Addis Ababa University

School of Graduate Studies

Evaluation of Wound Healing Activity of Rhizomes of *Rumex abyssinicus* J. in Mice

By:

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A Thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Experimental Pharmacology

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Acronyms

AAU- Addis Ababa University

BP- British Pharmacopoeia

CMC- Carboxyl methyl cellulose

ECM- Extracellular matrix

MMP- Matrix Metalloproteinases

OECD- Organization for Economic Cooperation and Development

PDGF- Platelet derived growth factor

PGE_2- Prostaglandin E_2

ROS- Reactive oxygen species

SEM- Standard error of the mean

TIMP- Tissue inhibitor of metalloproteinase

TNF- Tumor necrosis factor

Abstract

Rumex abyssinicus Jacq (Polygonaceae) is a perennial herb that is widely distributed in the highlands from North Africa to Ethiopia. In the Ethiopian traditional medicine, the rhizomes of *R. abyssinicus* ("Mekmeko" in Amharic) are used to treat various ailments. Combined with other plants, the rhizomes of *R. abyssinicus* are also used to treat wound. However, there is no scientific study justifying the use of *R. abyssinicus* on wound healing. Thus, the present study provides a scientific evaluation for the wound healing potential of 80% methanolic extract of *R. abyssinicus* rhizomes in mice.

The extraction of the rhizomes of *R. abyssinicus* was carried out using 80% methanol. The hydroalcoholic extracts were studied for wound healing activity topically by incorporating in simple ointment base B.P. in concentration of 5% (w/w) and 10% (w/w). For the study of *in vivo* antiinflammatory activity, the hydroalcoholic extract was dissolved in 1% carboxyl methyl cellulose. Two models were used for wound healing activity in mice viz. excision and incision. Carrageenan induced hind paw oedema model was used for antiinflammatory study. Parameters such as wound contraction, period of epithelization and hydroxyproline content were studied in case of the excision wound model, while incision wound model was evaluated by determining tensile strength. The hydroalcoholic extract of the rhizomes of *R. abyssinicus* was given orally at dose of 250, 500 and 750 mg/kg in carrageenan induced hind paw oedema model and oedema was evaluated by determining mean increase in paw volume and percentage inhibition of inflammation.

Treatment of wound with ointment containing 5% and 10% (w/w) hydroalcoholic extract exhibited significantly increased wound contraction rate, shorter epithelization time, higher skin breaking strength and increased hydroxyproline content ($p < 0.05$-0.001) in the two experimental model as compared to control. 10% (w/w) hydroalcoholic extract ointment showed better wound healing property than the 5% (w/w) ointment and its effect was comparable to that of the reference standard (nitrofurazone). *R. abyssinicus* rhizomes hydroalcoholic extract also produced dose-related significant reductions ($p < 0.05$-0.001) of inflammation as compared to control by reducing paw oedema volume induced by carrageenan.

The results of this study demonstrated that the hydroalcoholic extract of the rhizomes of *R. abyssinicus* facilitated wound healing at least in part via its antiinflammatory activity, supporting its traditional claim as wound healing agent.

Key words: *Rumex abyssinicus*, Excision model, Incision model, Antiinflammatory, Wound healing activity

1 Introduction

1.1 Wound

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues. It may be closed (e.g. bruises, ruptures and sprains) or open (e.g. abrasions, lacerations, avulsions, ballistics, hernias and excised or surgical wounds). Open wounds are by far the most common and are characterized by break in the skin. They can be caused accidentally or intentionally or be the result of a disease process (Pillai *et al.*, 2010; Nagori and Solanki, 2011).

Wounds can be broadly categorized as acute or chronic wound. An acute wound is defined as one that proceeds through an orderly and timely reparative process to establish sustained anatomic and functional integrity and comprises a series of overlapping phases (Franz *et al.*, 2008). They are caused by external damage to intact skin and include surgical wounds, bites, burns, minor cuts, abrasions and more severe traumatic wounds such as lacerations and those caused by crush or gunshot injuries (Li *et al.*, 2007).

Conversely, chronic wounds are defined as wounds that have not proceeded through orderly and timely reparation to produce anatomic and functional integrity after 3 months. All wound types have the potential to become chronic and, as such, chronic wounds are classified by cause. Venous or arterial insufficiency, diabetes, and local pressure effects are the most common pathophysiological causes, whereas systemic factors, such as compromised nutritional status, infection, and altered immunological status further contribute to poor wound healing (Bowler, 2002).

1.2 Scope of wound problems

Chronic wound is a common and important medical problem that causes significant morbidity (Etufugh *et al.*, 2007). Studies from Northern European countries reported that up to 85% of amputations are preceded by foot ulcers (Rathur and Boulton, 2007). An estimated 1% to 2% of the populace in developing countries, such as Sub-Saharan African and South Asian countries, will experience a chronic wound during their lifetime. The prevalence of chronic wounds in the community was reported as 4.5 per 1000 population, whereas that of acute wounds was nearly double at 10.5 per 1,000 populations. These wounds predominantly affect

patients aged older than 60 years. The poor hygienic condition in some third world countries is the main cause of this problem (Sasidharan *et al.*, 2010; Siddiqui and Bernstein, 2010).

In addition to the numbers of chronic wounds in the world, there are a large number of acute wounds, adding to society's wound burden. Every year in the US, it is estimated that greater than 1.25 million people have burns, and greater than 5 million people suffer from a non-healing wound. When acute wound healing does not progress in an orderly and timely manner, complications can occur. Incisions can dehisce; hernias can form; anastomoses can leak; and fistulae can develop (Robson *et al.*, 2001; Meier and Nanney, 2006).

The costs to society for caring of patients with wounds are difficult to determine. In European countries, the treatment cost of chronic wounds has been estimated to range from 1% to 2% of the annual health care budget. In US, management of chronic wounds cost an estimated $1 billion/year (Pierce *et al.*, 1995). The market for wound care products exceeds $7 billion. Global wound care expenditures amount to $13 to $15 billion annually (Siddiqui and Bernstein, 2010). 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 (Robson *et al.*, 2001). Besides, most people in developing countries who suffer from an infected wound cannot afford to purchase modern drugs, which are very expensive and might have side effects, and they cannot afford also hospital costs (Sasidharan *et al.*, 2010). These increase mortality and morbidity associated with wound to greater extent.

Patients incur numerous direct and indirect medical costs during wound treatment. Above all psychosocial damage incurred by patients is incalculable. They also face disability that results in lost wages, decreased productivity and a diminished quality of life. Another large problem is related to infections. Cellulitis, abscess formation, osteomyelitis, gangrene and even sepsis may occur as a result of an infected wound. Furthermore, chronic wounds have the potential for malignant transformation (i.e., Marjolin's ulcer) (Menke *et al.*, 2007). There are no much data concerning the current status of wound problem in Ethiopia. Majority of the study focuses on microbial infection of the skin and others. However, Ethiopia is one of the poor Sub-Saharan African countries whose population face chronic wound at least once in their life time.

1.3 Wound healing

1.3.1 Wound healing physiology

Wound healing is defined by the wound healing society as a complex dynamic process that results in the restoration of anatomic continuity and function (Lazarus *et al.*, 1994). The healing process consists of a highly orchestrated sequence of events but yet with overlapping stages (Mutsaers *et al.*, 1997). There are anywhere from 3 to 5 stages of wound healing, depending on how the various biologic mechanisms are linked (Strodtbeck, 2001). The steps are reviewed below.

Haemostasis: Tissue injury provokes immediate activation of the extrinsic and intrinsic coagulation pathways. Within minutes of injury, platelet activation products and intense vasoconstriction lead to clot formation with haemostasis. Vasodilatation and increase in capillary permeability follows, possibly as a result of release of histamine from activated platelets. This allows serum rich in proteins such as fibronectin, fibrinogen and fibrin to leak into the interstitial space, where these combine with the clot to produce a fibrin plug that temporarily closes the wound (Williamson and Harding, 2004). The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing (Nayak, 2006).

Inflammation: The inflammatory phase is characterized by its cardinal signs: redness, warmth, swelling, pain, and loss of function (Wild *et al.*, 2010). Many substances in the injured area, such as fibrin degradation products, leukotrienes, complement products, bacterial peptides, and platelet derived growth factors (PDGF) and transforming growth factor (TGF) that have been released from platelets, act as general leukocyte attractant mediators that support the recruitment of leukocytes. The neutrophils are the first blood cell types that enter the area of injury (Tsirogianni *et al.*, 2006). The neutrophils engulf debris and microorganisms, providing the first line of defense against infection (Monch *et al.*, 2004). After clearing any invading bacteria, the neutrophils undergo spontaneous apoptosis or are phagocytosed by wound macrophages (Solanki and Jain, 2011).

In the absence of infection, the existing monocytes differentiate into macrophages and become the major phagocytic cell at the injury site. If macrophage infiltration is prevented, then healing is severely impaired. Macrophage tasks include phagocytosis of any remaining

pathogenic organisms and other cell and matrix debris (Inkinen, 2003). Macrophages also have a number of functions, including mediating angiogenesis, synthesizing nitric oxide and forming fibrous tissue (Stechmiller, 2010). Thus, they are essential for the transition from the inflammatory to the repair phase because of their essential role in wound (Williamson and Harding, 2004).

Proliferation and repair: The proliferative phase of wound healing usually occurs on the fourth day after wounding and is characterized by the early appearance of fibroblasts in the wound bed. There are 4 major steps in this phase: (i) angiogenesis, (ii) reepithelization (the process of restoring an intact epidermis after cutaneous injury), (iii) granulation, and (iv) tissue formation and collagen deposition (Li *et al.*, 2007; Schreml *et al.*, 2010; Stechmiller, 2010). Wound (tensile) strength begins to develop during this stage. Key cells for these processes are the fibroblast and keratinocyte (Strodtbeck, 2001). Fibroblasts migrate inwards from the wound margins stimulated by many chemical activators and messengers, mostly released by macrophages, which dominate towards the end of the inflammatory phase. Fibroblasts themselves secrete a variety of cytokines, allowing other vital cells to proliferate and aid the healing process. Such cells include endothelial cells and angiocytes. Expansion of these cell numbers contributes to a process known as angiogenesis, the generation of new blood vessels (Nigam *et al.*, 2010). Hypoxia and acidosis stimulate angiogenesis. Within the wound bed, fibroblasts produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM) (Guo and Dipietro, 2010). Fibroblasts also participate in the process of wound contraction after differentiation into myofibroblasts. 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. The new granulation tissue contains type I, III and V collagen fibers. Thirty percent of the collagen is type III collagen, which does not contribute to restoring tensile strength in the wound (Strodtbeck, 2001).

Remodeling: The tissue remodeling phase starts as early as a few days after injury and lasts up to 2 years thereafter. After the main steps of the proliferative phase are fulfilled, the density of cells, such as macrophages, keratinocytes, fibroblasts and myofibroblasts is reduced by apoptosis. Keratinocytes are the first cells to undergo programmed cell death;

myofibroblasts are the second so that the accelerated proliferation and migration normalizes. Gradually, the provisional collagen (type III initial new collagen synthesized in the proliferative phase) is replaced by the more stable collagen type I that is produced strictly oxygen dependently by fibroblasts and is deposited in a physiological alignment. The healing wound gains increased wound tensile strength. The collagen fibres contract so that the wound tissue shrinks (Schreml *et al.*, 2010).

1.3.2 Extracellular matrix and wound healing

Cell migration, polarity and orientation are influenced by the constituents of the ECM. Therefore, the regulation of ECM deposition is a key event in many physiological and pathological conditions. In cutaneous healing the ECM acts as an "adhesive substrate" for both regeneration and repair. Excessive deposition of connective tissue, however, is the pathological hallmark of fibrosis and hypertrophic scars and keloids. A tight balance between connective tissue synthesis and breakdown is, therefore, required for the normal functioning of all tissues (Eckes *et al.*, 2000).

The ECM is a complex mixture of matrix molecules, including the glycoproteins, fibronectin, collagens, laminins, and proteoglycans (Liu and Velazquez, 2010). Collagen is the component of the ECM that played the greatest role in wound healing and many researches done nowadays on wound healing focus on it.

Collagen

Collagen makes up for 70% of the total nitrogen content of skin proteins. It is, therefore, the most abundant protein in human body (Dioguardi, 2008). It is involved in a number of important biological functions, such as cell attachment, chemotaxis, platelet aggregation and filtration through basement membrane. It also plays important roles in the healing of wound and fractures (Inkinen, 2003).

Collagen has a unique amino acid composition with high concentration of glycine, hydroxyproline and hydroxylysine. These amino acids are typical of collagen structures and are very rarely found in other proteins (Dioguardi, 2008). There are more than 30 collagens and collagen-related protein (Kadler *et al.*, 2008). Different collagen types have been detected in human skin. The main collagens are the interstitial types I, II and III, which form a network

of fibres in the interstitium of tissues, linking cells to other structural components as well as to each other (Mutsaers *et at.*, 1997).

In healthy adults, type I collagen accounts for approximately 80% of collagens and type III collagen constitutes 10% of collagens in the dermis. During early wound healing, however, type III collagen is the predominant collagen synthesized by fibroblasts in granulation tissue (Li *et al.*, 2007; Li and Wang, 2009). With wound closure, a gradual turnover of collagen occurs as type III collagen undergoes degradation and type I collagen synthesis increases. Type I collagen synthesis coincides with increased wound breaking strength. The process of this conversion of the dermis is accomplished through a tightly controlled synthesis of new collagen and lysis of old collagen, mainly carried out by the actions of matrix metalloproteinases (MMPs) (Visse and Nagase, 2003).

1.3.3 Matrix metalloproteinases

MMPs comprise a family of ECM degrading enzymes that play a role in many aspects of wound healing (McKeown *et al.*, 2007). They participate in regulating mechanisms in all of wound repair processes. For example, inflammation is shaped by cytokines and chemokines, which arise largely from resident cells (epithelium, endothelium, fibroblasts, etc.). MMPs can activate these mediators by cleaving them from the cell surface or processing them to increase their activity, or degrade them, thereby inhibiting inflammatory signals. As well, MMPs are able to cleave components of cell–cell junctions and cell–matrix contacts within the epithelium to promote re-epithelialization. Furthermore, MMPs are involved in remodeling the scarred ECM either directly by proteolytic degradation of proteins, such as collagens, or indirectly via their ability to affect cell behavior. Alteration of the ECM is integral to the resolution of wound healing but also has implications in regulation of inflammation. Thus, MMPs are key regulators of multiple aspects of tissue repair (Gill and Parks, 2008).

MMPs are produced by several types of cells including fibroblasts, keratinocytes, macrophages and eosinophils (Brandner *et al.*, 2007). Depending on substrate specificity, amino acid similarity and identifiable sequence modules, the family of MMPs can be classified into the following distinct subclasses: collagenases**,** gelatinases**,** stromelysins, matrilysins, macrophage metalloelastase and membrane type matrix metalloproteinases (MT-MMP) (Inkinen, 2003; Visse and Nagase, 2003). There is a difference in MMP expression

between acute and chronic wounds, with chronic non-healing wounds expressing high levels of MMP and low levels of TIMPs suggesting excessive MMP expression may prevent healing (Brandner *et al.*, 2007). Proteolytic degradation of ECM is an essential part of wound repair and remodeling, but excessive levels of MMPs may degrade ECM, preventing cellular migration and attachment (Schultz *et al.*, 2005).

1.3.4 Growth factors in wound healing

Re-epithelization and granulation tissue formation during repair are mediated by a wide variety of growth and differentiation factors (Padgett *et al.*, 2007). The most important growth factors are: PDGF which is the principal inducer of matrix proteins that are important for creating the provisional matrix that initially fills the void created by tissue damage. Transforming growth factor- β (TGF- β 1, - β 2, and- β 3), produced by macrophages during the early phase of wound healing, mediates the chemotaxis of monocytes, lymphocytes, polymorphonuclear neutrophils (PMNs) and fibroblasts. It is also a potent stimulant of ECM production *in vivo* and is involved in the synthesis of collagen by fibroblasts. Fibroblast growth factors (FGF) are the major growth factor family involved in angiogenesis, directing endothelial cell migration, proliferation and plasminogen activator synthesis. Vascular endothelial growth factor (VEGF) is also known to promote angiogenesis. Insulin/(insulin-like growth factor (IGF)-1 has been shown to produce favourable effects on wound healing by enhanced tensile strength, increased hydroxyproline content and enhanced grade of cellular immunity (Monaco and Lawrence, 2003; Santoro and Gaudino, 2005; Kapoora *et al.*, 2006).

1.4 Factors affecting wound healing

Several clinical conditions and factors are known to impair wound healing and these include hypoxia, infection, certain drugs, dietary deficiencies of protein, vitamins and minerals (Odimegwu *et al.*, 2008). Wounds will heal in a short period of time if the factors inhibiting wound healing are adequately identified and managed. Some of the factors are:

Infection: Wounds are known to be easy portals for infections and provide suitable medium for the proliferation of microbial organisms (Odimegwu *et al.*, 2008). Wound infection is defined as the presence of replicating microorganisms within a wound that cause host injury.

It is an important cause of morbidity and account for 70-80% mortality (James and Victoria, 2010).

Wound infections can be caused by different groups of microorganisms like bacteria, fungi and protozoa. However, different microorganisms can exist in polymicrobial communities especially in the margins of wounds and in chronic wounds (Zafar *et al.*, 2007). All open wounds may be considered contaminated but not infected (Robson *et al.*, 2001). The critical number of organisms able to cause a wound infection has been shown to be 10^5 organisms per gram of tissue. If greater than 10^5 organisms are present per gram of tissue, the chances of successful wound closure are low (on the order of 19%). If, on the other hand, less than 10^5 organisms per gram of tissue are present, then the likelihood of successful wound closure is approximately 94% (Stadelmann *et al.*, 1998; McGuckin *et al.*, 2003).

The contaminants are from the surrounding skin, the local environment and endogenous patient sources (Bowler, 2002; Siddiqui and Bernstein, 2010). The most common source of infections is microorganisms on patient skin, an estimated 50% of which are caused by the skin bacterium *Staphylococcus aureus* (Rosso *et al.*, 2011).

The infecting microorganism may belong to aerobic as well as anaerobic group. Most commonly isolated aerobic microorganism include: *S. aureus*, *Coagulase-negative staphylococci* (CoNS), *Enterococci*, *Escherichia coli, Pseudomonas aeruginosa, Klebsieila pneumoniae, Enterobacter* species, *Proteus mirabilis,* other streptococci*,* Candida with 80% *Candida albicans,* Group D streptococci and Acinetobacter. Other gram-positive aerobes and anaerobes also cause wound infections (Ladal *et al.*, 1999; Zafar *et al.*, 2007). *S. aureus* from gram positive and *P. aeruginosa* from gram negative are the most common pathogens which infect the skin (Patel *et al.*, 2009).

Chronic wounds tend to have a low tissue oxygen level that facilitates the growth of anaerobes. As a result, chronic wounds have a statistically higher proportion of anaerobes than acute wounds. Common anaerobic colonizers include Prevotella, Bacteroides, Peptostreptococcus and Porphyromonas. More than 95% of chronic wound infections contain anaerobes along with aerobes such as *S. aureus*, *Enterococcus* spp., and *coliforms* (Siddiqui and Bernstein, 2010).

Wound hypoxia: Oxygen plays an important role in each stage of the wound healing process. It controls the migration and proliferation of fibroblasts. Angiogenesis and leukocytes require oxygen (Thackham *et al.*, 2008). Therefore, oxygen delivery is a critical element for the healing of wounds. Local tissue hypoxia is a potent stimulus for the migration of fibroblasts and endothelial cells into the wound center. However, wound healing is impeded if hypoxia persists. In an environment of 30 to 40 mm Hg of oxygen, fibroblasts cannot replicate and collagen production is severely limited. Wound hypoxia also predisposes the wound to bacterial invasion which significantly impedes wound healing. Wound hypoxia may be worsened by many clinical conditions, such as poor cardiac output, peripheral vascular disease, diabetes mellitus, past irradiation, tobacco consumption and chronic infection (Stadelmann *et al.*, 1998).

Formation of free radicals: Free radicals are produced by neutrophils and non-phagocytic cells. While they fight pathogens, neutrophils release inflammatory cytokines and enzymes that damage cells. One of their important jobs is to produce reactive oxygen species (ROS) to kill bacteria, for which they use an enzyme called myeloperoxidase. The enzymes and ROS produced by neutrophils and other leukocytes damage cells and prevent cell proliferation and wound closure by damaging DNA, lipids, proteins, enzyme inactivation, including free-radical scavenger enzymes, the ECM and cytokines that speed healing. Neutrophils remain in chronic wounds for longer than they do in acute wounds. Agents that demonstrate significant antioxidant activity may, therefore, preserve viable tissue and facilitate wound healing (Leach, 2008).

Drugs: Many medications, such as those which interfere with clot formation or platelet function, or inflammatory responses and cell proliferation have the capacity to affect wound healing (Guo and Dipietro, 2010). Among the most frequently encountered medications/drugs that impede wound healing are systemic steroids (Lama and Fechtner, 2003) through reduction of neutrophils and inhibition of leukocyte and tissue macrophage function, and nonsteroidal antiinflammatory drugs (NSAIDs) (Furst and Ulrich, 2007) by inhibition of platelet function and aggregation, and chemotherapeutic drugs (Guo and Dipietro, 2010) by inhibition of cellular metabolism, rapid cell division and angiogenesis.

Vitamin deficiencies: Vitamin A benefits the wound by enhancing the early inflammatory phase, including increasing the number of monocytes and macrophages at the wound site, modulating collagenase activity, supporting epithelial cell differentiation, and improving localization and stimulation of the immune response. Vitamin C is necessary for collagen formation, proper immune function, and as a tissue antioxidant. Vitamin E functions as the major lipophilic antioxidant, preventing peroxidation of lipids (Douglas and Miller, 2003).

1.5 Animal models for wound healing study

Several animal models have been established to serve as an experimental basis to determine molecular and cellular mechanisms underlying and controlling an undisturbed wound healing process (Frank and Kämpfer, 2003). The small mammals are beneficial for multiple reasons. They are inexpensive, easily obtainable, require less space, food and water, easy to maintain, and can be genetically modified (Thakur *et al.*, 2011). They limit variations in temperature, pH, oxygen and nutrient supply, etc., which are usually encountered in the *in vitro* methods (Jia *et al.*, 2008). Excision, incision, dead space and burn wound models can be employed to study wound healing. Mice and rats are some of the various small animals used in those wound healing models.

i) Excision wound model

As the name implies, such wounds involve the removal of a significant volume of the target tissue by excising skin areas from the backs of the animals. The wounding is done with fine scissors, punch or scalpel, and the cut removes the epidermal, dermal, and subcutaneous layer including the panniculus carnosus (Frank and Kämpfer, 2003).

Excisional wounds can be covered with occlusive dressings, which retain the exudates (wound fluid) as a means of assessing the status of various soluble factors in the wound environment, such as nutrients, proteinases, cytokines, and tissue degradation products. This model, specially full-thickness excision, offers the advantages of significant wound volume, involvement of all dermal components, epithelialization only from the wound margins, and the ability to analyze chemistry, histology, and cell populations in the wound site. Healing rates are often monitored on the basis of total excisional volume (or cross-sectional area) filled with granulation tissue (neodermis), rate of contraction of wound, extent of re-

epithelization, histological organization of connective tissue, angiogenesis, and biochemical content of collagen or proteoglycans (Davidson, 1998; Sherratta and Dallon, 2002).

ii) Incision wound model

Incision models have been used for decades to test the "tensile strength" of healing incisions. An incision is made through the skin and either closed with staples or sutures, or allowed to heal spontaneously. The "breaking strength" is the force required to break the skin. The breaking strength of any exogenous treatment is then compared to that of the control animals (Greenhalgh, 2005). Sharp blade, electro-cautery blades and laser surgical devices are used while making an incision. It is less adequate for histological assessment of healing because of the limited volume/area of wound healing activity; for the same reasons, it is poor for evaluation of tissue biochemistry or epithelization (Davidson, 1998).

iii) Dead-space wound model

Dead-space models are used for studying granulation and reparative tissue ingrowth. All such models function by creating an artificial tissue space into which plasma infuses. This leads to development of a fibrin clot and subsequent formation of granulation tissue. Depending on the implant material, further maturation into scar may occur, and a connective tissue capsule comprised of several collagenous fascias usually surrounds the implant (Efron and Barbul, 2003). Implant materials vary widely: viscose/cellulose sponges, polyvinyl alcohol sponges, and so on. In general, they are chosen to be relatively inert and non-biodegradable so that the implant site has minimal inflammation (Davidson, 1998).

This model is ideal for biochemical, immune-histochemical and molecular analysis because wound cells, wound fluid, ECM, DNA, and RNA can all be harvested from the implant. Additionally, this model can reflect the systemic (nutritional, pharmacological) or local (growth factors, gene induction, gene therapy) manipulation of the healing process (Efron and Barbul, 2003).

Limitations of the dead-space models include the interference of the implant with normal scar maturation, probably by the uncoupling of physical interactions among cells, the lack of epithelial components, and the likelihood of an eventual foreign body response. Thus, studies

with most implant materials are only relevant for the first three to four weeks of the repair process (Davidson, 1998; Efron and Barbul, 2003).

iv) Burn wound model

There have been all types of burn models that are used for examining wound healing and the systemic response to thermal injury. Both partial-thickness and full-thickness models have been developed. The burning agent can be a scald, flame, or heated piece of metal (usually placed in boiling water) to produce a contact burn. Wound contraction, epithelialization periods and scar formation are used to evaluate the healing of this wound. A practicable, reliable and reproducible model for infliction of partial skin thickness burn lesions in rabbits is a possible example of burn wound model. The model is dedicated to experimental studies investigating the influence of drugs on burn wounds (Greenhalgh, 2005).

There are also several *in vitro* models of tissue repair that can assist with answering specific mechanistic questions related to wound repair**.** Chick chorioallantoic membrane (CAM) assay, fibroblast bioassay, and keratinocytes assay are among them (Thakur *et al.*, 2011).

1.6 Management of wounds

1.6.1 Antimicrobial agents

The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to repair and regeneration (Mohanty *et al.*, 2010). The term "antimicrobial agents" comprises disinfectants, antiseptics, and antibiotics. Antiseptics are chemical agents that are broadly toxic to microbes, whereas antibiotics are narrow-spectrum antimicrobial agents with specific intracellular targets (Siddiqui and Bernstein, 2010).

All wounds harbor microbes. Techniques to rid the wound of microbes can include the use of expensive silver-containing dressings, the application of topical antibiotics or antiseptics, or even the routine use of long-term systemic antibiotics (Esimone *et al.*, 2005; Joseph, 2011).

Topical antimicrobial therapy is one of the most important methods of wound care (Odimegwu *et al.*, 2008). Because, the goal of topical antimicrobial therapy in wound care is to control microbial colonization and subsequent proliferation thus promoting the healing of

the wounds (Odimegwu *et al.*, 2008; Sasidharan *et al.*, 2010). They are chosen based on their ability to destroy or inhibit the growth of pathogenic organisms, while the tissue is left unharmed (Thakare *et al.*, 2011).

Antibiotics available for topical use are numerous. They are available in the form of lotions, creams, ointments, powders, gels, foam and sprays. Topical antibiotics are of low risk of systemic side effects, have a higher concentration in the affected area, low quantity of drug usage, and none interference with intestinal flora. Topical preparations that may be helpful include amikacin (in gel or cream), bacitracin, chloramphenicol, clindamycin (cream, lotion, and foam), gentamicin (available in form of ointment or cream), nitrofurazone (available as a 0.2% cream, solution, or soluble dressing) and polymyxin B (Elmetti, 2008).

Despite the availability of drugs, many wounds still fail to heal and remain a significant burden for patients and caregivers alike. The primary reason is resistance to drug. Antimicrobial resistance costs money and human lives. It is a global problem with the particular level of resistance varying from one locality to another (Meier and Nanney, 2006). The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents (Okeke *et al.*, 2005).

1.6.2 Traditional medicines in wound healing

Traditional medicines include herbal medicines composed of herbs, herbal materials, herbal preparations, and finished herbal products, that contain active ingredients, parts of plants, or other plant materials, or combinations thereof. Traditional medicines may also use animal parts and/or minerals (Robinson and Zhang, 2011).

Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns (Kokane *et al.*, 2009). Between 70% and 95% of citizens in the majority of developing countries, and 70% to 90% of populations in some industrialized nations use traditional medicine as primary health care to address their health-care needs and concerns (Robinson and Zhang, 2011). Among users of herbal remedies, more than 80% uses herbal remedies for their ailments, especially for wound management, as they provide a moist environment suitable for wound (Annan and Houghton, 2008; Patel *et al.*, 2009).

Herbal medicines are an important part of the culture and traditions of African people (Fennell *et al.*, 2004). Populations using traditional medicine for primary care in African countries accounts for 90% in Ethiopia, 75% in Mali , 70% in Rwanda, 60% in Tanzania, and 60% in Uganda (Robinson and Zhang, 2011).

Especially traditional medicine has been practiced in Ethiopia since long time ago. The country has about 800 species of plants that are used in the traditional health care system to treat nearly 300 mental and physical disorders (Teklehaymanot *et al.*, 2007). Several reports indicate that skin disorders are very common in Ethiopia (Tadeg *et al.*, 2005). Thus, traditional medicine still remains the main resource for a large majority of the people in Ethiopia for treating health problems. In general, traditional medical consultancy has a much lower cost, including the consumption of the medicinal plants required than modern medical attention (Mesfin *et al.*, 2009).

Natural products are largely preferred because of their widespread availability, their potent pharmacological activities, low toxicity and effectiveness as crude preparations (Fennell *et al.*, 2004; Sasidharan *et al.*, 2010). It is estimated that at least 25% of all modern medicines are derived, either directly or indirectly, from medicinal plants, primarily through the application of modern technology to traditional knowledge. In the case of certain classes of pharmaceuticals, such as antitumoral and antimicrobial medicines, this percentage may be as high as 60% (Robinson and Zhang, 2011). For example of the 104 new drugs developed over 37 years, 60 originated from plants used in traditional medicine of China. Furthermore, the discovery of modern drugs such as quinine, vincristine, digoxin and digitoxin, emetine, artemisinine, etc. from medicinal plants signify the huge potential that still exists for the production of many more novel pharmaceuticals. Thus, there has recently been a resurgence of interest in the development of drugs from plants, especially from those of the developing countries that have a rich heritage of botanical ethnopharmacopoea (Geyid *et al.*, 2005). Therefore, the medicinal properties of plants have been investigated in the light of recent scientific developments throughout the world.

Only 1 to 3% of drugs listed in Western Pharmacopoeia are intended for use on the skin and on wounds; but at least one third of herbal remedies are for such uses (Sasidharan *et al.*, 2010). Many of the available drugs are not only expensive but also pose problems such as

allergy, and drug resistance (Prasad and Dorle, 2006; James and Victoria, 2010). These affect mostly developing countries like Ethiopia where most of the people are illiterate and poor.

Efforts are being made all over the world to discover agents that can promote healing and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications (James and Victoria, 2010; Pillai *et al.*, 2010). Hence, plant products are seen as alternative solutions to the problem of wound treatment (Fennell *et al.*, 2004).

There are a number of plants which have been reported for their wound healing activity. Incidentally almost all the plants which have been reported pharmacologically are being used traditionally as well. Recent studies with significant finding proved plants like *Alternanthera sessilis, Carica papaya, Catharanthus roseus, Cecropia peltata, Clerodendrum serratum Euphorbia hirta, Euphorbia nerrifolia, Ginkgo biloba, Lycopodium serratum, Morinda citrifolia, Ocimum sanctum, Pterocarpt santalinus, Sesame indicum,* and *Trigonella foenum-graecum* as wound healing agents (Raina *et al.*, 2008; Nagori and Solanki, 2011). One plant commonly used in Ethiopian traditional medicine for wound healing is *Rumex abyssinicus* Jacq.

1.7 *Rumex abyssinicus* Jacq

Rumex abyssinicus Jacq (Fig 1) is a perennial herb that grows up to 3-4 m. It belongs to the family Polygonaceae. It is widely spread medicinal plant in the highlands of tropical Africa and is a common weed of cultivated lands or disturbed grounds ranging from North Africa to Ethiopia (Mekonnen *et al.*, 2010). In the Ethiopian traditional medicine, the rhizomes of *R. abyssinicus* (“Mekmeko” in Amharic) are used to treat malaria, gonorrhoea, poisoning, hepatitis, constipation, sciatic neuralgia, hypertension, migraine, rheumatism, breast cancer, stomach distention, earache, liver diseases, hemorrhoids, typhus, rabies and wound (Abebe and Ayehu, 1993; Abebe *et al.*, 2003).

Previous report indicates that the 80% methanol extract of the rhizomes of *R. abyssinicus* possess antimicrobial and *in vitro* antiinflammatory activities (Getie *et al.*, 2003). Similarly, extracts of the plant have been proved to have diuretic and analgesic (Mekonnen *et al.*, 2010), anthelmintic (Raju and Yesuf, 2010) and antimalarial (Muganga *et al.*, 2010) activities. To date, however, no scientific report could be found in the literature concerning the wound healing effects of the plant in spite of the ethnobotanical claim for its wound healing

properties. It was, therefore, considered worthwhile to conduct scientific experiments to prove whether extracts of the plant have genuine wound healing activity or not.

Fig. 1 Photograph of *Rumex abyssinicus* J.

2 Objective

2.1 General objective

The aim of this study is to evaluate the wound healing activities of the 80% methanolic extract of *R. abyssinicus* rhizomes in experimental animals.

2.2 Specific objectives

The specific objectives of the study are:

- To study acute dermal toxicity of the hydroalcoholic extract in rat;
- To study the antiinflammatory effect of the hydroalcoholic extract using carrageenan induced paw oedema in mice; and
- To evaluate the wound healing effect of the 80% methanolic extract of the plant by using excision and incision wound models in mice.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals

Chemicals like methanol (Aston Fields Road Whitehouse Industrial Estate, USA), denatured alcohol, diethyl ether (Labmark Chemicals Pvt. Ltd, India), nitrofurazone 0.2% (Shanghai General Pharmaceuticals Co., Ltd, China), carrageenan (Sigma-Aldrich Steinheim, Germany), carboxy methyl cellulose, cetosearyl alcohol, copper (ii) sulphate, hard paraffin, hydrochloric acid, hydrogen peroxide, sulphuric acid, wool fat, white soft paraffin (BDH Laboratory Supplies Poole, England), ketamine hydrochloride (Rotex Medica, Germany), sodium hydroxide (Bio-Lab Laboratories Ltd, Israel), hydroxyproline, *p*-dimethylminobenzaldehyde (Merck Specialties Private Limited, Mumbai/India) and indomethacine (Greenfield Pharmaceuticals, China) were used as received.

3.1.2 Plant material

The fresh rhizomes of *R. abyssinicus* were collected in the month of November 2010 in and around Menagesha forest, West of Addis Ababa, Ethiopia. The plant was identified at the National Herbarium, College of Natural Sciences, Biology Department, Addis Ababa University where a voucher specimen was deposited (collection no. E001/10).

3.1.3 Experimental animals

Healthy adult Swiss albino mice of either sex (25-30 g, and 6-8 weeks of age) procured from the animal house of the Ethiopian Health and Nutrition Research Institute (EHNRI) and adult Wistar albino rats of both sex (200-300 g, aged 3-4 months) obtained from the animal house of the School of Pharmacy, AAU, were used for the study. The animals were housed individually in their cages under standard environmental conditions (25 ± 2 °C, 55 ± 5% relative humidity, and 12 h light and dark cycles). Animals were caged individually to prevent damage that may be inflicted upon each other and were maintained on standard pellet diet and water *ad libitum* throughout the experiment. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996). At the end of each experiment the animals were sacrificed by cervical dislocation.

3.2 Methods

3.2.1 Extraction of plant material

Rhizomes of *R. abyssinicus* were first washed with distilled water using brush to remove soil attached to them and sliced to smaller pieces before they were dried in the shade for three weeks. The dried rhizomes were coarsely powdered in a grinder and 500 g of the powder were macerated with 80% methanol for three days in conical flask with occasional stirring and shaking. The extract was then filtered (Whatman No. 1). The residue was further macerated with the hydroalcohol for three more days to exhaustively extract the plant material and filtered. The combined filtrate was evaporated in a ventilated oven at 40 ºC until dried. The resulting dry extract was weighed to calculate the percentage yield, which was 16.19% (w/w). The extract was stored in a refrigerator for the preparation of topical formulation (ointment).

3.2.2 Ointment formulation

The following formula was used from British Pharmacopoeia (BP, 1988) in order to prepare simple ointment base for 80% methanol extract ointment formulation.

Ingredients	**M.F**	**R.F**
Wool fat	50 g	10g
Hard paraffin	50 g	10g
White soft paraffin	850g	170g
Cetostearyl alcohol	50g	10g
	1000g	200g

M.F= Master Formula, R.F= Reduced Formula

200 g of simple ointment base was prepared by using reduced formula from BP. 10 g of hard paraffin was placed in a beaker and melted over water bath. It was removed from the heat after complete melting and the other ingredients such as cetostearyl alcohol (10 g), white soft paraffin (170 g) and wool fat (10 g) were added in descending order of melting point, respectively. All the ingredients were melted over a water bath with constant stirring until they became homogeneous. The mixture was removed from the heat and stirred until cold (Ansel, 1985; Langley and Belcher, 2008).

To prepare hydroalcoholic extract ointment, first the extract was powdered in a mortar and pestle. Then 5 g and 10 g of the powdered extract was incorporated into portion of simple ointment base to prepare 5% and 10% (w/w) ointment, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture. The remainder of simple ointment base was gradually added and mixed thoroughly. Finally, the extract ointment was transferred to a clean container for topical application during the experiment (Ansel, 1985; Deshmukh *et al.*, 2009).

3.2.3 Acute dermal toxicity

Acute oral toxicity was previously done by Mekonnen et al. (2010). To study acute dermal toxicity five male and five female rats were randomly chosen and assigned to treatment groups. The animals were acclimatized to the laboratory condition for five days prior to test. The hair was removed from the back trunks of the animals by shaving 24 h prior to hydroalcoholic extract ointment application. Hydroalcoholic extract ointment of the highest concentration of 10% (w/w) was applied on the shaved back of the rats within a range that was approximately 10% of the body surface area using limit test dose of 2 g/kg (Kokane *et al.*, 2009). The ointment was applied thinly and uniformly to the entire application site for a period of 24 h. The application site was covered with porous gauze, and secured with non-irritating tape so as to preserve contact with the skin. After 24 h the gauze was removed gently from the skin and observed for inflammation. Cage side observation like changes in eyes, tremors, convulsion, salivation and diarrhea was also made for the following 14 days (OECD (402) guidelines, 1987).

3.2.4 Grouping and dosing of animals

Four groups of mice containing six in each were used for excision model. Animals in Group I were treated with nitrofurazone (0.2%) ointment. Group II and III received 10% (w/w) and 5% (w/w) hydroalcoholic extract ointments, respectively, and the animals in group IV were treated with simple ointment. Five groups of mice containing six in each were used for incision wound model. The animals of group I-IV were treated in a similar fashion with excision wound model but animals in group V were not treated with any material. Strength of the medicated ointment was selected based on pilot study performed prior to the main work.

For the assessment of antiinflammatory activity three dose levels were chosen based on acute oral toxicity results (Mekonen *et al.*, 2010). The dose was calculated based on Deshmukh et al. (2009) with slight modification in such a way that, the middle dose (500 mg/kg) was approximately one-tenth of the maximum dose used during acute toxicity studies, the low dose (250 mg/kg) was 50% of the one-tenth dose, and the high dose (750 mg/kg) was selected base on pilot study prior to the actual work. Five groups of mice containing six in each were used. 1% carboxyl methyl cellulose (CMC), 250 mg/kg, 500 mg/kg, 750 mg/kg of hydroalcoholic extract and indomethacine (10 mg/kg) were administered orally to group I-V, respectively.

3.2.5 Excision wound model

Mice were anesthetized by administering ketamine subcutaneously (1 ml/kg) before wound creation (Shetty *et al.*, 2006). The particular skin area was shaved by using a shaving machine and an excision wound was inflicted by cutting away 300 mm^2 full thickness of skin marked with thin permanent marker from a predetermined shaved area 10 min after the administration of ketamine (Kokane *et al.*, 2009). Excision was done using sharp and small sterilized scissors, and forceps. The wound was left undressed to the open environment. The mice were divided into four groups (6 animals per group) randomly and each mouse was placed in a separate cage (Rashed *et al.*, 2003). The wounding day was considered as day 0 (Fig 2). The standard, extract and simple ointment were applied topically to the respective groups till the wound was completely healed. In this model, wound contraction, epithelization period and hydroxyproline content were monitored. Wound contraction was measured as percent contraction every 2 days after wound formation (Lodhi *et al.*, 2006).

Fig. 2 Excision wound on day 0.

i) Measurement of wound contraction

An excision wound margin was traced by following the progressive changes in wound area, excluding the day of wounding. The wound healing progress was evaluated by measuring wound areas on days 3, 5, 7, 9, 11, 13, 15 and 17 for all the groups using a transparency sheet and a permanent marker. The tracing was then shifted to graph paper, from which the wound surface area was evaluated, each square representing 1 mm^2. The evaluated surface area was then employed to calculate the percentage of wound contraction, taking initial size of the wound, 300 mm^2 as 100%, by using the following formula (Shivhare *et al.*, 2010):

$$\% \text{ Wound contraction} = \frac{\text{Initial wound size - specific day wound size}}{\text{Initial wound size}} X 100$$

ii) Epithelization time measurement

It was evaluated by noting the number of days required for falling of the scabs without any residual of raw wound behind (Wesley *et al.*, 2009).

iii) Hydroxyproline content determination

The Neuman and Logan (1950) technique for the determination of hydroxyproline in protein hydrolysates consists of (i) oxidation of hydroxyproline with hydrogen peroxide in the presence of alkaline copper sulphate, (ii) destruction of excess of peroxide by heat and (iii) reaction of the oxidation product with *p*-dimethylaminobenzaldehyde by heating in the presence of dilute sulphuric acid to produce a red colour, the intensity of which is compared with a standard (Leach, 1960).

For standard hydroxyproline preparation 0.05 g of hydroxyproline was dissolved in water and diluted to about 400 ml with water. Twenty ml of conc. HCl were added and the solution was made up to 500 ml with water. The solution (100 µg /ml) was diluted to give 5, 10 and 15 µg of hydroxyproline/ml. Triplicate solution of each of these concentrations and blank solutions was prepared. One ml of 0.05 M $CuSO_4$ was placed into each tube, followed by addition of 1 ml of 2.5 N NaOH, and the tube contents were mixed by gentle swirling of the liquid. The tubes were placed in a water bath at 40 ºC for about 3-5 min and then 1 ml of 6% hydrogen peroxide was added, which was immediately mixed by swirling the contents of the tube before addition to the next tube. Then, the tubes were left in the bath for 10 min, but were

occasionally removed from the bath and the contents swirled. The tubes were cooled with tap water, then 4 ml of 3 N H_2SO_4 and 2 ml of 5% *p*-dimethylaminobenzaldehyde solution were added by swirling after each addition. Caps were placed on the tubes, which were kept in a water bath at 70 ºC for 16 min; the solutions were cooled, mixed and their absorbance measured against the blank solution at a wavelength of 572 nm in 1 cm cells (Leach, 1960). The following calibration curve (Fig 3) was obtained.

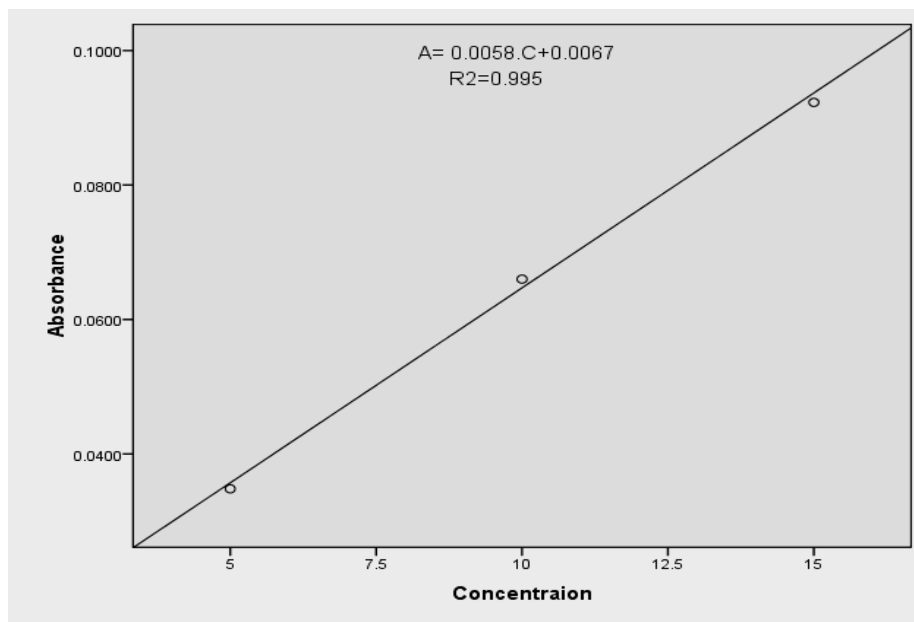

Fig. 3 Calibration curve for standard hydroxyproline.

To determine the hydroxyproline content in mice tissue, circular wound with approximate area of 300 mm^2 was created using the procedure described in excision wound model. The wounds were treated with topical application of ointments as described above for 10 days (Kokane *et al.*, 2009). On the 11th day, the animals were killed under anaesthesia using high dose of diethyl ether. The wound tissue was excised; its weight was recorded and stored in a refrigerator using 10% formalin. On the day of the experiment, the tissue was dried in oven at 60 ^{0}C for 12 h and the dry weight was again noted. It was hydrolyzed in 6 N HCl for 24 h at 110 ^{0}C in sealed glass tubes. The hydrolysate was neutralized to pH 7 (Sanwal and Chaudhary, 2011). Then, 1 ml of the supernatant solution was taken from each tissue hydrolysate. Absorbance of the hydrolysate was read using the same procedure done during standard hydroxyproline absorbance measurement. The amount of hydroxyproline in the

samples was calculated by using the following equation which was obtained from the calibration curve (Fig 3) of the standard hydroxyproline:

$$A=0.0058.C + 0.0067$$

Where A is absorbance and C is concentrations

3.2.6 Incision wound model

Anesthesia was done in the same manner described for excision wound model (Section 3.2.5). The dorsal fur of the mice was shaved with shaving machine. A 3 cm long longitudinal paravertebral incision was made through the skin and cutaneous tissue. Then, the parted skin was sutured 1 cm apart using a surgical thread (no. 000) and curved needle (no. 11) (Fig 4) (Rawat and Gupta, 2011). The continuous thread on both wound edges were tightened for good closure of the wounds. The animals were randomly divided into five groups of 6 mice each and the treatment of the experimental animals was similar to that for the excision wound experiments except the fifth group which was left with no material application. The sutures were removed on day 8 post-incision and treatment was continued up to 9th day (Wang *et al.*, 2011). Tensile strength was measured by continuous water flow technique (Lee, 1968).

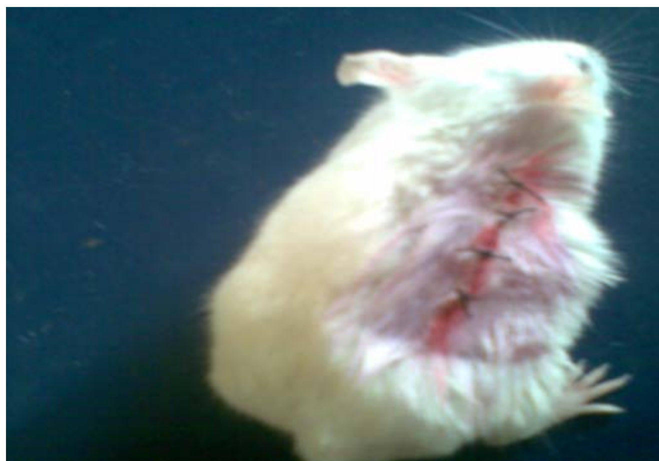

Fig. 4 Incision wound on day 0.

Measurement of tensile strength

On the 10th post-wounding day each mouse was anaesthetized using diethyl ether. The anesthetized animals were secured to the table using thread as shown in Fig 5. The two forceps were firmly applied to the healed tissue on the incised part of the skin on to the line

facing each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light weight measuring graduated container through a string run over to a pulley. Water was allowed to flow from the reservoir slowly and continuously into the container. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. As soon as wound gaping appeared, water flow was stopped, and the volume of water collected in the container (approximately equal to its weight) was determined and noted as a measure of breaking strength in grams. Three readings were recorded for a given incision wound (Thakur *et al.*, 2011). Percentage of tensile strength for extract and reference drug with respect to negative control treated with simple ointment (s.o), and percent strength of negative control with simple ointment (s.o) with respect to negative control left untreated (l.u) was measured using the following formula (Akkol *et al.*, 2011):

$$\text{Tensile strength (TS) of extract } \% = \frac{\text{TS extract - TS s.o}}{\text{TS s.o}} X100$$

$$\text{Tensile strength of reference } \% = \frac{\text{TS reference - TS s.o}}{\text{TS s.o}} X100$$

$$\text{Tensile strength of s.o } \% = \frac{\text{TS s.o - TS l.u}}{\text{TS l.u}} X100$$

Fig. 5 Water flow technique: for the measurement of tensile strength of skin.

3.2.7 Antiinflammatory activity

Swiss albino mice of either sex were used to determine the antiinflammatory activity of *R. abyssinicus* by using mouse paw oedema model. Following overnight fasting with free access

to water, the basal volume of the right hind paw of each mouse was determined before administration of any drug using plethysmometer (Ugo Basile, Italy) (Padilha *et al.*, 2010). After determination of the basal volume, the animals were divided into five groups such that the mean volumes of the different groups were similar. Vehicle (CMC), *R. abyssinicus* hydroalcoholic extract and standard drug were administered orally to their respective groups 1 h before carrageenan injection. The extract and the standard drug were dissolved in 1% CMC to prepare an oral suspension. Paw swelling was induced by subplantar injection of 0.05 ml of a solution of 1% carrageenan in 0.9% saline (w/v) into the right hind paw. The inflammation was quantified by measuring the volume displaced by the paw, in a plethysmometer 1, 2, 3 and 4 h after carrageenan injection (Recio *et al.*, 1995; Marrassini *et al.*, 2010). Results were represented as the paw volume (ml) variation with respect to the basal values. The percentage inhibition of oedema for each group was calculated using the following formula (Mahomed and Ojewole, 2004):

$$\text{Percentage inhibition of edema} = \frac{\text{Co - Ct}}{\text{Co}} X100$$

Where C_o is the average inflammation (hind paw oedema) of the control group at a given time; and C_t is the average inflammation of the plant extract or indomethacine treated mice at the same time.

3.2.8 Statistical analysis

Raw data obtained from both wound and mouse paw oedema models were expressed as mean±SEM and the treated group was compared to each other and with control group. The results were compared statistically by one-way ANOVA followed by Post Hoc Tukey test using SPSS 17.0 software, to analyze the differences between different groups. The data were considered significant at $p < 0.05$.

4 Results

4.1 Acute dermal toxicity

Maximum concentration of hydroalcoholic ointment (10% w/w) administered using a limit dose of 2 g/kg was found to be safe. After 24 h, the application site did not show any sign of inflammation (Fig 6). There were no signs toxicity seen when the animals were monitored for 48 h. There was also neither mortality nor any sign of toxicity observed in rats when monitored for 14 days after administration of the extract.

A B

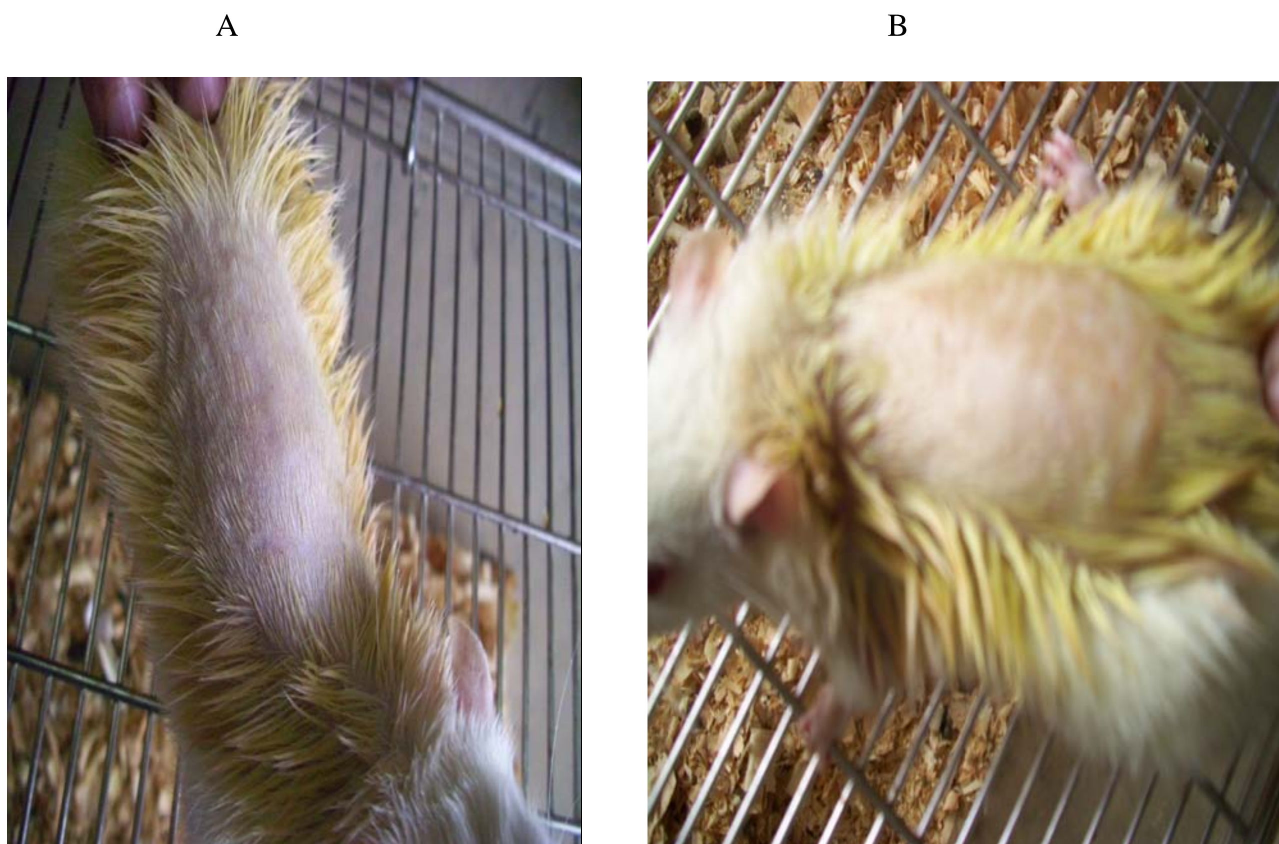

Fig. 6 Photograph of acute dermal toxicity test result. A) Male rat, B) Female rat.

4.2 Wound healing study

4.2.1 Excision wounds

i) Wound contraction

The progress of wound contraction induced by treatment of 5% and 10% (w/w) *R. abyssinicus*

rhizome hydroalcoholic extract ointment, simple ointment base and nitrofurazone is shown in Table 1 and Fig 7. Contraction of the excision wound was promoted till day 15 in extract and standard ointment treated groups. The plant extracts facilitated wound contraction significantly at both dose levels from 7th day to 15th day as compared to control.

The 10% (w/w) hydroalcoholic extract ointment treated group showed significant ($p< 0.001$) wound contraction starting from the third day onwards, in comparison with the control group. On the 3rd and 5th day, the 10% (w/w) extract containing ointment treated group showed significant ($p< 0.01$) and better wound contraction than those treated with 5% (w/w) ointment. As shown in Table 1, there was no significant difference in activity between the 10% extract and the standard drug. But, higher rate of wound closure was observed with 10 % (w/w) ointment as shown in Fig 7. The maximum rate of wound contraction was seen on the 9th, 11th, and 13th day which was 96.3, 99.4, and 100%, respectively. The corresponding figures for the standard drug treated group were 94.2 (day 9), 98.4 (day 11) and 99.4% (day 13).

The animals treated with 5% (w/w) hydroalcoholic extract ointment showed insignificant wound contraction on the 3rd and 5th day as compared to control group even though the area of the wound was reduced to 179.3±23.3 and 150±15.0 mm^2, respectively, of their original size (300 mm^2). However, this ointment showed significant ($p < 0.01$) wound contraction effect on the 7th, 9th, 11th and 13th day in comparison with control group. Starting from the 7th day, the 5% (w/w) treated group did not have significant differences either with 10% (w/w) ointment treated or nitrofurazone treated group. The percentage wound closure on the 9th, 11th and 13th days was 90.9, 96.7, and 99.3%, respectively. The 5% (w/w), 10 % (w/w) and nitrofurazone ointment treated groups possess very much close percent wound closure on the 13th day post wound period which was 99.3, 100 and 99.4%, respectively. Significant ($p < 0.001$) wound contraction was also observed for standard treated group on the 3rd day onward as compared to control.

A healing pattern with complete wound closure was observed in standard, 10% (w/w) extract ointment and 5% (w/w) extract ointment treated groups within 14, 13, and 15 days respectively, while it took about 17 days for complete wound closure in the control group.

Table 1: Effect of topical application of the 80% methanolic extract of the rhizomes of *Rumex abyssinicus* on wound contraction of excision wound model in mice.

	Wound area (mm^2) post-wounding days						
Treatment	3	5	7	9	11	13	15
SO	234.2±11.8	202.0±10.6	159.1±13.0	110.0±8.5	33.5±5	20.5±2.7	9.2±3.0
5% HE	179.3±23.3	150±15.0	$80.2±21.7^{a2}$	$27.3±11.4^{a2}$	$10±6^{a2}$	$2±1.4^{a3}$	0^{a2}
10% HE	$82.4±8.1^{a3,c2}$	$72.67±9.2^{a3,c2}$	$39.1±8.1^{a3}$	$11.0±3.0^{a3}$	$1.8±.5^{a3}$	0^{a3}	-
NF	$130.7±17.0^{a2}$	$108.5±17.4^{a3}$	$54.5±10.7^{a3}$	$17.2±4.8^{a3}$	$4.8±2^{a3}$	$1.67±1.3^{a3}$	0^{a2}

SO, simple ointment base; HE, hydroalcoholic extract; NF, nitrofurazone, n = 6 animals in each group; Values are expressed as mean ± SEM, one way ANOVA. ^{a} against control, ^{c} against 5% (w/w) hydroalcoholic extract, $^{2}p < 0.01$, $^{3}p < 0.001$.

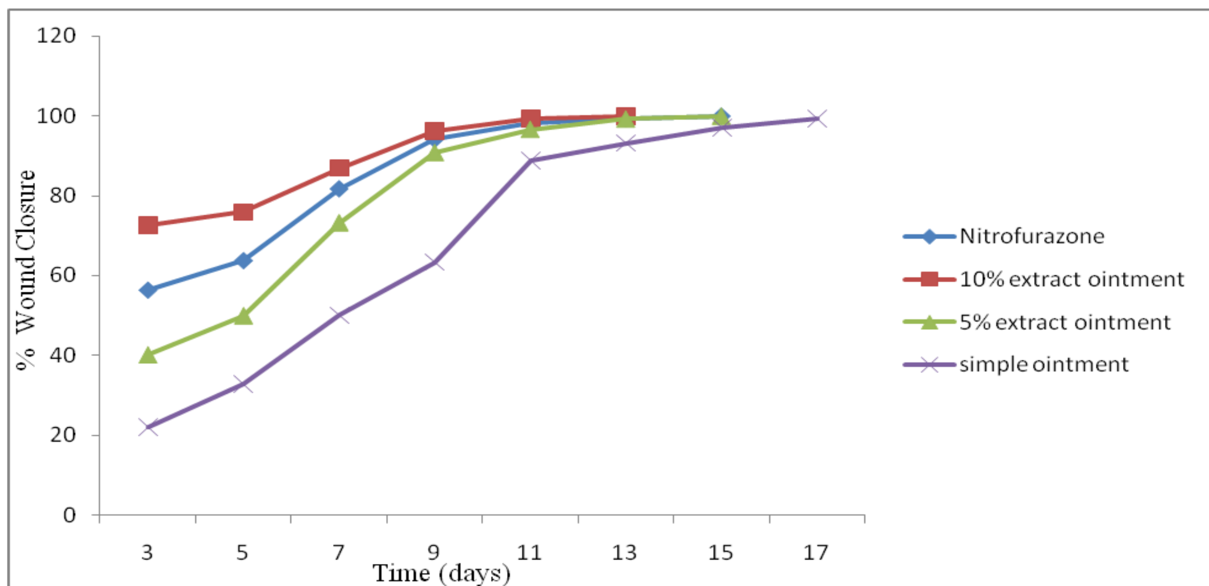

Fig. 7 Effects of the 80% methanolic extract of *Rumex abyssinicus* rhizomes on the percentage wound closure of excision wound model.

ii) Epithelization period

Time for complete epithelization was short in extract ointment and nitrofurazone treated groups as compared to control (Table 2). Animals treated with 10% (w/w) extract and nitrofurazone ointments showed significant decrease (23.1% and 22.1%, respectively, $p < 0.001$) in epithelization period as evidenced by shorter period for fall of eschar. Likewise, 5% (w/w) ointment treated group exhibited significant decrease (16.3%, $p < 0.05$) in epithelization period as compared to control group. However, the difference between epithelization periods of the extract ointments treated groups between each other and with standard treated group was not statistically significant.

Table 2: Effect of topical application of the 80% methanolic extract of the rhizomes of *Rumex abyssinicus* on epithelization period.

Groups	Epithelization period (days)
Simple ointment base	17.33±0.333
5% hydroalcoholic extract	14.50 ± 0.764^{a2}
10% hydroalcoholic extract	13.33 ± 0.333^{a3}
Nitrofurazone	13.50 ± 0.671^{a3}

n = 6 albino mice per group; values represents mean±SEM, one way ANOVA.
^{a} against control, $^{2}p < 0.01$, $^{3}p < 0.001$.

iii) Hydroxyproline content

Table 3 shows the hydroxyproline content in the wound tissues of the four groups on day 11. Hydroxyproline levels in 5% and 10% (w/w) ointment treated groups were significantly increased ($p < 0.01$ and $p < 0.001$, respectively), when compared to control. The mice treated with nitrofurazone ointment also exhibited significantly higher hydroxyproline content (p <

0.001) in comparison to controls. Hydroxyproline content of extract treated mice was not significantly different compared to standard.

Table 3: Hydroxyproline content of topical application of the 80% methanolic extract of *Rumex abyssinicus* on excision wounds.

Groups	Hydroxyproline (mg/100g)
Simple ointment base	10.60±0.92
5% hydroalcoholic ointment	13.95±0.89[a1]
10% hydroalcoholic ointment	18.47±0.70[a3]
Nitrofurazone	21.62±0.86[a3]

Values are expressed as mean±SEM (n= 6). [a] against control, [1]$p < 0.05$, [3]$p < 0.001$.

4.2.2 Incision wounds

Tensile strength of incision wound model

The mean tensile strength in the group treated with simple ointment base BP tended to increase by about 19.4%, which failed to reach statistical significance, compared to untreated controls. By contrast, tensile strength was significantly increased by about 57.2% ($p< 0.001$) and 79.1% ($p<0.001$) by treatment with 10% of the extract and nitrofurazone ointment, respectively (Table 4). The tensile strength of 5% (w/w) extract ointment treated group also showed a lesser but significant ($p < 0.05$) increase in tensile strength compared to treated control group.

The tensile strength of standard drug treated animals was significantly higher ($p < 0.01$) than the 5% extract treated animals. But there was no significant difference between 10% and 5% (w/w) hydroalcoholic extract ointment treated groups.

Table 4: Effect of topical application of the 80% methanolic extract of *Rumex abyssinicus* on tensile strength of incision wound model.

Group	Tensile strength (g) (mean± SEM)	%Tensile strength
Untreated control	205.35±19.451	-
SO	244.67±17.111	19.4%
5% (w/w) HE	333.33±18.196^{a1}	36.2%
10% (w/w) HE	384.50±19.619^{a3}	57.2%
Nitrofurazone	438.17±19.123^{c2,a3}	79.1%

SO, simple ointment base, HE, hydroalcoholic extract, n = 6 swiss albino mice per group

^{a} against control treated with simple ointment base, ^{c} against 5% hydroalcoholic extract, $^{1}p < 0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

4.3 Antiinflammatory test

The antiinflammatory activity of the extract is summarized in Table 5. One hour after administration of carrageenan, neither the extract nor the standard drug showed significant antiinflammatory activity as compared to the control. The two higher doses of *R. abyssinicus* extract (500 and 750 mg/kg) started suppression of oedema significantly ($p < 0.001$) after 2 h of carrageenan injection as compared to control showing 32.86% and 38.57% inhibition of oedema, respectively. But the lower dose (250 mg/kg) began significant suppression ($p < 0.05$) after 3 h of oedema induction. The group which received the standard drug showed significant inhibition of inflammation starting from 2 h post carrageenan injection. The inhibitory values of oedema at 3 h post carrageenan were 20.29, 36.23 and 42.03% for 250, 500 and 750 mg/kg of the extract, respectively. The percentage inhibition of oedema by standard drug was 65%.

There were significant differences between 250 and 750 mg/kg doses at 2 h ($p < 0.05$), 3 h ($p < 0.01$) and 4 h ($p < 0.001$) post carrageenan administration. The 500 mg/kg showed

significant difference from the 250 mg/kg dose at 4 h ($p < 0.001$) post inflammation induction. It was also observed that the standard drug showed significant difference from 250 mg/kg at 2 h, 3 h and 4 h ($p < 0.001$) post carrageenan injection, while the difference was not significant as compared to 500 mg/kg and 750 mg/kg doses post carrageenan administration.

Table 5: Antiinflammatory effects of orally administerd *Rumex abyssinicus* rhizomes 80% methanol extract on carrageenan-induced mice paw oedema.

Groups	Mean increase in the paw volume (ml)				
	Basal	1 h	2 h	3 h	4 h
Control	0.50±0.04	0.61±0.02	0.7±0.03	0.69±0.04	0.65±0.03
Extract (250 mg/kg)	0.50±0.02	0.60±0.03 (1.64%)	0.58±0.04 (17%)	0.55±0.02^{a1} (20.29%)	0.51±0.02^{a2} (21.54%)
Extract (500 mg/kg)	0.50±0.03	0.55±0.02 (9.84%)	0.47±0.04^{a3} (32.86%)	0.44±0.02^{a3} (36.23%)	0.30±0.03^{ac3} (53.85%)
Extract (750 mg/kg)	0.51±0.01	0.48±0.04 (21.31%)	0.43±0.02^{a3,c1} (38.57%)	0.40±0.04^{a3,c2} (42.03%)	0.25±0.02^{ac3} (61.54%)
Indomethacine (10 mg/kg)	0.53±0.03	0.48±0.03 (24.6%)	0.35±.02^{ac3} (50%)	0.24±0.02^{ac3} (65%)	0.10±0.02^{ac3} (84.6%)

Each value denotes the mean±SEM with % inhibition depicted in the parenthesis, n = 6 swiss albino mice per group; ^{a} against control, ^{c} against 250 mg/kg of hydroalcoholic extract, $^{1}p < 0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

5 Discussion

Traditionally, the rhizomes of *R. abyssinicus* are used for wound healing activity mixed with butter. Butter is non polar agent. Thus, the rhizomes of *R. abyssinicus* were extracted with 80% methanol in order to get hydrophilic and charged products. Applying the extract directly on the affected wound can't bring the desired effect as they don't stay longer on the wounded skin of the experimental animals. Semi-solid preparation like ointment is necessary to achieve a sustained drug release at the application sites. Hence, a hydrophobic base (simple ointment) was selected based on *R. abyssinicus* rhizomes traditional wound healing use having assumption that a non polar extract would be released better from the polar base and vice versa (Manjunatha *et al*, 2005).

The ointment base has multiple roles. The hydrocarbon bases such as hard paraffin and white soft paraffin are used to form an occlusive barrier on the skin that prevents escape of moisture from the skin into the environment. As a result moisture accumulates between skin and ointment layer that causes hydration of the stratum corneum. Additionally, the moisture layer provides a medium for dissolution of the drug that is otherwise dispersed as fine particles in the ointment base. Wool fat and Cetostearyl alcohol are thickeners. And they are used for stabilization of ointment (Ansel, 1985).

In the present study, two different models were used to assess the effects of hydroalcoholic extract ointment of *R. abyssinicus* on the various wound phases which run concurrently but independent of each other. Because the use of a single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing, two or more models are used in wound healing studies. Even though a large effort has been made to study *in vitro* wound healing activity, *in vivo* studies are still remain indispensable for wound healing activity investigation as wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state (Abdulla *et al.*, 2010).

Wound healing involves various phases. Initially involves acute inflammatory phase followed by the synthesis of collagen and other extracellular macromolecules, which are later removed to form a scar. Drugs, which influence one phase, may not necessarily influence another (Paschapur *et al.*, 2009). Thus, the development of an ideal wound healing drug is still a

challenge to the medical scientists. The ideal drug should fulfill the criteria such as rapid contraction of wound leading to quick healing, reduction of wound epithelization and appreciable gain of tensile strength. Biochemically, the tissue DNA, RNA, total protein, and hydroxyproline will be the marker of good healing property of the drug (Kanti *et al.*, 2001).

Wound contraction, a part of the proliferative phase of wound healing, can be defined as the centripetal movement of the edges of a full-thickness wound to facilitate closure of the defect (Tang *et al.*, 2007). In excision wound healing model the hydroalcoholic extract of the rhizomes of *R. abyssinicus* showed significant increase in percentage closure of excision wounds. The wound closure time was shorter and the percentage of wound contraction was much higher in the 10% (w/w) extract ointment treated group which was almost similar to that of nitrofurazone treated group. The 5% (w/w) extract ointment treated mice show significant wound healing activity starting from the 7th day onwards. The higher wound contraction rate of the 10% (w/w) extract ointment may be due either to its higher antiinflammatory effect or induction of macrophage cell proliferation (Getie *et al.*, 2003) than the 5% (w/w) extract ointment. The faster rate of contraction with the standard drug may be attributed to its antimicrobial effect. The wound contractions observed indicate that the rhizomes of *R. abyssinicus* possess a definite prohealing action, since 88% of the healing of wound occurred due to contraction, and the other 22% occurred due to scar formation (Ejaz *et al.*, 2009). Wound contraction indicates rate of reduction of unhealed area during the course of treatment. The greater the reduction, the better is the efficacy of medication. In other words the wound will close at fast rate if the medication is more efficient (Prasad and Dorle, 2006).

The epithelization time was significantly reduced from 17 days (control) to 15, 14 and 13 days for 5% extract, nitrofurazone and 10% extract ointment treated groups, respectively. The occurrence of enhanced epithelization and wound contraction could be due to the ability of *R. abyssinicus* extracts to enhance collagen synthesis as evidenced by the effect of the extract on hydroxyproline content, as wound contraction begins almost concurrently with collagen synthesis. The rate of contraction depends on the degree of tissue laxity and shape of the wound (Pillai *et al.*, 2010). The plant's ability to facilitate the proliferation of epithelial cells (Getie *et al.*, 2003) could be another reason for enhanced wound contraction by enhanced epithelial migration as it increases the viability of epithelial cells. Collagen also plays an

important role in haemostasis and epithelization at a later phase of wound healing (Wang *et al.*, 2011).

The importance of collagen in wound healing has been appreciated for a long time for the simple reason that the ultimate result of most repairs in the higher vertebrate is the formation of scar tissue composed of collagenous fibers (James and Friday, 2010). The re-arrangement of ground collagen fibers can ultimately influence the quality of scars. The ECM not only functions as a reservoir for growth factors and signaling molecules, but also acts as a framework upon which endothelial cells can migrate during angiogenesis. Contraction is caused by the re-arrangement of collagen fibers through the action of fibroblasts. The dislocation forces created by these cells within the connective tissue would lead to the re-orientation of collagen fibers into thicker bundles as well as their contraction (Ejaz *et al.*, 2009).

Earlier studies have shown that antimicrobial activity of various plants support wound healing (Deshmukh *et al.*, 2009). Increased rate of wound contraction and decrease in period of epithelization in the animals treated with the 80% methanolic extract of *R. abyssinicus* in excision wound model may also be due to their antimicrobial activity. Antimicrobial activity of the 80% methanol extract of the rhizomes of *R. abyssinicus* against *S. pyogenes* and *S. aureus* was reported by Getie et al. (2003).

Postoperative wounds are commonly known to be complicated by infection (Deshmukh *et al.*, 2009). Infection can seriously delay healing process by disrupting the normal clotting mechanisms, promoting disordered leukocyte function and ultimately delaying angiogenesis (Annan and Houghton, 2008). Microbes can also cause poor quality granulation tissue formation, reduced tensile strength of connective tissue, impaired epithelization and odour (Maryanne *et al.*, 2003). Both microbes and endotoxins can lead to a prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. This can cause a chronic inflammatory state that promotes the development of MMPs, thus inhibiting wound healing. *P. aeruginosa* and *S. aureus* appear to play an important role in bacterial infection in wounds (Esimone *et al.*, 2005; Guo and Dipietro, 2010; Joseph, 2011).

In the group treated with simple ointment base, very slow epithelial reorganization and wound closure was observed. But the *R. abyssinicus* as well as standard drug ointment treated

wounds were clean with healthy tissues. This could be due to the presence of microorganisms and their metabolites in the control group, which inhibit wound contraction and deteriorate the wound healing activity.

The other contributing factor for the wound healing effect of the plant is its antiinflammatory activity. Inflammation is a normal part of the wound healing process. The normal function of inflammation in an acute wound is to prepare the wound bed for healing by removing necrotic tissue, debris, and bacterial contaminants as well as recruiting and activating fibroblasts. Under normal conditions, inflammation is a self-limiting process (Menke *et al.*, 2007). Excessive inflammation, however, impedes wound healing.

The present *in vivo* study revealed antiinflammatory activity of the 80% methanolic extract of *R. abyssinicus* in carrageenan induced paw oedema model in mice. Carrageenan induced hind paw oedema model has been used widely for the discovery and evaluation of antiinflammatory drugs, since the relative potency estimates obtained from most drugs tend to reflect clinical experience (Padilha *et al.*, 2010). The extract showed highly significant antiinflammatory effect in a dose dependent manner as shown in Table 5.

Carrageenan is a sulphated polysaccharide obtained from sea weed and is commonly used to induce acute inflammation. Inflammation induced by carrageenan develops immediately following injections. It produces three distinct phases. In the first phase (0-1.5 h) histamine and serotonin are the mediators involved. The second phase (1.5-2.5 h) is mediated by bradykinin, while prostaglandins are implied in the third phase (2.5-5 h). The third phase of oedema is sensitive to most clinically effective antiinflammatory drugs, which have been frequently used to assess the antioedematous effect of natural products (Di Roso *et al.*, 1971; Marrassini *et al.*, 2010). This investigation showed that the 80% methanol extract of *R. abyssinicus* had no antiinflammatory activity during the 1st h but percent inhibition of inflammation was highest during the 3rd and 4th h after inflammation was induced. The lower dose was unable to produce any effect until the third hour and the others produced in the second hour. The reason could be the lower dose might not be able to achieve maximum plasma concentration at 2 h for the second phase inhibition.

The percent inhibition of oedema of the extract of the rhizomes of *R. abyysinicus* and indomethacine is higher in the late phase due to the effect of phase one mediators are less

responsive to steroidal and non-steroidal antiinflammatory agents. Thus, it is possible to propose that the antiinflammatory effect observed may be due to the ability of the extract to inhibit the release and/or the activity of bradykinin and/or prostaglandins. This activity was supported by *in vitro* antiinflammatory study reported by Getie et al. (2003). This report indicated that the antiinflammatory effect of the 80% methanol extract of the rhizomes of *R. abyssinicus* is by inhibition of PGE_2 synthesis.

Previous reports indicate that a number of plants with antiinflammatory activity do also possess wound healing effect. These include *Centaurea iberica* Trev (Koca *et al.*, 2009), *Curcuma aromatica* (Kumar *et al.*, 2009), *Memecylon edule* Roxb (Nualkaew *et al.*, 2009) and *Prosthechea michuacana* (Gutierrez and Solis, 2009). This arises from the fact that prolonged inflammation leads to an increased level of MMPs, a family of proteases that can degrade the ECM. Hence, the mitogenic activity of cells is suppressed (Menke *et al.*, 2007; Guo and Dipietro, 2010). Prostaglandins are also involved in chemotaxis of leukocytes mainly neutrophils that contribute to inflammatory response by producing oxygen derived free radicals that damage wound tissue (Serhan *et al.*, 2008). Thus, prostaglandin inhibition benefit wound healing by reducing damaging effect of neutrophils. Even though many antiinflammatory agents were used for wound treatment, they are well known to inhibit wound repair *via* global antiinflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis (Guo and Dipietro, 2010).

The force required to open the healing wound is known as tensile strength. It is used to measure the completeness of healing. It also indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue (Shivhare *et al.*, 2010). In incision wound, significant increase was observed in skin breaking strength. 10% and 5% (w/w) extract ointment treated animals required a force which was stronger than simple ointment base treated animals to open the wound on 10th day. The wound of standard ointment treated group required even stronger force compared to simple ointment base treated control. Less amount of force was needed to open wounds of simple ointment base treated animals compared to untreated ones. The tensile strength of wounds treated with nitrofurazone was the highest, but the difference between this and those treated with 10% ointment formulation was not statistically significant. It may be inferred that nitrofurazone

and 10% extract ointment exert more or less similar effects on the tensile strength of the healing tissue.

The healing process depends to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation (Prasad and Dorle, 2006). In incision wound, the increase in tensile strength of treated wounds may be due to the increase in both remodeling of collagen, and the formation of stable intra- and intermolecular crosslink. The collagen molecules synthesized are laid down at the wound site and become crosslinked to form fibres (Wang *et al.*, 2011). In addition, several studies reported an increase in wound tensile strength depends on factors, in addition to collagen deposition, such as matrix deposition and cell migration. *R. abyssinicus* has been reported to have cell proliferation effect (Gete *et al.*, 2003), which may contribute to its ability to increase matrix deposition for significant increment of tensile strength.

Hydroxyproline is one of the biomarkers indicating wound healing process as evidenced by increase in its content on the 11th day of treatment. It is an amino acid found in abundant in collagen and breakdown of collagen liberates free hydroxyproline and its peptides (Nayak *et al.*, 2006). It is known that collagen accumulation is the sum of synthesis and destruction and both occur simultaneously during wound healing process (Pillai *et al.*, 2010). Thus, measurement of hydroxyproline, which comes from the breakdown of collagen, has been used as an index of collagen turnover (Pillai *et al.*, 2010). High concentration of hydroxyproline indicates faster rate of wound healing (Prasad and Dorle, 2006). In the present study, hydoxyproline content was significantly increased with 10% and 5% extract ointment treated groups. Therefore, the increased amount of hydroxyproline in extract treated groups underlines increased collagen content. The decreased collagen content in the negative control group might be due to a prolonged inflammatory phase where the degradation of collagen is greater than its synthesis.

A number of studies indicate that plant products are potential agents for wound healing and largely preferred because of their widespread availability, absence of unwanted side effects and their effectiveness (Tang *et al.*, 2007). Botanical remedies provide two advantages over single compound drugs: primary active compounds in plants are synergized by secondary compounds and secondary compounds ease the side effects caused by primary active

compounds. Thus, the presence of more than one compound in a plant extract could contribute to a net pharmacological response of the extract (Akkol *et al.*, 2009).

In recent studies various plant extracts have been proved to possess wound healing effect as a result of the different phytochemical constituents they contain. Previous phytochemical analysis of the 80% methanol extract of the rhizomes of *R. abyssinicus* revealed that tannins, saponins, flavonoids, steroids and anthraquinones are the major constituents of the plant (Mekonnen *et al.*, 2010). Phenolics (such as flavonoids and tannins) obtained from the hydroalcoholic extract of *Rumex crispus* (Coruh *et al.*, 2008), *R. maderensis* (Tavares *et al.*, 2010), *R. acetosa*, *R. patientia* (Sreelekshimi *et al.*, 2007), *Polygonum convolvulus*, *Rheum undulatum*, *and R. acetosella* (Tseye-Oidov *et al.*, 2010) are reported to have antioxidant, antiinflammatory and antibacterial activities, which seem to be responsible for wound contraction and increased rate of epithelization. Chrysophanol, emodin and physcion were also previously isolated from the rhizomes of *R. abyssinicus* (Fassil *et al.*, 1984). Emodin (Tang *et al.*, 2007; Omoregie *et al.*, 2010) and chrysophanol (García-Sosa *et al.*, 2006; Sheeba *et al.*, 2009; Kim *et al.*, 2010) isolated from other plant species are reported to have antimicrobial, antiinflammatory and wound healing activities. Majority of Rumex species have also been reported to have antimicrobial action due to the presence of physcion and rumicin (Abebe *et al.*, 2003).

Therefore, it is likely that the wound healing and antiinflammatory property of *R. abyssinicus* may be attributed to its phytoconstituents which work either individually or may have synergistic effects. The effects produced by the *R. abyssinicus* hydroalcoholic extract ointment, in terms of wound contracting ability, wound closure time and tensile strength of the wound are comparable to the effects produced by the leaf extracts of *Hypericum patulum* (Mukherje *et al.*, 2000), and *Ficus religiosa* (Nayeem *et al.*, 2009). The tannins, steroids and flavonoids present in *Hypericum patulum* and *Ficus religiosa* have been reported to be the wound healing components of these plants.

6 Conclusions and Recommendations

6.1 Conclusions

In the present study, the different phases of wound repair, including collagen synthesis, wound contraction and epithelization were improved by ointments prepared from the 80% methanolic extract of the rhizomes of *R. abyssinicus* as compared to the control group. Hence, the present research results support the traditional claims of the plant for the treatment of wounds. This work also showed that the hydroalcoholic extract of *R. abyssinicus* was endowed with significant antiinflammatory activities that explain at least in part its wound healing activity.

6.2 Recommendations

- Chronic toxicity studies should be performed.
- The results of the present study should be corroborated with histopathological studies
- The plant should be further fractionated and the possible active components isolated to identify which fractions or component (s) of the plant extract responsible for wound healing activity.
- As chronic wounds such as diabetic wounds are major global burden, it is worthwhile to study the activity of the plant on chronic wounds.

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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 our approval as University Advisors.

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1. Dr. Ephrem Engidawork	__________________
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