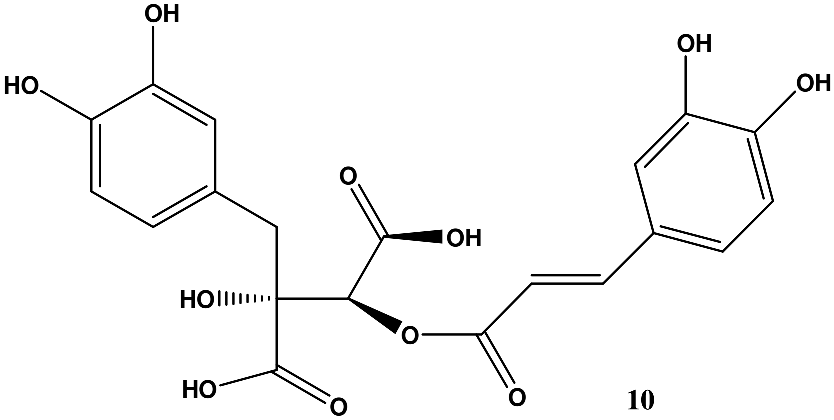

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Figure 1.1 (continued)

2. OBJECTIVES OF THE STUDY

2.1 General objective

- ✓ To study the wound healing activities of the crude and fractionated extracts of the leaves of *A. abyssinicus*.

2.2 Specific objectives

- ✓ To evaluate the wound healing, antiinflammatory, antimicrobial and DPPH radical scavenging activities of the 80% methanol extract of *A. abyssinicus* using different wound models (excision, incision, and dead space) for wound healing, carageenan induced hind paw oedema method for antiinflammatory activity, agar well diffusion method for antimicrobial test and DPPH radical scavenging activity test for antioxidant activity.
- ✓ To fractionate the crude 80% methanolic extract using chloroform, acetone, methanol and water and to study the wound healing, antiinflammatory, antimicrobial and DPPH radical scavenging effect of each fraction.
- ✓ To isolate the active compound (s) from the fraction that shows the highest activity.
- ✓ To elucidate the structure of the active compound (s).
- ✓ To evaluate the wound healing, antiinflammatory, anti microbial and DPPH radical scavenging activity of the isolated compound (s).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Wistar rats of either sex weighing 125-175 g and Swiss Albino mice with body weight 25-30 g were used for the experiments. The animals were procured from the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. Animal quarters were maintained at a temperature of 22±2^{O}C with 12-h light/12-h dark cycle.

3.1.2 Microorganisms

The antibacterial assay was carried out against the following Gram-positive bacterial strains: *S. aureus* (ATCC 25913), *S. pyogenes* (Patient isolate 09/10) and Gram negative bacterial strains: *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853)

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3.1.3 Chemicals

The following chemicals and solvents were used. Silica gel for thin layer chromatography F_{254} (0.2 mm, E. Merk, Stockholm), carrageenan (Sigma Chemical Co. St. lewis, USA), hydrochloric acid (BDH Laboratory Supplies Poole, England), sodium hydroxide (BDH Laboratory Supplies Poole, England), indomethacin, para-dimethyl-amino-benzaldehyde (E. Merk, Stockholm), copper sulphate (Bio-Lab laboratories LTD, Jerusalem), *n*-buthanol (Research-Lab fine chemical industries, India), acetic acid (BDH Laboratory Supplies Poole, England), methanol (BDH Laboratory Supplies Poole, England), chloroform (E. Merk, Stockholm), acetone (LOBA CHIME pvt. Ltd, India), dimethyl sulphoxide (DMSO) (Riedel-Dehaen, Germany), wool fat, hard paraffin, white soft paraffin, cetosteryl alcohol, carboxy methyl cellulose (CMC), hydroxypropyl methyl cellulose (HPMC), diazepam (Intas Pharmaceuticals, India), ketamine hydrochloride (Rotex Medica, Germany), 0.2% nitrofurazone ointment, $K_3Fe(CN)_6$, $FeCl_3$ (Hayashi Pure Chemicals, Japan), gelatin, H_2SO4 (BDH Laboratory Supplies Poole, England), hydrogen peroxide (BDH Laboratory Supplies Poole, England), isopropanol, DPPH

(Sigma Chemical Co St. lewis, USA), ascorbic acid, adhesive plaster (Omniplas®, Paul Hartman, Germany), surgical blade and suturing silk.

3.2 Methods

3.2.1 Collection of plant material

The leaves of *A. abyssinicus* (Hochst.) Radlk were collected in December 2009 from the compound of Science faculty, Addis Ababa University. The authenticity of the plant material was confirmed by Ato Melaku Wondaferash, the National Herbarium, Department of Biology, Addis Ababa University, where voucher specimen was deposited (collection number A0001).

3.2.2. Extraction

Air-dried leaves of *A. abyssinicus* (100 g) were extracted using 80% (v/v) methanol by maceration. Maceration was carried out for 72 h with intermittent agitation. The extract from each plant was filtered (Whatman No. 3, Whatman Ltd. England) and the marc remacerated 3X for 72 h and filtered. The combined filtrates were concentrated using Rota Vapor (Büchi Rota Vapor R-205, Switzerland). It was dried in a vacuum oven and kept in a desiccator until used.

3.2.3 Preparation of solvent fractions

The dried hydroalcoholic extract was placed in a thimble and extracted exhaustively with chloroform, acetone and methanol sequentially in a Soxhlet apparatus and the final residue in the thimble was dissolved in water as an aqueous fraction. Each solvent were evaporated to dryness at a reduced pressure.

3.2.4 Thin layer chromatography (TLC)

Analytical and preparative TLC procedures utilized adsorption chromatography. Normal phase analytical TLC utilized silica gel 60 F254 precoated plates (0.20 mm) (E. Merk, Dermstat). For preparative TLC, glass measuring 20 × 20 cm were prepared using a slurry of silica gel G suspended in distilled water (1:2 w/v) and were spread to a thickness of 0.50 mm. The plates were activated for one h at 110^{O}C and allowed to cool to room temperature and humidity before use. Butanol:acetic acid: water (4:1:5), upper phase was used as a solvent system. For analytical TLC, the developed chromatograms were air dried and then examined under UV 254 nm and UV 366 nm (UNIC–2100 UVspectrophotometer, USA) prior to spraying. Plates were then sprayed with natural product reagent A (poly ethylene glycol 4000). In the case of preparative TLC, the chromatograms were air dried after development and bands corresponding to major compounds were scraped off based on their absorbance of long wave UV light (UV 366 nm) and retention factor (Rf) values obtained from analytical TLC. The scrapings were then washed repeatedly with methanol, filtered and concentrated.

3.2.5 Structural elucidation of compound (s) from the active fraction

For structural elucidation, NMR spectra were recorded on JEOL JNM-L-400 spectrometer instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature using DMSO-d_6. A region from 0 to 20 ppm for ^{1}H and 0 to 205 ppm for ^{13}C was employed for scanning. Signals were referred to internal standard tetramethylsilane (TMS). Chemical shifts are reported in δ units.

3.2.6 Formulation of test substances using different bases

The test substances were formulated using either simple ointment base or HPMC gel according to the master formula indicated below.

i) Preparation of ointment using the 80% methanol leaf extract of *A. abyssinicus*

Simple ointment was prepared according to formula given in British Pharmacopoeia as indicated in Table 3.1 (BP, 1988).

All the ingredients were mixed, heated gently with stirring until homogenous and stirred until cool. Medicated ointments were prepared by mixing weighed amounts of the powdered extracts with the simple ointment base by levigation on the surface of ointment slab to make the ointment of uniform consistency and smooth texture (Ansel, 1985).

Table 3.1. Master formulae for preparation of simple ointment base.

Ingredients	Master Formula (g)	Reduced Formula (g)
Wool fat	50	10
Hard paraffin	50	10
White soft paraffin	850	170
Cetostearyl alcohol	50	100
	1000	200

ii) Preparation of medicated HPMC gel

HPMC (7.5 %) in distilled water was boiled, after cooling weighed amount of extracts were incorporated by levigating on the surface of a smooth ointment slab to make the desired percentage of each medicated jel. In all cases smooth and uniform gels were packed in wide-mouthed ointment jars and were stored in cool until used.

3.2.7 Acute toxicity test

i) Acute dermal toxicity test

Skin irritation test for the different test substances was conducted on rabbits by using occluded dermal irritation test (Robinson and Perkins, 2002). The skin of each rabbit was shaved at two different positions on the dorsal side, each about 1200 mm^2 area. The first area was kept as control, to which non medicated ointment was applied. The second area was applied with the test substance (Gfeller *et al.*, 1985). On day one of the test period the preparations were evenly applied on the shaven area of the animals' skin. Immediately the area was covered by dressing gauze over which a plastic sheet (occlusive material) was placed and altogether the covering was loosely held in contact with the skin by means of a non-irritating adhesive tape (Teshome *et al.*, 2008). After 24 h of exposure period, the elastic bandage, the adhesive plaster, the plastic sheet and the gauze were removed taking care not to damage the skin and the test site was rinsed with distilled water. The animals were examined for the presence of erythema and edema according to Draize dermal irritation scoring system at grading intervals of 1, 24, 48 and 72 h (Draize, 1959). The degree of erythema and edema were determined based on the scores given in Table 3.2.

Primary irritation index (PII) which is a parameter that indicates the potential of a given substance for skin irritation was also calculated for the different test substances by summing up all the erythema and oedema scores of all the 4 time intervals of grading (1 h, 24 h, 48 h, and 72 h) and dividing by the number of test sites (2) multiplied by the grading interval (4) (Teshome *et al.*, 2008). According to Draize classification substances scoring PII of < 2 are mildly irritant, 2-5 moderately irritant, > 5 severely irritant.

Table 3.2. Draize dermal irritation scoring system.

Erythema and eschar formation	Value	Edema formation	Value
No erythema	0	No oedema	0
Very slight erythema	1	Very slight oedema	1
Well defined erythema	2	Slight oedema	2
Moderate to severe erythema	3	Moderate oedema	3

ii)Acute oral toxicity test

Swiss albino mice of either sex weighing 30-40 g were used for acute oral toxicity study. Oral toxicity study was conducted as per the protocol drawn under Organization for Economic Cooperation and Development (OECD) guidelines 420 in Swiss albino mice starting at a dose level 2000 mg/kg up to 5000 mg/kg of the extract (Karodi *et al.*, 2009). First, the animals were dosed with the extract and observed for signs of acute toxicity within 48 h periodically. Then, they were further watched for 14 days for signs of acute toxicity like diarrhea, seizure, weight reduction etc.

3.2.8 Wound healing activity tests

i) Excision model

Rats containing six in each group were anaesthetized by open mask method with anesthetic ether. Each rat was depilated at the back and one excision was inflicted by cutting away 500 mm^2 full thickness of skin of a predetermined area (Figure 3.1). The rats were left undressed to the open environment. Then the positive control (0.2% w/v nitrofurazone ointment) or the negative control (simple ointment BP/HPMC gel) or the test samples were administered till the wound gets completely healed. Then wound contraction and epithelisation time were monitored. Epithelisation time was noted as the number of days after wounding required for the scar to fall off leaving no raw wound behind. Wound contraction was calculated as percent reduction in wound area. The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper every alternative date. To determine the changes in healing of wound, measurement of wound area on graph paper were expressed as unit (mm^2) (Saha *et al*., 1997).

Fig 3.1. A circular excision wound on day 0.

ii) Incision model

Six mice in each group were anaesthetized and one paravertebral-long incision was made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline of the depilated back of the mice (Fig 3.2). Full aseptic measures were not taken and no local or systemic antimicrobials were used throughout the experiment (Udupa *et al*., 1995). Then the negative control (1% CMC), different concentrations of test substances suspended in 1% CMC was orally administered once daily for 9 days. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals; surgical thread (No. 000) and a curved needle (No. 11) were used for stitching. The wound was left undressed. The sutures were removed on the 7th day. On the tenth day the mice were again anaesthetized and each mouse was placed on the middle of the board towel. The clamps were then carefully attached to the skin on the opposite sides of the wound at a distance of 0.5 cm away from the wound. The longer pieces of the fishing line were placed on the pulley and finally on to the polyethylene bottle and the position of the board was adjusted so that the bottle receive a rapid and constant rate of water from a large reservoir until the wound began to open. The amount of water in the polyethylene bag was weighed and considered as an indirect measure of the tensile strength of the wound. The tensile strength of the extract treated wounds was compared with controls. The tensile strength increment indicates better wound healing stimulation by the applied test substance. Tensile strength was calculated using the following formula (Reddy *et al*., 2008):

$$\text{Tensile strength} = \frac{\text{Breaking strength}}{\text{Cross-sectional area of skin (mm}^2\text{)}}$$

iii) Dead space model

Dead space wounds were inflicted by implanting sterile cotton pellets (5 mg) on one side of the groin on the ventral surface of each mouse as described by Neuman and Logan (1950). The animals were randomly divided into groups of six each. The control group animals were provided with 1% CMC. The test group mice were given different concentrations of the test substances dispersed in 1% CMC orally. On the 10th post wounding day, the granulation tissue formed on the implanted cotton pellets was carefully removed under anesthesia. These tissue samples were dried at 60°C for 12 hrs. The dried tissue was added with 5ml 6N HCl and kept at 110°C for 24 hrs. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline.

a) Estimation of hydroxyproline

Dry granulation tissue from both control and treated groups were used for estimation of hydroxyproline. Hydroxyproline present in the neutralized acid hydrolysate was oxidized by hydrogen peroxide in the presence of copper sulfate, and subsequently complexed with p-dimethylaminobenzaldehyde to develop a pink colour that was measured spectrophotometrically at 572 nm.

Figure 3.2. Incision wound on day 0.

b) Preparation of calibration curve

Standard L-hydroxyproline (0.05 g) was dissolved in water and diluted to about 400 ml. Concentrated HCl (20ml) were added and the solution made up to 500 ml with water. Then 100 µg/ml solution was diluted to give concentrations of 5, 10, 15, 25 and 50µg of hydroxyproline/ml.

Nineteen test tubes were arranged sequentially. In each of test tubes 1-3, l ml of 5 µg/ml, test tubes 4-6, 1 ml of 10µg/ml, test tubes 7-9, 1 ml of 15µg/ml, test tubes 10-12, 1 ml of 25 µg/ml and to test tubes 13-15, 1 ml of 50µg/ml of hydroxyproline solutions were added. To each of test tubes 16-18, 1 ml of the test solution and to test tube 19, 1 ml of water was added. 1 ml of 0.05 M $CuSO_4$ was added in to each test tube followed by 1 ml of 2.5 N NaOH with gentle mixing. The tubes were placed on a water bath at 40°C for 3-5 minutes. 1 ml of 6% hydrogen peroxide was added, which was immediately mixed by swirling the contents before addition is made to the next tube. The tubes were left on the water bath for 10 minutes with occasional swirling and then cooled with tap water. Finally 4 ml of 3N H_2SO_4 and 2 ml of 5% p-dimethylaminobenzaldehyde solutions were added with mixing and swirling after each addition. Caps were placed on the tubes, which are kept on a water bath at 70°C for 16 min.; the solutions were then cooled, mixed and their extinctions measured against the blank solution at a wavelength of 572 nm in 1 cm cells. The average reading for each set of tubes was used in the calculation. Standard absorbance versus concentration curve was drawn (fig 3.3) and based on the curve, hydroxyproline concentration of the unknown was determined.

3.2.9 *In vivo* antiinflammatory activity testing

In vivo antiinflammatory activity was evaluated on the basis of inhibition of carrageenan-induced mice hind paw oedema as described by Dongmo *et al*. (2003). The extracts/ fractions or isolated compounds, indomethacin, vehicle were given orally to the experiment, reference and control groups, respectively. The oedema inducing agent, i.e. 0.1 ml of 1% carrageenan in normal saline was then injected into the plantar surface of the left hind paw 30 min after oral administration of the test substances. The volumes of injected paws were measured before, and 60, 120, 180 and 240 min after injection of carrageenan using Ugo Basile plethysmometer (Italy, model 7140). Animals of either sex were used in the experiment and each group was composed of six mice (three male and three female). The increase in paw volume, i.e. inflammation (%I) was calculated according to the equation given by Delporte *et al*. (1998):

$$\%I = \frac{Vf - Vi}{Vi} \times 100$$

Where Vf and Vi are the final and initial paw volumes of each animal, respectively. The mean %I was then calculated and a curve of mean %I versus time was plotted. In addition, the anti-inflammatory effect (%A) was calculated according to the formula given below (Delporte *et al*., 1998) and data were presented as mean ± standard error of the mean (SEM).

$$\%A = \frac{\%I_c - \%I_e}{\%I_c} \text{ x } 100$$

Where I_c and I_e are the mean inflammation values reached in control and experimental groups respectively

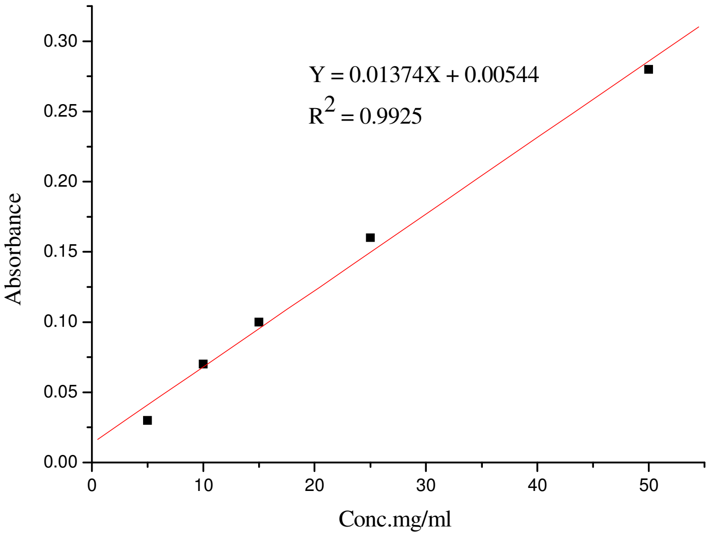

Fig 3.3. Standard curve for determination of hydroxyproline concentration.

3.2.10 Antioxidant activity testing

The hydrogen-donating ability of the extract, fractions and isolated compounds was examined on the basis of the method of Blois (1958) in the presence of DPPH stable radical. The samples and the positive control, vitamin C, were diluted with methanol to prepare sample solution equivalent to 1000, 500, 250, and 125 µg of dried sample/ml solutions. 5 ml of 0.004% DPPH solution was pipetted into each test tube followed by the addition of 50 µl of the sample solution. The mixture was incubated at 37°C for 30 min. Absorbance of the methanolic DPPH-tincture was measured at 517 nm (Jenway Model 6500 spectrophotometer). The inhibition percentage (%) of radical scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = ((A\text{o} - A\text{s}) / A\text{o}) \times 100$$

Where Ao is absorbance of the control and As is absorbance of the sample at 517 nm. The IC_{50} values were calculated by linear regression of plots, where the abscissa represented the concentration of tested plant extracts and the ordinate represented the average percent of scavenging capacity from three replicates.

3.2.11 Antimicrobial activity testing

3.2.11.1 Preparation of the samples

The test substances were dissolved in DMSO at concentrations 5, 10, 25, 50, 100, 200, 400 and 800 mg/ml. Negative and positive controls were also prepared. The antibacterial activity was evaluated using standard and isolated strains which included the Gram-positive bacteria *S. aureus* (ATCC 25913) and *S. pyogenes* (Patient isolate 09/10). The Gram negative bacteria used were *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853).

3.2.11.2 Preparation of media

A weighed amount of Muller Hinton agar (38 g) was put in 1 liter of distilled water. The pH of the mixture was adjusted to 7.3 using 1N NaOH. The mixture was heated by agitation to dissolve the contents. Each test tube containing 10ml of the mixture was plugged with cotton and was autoclaved (Dixon Surgical, England) at 121°C for 15 min and allowed to cool to 40^oC.

3.2.11.3 *In vitro* antimicrobial activity test

The agar well diffusion method was used to test the standard and clinically isolated strains of microbes. The bacterial specimens were transferred to sterile test tubes aseptically and mixed with the agar that was maintained in a molten state and poured into Sterilized Petri plates. After congealing, the seeded agars were punched out with a sterile hole borer at equally spaced out positions to make two wells with diameters of 5 mm facing each other. The first hole was filled with ciprofloxacin and the second hole was filled with 50 µl of different dilutions of the test substance dissolved in DMSO using micropipette. The plates were then left at room temperature for two hrs to allow diffusion over microbial growth and then incubated at 37°C for 24 hrs. The antimicrobial activity was evaluated by determining the mean zone of inhibition (MZI) using transparent ruler.

3.2.12 Statistical analysis

Results obtained have been expressed as mean ± SEM and were compared with the corresponding control group by applying analysis of variance (ANOVA) test followed by dunnett test (comparing all vs. control), to compare the mean of each dose group to the control. $P < 0.05$ was the probability level taken to determine statistical significance. Statistical analysis was done using Graph Pad Instat®.

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List of acronyms

ABTS	2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonate
ATCC	American type culture collection
C5a	Complement 5a
CMC	Carboxy methyl cellulose
DMPD	N, N-dimethyl-*p*-phenylenediamine
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EHNRI	Ethiopian health and nutrition research institute
FGF	Fibroblast growth factor
HPMC	Hydroxy propyl methyl cellulose
IC_{50}	Fifty percent inhibitory concentration
IGF	Insulin like growth factor
IL	Interleukin
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
MZI	Mean zone of inhibition
NSAIDs	Non steroidal anti-inflammatory drugs
OECD	Organization for Economic Cooperation and Development
PDGF	Platelet derived growth factor
PII	Primary irritation index
Rf	Retention factor
ROS	Reactive oxygen species
SEM	Standard error of the mean
TBA	Thiobarbituric acid
TEAC	Trolox equivalent antioxidant capacity
TGF	Transforming growth factor
TMS	Tetramethylsilane
TNF	Tumor necrosis factor alpha

Abstract

The leaves of *Allophylus abyssinicus* (Hochst.) Radlk. (family: Sapindaceae) are traditionally used for the treatment of wounds, burns and in arresting bleeding and in skin diseases in the Ethiopian folk medicine. However, there is no prior scientific study regarding phytoconstituents and biological activities. Therefore; the present study was aimed at evaluation of wound healing activity of the plant.

In the present study, the hydro-alcoholic extract, the different solvent fractions and isolated bands from the methanolic fraction of the leaves of *A. abyssinicus* were studied for wound healing activity by incorporating in simple ointment base B.P. at different concentration. Wound healing activity was studied in rodents viz. excision, incision and dead space wound models. In case of the excision wound model, wound contraction and period of epithelization were studied while incision wound model was evaluated by determining tensile strength. The content of hydroxyproline was determined in case of dead space wound model. To determine the healing mechanism; antimicrobial, anti-inflammatory (carageenan induced paw oedema) and antioxidant (DPPH scavenging) activities were studied.

Treatment of wounds with ointment containing 5% and 10% (w/w) of the hydroalcoholic extract exhibited significant ($P < 0.001$) wound healing activity in excision wound model. The methanolic fraction was found to be the most active in all the test models after fractionation using chloroform, acetone, methanol and water at 200mg/kg. Further preparative TLC done on the methanolic fraction gave three bands and band three was found to be the most active in excision (2%), incision and dead space wound models at 25 and 50 mg/kg. It also showed the highest antiinflammatory, antimicrobial and antioxidant activity. Its structure tentatively deduced to be 2-(2,3,4,6,8,8a-hexahydro-oxopyrano[3,4-b] pyran-4yl oxy) propanoic acid.

In conclusion, the leaves of *A. abyssinicus* were shown to have promising wound healing activity. This could probably be due its antimicrobial, antiinflammatory and antioxidant effect.

4 RESULTS AND DISCUSSION

4.1 Extraction

The dried leaves of *A. abyssinicus* were extracted by maceration using 80% methanol to obtain the total extract. Solvent fractions were prepared by successive soxhlet extraction using chloroform, acetone, methanol and water. The percentage yields of the extracts are given in Table 4.1.

Table 4.1: Percentage yields of the hydroalcoholic extract and solvent fractions of *Allophylus abyssinicus.*

Extract/Fraction	**Percentage yield (w/w)**
Chloroform	4
Acetone	2
Methanol	11
Water	9.5
80% methanol	16

The total extract obtained by soxhlet extraction was found to be more than the hydroalcoholic extract. This could be due to employment of heat in the former method that increases the solubility of the components.

4.2 Oral acute toxicity study

No death or perception of adverse reactions was observed on the animals within the fourteen days follow up period. This is an indication that the extracts, the fractions and the compounds were not toxic at the doses employed in this study.

4.3 Skin-irritation test

In skin irritation test, no irritation symptoms were developed over the test period. Neither erythema formation nor skin swelling were developed during a 72 hr time period for all test substances. Hence the PII is found to be zero.This indicates that all the test substances from the leaves of *A. abyssinicus* do not have irritant property.

4.4 Wound healing activity of the 80% methanolic extract and fractions

4.4.1 Excision model

In excision wound healing model, the 80% methanolic extract of the leaves of *A. abyssinicus* was formulated by using simple ointment and HPMC gel as a base. As presented in Table 4.2, the hydroalcoholic extract showed significant increase in percentage closure of excision wounds and enhanced epithelization. This effect was observed in a dose dependant manner. Especially the 80% methanolic extract levigated in simple ointment at 10% concentration shows comparable activity with that of the positive control. The same concentration of the 80% methanol extract in HPMC gel has lower activity. This could be due to the better release of the ingredients from the simple ointment than from HPMC jel. It can also indicate that the components responsible for activity may probably be polar. Further fractionation using chloroform, acetone, methanol and water gives different yields as indicated in Table 4.1. For better release the chloroform and acetone fractions were formulated as HPMC gel and the methanol and aqueous fractions in simple ointment.

The methanolic and aqueous fractions show significant wound healing activity in a dose dependant manner on excision wound healing model comparable with that of nitrofurazone (Table 4.3). The percentage contraction on day eight post treatment was significant for methanolic fractions and nitrofurazone as compared with the negative control. The remaining fractions possessed better wound contraction than the respective negative controls but statistically insignificant. On day twelve and day sixteen all the fractions displayed significant wound contraction rate as compared with the control. This indicates that the components are active mainly in the late phase of the wound healing cascade. The period of epithelization for the 10% methanol and aqueous fractions was found to be 17.54 ± 0.33 and 19.34 ± 0.76, respectively. These were comparable with 0.2% nitrofurazone (16.33 ± 0.61) and extremely significant as compared with simple ointment treated group. The 10% chloroform, 10% acetone and 5% methanol ointments also showed significant period of epithelization compared with the respective negative control groups.

Table 4.2. Effect of topical application of ointments containing 80% methanolic extract of the leaves of *Allophylus abyssinicus* on wound contraction of excision wound model.

Group	**Day 0**	**4th day**	**8th day**	**12th day**	**16th day**	**Period of Epithelization (days)**
0.2% NF	510.67± 3.73	313.46±3.14 (38.62)	163.20±3.42 (68.04)***	63.94±1.30 (87.48)***	10.32±1.98 (97.98)***	15.33±0.61***
5% ME in SO	514.22±3.45	348.67±3.21 (32.19)	218.67±4.32 (57.48)	120.76±2.71 (76.52)***	47.11±0.32 (90.84)***	20.97±0.76**
10% ME in SO	520.40±4.23	340.43±1.94 (34.58)	187.23±3.12 (64.02)**	72.11±1.69 (86.14)***	14.22±0.32 (97.27)***	16.68±0.43***
5% ME in HPMC	522.12±5.43	415.54±5.64 (20.41)	307.65±4.87 (41.08)	245.56±3.65 (52.97)	194.23±1.80 (62.80)*	22.76±0.62
10% ME in HPMC	505.34±4.54	385.09±4.29 (23.80)	267.43±3.07 (47.08)	162.98±3.07 (67.75)***	97.76±1.32 (80.65)***	21.76±0.71***
SO	516.50±2.34	374.76±2.08 (27.44)	304.72±2.40 (41.00)	260.47±1.75 (49.57)	208.00±2.45 (59.73)	24.78±0.49
HPMC	498.21±6.75	412.76±5.01 (17.15)	345.98±4.60 (30.56)	280.65±3.54 (43.67)	224.87±1.71 (54.86)	25.55±0.83

Values are expressed as mean ± S.E.M (n = 6), percentage of contraction are in parenthesis, $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control; NF = nitrofurazone, ME in SO = 80% methanolic extract in simple ointment, ME in HPMC = 80% methanolic extract in hydroxy propyl methyl cellulose, SO = simple ointment, HPMC = hydroxy propyl methyl cellulose.

4.4.2 Incision model

In the wound healing process, deposition of newly synthesized collagens at the wound site increases the collagen concentration per unit area and hence the tissue tensile strength (Deshmukh *et al*., 2009).

Table 4.3. Effect of topical application of ointments containing different solvent fractions of the leaves of *Allophylus abyssinicus* on wound contraction of excision wound model.

Group	**Day 0**	**4th day**	**8th day**	**12th day**	**16th day**	**Period of Epi. (days)**
5%CHL	523.75±5.34	426.72±4.05 (18.53)	336.41±3.85 (35.77)	201.54±1.91 (61.52)**	124.43±0.78 (76.24)***	22.78±0.63
10%CHL	521.43±5.43	411.76±4.82 (21.03)	288.42±3.76 (44.69)	164.96±2.75 (68.36)***	69.71±1.65 (86.63)***	21.34±0.73*
5% ACN	505.33±5.32	407.32±4.71 (19.40)	300.12±3.02 (40.61)	189.28±2.19 (62.54)**	106.37±1.36 (78.95)***	22.01±0.46
10%ACN	518.34±5.54	399.84±4.71 (22.86)	267.65±3.23 (48.36)	145.32±1.87 (71.96)***	53.82±0.76 (89.62)***	20.96±0.62*
5% ME	516.76±6.43	392.65±4.54 (24.02)	243.76±3.70 (52.83)*	139.91±4.34 (72.93)***	56.91±3.03 (88.99)***	21.02±0.42*
10% ME	490.21±5.74	352.37±4.45 (28.12)	198.34±4.78 (59.54)***	89.09±1.34 (81.83)***	27.22±0.32 (94.44)***	17.54±0.33***
5% WA	498.56±6.43	404.98±5.44 (18.77)	288.43±3.08 (42.15)	176.34±3.28 (64.63)*	93.32±1.98 (81.28)***	21.44±0.65
10% WA	524.55±5.22	367.91±4.04 (29.86)	239.23±3.21 (54.39)**	103.72±2.93 (80.23)***	39.87±1.01 (92.40)***	19.34±0.76***
0.2% NF	510.67 ±3.73	313.46±3.14 (38.62)*	163.20±3.27(68.04)***	63.94±1.30 (87.48)***	10.32±1.98 (97.98)***	16.33±0.61***
SO	526.32±3.43	435.18±2.91 (17.32)	345.23±2.34 (34.41)	250.75±1.38 (52.36)	176.32±0.98 (66.50)	23.83±0.48
HPMC	519.76±4.34	427.34±4.21 (17.78)	347.38±3.01 (33.17)	276.62±1.98 (46.78)	197.12±1.01 (62.07)	24.11±0.46

Values are expressed as mean ± S.E.M (n = 6), percentage of contraction are in parenthesis. *P < 0.05 vs. control, **P < 0.01 vs. control, ***P < 0.001 vs. control. CHL = chloroform fraction, ACN = acetone faction, ME = methanolic fraction, WA = aqueous fraction, NF = nitrofurazone, SO = simple ointment, HPMC = hydroxy propyl methyl cellulose, period of epi = period of epithelization.

Table 4.4. Compares the tensile strength of the healing skin treated with different formulations for 10 days. The wound, which was untreated, had the minimum strength (267.64 g). The tensile strength of the tissue treated with other formulations was significantly higher in treated than untreated wounds. The tensile strength of the wound treated with 200 mg/kg of the methanolic fraction was the highest. Those which received

200 mg/kg of the methanolic and acetone fractions showed higher and statistically significant tensile strength when compared with the remaining groups. The chloroform fraction at 100 mg/kg did not show significant increase in tensile strength. Increased tensile strength indicates increase in collagen synthesis and strength by the formation of inter and intra-molecular cross links which facilitate wound healing (Reddy *et al.*, 2002).

Table 4.4. Effect of oral administration of the different solvent fractions obtained from the leaves of *Allophylus abyssinicus* on tensile strength of the skin having incision wound.

Amount of extract/fraction (mg/kg)	**Average tensile strength**
Negative control (1% CMC)	267.64±6.28
80% methanol extract (200mg/kg)	386.91±5.67***
80% methanol extract (400mg/kg)	451.32±4.76***
Chloroform fraction (200mg/kg)	301.43±5.40**
Chloroform fraction (100mg/kg)	286.09±4.32
Acetone fraction (200mg/kg)	360.43±6.83***
Acetone fraction (100mg/kg)	322.87±5.91***
Methanol fraction (200mg/kg)	412.87±6.43***
Methanol fraction (100mg/kg)	384.32±5.57***
Water fraction (200mg/kg)	367.54±5.98***
Water fraction (100mg/kg)	332.87±6.21***

Values are expressed as mean ± S.E.M (n = 6). Dose levels are in parenthesis, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control.

4.4.3 Dead space model

Wound healing mainly depends on the repairing ability of the tissue, type and extent of damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen and small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into fibroblast, which start migrating into the wound gap along with the fibrin strands. The collagen composed of amino acid (hydroxyproline) is the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides (Nayak and Pereira, 2006). Hence estimation of hydroxyproline in the granulation tissue may throw light on the maturation and healing process (Azeez1 *et al.*, 2007). The data depicted in Table 4.5 showed that the hydroxyproline content of the granulation tissue of the animals treated with 400mg/kg of the 80% methanolic extract (P < 0.001) and 200mg/kg of the methanolic fraction (P < 0.01) have significant increase in hydroxyproline content than the rest groups.

Table 4.5. Effect of oral administration of the 80% methanolic extract and the different solvent fractions obtained from the leaves of *Allophylus abyssinicus* on dead space wound model.

Amount of extract/fraction (mg/kg)	**Hydroxyproline (mg/g tissue)**
Negative control (1% CMC)	21.53 ± 4.23
80% methanol extract (200mg/kg)	46.76± 10.65
80% methanol extract (400mg/kg)	$63.25 \pm 4.12^{***}$
Chloroform fraction (200mg/kg)	28.09±3.43
Chloroform fraction (100mg/kg)	23.66±5.92
Acetone fraction (200mg/kg)	42.37±4.27
Acetone fraction (100mg/kg)	34.87±3.65
Methanol fraction (200mg/kg)	$56.66 \pm 4.67^{**}$
Methanol fraction (100mg/kg)	38.69±5.99
Aqueous fraction (200mg/kg)	44.65±4.09
Aqueous fraction (100mg/kg)	33.93±6.32

Values are expressed as mean ± S.E.M (n = 6). Dose levels are in parenthesis, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control.

4.5 Antiinflammatory activity of the 80% methanolic extract and fractions

Chronic ulcers will not heal until the chronic inflammation is reduced (Diegelmann and Evans, 2004). The inflammation in a chronic wound serves only to cause further injury and promote inflammation (Menke *et al.,* 2007). In the present study carrageenan induced oedema was used as a prototype to induce inflammation. The development of oedema has been described as biphasic. The initial phase is due to release of histamine, serotonin and kinins in the first hour after injection of carrageenan. The more pronounced second phase is related to the release of a prostaglandin-like substance in 2–3 hrs. Indomethacin is known to show significant antiinflammatory effect, which may be due to inhibition of the mediators of inflammation induced by the phlogogenic stimuli (Lalitha and Sethuraman, 2010).

In the present study, all the test substances except the chloroform fraction exerted anti-inflammatory effects, with respect to the control at a dose level of 200 mg/kg (Figure 4.1). Indomethacin is found to be the most effective both in the first and the second phase of inflammation. In the first h of inflammation, the methanolic fraction and indomethacin showed significant antiinflammatory activity as compared with the control group (P < 0.05 and P <0.001, respectively). In the subsequent hours of inflammation indomethacin showed statistically significant activity as compared with the rest of the test substances. The 80% methanolic extract, the acetone, aqueous and methanolic fractions showed significant activity when compared with that of the control group in the second hr. In the third and fourth hrs, all the test substances except the aqueous and chloroform fractions displayed statistically significant antiinflammatory activity (Table 4.6). This could be by blocking the release of prostaglandins.

Table 4.6: Inhibition of carageenan induced mice paw edema by the 80% methanolic extract and the different solvent fractions from the leaves of *Allophylus abyssinicus.*

Treatment	Dose (mg/kg)	%A ± SEM (n = 6)			
		1hr	2hrs	3hrs	4hrs
80% methanol extract	200	36.00 ± 4.40	41.67 ± 8.12***	32.35 ± 5.10***	21.21 ± 6.11*
Chloroform fraction	200	2.00 ± 5.08	5.56 ± 4.63	-2.94 ± 9.02	-9.09 ± 1.81
Acetone fraction	200	28.00 ± 0.84	44.44± 8.50***	32.35± 0.93**	24.24 ± 5.98**
Methanol fraction	200	44.00 ± 3.17*	47.22± 2.74***	38.24± 2.56***	30.30 ±2.71***
Aqueous fraction	200	4.00 ± 0.82	27.78± 4.34**	11.76 ± 4.83	15.15 ± 7.54
Indomethacin	10	64.00 ± 5.4***	69.44 ± 6.22***	64.71± 10.40***	60.61± 2.81***

Values are expressed as mean ± S.E.M, *P < 0.05 , **P < 0.01, ***P < 0.001

The methanolic fraction and indomethacin seems to block both phases, that means blocking histamin and serotonin release in the first phase and preventing the release of some of the inflammatory mediators by blocking prostaglandin's action in the second phase. Although the methanolic fraction was found to be effective in both phases, its effect was not as pronounced as indomethacin. So, it is very likely that the antiinflammatory components of the plant reside in the methanolic fraction.

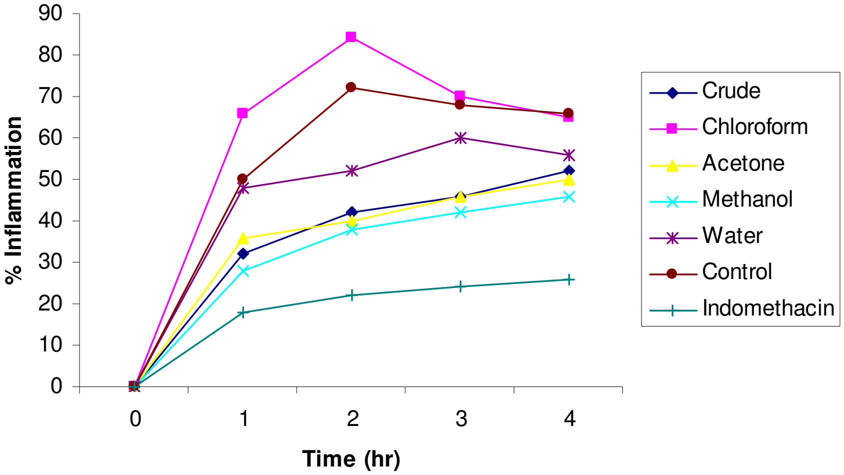


Figure 4.1: Effect of oral administration of various extracts of *Allophylus abyssinicus* (200 mg/kg) and indomethacin (10 mg/kg) on carrageenan induced paw oedema.

4.6 Antioxidant activity of the hydroalcoholic extract and fractions

DPPH (**11**) is a stable radical used to study radical scavenging effect of substances. Methanolic solution of DPPH has deep purple colour with maximum absorption at 517 nm. Substances that can donate hydrogen atom change the purple colour to colourless or yellow. Thus antioxidant molecules can quench DPPH radicals and convert them to colourless or yellow coloured; 2, 2-diphenyl-1-hydrazine **(12)** resulting in the decreasing of absorbance at 517 nm (Molyneux, 2004; Jayasri *et al.*, 2009). Figure 4.1 shows the reduction of DPPH radical to DPPH-H.

Oxidative stress and free radicals have been implicated in impaired wound healing (Shetty *et al.*, 2008). When a wound occurs, oxygen free radicals, also called reactive oxygen species (ROS), are created. These ROS molecules are unstable, containing unpaired electrons which are highly reactive, seeking to steal and bond with electrons from other molecules, and thus creating more ROS's. In severe cases, wound healing can be halted to such an extent that the wound becomes susceptible to contamination, and problems such as infection (Keller *et al.*, 2006).

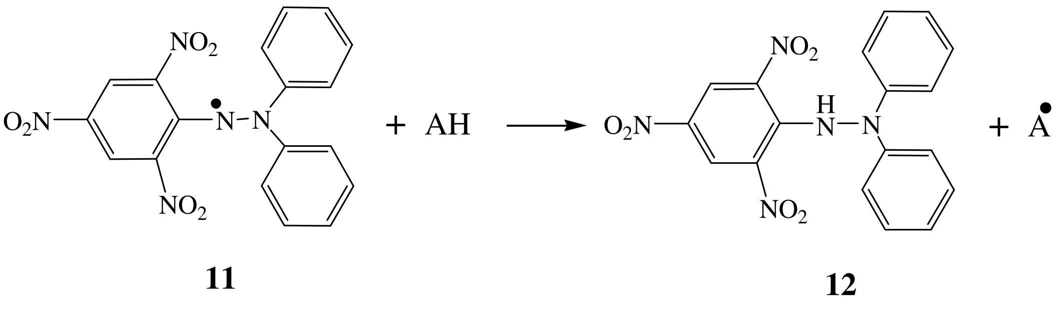


Figure 4.2: Mechanism of DPPH radical scavenging.

In this study the 80% methanolic extract and the different solvent fractions of *A. abyssinicus* reduced DPPH in concentration dependent manner. Among these, the methanolic fraction was the strongest with a fifty percent inhibitory concentration (IC_{50}) value of 292.77 µg/ml. Ascorbic acid, which was used as a standard, showed an IC_{50} value of 5.3 µg/ml. The chloroform fraction did not show significant activity in a concentration dependant manner. The IC_{50} value of each of the test substances is shown in Table 4.7. Figure 4.3 also shows the radical scavenging effect of the methanolic fraction which was better when compared with the remaining solvent fractions.

Table 4.7: DPPH free radical scavenging activity of the 80% methanolic extract and the different solvent fractions obtained from the leaves of *Allophylus abyssinicus.*

Test substance	**IC_{50} (µg/ml)**
80% methanol extract	374.96
Acetone fraction	494.50
Methanol fraction	292.77
Aqueous fraction	522.65
Ascorbic acid	5.3

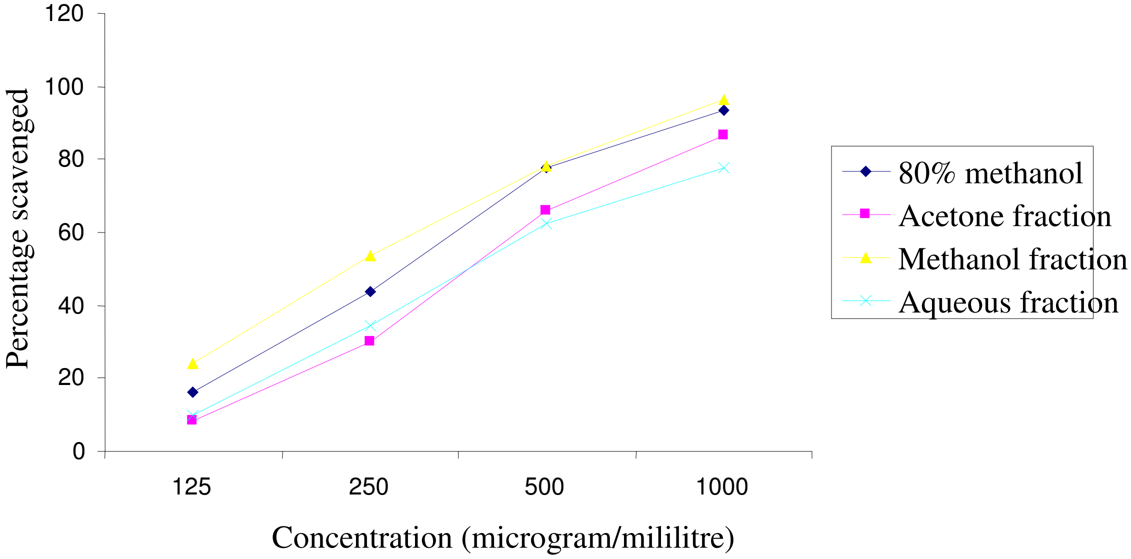


Figure 4.3: DPPH radical scavenging activities of the 80% methanolic extract and the different solvent fractions obtained from the leaves of *Allophylus abyssinicus.*

4.7 Antimicrobial activity of the crude extract and fractions

Probably the most common cause of healing delays is wound infection. Bacteria prolong the inflammatory phase and interfere with epithelialization, contraction, and collagen deposition. The endotoxins themselves stimulate phagocytosis and release of collagenase, which contributes to collagen degradation and destruction of surrounding, previously normal tissue. Treatment to decrease the bacterial count therefore limits the amount of inflammation and allows closure of the wound (Ethridge *et al*., 2007). Common bacterial skin infections include *E. coli*, *P. aeroginosa*, *S. aureus*, *S. pyogenes* with others (Pattanayaka and Sunitab, 2008). In this study the 80% methanolic extract and the different solvent fractions obtained from the leaves of *A. abyssinicus* were tested for their antibacterial activity against a range of standard and isolated strains which included the Gram-positive bacteria: *S. aureus* (ATCC 25913), *S. pyogenes* (Patient isolate 09/10) and the Gram negative bacteria: *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853). As shown in Table 4.8, all the test substances were active against all the test organisms at different concentration levels. The minimum inhibitory concentration (MIC) of all the test samples with the exception of the chloroform fraction against *S. aureus* was 200 µg/ml. similarly, *E. coli* was inhibited by all the test sample at the same concentration level. The hydroalcoholic extract and the methanolic fraction showed better activity against *S. pyogens,* as indicated by their MIC (200 µg/ml), than the remaining test substances (MIC = 400 µg/ml). The acetone, methanol and aqueous fractions also displayed good activity against *P. aerigunosa*, which is the most common wound causative bacterial strain.

The mean zone of inhibition at 200 µg/ml for the different test substances and the standard antibiotic ciprofloxacin is summarized in Table 4.9. By and large, the methanol fraction was found to be the most active extract against the bacterial strains used in this study followed by the hydroalcoholic extract. The mean zone of inhibition against *S. pyogens* was found to be 17.0 and 18.0 for the 80% methanolic extract and ciprofloxacin, respectively. The methanolic fraction showed comparable activity with that of ciprofloxacin against *P. aeruginosa* as indicated by the mean zone of inhibition. These results appear to suggest that the wound healing effect of the leaves of *A. abyssinicus* is mainly through their antimicrobial effect.

Based on the results obtained from the *in vivo* and *in vitro* wound healing tests performed on the different fractions of *A. abyssinicus*, the methanolic fraction appears to have good wound healing activity. This led to the isolation of compounds responsible for the activity from the methanolic fraction. Separation was done by using preparative TLC and the isolated bands were tested for their *in vitro* and *in vivo* wound healing activity.

Table 4.8. Determination of minimum inhibitory concentration (MIC) of the 80% methanolic extract and the different fractions obtained from the leaves of *Allophylus abyssinicus.*

Bacteria	Test substance	Growth in nutrient agar containing different concentrations of test substances in µg/ml								
		0^*	5	10	25	50	100	200	400	800
S. aureus	80% methanolic extract	+	+	+	+	+	+	-	-	-
	Chloroform fraction	+	+	+	+	+	+	+	-	-
	Acetone fraction	+	+	+	+	+	+	-	-	-
	Methanol fraction	+	+	+	+	+	+	-	-	-
	Aqueous fraction	+	+	+	+	+	+	-	-	-
E. coli	80% methanolic extract	+	+	+	+	+	+	-	-	-
	Chloroform fraction	+	+	+	+	+	+	-	-	-
	Acetone fraction	+	+	+	+	+	+	-	-	-
	Methanol fraction	+	+	+	+	+	+	-	-	-
	Aqueous fraction	+	+	+	+	+	+	-	-	-
S. pyogenes	80% methanolic extract	+	+	+	+	+	+	-	-	-
	Chloroform fraction	+	+	+	+	+	+	+	-	-
	Acetone fraction	+	+	+	+	+	+	+	-	-
	Methanol fraction	+	+	+	+	+	+	-	-	-
	Aqueous fraction	+	+	+	+	+	+	+	-	-
P. aerigunosa	80% methanolic extract	+	+	+	+	+	+	+	-	-
	Chloroform fraction	+	+	+	+	+	+	+	-	-
	Acetone fraction	+	+	+	+	+	+	-	-	-
	Methanol fraction	+	+	+	+	+	+	-	-	-
	Aqueous fraction	+	+	+	+	+	+	-	-	-

NA-not active (0^*-control (DMSO); + growth; - No growth.

Table 4.9. Comparison of zones of inhibition of the 80% methanolic extract and the different fractions obtained from the leaves of *Allophylus abyssinicus* with that of standard antibacterial agent ciprofloxacin at 200 µg/ml.

Bacteria	Test substance	Diameters of zones of inhibition in mm around discs of diameter 6 mm at a concentration of 200 µg/ml	
		Test substance	Ciprofloxacin
S.aureus	80% methanolic extract	13.0	19.5
	Chloroform fraction	NA	19.5
	Acetone fraction	12.0	19.5
	Methanol fraction	13.5	19.5
	Aqueous fraction	12.5	19.5
E.coli	80% methanolic extract	17.0	18.0
	Chloroform fraction	10.5	18.0
	Acetone fraction	12.5	18.0
	Methanol fraction	14.0	18.0
	Aqueous fraction	11.5	18.0
S. pyogenes	80% methanolic extract	15.5	23.0
	Chloroform fraction	NA	23.0
	Acetone fraction	NA	23.0
	Methanol fraction	16.5	23.0
	Aqueous fraction	NA	23.0
P. aerigunosa	80% methanolic extract	NA	16.0
	Chloroform fraction	NA	16.0
	Acetone fraction	7.0	16.0
	Methanol fraction	12.0	16.0
	Aqueous fraction	NA	16.0

NA-not active

4.8 Thin layer chromatography

Repeated TLC analysis of the 80% methanolic extract of the leaves of *A. abyssinicus* using solvent system butanol: acetic acid: water (4:1:5), upper phase, afforded three major spots with R_f values of 0.36, 0.51 and 0.60. As shown in figure 4.4, The spots were designated as AL-1, AL-2 and AL-3, respectively.

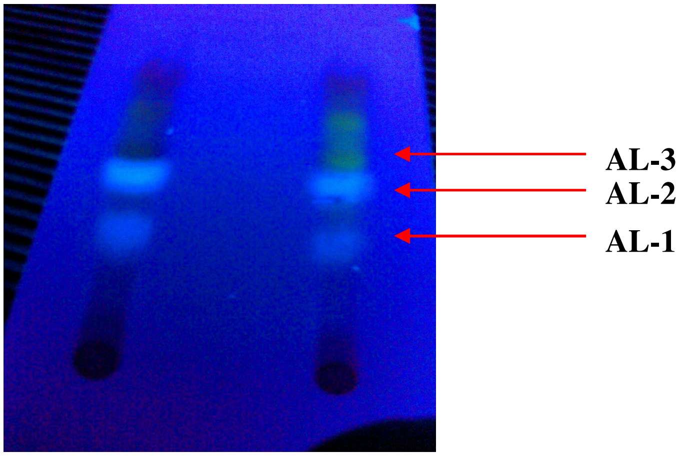


Figure. 4.4: Analytical TLC of the methanolic fraction obtained from the leaf extract of *Allophylus abyssinicus* using solvent system butanol: acetic acid: water (4:1:5), upper layer.

4.9 Wound healing activity of isolated substances

4.9.1 Excision wound model

Table 4.10 shows that all the isolated test substances formulated at a concentration of 2% in simple ointment possess significant reduction in wound area ($P < 0.001$) as compared with the negative control i.e. simple ointment treated group. The positive control, 0.2% nitrofurazone, shows significant wound contraction as compared with AL-1 and AL-2 ($P < 0.001$) at all days of treatment. AL-3 shows statistically comparable wound contraction and period of epithelization with that of nitrofurazone on the later periods of treatment.

4.9.2 Incision wound model

The isolated substances were tested at a dose of 25 and 50 mg/kg for this model. As summarized in Table 4.11, all the isolated substances at a dose of 50 mg/kg increased tensile strength as compared with the control group that were treated with 1% CMC ($P < 0.001$). AL-2 and AL-3 also showed statistically significant increase in tensile strength as compared with the control group at a dose level of 25 mg/kg. However the effect of AL-3 (25 mg/kg) was comparable to that of AL-2 (50 mg/kg). This indicates that AL-3 has a major share for the wound healing effect of *A. abyssinicus.*

Table 4.10. Effect of topical application of ointments containing the isolated substances from the methanoic fraction of the leaves of *Allophylus abyssinicus* on wound contraction of excision wound model.

Group	**Day 0**	**4th day**	**8th day**	**12th day**	**16th day**	**Period of epi. (days)**
AL-1 (2%)	516.00±7.73	394.34±6.71 (23.58)***	265.43±5.81 (48.56)***	154.87±3.66 (69.99)***	67.54±1.93 (86.91)***	20.78±0.45***
AL-2 (2%)	495.27±5.85	358.45±5.44 (27.63)***	203.81±4.32 (58.85)***	98.00±3.26 (80.21)***	38.09±1.09 (92.31)***	17.87±0.2***
AL-3 (2%)	512.98±6.12	340.12±6.09 (33.70)***	176.34±4.20 (65.62)***	76.32±3.18 (85.12)***	15.65±1.29 (96.95)***	15.95±0.45***
NF (0.2%)	516.43 ±4.86	325.72±4.11 (36.93)***	189.35±3.09 (63.33)***	76.12±2.44 (85.26)***	18.64±1.08 (96.39)***	16.32±0.66***
SO	526.32±3.43	435.18±2.91 (17.32)	345.23±2.34 (34.41)	250.75±1.38 (52.36)	178.09±1.08 (66.16)	23.34±0.71

Values are expressed as mean ± S.E.M (n = 6), percentage of contractions are in parenthesis, $^{***}P < 0.001$ vs. control, SO = simple ointment, Period of epi. (days) = period of epithelization.

4.9.3 Dead space wound model

Table 4.12. summarizes the increase in the synthesis of hydroxyproline for the different substances isolated from the leaves of *A. abyssinicus*. AL-2 and AL-3 at a dose level of 50 mg/kg increase hydroxyproline synthesis significantly with P value less than 0. 001 as compared with the control. AL-1 at the respective dose level did not show statistically significant increase in the level of hydroxyl proline as compared with the control group.

Table 4.11. Effect of oral administration of the different isolated substances from the methanoic fraction of the leaves of *Allophylus abyssinicus* on tensile strength of the skin having incision wound.

Group	**Average tensile strength**
AL-1 (50 mg/kg)	294.46±3.88***
AL-1 (25 mg/kg)	273.63±10.46
AL-2 (50 mg/kg)	301.38±6.78***
AL-2 (25 mg/kg)	218.50±4.97***
AL-3 (5 0mg/kg)	386.32±7.04***
AL-3 (25mg/kg)	289.47±8.37***
1% CMC	267.64±6.28

Values are expressed as mean ± S.E.M (n = 6). Dose levels are in parenthesis, $^{***}P < 0.001$ vs. control.

Table 4.12. Effect of oral administration of the different isolated substances from the methanoic fraction of the leaves of *Allophylus abyssinicus* on dead space wound model.

Group	**Hydroxyproline (mg/g tissue)**
1% CMC	21.53 ± 4.23
AL-1 (50 mg/kg)	27.44±5.50
AL-1 (25 mg/kg)	23.45±6.36
AL-2 (50 mg/kg)	36.76±6.12***
AL-2 (25 mg/kg)	22.48±3.59
AL-3 (50 mg/kg)	40.37±3.76***
AL-3 (25 mg/kg)	29.37±2.69

Values are expressed as mean ± S.E.M (n = 6). Dose levels are in parenthesis, $^{***}P < 0.001$ vs. control.

4.10 Antiinflammatory activity of isolated substances

The result of the antiinflammatory test carried out on the isolated substances from the methanolic fraction of *A. abyssinicus* showed that at the tested doses AL-3 has comparable activity with that of indomethacin (Figure 4.5). Table 4.13 also summarizes that AL-3 (50 mg/kg) and indomethacin (10 mg/kg) possess potent antiinflammatory activity at all phases of inflammation with P value less than 0.001 as compared with the control group. On the other hand AL-2 displayed significant activity in the second, third and fourth hrs of inflammation. In the third and fourth hrs, AL-2 exhibited comparable antiinflammatory activity to that of the positive control. Indicating that its mechanism of action is mainly due to blockage of prostaglandin-like substances.

Table 4.13: Inhibition of carageenan induced mice paw oedema by the test substances isolated from the methanolic fraction of *Allophylus abyssinicus*.

Treatment	Dose (mg/kg)	% ± SEM (n = 6)			
		1hr	2hrs	3hrs	4hrs
AL-1	50	2.13 ± 0.92	13.16 ± 6.72	6.98 ± 2.02	12.12 ± 3.31
AL-2	50	-2.13 ± 3.01***	34.21 ± 3.86***	55.81 ± 5.92***	45.45 ± 2.03
AL-3	50	51.06 ± 4.72***	60.53 ± 1.84***	58.14 ± 2.61***	63.63 ± 6.52***
Indomethacin	10	55.32 ± 2.54***	65.79± 7.0***	60.47± 0.75***	57.58± 3.91***

Values are expressed as mean ± S.E.M (n = 6), $^{***}P < 0.001$

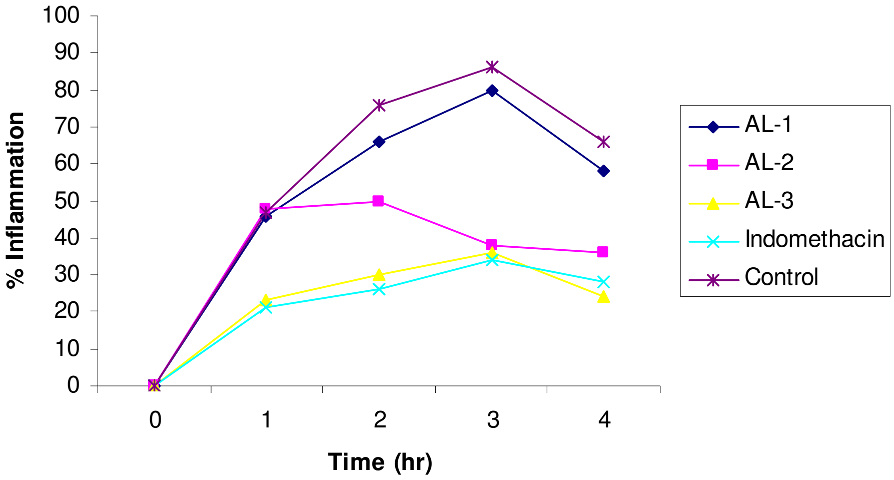


Figure 4.5: Effects of oral administration of the substances isolated from the methanolic fraction of *Allophylus abyssinicus* in comparison with that of indomethacin on carageenan induced paw oedema.

4.11 Antioxidant activity of isolated substances

All the test substances isolated from the methanolic fraction of *A. abyssinicus* were found to scavenge the stable radical, DPPH, significantly in a dose dependant manner. AL-3 showed the highest radical scavenging effect with an IC_{50} value 14.19 µg/ml, while the positive control ascorbic acid gave an IC_{50} value of 5.18 µg/ml in the same experiment. These results suggest that the wound healing activity of *A. abyssinicus* observed in excision, incision and dead space models could be partly due to its antioxidant activity. Figure 4.6 shows the percentage scavenged versus concentration curve of the isolated substances in comparison with that of ascorbic acid. Similarly the IC_{50} values of isolated substances are summarized in Table 4.14. and the positive control ascorbic acid.

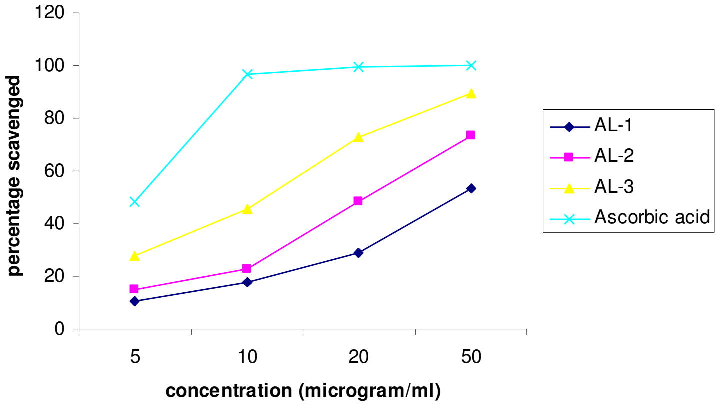


Figure 4.6: DPPH radical scavenging activities of the different test substances isolated from the methanoic fraction of the leaves of *Allophylus abyssinicus*.

Table 4.14: DPPH free radical scavenging activity of the different isolated substances from the methanoic fraction of the leaves of *Allophylus abyssinicus.*

Test substance	AL-1	AL-2	AL-3	Ascorbic acid
IC 50 (µg/ml)	42.27	29.25	14.19	5.18

4.12 Antimicrobial activity of isolated substances

As shown in Table 4.15 all the substances isolated from the methanolic fraction of *A. abyssinicus* displayed varying activity against the test organisms used in this study. In particular, AL-3 exhibited potent activity against *P. aerigunosa* which is known to be the most common cause of wound infection. Table 4.16 summarizes the zones of inhibition for the isolated substances at 200 µg/ml in comparison with standard antibiotic, ciprofloxacin. Inspection of the overall antimicrobial data of the isolated substances revealed that AL-3 has a promising activity profile that was comparable with that of ciprofloxacin at the tested dose. The results further confirm that the wound healing effect of *A. abyssinicus* is associated partly with its antimicrobial activity.

Table 4.15. Determination of minimum inhibitory concentrations of the different isolated isolated substances from the methanoic fraction of the leaves of *Allophylus abyssinicus.*

Test organism	Test substance	Growth in nutrient agar containing different concentrations of test substances in µg/ml								
		0*	5	10	25	50	100	200	400	800
S.aureus	AL-1	+	+	+	+	+	+	-	-	-
	AL-2	+	+	+	+	+	-	-	-	-
	AL-3	+	+	+	+	+	-	-	-	-
E.coli	AL-1	+	+	+	+	+	-	-	-	-
	AL-2	+	+	+	+	-	-	-	-	-
	AL-3	+	+	+	+	-	-	-	-	-
S. pyogenes	AL-1	+	+	+	+	-	-	-	-	-
	AL-2	+	+	+	-	-	-	-	-	-
	AL-3	+	+	+	-	-	-	-	-	-
P. aerigunosa	AL-1	+	+	+	+	+	+	+	+	-
	AL-2	+	+	+	+	+	+	+	+	-
	AL-3	+	+	+	+	+	-	-	-	-

+ Growth; - No growth

Table 4.16. Comparison of zones of inhibition of different isolated substances at 200µg/ml from the methanolic fraction of the leaves of *Allophylus abyssinicus* with that of standared anti-bacterial agent ciprofloxacin.

Test organism	Test substance	Diameters of zones of inhibition in mm around discs of diameter 6mm at a concentration of 200 µg/ml	
		Test substance	**Ciprofloxacin**
S.aureus	AL-1	11.0	19.5
	AL-2	13.5	19.5
	AL-3	14.5	19.5
E.coli	AL-1	13.0	18.0
	AL-2	14.0	18.0
	AL-3	16.0	18.0
	AL-1	12.5	23.0
S, pyogenes	AL-2	16.0	23.0
	AL-3	19.5	23.0
P. aerigunosa	AL-3	12.5	16.0

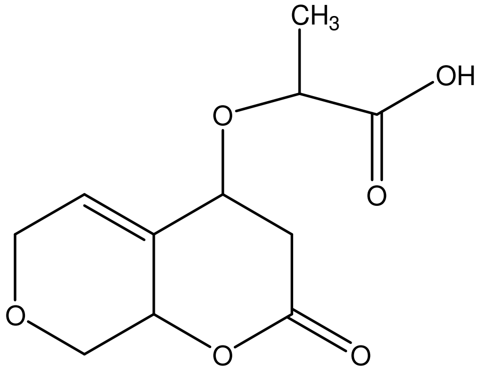
4.13 Structural elucidation

AL-3 was appeared yellow under UV light of 366 nm. The structure of this compound was partially established on the basis of the ^{1}H and ^{13}C NMR spectral data as shown in Table 4.17. Analysis of the ^{1}H NMR spectrum indicated the presence of a proton at δ 6.38 assignable to the olefinic proton and three oxymethines at δ 3.70, 4.08 and 5.27 ppm. Methyl protons appeared at δ 1.18 and a signal at δ 2.47 was assigned for methylene protons. The signals at δ 3.66 and 4.08 were assigned for Oxymethylene protons. The ^{13}C NMR with DEPT data revealed one methyl (δ 24.8), three methylene (two oxymethylenes), four methines (three oxymethines and one olefinic methine) and three quaternary carbons including two carbonyl carbons.

Table 4.17: ^{1}H and ^{13}C spectral data of AL-3.

Assignments	^{1}H (δ, ppm)	^{13}C (δ, ppm)
1	-	-
2	-	173.4
3	2.47	32.9
4	4.08	66.5
5	6.38	132.9
6	4.08	72.7
7	3.68	60.7
8	5.27	67.4
9	-	135.2
10	3.70	72.6
11	-	177.6
12	1.18	24.8

From the data presented above the structure of AL-3 was tentatively deduced as 2-(2,3,4,6,8,8a-hexahydro-oxopyrano[3,4-b] pyran-4yl oxy) propanoic acid **(13)**.



13

Figure 4.7: Proposed structure of AL-3.

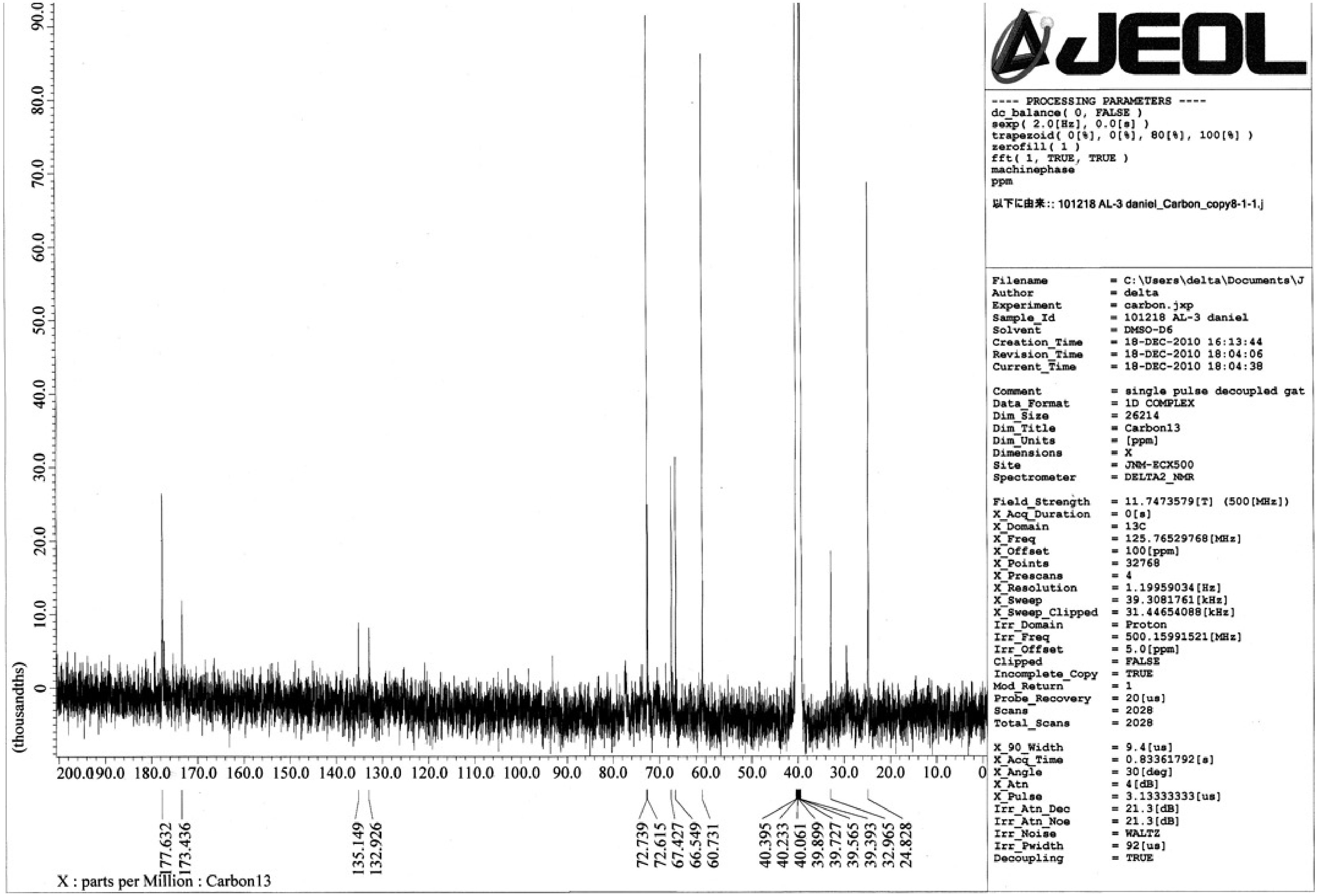
5 CONCLUSIONS

- ✓ The present study supports the traditional use of *A. abyssinicus* leaves for the treatment of wounds.
- ✓ The wound healing activity of the plant could be due to its antimicrobial, antiinflammatory, antioxidant or improved collagen synthesis or a combination of these.
- ✓ The wound healing activity of *A. abyssinicus* resides mainly on the methanolic fraction.
- ✓ The compound isolated from the leaves of *A. abyssinicus* tentatively identified as 2-(2,3,4,6,8,8a-hexahydro-oxopyrano[3,4-b] pyran-4yl oxy) propanoic acid appears to be the major contributor for wound healing activity of *A. abyssinicus*.

6 SUGGESTIONS FOR FURTHER WORK

- ✓ It is suggested that the structures of the main compounds isolated from the active fractions be elucidated; the activity of minor compounds present in the active fraction be tested; complete acute, sub-chronic and chronic toxicity studies of isolated compounds be carried out.

Appendix I: ^{13}C spectrum of AL-3



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trapezoid( 0[%], 0[%], 80[%], 100[%] )
zerofill( 1 )
fft( 1, TRUE, TRUE )
machinephase
ppm

以下に由来:: 101218 AL-3 daniel_Carbon_copy8-1-1.j
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Author              = delta
Experiment          = carbon.jxp
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Solvent             = DMSO-D6
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Current_Time        = 18-DEC-2010 18:04:38

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Spectrometer        = DELTA2_NMR

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X_Points            = 32768
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X_Sweep_Clipped     = 31.44654088[kHz]
Irr_Domain          = Proton
Irr_Freq            = 500.15991521[MHz]
Irr_Offset          = 5.0[ppm]
Clipped             = FALSE
Incomplete_Copy     = TRUE
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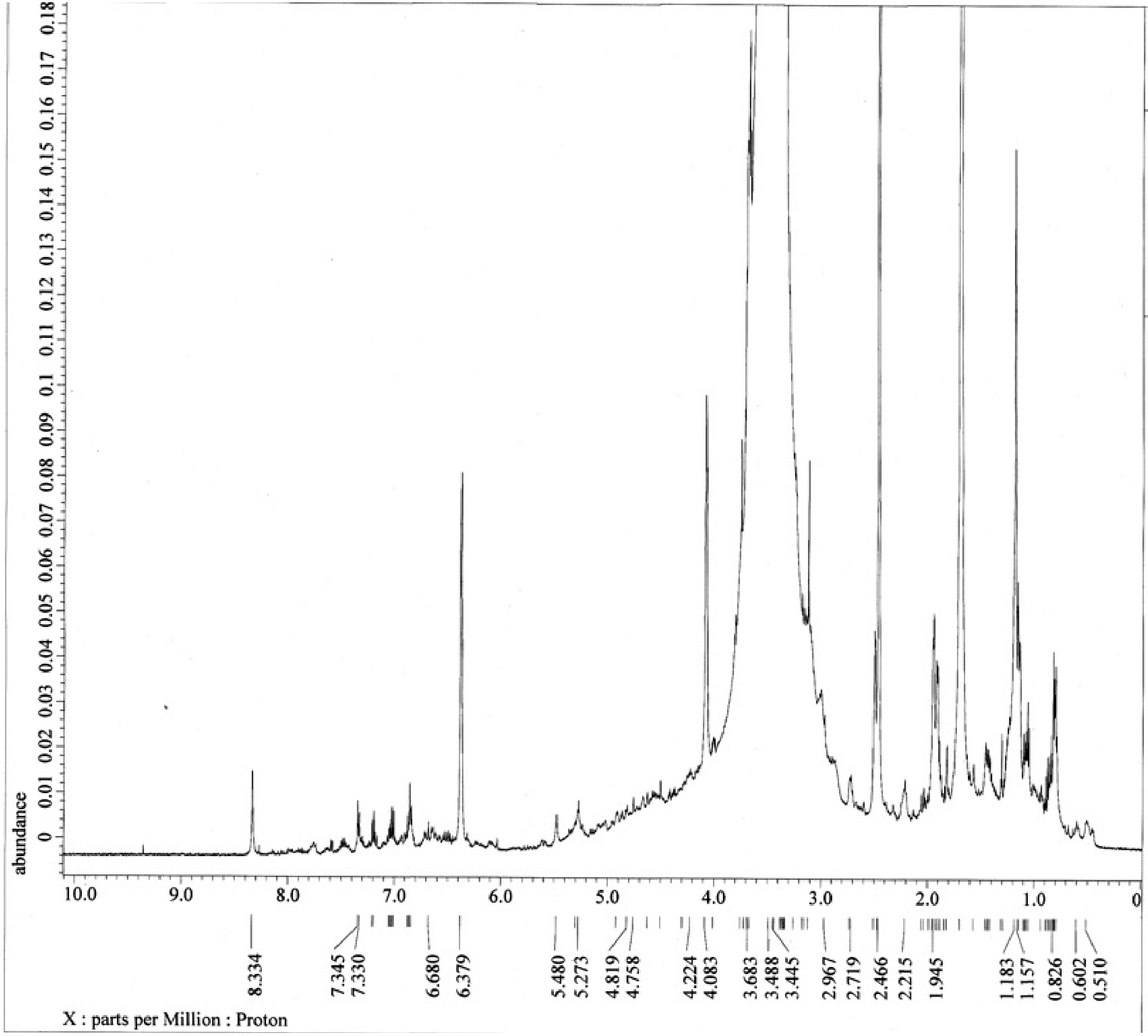
Appendix II: ^{1}H spectrum of AL-3



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ppm

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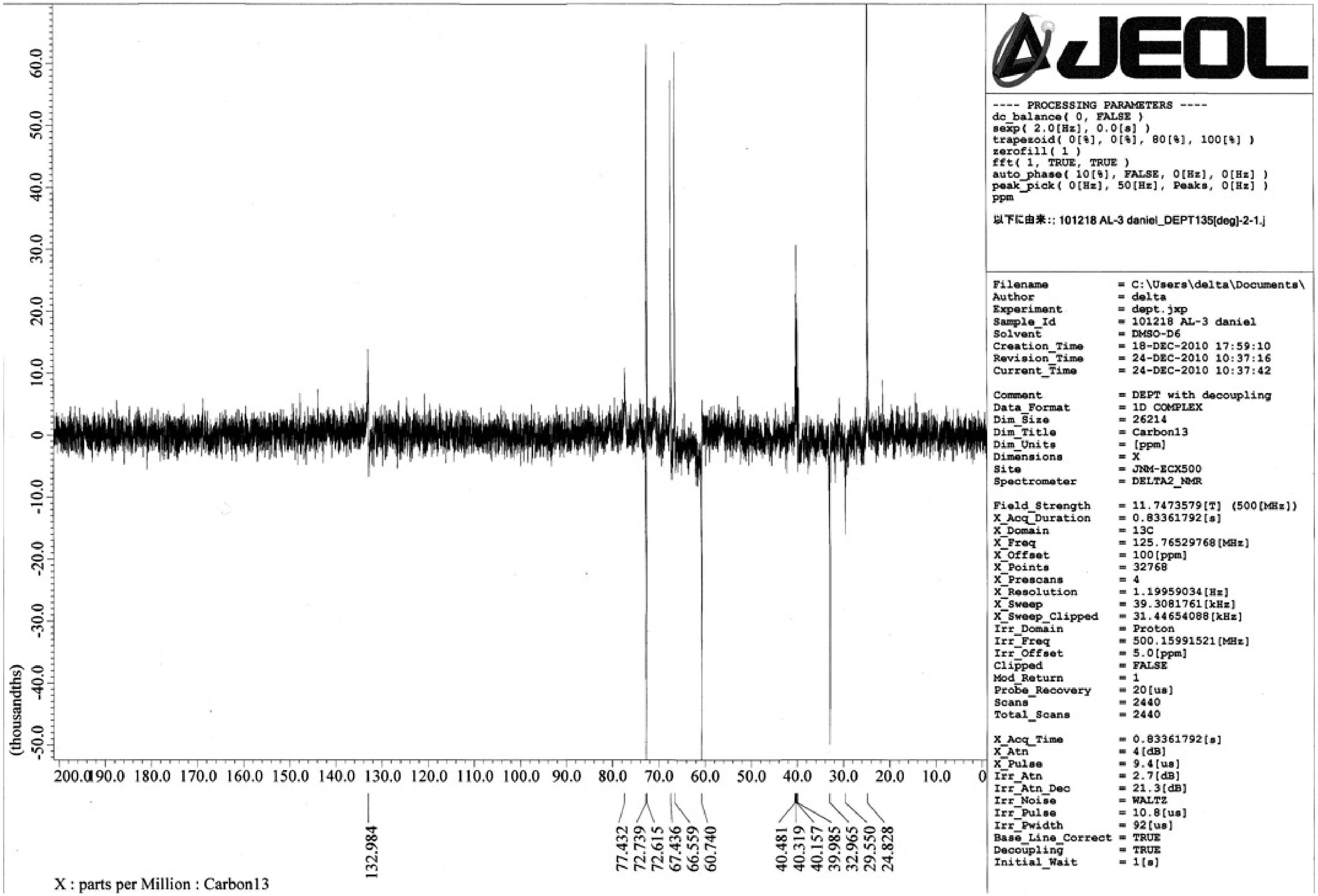
Appendix III: DEPT ^{135} spectrum of AL-3



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---- PROCESSING PARAMETERS ----
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sexp( 2.0[Hz], 0.0[s] )
trapezoid( 0[%], 0[%], 80[%], 100[%] )
zerofill( 1 )
fft( 1, TRUE, TRUE )
auto_phase( 10[%], FALSE, 0[Hz], 0[Hz] )
peak_pick( 0[Hz], 50[Hz], Peaks, 0[Hz] )
ppm

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Spectrometer        = DELTA2_NMR

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X_Points            = 32768
X_Prescans          = 4
X_Resolution        = 1.19959034[Hz]
X_Sweep             = 39.3081761[kHz]
X_Sweep_Clipped     = 31.44654088[kHz]
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Decoupling          = TRUE
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REFERENCES

Abu-Al-Basal MA (2010). Healing potential of *Rosmarinus oficinalis* L. on full-thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/c mice. *J. Ethnopharmacol.* 131:2:443-450.

Ahmad NS, Mohd SA, Ahmad AY (2005). The Effects of *Carica papaya* Linn. latex on the healing of burn wounds in rats. *MJHS.* 3:2: 39-47.

Annan K, Dickson R (2008). Evaluation of wound healing actions of *Hoslundia opposita* vahl, *Anthocleista nobilis* g. Don. and *Balanites aegyptiaca. J Sci and Tech.* (Ghana) 28:26-38.

Ansel HC (1985). Introduction to pharmaceutical dosage forms, 4th ed., Lea and Febiger, Philadelphia. PP 299-301.

Anthony JA, Oluseyi MA, Adeolu AA, Margaret OS (2009). Anti-inflammatory and analgesic activity of the methanol extract of *Malva parviflora Linn* (*Malvaceae*) in rats. *Afr J Biotechnol.* 9:8:1225-1229.

Arnold M, Barbul A (2006). Nutrition and wound healing. *Plast Reconstr Surg.* 117:S7:42S–58S.

Azeez S, Amudhan S, Adiga S, Rao N, Rao N, Udupa L (2007): Wound healing profile of *Areca catechu* extracts on different wound models in wistar rats. *KMJ.* 39:48-52.

Barrientos S, Stojadinovic O, Golinko M, Brem H, Tomic-Canic M (2010). Growth factors and cytokines in wound healing. *Wound Repair Regen.* 16:5:585–601.

Barua C, Talukdar A, Begum S, Sarma D, Pathak D, Barua A, Bora R, (2009). Wound healing activity of methanolic extract of leaves of *Alternanthera brasiliana* kuntz using *in vivo* and *in vitro* model. *Indian J Exp Biol.* 47:1001-1005.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. *Nature* 181: 1199-1200.

Burgalassi S, Raimondi L, Pirisino R, Banchelli G, Boldrini E, Saettone MF (2000). Effect of xyloglucan (tamarind seed polysaccharide) on conjunctival cell adhesion to laminin and on corneal epithelium wound healing. *Eur J Ophthalmol.* 10: 71-76.

Burgess C (2008). Topical vitamins. *J Drugs Dermatol.* 7:S7:S2–S6.

Calvin M (1998). Cutaneous wound repair. *Wounds* 10:12–15.

Campos AC, Groth AK, Branco AB (2008). Assessment and nutritional aspects of wound healing. *Curr Opin Clin Nutr Metab Care* 11:281–288.

Chah KF, Eze CA, Emuelosi CE, Esimone CO (2006). Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. *J Ethnopharmacol.* 104: 164 -167.

Choi SW, Son BW, Son YS, Park YI, Lee SK, Chung MH (2001). The wound-healing effect of a glycoprotein fraction isolated from *Aloe vera*. *Brit J Dermatol.* 145: 535-545.

Chung AS, Lee J, Ferrara N (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer* 10:05-514**.**

David M (2008). Regeneration of human scar tissue with topical iodine. *Thyroid Sci.* 3: 1-9.

Davis GE, Senger DR (2005). Biosynthesis, Remodeling, and Functions During Vascular Morphogenesis and Neovessel Stabilization. *Circ Res.* 97:1093-1107.

Dealey C (2005). The care of wounds: a guide for nurses, 3rd ed., Blackwell Publishing Ltd., UK, PP 9-10.

Delporte C, Backhouse N, Negrete R, Salinas P, Rivas P, Cassels BK, San Feliciano A (1998). Antipyretic, hypothermic and anti-inflammatory activities and metabolites from *Solanum lingustrinum* Lood. *Phytother Res.*12:118–122.

Deshmukh PT, Fernandes J, Atul A, Toppo E (2009). Wound healing activity of *Calotropis gigantea* root bark in rats. *J. Ethnopharmacol.* 125:178–181.

Diallo D, Sogn C, Samake FB, Paulsen BS, Michaelsen TE, Keita A (2002). Wound healing plants in Mali, the Bamako region. An ethnobotanical survey and complement fixation of water extracts from selected plants. *Pharm Biol.* 40: 117–128.

Diegelmann RF, Evans MC (2004). Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci*. 9:283-289.

DiPietro LA, Burns AL (2003). Wound Healing: Methods and Protocols. 10th ed, Humana press inc, New Jersey. PP 1-20.

Dongmo AB, Kamanyi A, Dzikouk G, Nkeh CAB, Tan PV, Nguelefack T, Nole T, Bopelet M, Wagner H (2003). Anti-inflammatory and analgesic properties of the stem bark extract of *Mitragyna ciliate* (Rubiaceae) Aubr´ev. & Pellegr. *J. Ethnopharmacol.* 84:17– 21.

Draize JH (1959). Dermal toxicity, Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. Austin, Texas, The staff of the division of Pharmacology of the Federal Food and Drug Administration. PP 46-48.

Eaglstein WH (2001). Moist Wound Healing with Occlusive Dressings: A Clinical Focus. *Dermatol Surg*. 27:2: 175–182.

Eckes B, Aumailley M, Krieg T (1996). Collagens and the reestablishment of dermal integrity, in Clark RAF. The Molecular and Cellular Biology of Wound Repair, 2 nd ed., Plenum Press, New York. PP 493– 512.

Eming SA, Sabine A, Thomas K, Jeffrey MD (2007). Inflammation in wound repair: Molecular and Cellular Mechanisms. *J Invest Dermatol.* 127:514-525.

Esimone C, Ibezim E, Chah K (2005). The wound healing effect of herbal ointments formulated with *Napoleona imperialis*. *J Pharmaceut Allied Sci*. 3:294 -299.

Esimone C, Nworu C, Jackson C (2009). Cutaneous wound healing activity of herbal ointment containing the leaf extract of *Jatropha curcas L. Int. j. appl. res. nat. prod.* 1:1-4.

Ethridge RT, Leong M, Phillips LG (2007). Wound Healing. In Townsend CM: Sabiston Textbook of Surgery. 18th ed, Elsevier. Saunders, Philadelphia PP 1-37.

Ferguson MW, O'Kane S (2004). Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos Trans R Soc Lond B Biol Sci*. 359:1445:839-850.

Franklin IJ, Dawson PM (2008). Pocket Essentials of Clinical Surgery, saunders Ltd, 1st ed., China. PP 355.

Franz MG (2006). Complications of wound healing. In: Mulholland MW, Doherty GM, eds. Complications in Surgery. 1st ed, Lippincott Williams & Wilkins, Philadelphia. PP 111.

Fu SC, Hui CW, Li LC, Cheuk YC, Qin L, Gao J, Chan KM (2005). Total flavones of *Hippophae rhamnoides* promote early restoration of ultimate stress of healing patellar tendon in a rat model. *Med Eng Phy.* 27: 313-321.

Garrett B (1998). Re-epithelialisation. *J Wound Care* 7:358–359.

Gfeller W, Kobel W, Seifert G (1985. Overview of animal test methods from skin irritation. *Food Chem Toxicol.* 23:2: 165-168.

Ghasemi A, koohpayeh A, Karimi I (2010). The wound healing activity of flower extracts of *Punica granatum* and *Achillea kellalensis* in wistar rats. *Acta Pol. Pharm. Drug Res.* 67:107-110.

Ghosh K, Ren XD, Shu XZ, Prestwich GD, and Clark RAF (2006). Fibronectin Functional Domains Coupled to Hyaluronan Stimulate Adult Human Dermal Fibroblast Responses Critical for Wound Healing. *Tissue Eng.* 12: 3:601-13.

Gosain A, DiPietro LA *(*2004*).* Aging and wound healing. *World J Surg.* 28*:*321–326.

Guo S, Dipietro LA (2010). Factors affecting wound healing. *JDR.* 89:3:219-229.

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008). Wound repair and regeneration. *Nature* 453**:** 314-21.

Hinz B, Gabbiani G (2003). Mechanisms of force generation and transmission by Myofibroblasts. *Curr Opin Biotechnol.* 14:5: 538-546.

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA (2004). Vascular Endothelial Growth Factor and Angiogenesis. *Pharmacol Rev.* 56: 4:549-580.

Houghton P, Hylands P, Mensah A, Hensel A, Deters A (2005). *In vitro* tests and ethnopharmacological investigations: Wound healing as an example: *J. Ethnopharmacol.* 100: 100–107.

Jagetia GC, Rajanikant GK (2004). Role of curcumin, a naturally occurring phenolic compound of turmeric in accelerating the repair of excision wound, in mice whole body exposed to various doses of gamma-radiation. *J Surg Res.* 120: 127-138.

Janis JE, Kwon RK, Lalonde DH (2010). A practical guide to wound healing. *Plast Reconstr Surg* 125:6:230e-44e.

Jayasri MA, Mathew L and Radha A. (2009). A report on the antioxidant activity of leaves and rihizomes of *Costus pictus* D.Don. *Int. J. Integ. Biol.* 5:1, 20-26.

Junkera JPE, Kratza C, Tollbäckb A, Kratza G (2008). Mechanical tension stimulates the transdifferentiation of fibroblasts into myofibroblasts in human burn scars. *Burns* 34:7:942-946.

Kanczler JM, Oreffo ROC (2008). Osteogenesis And Angiogenesis: The Potential For Engineering Bone**.** *Eur Cell Mater.* 15: 100-114.

Karen M, Lillian BN (2006). Emerging new drugs for wound repair. *Expert Opin Emerg Drugs* 11: 23-37.

Karodi R, Jadhav M, Rub R, Bafna A (2009). Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. in mice. *Int. j. appl. res. nat. prod.* 2: 12-18.

Keller U, Kümin A, Braun S, Werner S (2006). Reactive Oxygen Species and Their Detoxification in Healing Skin Wounds. *J Invest Derm Symp.* 11:106–111.

Kerr J (2002). The use of essential oils in healing wounds. *Int J Aromather.* 12: 202 – 206.

Keylock KT, Vieira VJ, Wallig MA, DiPietro LA, Schrementi M, Woods JA (2008). Exercise accelerates cutaneous wound healing and decreases wound inflammation in aged mice. *Am J Physiol Regul Integr Comp Physiol.* 294*:*R179–R184.

Khanna S, Venojarvi M, Roy S, Sharma N, Trikha P, Bagchi D, Bagchi M, Sen CK (2002). Dermal wound healing properties of redox-active grape seed proanthocyanidins. *Free Rad Biol Med.* 15: 1089-1096.

Kjær M (2004). Role of Extracellular Matrix in Adaptation of Tendon and Skeletal Muscle to Mechanical Loading. *Physiol Rev*. 84: 2:649-698.

Kose O, & Waseem A (2008). Keloids and hypertrophic scars: are they two different sides of the same coin? *Dermatol. Surg.* 34:336 - 346.

Krishnan P (2006). The scientific study of herbal wound healing therapies: Current state of play. *Curr Anaes Crit Care.* 17: 21–27.

Kumar A, Chomwal R, Kumar P, Sawal R (2009). Anti inflammatory and wound healing activity of *Curcuma aromatica* salisb extract and its formulation. *J Chem Pharm Res.* 1:1: 304-310.

Kumar B, Vijayakumar M, Govindarajan R, Pushpangadan P (2007). Ethnopharmacological approaches to wound healing – Exploring medicinal plants of India, *J. Ethnopharmacol.* 114: 103-113.

Kunwar PM, Bussmann RW (2008). Ethnobotany in the Nepal Himalaya. *J Ethnobiol Ethnomedicine* 4:24–29.

Kusano A, Seyama Y, Nagai M, Shibano M, Kusano G (2001). Effects of fukinolic acid and cimicifugic acids from *Cimicifuga species* on collagenolytic activity. *Biol Pharm Bull.* 24: 1198-1201.

Lalitha KG, Sethuraman MG (2010). Anti-inflammatory activity of roots of *Ecbolium viride* (Forsk) Merrill. *J. Ethnopharmacol.* 128: 248–250.

Lee PHA, Trowbridge JM, Taylor KR, Morhenn VB, Gallo RL (2004). Dermatan Sulfate Proteoglycan and Glycosaminoglycan Synthesis Is Induced in Fibroblasts by Transfer to a Three-dimensional Extracellular Environment. *J Biol Chem*. 279: 47: 48640–48646.

Letts MG, Villegas LF, Marcalo A, Vaisberg AJ, Hammond GB (2006). *In vivo* wound healing activity of olanolic acid derived from the acid hydrolysis of *Andredera diffusa*. *J Nat Prod.* 69: 978-979.

Lorenz HP, Longaker MT (2008). Wounds: Biology, Pathology, and Management. *Surgery* 1:191-208.

Malviya N, Jain S (2009). Wound healing activity of aqueous extract of *Radix paeoniae* root. *Acta Pol. Pharm. Drug Res*. 66: 543-547.

Mantle D, Gok MA, Lennard TWJ (2001). Adverse and beneficial effects of plant extracts on skin and skin orders. *Adverse Drug React Toxicol Rev.* 20:89–103.

Manuskiatti W, Fitzpatrick RE (2002). Treatment response of keloidal and hypertrophic sternotomy scars: comparison among intralesional corticosteroid, 5-fluorouracil, and 585-nm flash lamp-pumped pulsed-dye laser treatments. *Arch. Dermatol*. 138:1149-1155.

Mariggio MA, Cassana A, Vinella A, Vincenti A, Fumarulo R, Lo Muzio L, Maiorano E, Ribatti D, Favia G (2009). Enhancement of fibroblast proliferation, collagen biosynthesis and production of growth factors as a result of combining sodium hayaluronate aminoacids. *Int J Immunopathol Pharmacol.* 22:2:485-492.

Mathieu D, Linke JC, Wattel F (2006). Non-healing wounds. In: Mathieu DE, Handbook on hyperbaric medicine. 1st ed, Springer, Netherlands. PP 401–427.

McDougall S, Dallon J, Sherratt J, Maini P (2006). Fibroblast migration and collagen deposition during dermal wound healing: mathematical modelling and clinical implications. *Philos Transact A Math Phys Eng Sci.* 364:1843:1385-1405.

Meehan M, O'Hara L, Morrison YM (1999). Report on the prevalence of skin ulcers in a home health agency population. *Adv Wound Care* 9:459-67.

Mehibub M, Chowdhury (2009). Dermatological Pharmacology. *Burn* 37: 232-234

Melkonian G, Munoz N, Chung J, Tong C, Marr R, TALBOT P (2002). Capillary Plexus Development in the Day Five to Day Six Chick Chorioallantoic Membrane Is Inhibited by Cytochalasin D and Suramin. *J. Exp. Zool.* 292:3:241–254.

Menke NB, Diegelmann RF (2006). Biochemical Pathways of Wound Healing: Implications for Development of Disease-Specific Diagnostics. *Adv Clin Chem.* 41:167-187.

Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF (2007). Impaired wound healing. *Clin Dermatol.* 25:19–25.

Mole DR, Ratcliffe PJ (2008). Cellular oxygen sensing in health and disease. *Clin Nephrol* 23:5:681-694.

Molnar JA (2007). Overview of Nutrition and Wound Healing. In Nutrition and Wound Healing. CRC Press, 1st ed. New York. PP 1-14.

Molyneux P. (2004) The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol.* 26:2:211-219.

Mrityunjoy M, Naira N, Jagadish K, Mohammed A (2007). Evaluation of *Tectona Granddis* leaves for wound healing activity. *Pak J Pharm Sci.* 20:2:120-124.

Nagori BP, Solanki R (2011). Role of medicinal plants in wound healing. *Res. J. Med. Plant* 5:392-405.

Nalwaya N, Pokharna1 G, Deb L, Kumar N, (2009). Wound healing activity of latex of *Calotropis gigantean. Int J Pharm Pharm Sci.* 1: 176-181.

Nayak BS, Kanhai J, Milne DM, Swanston WH, Mayers S, Mathew Eversley, Rao AVC (2010). Investigation of the Wound Healing Activity of *Carapa guianensis* L. (Meliaceae) Bark Extract in Rats Using Excision, Incision, and Dead Space Wound Models. *J Med Food* 13:5:1141-1146.

Nayak BS, Pereira LMP (2006). *Catharanthus roseus* flower extract has wound-healing activity in Sprague Dawley rats. *BMC Complement Altern Med* 6:41: doi:10.1186/1472-6882-6-41.

Neuman RE, Logan MA (1950). The determination of hydroxyproline. *J Biol Chem.* 184:299–30.

Okoli C, Ezike A, Akah A, Udegbunam S, Okoye T, Mbanu T, Ugwu E (2009). Studies on wound healing and antiulcer activities of extract of aerial parts of *Phyllanthus niruri* L. (Euphorbiaceae): *Am. J. Pharm. & Toxicol*. 4: 118-126.

Palpandi C, Ramasamy P, Rajinikanth T, Vairamani S, Shanmugam A (2010). Extraction of Collagen from Mangrove Archeaogastropod Nerita (Dostia) crepidularia Lamarck. *AEJSR.* 5:1: 23-30.

Paschapur M, Patil M, Kumar R, Patil S (2009). Evaluation of aqueous extract of leaves of *Ocimum kilimandscharicum* on wound healing activity in albino wistar rats. *International Journal of Pharm. Tech. Res.*1: 544-550.

Pattanayaka SP, Sunitab P (2008). Wound healing, antimicrobial and antioxidant potential of *Dendrophthoe falcata* (L.f) Ettingsh. *J. Ethnopharmacol.* 120: 241–247.

Phan TT, Wang L, See P, Grayer RJ, Chan SY, Lee ST (2001). Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous. *Biol Pharm Bull.* 24: 1373-1379.

Pieper B, Templin TN, Dobal M, Jacox A (1999). Wound prevalence, types, and treatments in home care. *Adv Wound Care* 12:117-26.

Principe P (2005). Monetising the pharmacological benefits of plants. US Environmental protection Agency, Washington D.C. PP. 1991.

Raina R, Prawez S, Verma PK and Pankaj NK (2008). Medicinal Plants and their Role in Wound Healing. *VetScan* 3:1:1-25.

Reddy BS, Reddy RKK, Naidu VGM, Madhusudhana K, Agwane SB, Ramakrishna S, Diwan PV (2008). Evaluation of antimicrobial, antioxidant and wound healing potentials of *Holoptelea integrifolia*. *J Ethnopharmacol.* 115:249-256.

Reddy JS, Rao PR, Reddy MS (2002). Wound healing effects of *Heliotropium indicum*, *Plumbago zeylanicum* and *Acalypha indica* in rats. *J. Ethnopharmacol.* 79: 249–251.

Robert F, Diegelmann, Melissa CE (2004). Wound healing: An overview of acute, fibrotic and delayed healing. *Front Biosci* 9: 283-289.

Robinson KM and Perkins AM (2002). A strategy for skin irritation testing. *American J Contact Dermatitis* 13:1: 21-29.

Robles DT, Berg D (2007). Abnormal wound healing: keloids. *Clin Dermatol.* 25:26–32.

Robson MC, Steed DL, Franz MG (2001). Wound Healing: Biologic Features and Approaches to Maximize Healing Trajectories. *Curr Probl Surg.* 38:2:72.

Rodriguez PG, Felix FN, Woodley DT, and Shim EK (2008). The Role of Oxygen in Wound Healing: A Review of the Literature. *Dermatol Surg*. 34:9:1159-1169.

Roho vsky S, D'Amore P (1997). Growth factors and angiogenesis in wound healing, in Ziegler T, Pierce G, Herndon D (eds): Growth Factors and Wound Healing: Basic Science and Potential Clinical Applications. 1st ed, Springer, U.S.A. PP 234–245.

Saha K, Mukherjee PK, Das J, Pal M, Saha BP (1997). Wound healing activity of *Leucas lavandulaefolia* Rees. *J Ethnopharmacol.* 56:139-144.

Sanchez-Moreno C (2002). Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Technol. Int*. 8:121–137.

Satish L, Kathju S (2010). Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing. *Dermatol Res Pract*, Article ID 790234 doi:10.1155/2010/790234.

Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P (2009). Wound healing in the 21st century. *J Am Acad Dermatol* 10.1016/j.jaad.2009.10.048.

Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, Romanelli M, Stacey MC, Teot L, Vanscheidt W (2003). Wound bed preparation: a systematic approach to wound management . *Wound Repair Regen.* 11:S1: S1–S28.

Shai A, Maibach HI (2005). Wound Healing and Ulcers of the Skin. Diagnosis and Therapy – The Practical Approach. 1st ed. Springer-Verlag, Berlin, Germany, PP 1-15.

Shepherd AA (2003). Nutrition for optimum wound healing. *Nurs Stand.* 18:55–58.

Shetty S, Udupa S, Laxminarayana Udupa L (2008). Evaluation of Antioxidant and Wound Healing Effects of Alcoholic and Aqueous Extract of *Ocimum sanctum* Linn in Rats. *eCAM* 5:1:95–101.

Shukla A, Rasik AM, Jain GK, Shankar R, Kulshrestha DK, Dhawan BN (1999). *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica*. *J. Ethnopharmacol.* 65: 1–11.

Srivastava P, Durgaprasad S (2008). Burn wound healing property of *Cocos nucifera*: An appraisal. *Indian J Pharmacol.* 40:4:144-146.

Stadelmann WK, Digenis AG, Tobin GR (1998). Physiology and Healing Dynamics of Chronic Cutaneous Wounds. *Am J Surg.* 176:S2A:26S–38S.

Strodtbeck F (2001). Physiology of wound healing. *Newborn Infant Nurs Rev.* 1:1: 43-52.

Sumitra M, Manikandana P, Suguna L (2005). Efficacy of *Butea monosperma* on dermal wound healing in rats. *Int J Biochem Cell Biol.* 37: 566–573.

Sussman G (2007). The impact of medicines on wound healing. *Pharmacist* 26:11:874-878.

Teshome K, Gebre-Mariam T, Asres K, Perry F (2008). Toxicity studies on dermal application of plant extract of *Plumbago zeylanica* used in Ethiopian traditional medicine. *J. Ethnopharmacol.* 117: 236-248.

Thiem B, Grosslinka O (2003). Antimicrobial activity of *Rubus chamaemorus* leaves. *Fitoterapia* 75:93–95.

Thornton C, Span M, Susan C, Taylor M, Jeffery M, Winberg M (2003). Topical antimicrobial agents in dermatology *clin. Dermatol.* 21:70-77.

Udupa AL, Kulkarni DR, Udupa SL (1995). Effect of *Tridax procumbens* extracts on wound healing. *Inter J pharmacog.* 33: 37-40

Van der Veera WM, Bloemenb MCT, Ulrichab MMW, Molemac G, van Zuijlenbd PP, Middelkoopab E, Niessena FB (2009). Potential cellular and molecular causes of hypertrophic scar formation. *Burns* 35:1:15-29.

Villegas LF, Marcalo A, Martin J, Fernandez ID, Maldonado H, Vaisberg AJ, Hammond GB (2001). (+)-epi- Alpha-bisabolol [correction of bisbolol] is the wound healing principle of *Peperomia galioides*: investigation of the *in vivo* wound-healing activity of related terpenoids. *Nat Prod.* 64: 1357-1359.

Vollsen K. (1989). Sapindaceae. In: Hedberg I and Edwards S (eds): Flora of Ethiopia and Eritrea. Vol. 3. The National Herbarium, Addis Ababa University, Addis Ababa & Uppsala. PP 490.

Williamson D, Harding K (2004). Wound healing. *Medicine* 32:12:1-7.

Woo K, Ayello EA, Sibbald RG (2007). The edge effect: current therapeutic options to advance the wound edge. *Adv Skin Wound Care* 20*:* 99–111