ADDIS ABABA UNIVERSITY
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Evaluation of anti-inflammatory and wound healing activities of the 80% methanol fruit extracts of *Dovyalis abyssinica* A. Rich. in mice

By

Hailu Abdissa (B. Pharm)

October, 2011

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By Hailu Abdissa

A Thesis submitted to the Department of Pharmacology and Therapeutics, School of Pharmacy, Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pharmacology.

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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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ACKNOWLEDGEMENTS

First of all I would like to express my sincere gratitude to my advisors Dr. Ephrem Engidawork and Dr. Kaleab Asres for their invaluable advice and follow up during my study.

My deeper gratitude also goes to the staff members of departments of Pharmacology and Therapeutics, Pharmacognosy and Pharmaceutical Chemistry and Pharmaceutics (School of Pharmacy) for their support during my work.

I am also thankful to Ato Melaku Wondafrash of the National Herbarium (Department of Biology, College of Natural Sciences, AAU) for identifying the plant specimen.

I am very much thankful to my family and friends for supporting me in one way or another during my study.

My acknowledgement also goes to Addis Ababa University for funding this study.

Everything was possible because of the Almighty God. Father God, You are worthy of my thanks and praise.

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ACRONYMS

AAU:	Addis Ababa University
bFGF:	Basic Fibroblast Growth Factor
BP:	British Pharmacopoeia
BPC:	British Pharmaceutical Codex
ECM:	Extracellular Matrix
FGF:	Fibroblast Growth Factor
HIF:	Hypoxia Inducible Factor
IGF-1:	Insulin Like Growth Factor One
IL-1:	Interleukin One
IL-8:	Interleukin Eight
KGF:	Keratinocyte Growth Factor
MEBO:	Moist Exposed Burn Ointment
MF:	Master Formula
MMP:	Matrix Metalloprotease
PDGF:	Platelet Derived growth Factor
RF:	Reduced Formula
ROS:	Reactive Oxygen Species
TGF-β:	Transforming Growth Factor Beta
TIMP:	Tissue Inhibitor of Metalloprotease
TNF-α:	Tumor Necrosis Factor Alpha
VEGF:	Vascular Endothelial Growth Factor

ABSTRACT

Dovyalis abyssinica locally known as "Koshim" is traditionally used to treat conditions such as hemorrhoids, ulcers and swelling of the throat. The toothbrush sticks are also reported to be used for oral hygiene. There is also a traditional claim that the fruits of *D. abyssinica* promote wound healing. However, there is no scientific evidence that justifies the traditional claims and therefore the present study was aimed at evaluating the wound healing and anti-inflammatory effects of the fruits wound healing mice models.

In this study, the fruits of *D. abyssinica* were evaluated by formulating the 80% methanol fruit extracts in simple ointment base in strengths of 5% and 10% for topical applications for excision as well as incision wound models. Extract solutions in saline in strengths of 100 mg, 200 mg and 400 mg/Kg body weight doses were also prepared and used for oral administration for anti-inflammatory activity tests. From the excision wound model, rate of wound contraction, epithelization period and hydroxyproline content were determined. From the incision wound model, tensile strength of the healing wound was evaluated. For the evaluation of the anti-inflammatory activity, carrageenen-induced hind paw edema model was employed and increase in paw size and % inhibition of edema were determined.

The 80% methanol fruit extracts exhibited a significant wound healing activity in both strengths compared with control as evidenced by an increase in % wound contraction ($p < 0.001$), a decrease in epithelization period ($p < 0.001$), and an increase in hydroxyproline content ($p < 0.001$) in excision wound model. In the same model, the 10% extract ointment resulted in a significant wound contraction at days 8, 12 and 16 and hydroxyproline content compared against both standard and 5% extract ointment ($p < 0.01$). In the incision wound model, both 5% and 10% extract ointments resulted in a significant increase in tensile strength compared with control ($p < 0.001$). The same extract also exhibited a significant anti-inflammatory effect compared with control particularly 3 to 5 h after extract administration as shown by a decrease in edema expressed as % reduction of edema. At the 3rd h, both 200 and 400 mg/kg body weight doses exhibited a higher effect compared against control ($p < 0.001$) while the 100 mg/kg extract dose resulted in a comparable effect compared with same ($p < 0.01$). At the 4th and 5th h, all the extract doses exhibited a significant effect compared with control ($p < 0.001$). It was also shown that the

400 mg/kg extract dose exhibited a significant effect compared with the 100 mg/kg extract dose ($p < 0.05$ at the 3rd and 5th h and $p < 0.01$ at the 4th h).

The 80% extract of the fruits of *Dovyalis abyssinica,* therefore, proves to support healing of wounds as the traditional claims as evidenced by an increase in wound contraction, hydroxyproline content and tensile strength and a decrease in epithelization period compared with control results. It also possesses a significant anti-inflammatory effect as shown by a significant decrease in edema compared with control results supporting the wound healing effect.

1. INTRODUCTION

1.1. Overview of wounds

Wound is a clinical problem as old as mankind and can be defined as loss of cellular or anatomic or functional integrity of a living tissue. Wounds can be categorized as bites, burns, surgical wound abrasion, laceration or acute inflammatory phase followed by synthesis of collagen and extracellular molecules which are later remodeled to form scar (Kokane *et al*., 2009).
Wound can also be broadly classified as acute and chronic. Acute wounds normally heal in a very orderly and efficient manner characterized by the wound healing phases described under section 1.2 (Diegelmann and Evans, 2004).

Chronic wounds, on the other hand, are those that failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity of injured site. These are rarely seen in healthy individuals and are usually associated with diseases like diabetes and obesity. Included under this category are foot ulcers and pressure ulcers that are complications of diabetes and spinal cord injuries, respectively. It is estimated that about 6.5 million patients are affected in USA accounting for loss of USD 25 billion for the clinical management of chronic wounds representing a tremendous burden in public health expenditure. In other developed countries, it has also been estimated that 1-2% of the population will experience a chronic wound during their lifetime posing a public health problem. In Scandinavian countries, 2-4% of the total healthcare expenses are lost for the clinical management of chronic wounds (Sen *et al*., 2009).

Another area of concern in the clinical management of wounds is the clinical management of burn wounds. Burns are one of the most common and devastating forms of trauma. Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. Data from the National Center for Injury Prevention and Control in the United States show that approximately 2 million fires are reported each year which result in 1.2 million people with burn injuries. Moderate to severe burn injuries requiring hospitalization account for approximately 100,000 of these cases, and about 5,000 patients die each year from burn related complications.

The survival rates for burn patients have improved substantially in the past few decades due to advances in modern medical care in specialized burn centers. Improved outcomes for severely burned patients have been attributed to medical advances in fluid resuscitation, nutritional support, pulmonary care, burn wound care, and infection control practices. As a result, burn-related deaths, depending on the extent of injury, have been halved within the past 40 years. In patients with severe burns over more than 40% of the total body surface area, 75% of all deaths are currently related to sepsis from burn wound infection or other infection complications and/or inhalation injury (Church *et al*., 2006).

The primary goals of treating wounds are rapid wound closure allowing the skin serve its functions of being a protective barrier and formation of functional and aesthetically satisfactory scar (Singer and Clark, 1999). A lot of research has been and is being undertaken to develop better wound healing agents that can keep up pace with problems encountered in clinical practice (Kokane *et al*., 2009).

The process of wound healing consists of different phases such as granulation, collagenization, collagen maturation and scar maturation that occur concurrently but independently of each other (Deshmukh *et al*., 2009).

1.2. The healing cascade

Cutaneous wound healing is a dynamic and highly coordinated physiological process that rapidly and efficiently restores skin integrity (Santoro and Guadino, 2005). It is a complex physiological process involving the interplay of a series of overlapping chemical, cellular and biological events (Singer and Clark, 1999). This process can be roughly divided into 3 overlapping phases of inflammatory reaction, proliferation and remodeling (Li *et al*., 2007).

The healing cascade begins immediately following injury when the platelets come in contact with collagen exposed as a result of injury (Diegelmann and Evans, 2004). As a result, a fibrin plug forms and inflammatory cells are recruited to the wound area (Falanga, 2005). This initiates and sets off the different phases of wound healing through which acute wounds pass in the

healing process, namely hemostasis, inflammation, proliferation (new tissue formation) and remodeling (Williamson and Harding, 2004). These phases are distinct but overlapping events that require extensive communication between cells (Mutsaers *et al*., 1997). In chronic wound, however, this normal progression is disrupted and slow healing or non-healing can occur (Williamson and Harding, 2004). Non-healing wounds (chronic wounds and chronic ulcers) are trapped in a constant inflammatory state due to a failure to progress through the normal stages of wound healing. This inflammation results in an abnormal wound microenvironment and results in significant, chronic disability with frequent relapse (Menke *et al*., 2008).

1.2.1. Hemostasis

The moment a tissue suffers an injury, blood constituents and vasoactive factors will leak into the wound site causing platelet aggregation and activating the clotting cascade and hemostasis resulting in the realease of clotting factors and deposition of a fibrin clot (Eming *et al*., 2007; Diegelmann and Evans, 2004). The fibrin clot serves as a matrix for the recruitment of inflammatory cells and at a later stage serves for the migration of fibroblasts and other cells that are involved in the healing response (Mutsaers *et al*., 1997). The platelets also release two important signals: platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) that are central in wound healing cascade (Diegelmann and Evans, 2004). In the absence of hemorrhage, however, platelets are not essential in wound healing. Numerous vasoactive mediators and chemotactic factors are generated by activated-complement pathways and by injured or activated parenchymal cells. These released chemical signals recruit inflammatory leukocytes to the site of injury (Singer and Clark, 1999).

1.2.2. The inflammatory phase

Once hemostasis is achieved and the platelets release PDGF and TGF-β, this will be immediately followed by a huge influx of inflammatory cells to the wound site marking the beginning of the inflammatory phase (Kapoor *et al*., 2006). PDGF initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts and stimulates the mitogenesis of fibroblasts

and smooth muscle cells. TGF-β elicits a rapid chemotaxis of macrophages and stimulates them to secret additional cytokines including fibroblast growth factor (FGF), PDGF, tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1). TGF-β further enhances fibroblast and smooth muscle cell chemotaxis and modulates collagen and collagenase expression. All these signals, although look like to be redundant, will result in a vigorous response of matrix producing cells to ensure a rapid deposition of connective tissue at the injury site during the proliferative phase that will immediately follow the inflammatory phase (Diegelmann and Evans, 2004; Tsirogianni *et al*., 2006).

The polymorphonuclear leukocytes (neutrophils) are the first cell types that enter the area of injury as a result of the chemotactic effects of the growth factors released by platelets and will predominate for the first three days after injury, with their number peaking at approximately 48 hours post injury (Diegelmann and Evans, 2004; Tsirogianni *et al*., 2006). The major function of neutrophils is to remove foreign materials, bacteria and non-functional host cells and damaged matrix components that may be present in the wound site by the process of phagocytosis (Diegelmann and Evans, 2004).

The mast cell is another marker cell of interest in wound healing. Mast cells release granules filled with enzymes, histamine and other active amines causing blood vessels surrounding the injured site to become leaky and thus allow the speedy passage of the neutrophils into the injury site (Artuc *et al*., 1999). Since mast cells are predominantly found in the vicinity of connective tissue vessels of skin and mucosa, this makes them easily available during tissue injury to play a role in the inflammatory response giving a way to wound healing (Ng, 2010).

By 48 h after injury, circulating and fixed tissue monocytes become activated to become wound macrophages and release PDGF and TGF-β that further attracts fibroblasts and smooth muscle cells to the wound site. They also remove non-functional host cells, bacteria-filled neutrophils, damaged matrix, foreign debris and any remaining bacteria from the wound site. Their presence also marks that the inflammatory phase is nearing an end and that the proliferative phase is beginning (Diegelmann and Evans, 2004; Tsirogianni *et al*., 2006).

1.2.3. The proliferative phase

Once the debris and infectious organisms are cleared off from the injury site by neutrophils and wound macrophages during the inflammatory phase, the repair response that includes formation of granulation tissue, re-epithelization, angiogenesis (neovascularization) and fibroplasia ensues (Tsirogianni *et al*., 2006; Li *et. al*., 2007). The granulation tissue, also called the provisional matrix, composed of endothelial cells, inflammatory macrophages, lymphocytes and new extracellular matrix (ECM) begins to fill the wound space 2-3 days after injury (Mutsaers *et al*., 1997; Tsirogianni *et al*., 2006). Activated macrophages release PDGF, TGF-β and fibroblast growth factor (FGF). FGF stimulates the fibroblasts to proliferate, migrate into the wound space and synthesize the glycosaminoglycans, proteoglycans and collagen for the new ECM. Fibroblasts become the dominant cell type reaching their peak number at 7-14 days after injury. By this time they start to produce basic fibroblast growth factor (bFGF), TGF-β, PDGF as well as keratinocyte growth factor (KGF) and insulin like growth factor-1 (IGF-1) that facilitate ECM synthesis (Tsirogianni *et al*., 2006).

Granulation tissue formation characterizes dermal reconstitution that begins approximately 3-4 days post injury and involves new blood vessel formation or angiogenesis and the accumulation of fibroblasts and ground matrices named fibroplasia (Li *et al*., 2007).

As the proliferative phase progresses, the TGF-β becomes a critical signal that regulates a host of fibroblast functions on ECM deposition (Diegelmann and Evans, 2004). First, it increases transcription of genes for collagen, proteoglycans and fibronectin thus increasing the overall production of matrix proteins. At the same time it decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates tissue inhibitor of metalloprotease (TIMP) synthesis (Hall *et al*., 2003).

The process of re-epithelization, restoration of an intact epidermis after cutaneous injury, is stimulated by the presence of epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) that are produced by activated wound macrophages, platelets and keratinocytes (Diegelmann and Evans, 2004; Li *et al*., 2007). It involves several processes such as migration of

adjacent epidermal keratinocytes into the wound site, proliferation of keratinocytes and differentiation of neoepithelium into a stratified epidermis and restoration of an intact basement membrane zone (Li *et al*., 2007). The role of EGF and TGF-α at this phase of wound healing has also been demonstrated by the observation that healing of a variety of wounds in animals and patients was enhanced by treatment that employs an exogenous administration of these growth factors (Schultz *et al*., 1991).

After the stratified epidermis is generated, an intact basement membrane zone is formed within 7-9 days after re-epithelization between the epidermis and dermis that is essential for the reestablishment of skin integrity and function. Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab resulting in removal (Li *et al*., 2007).

Angiogenesis refers to new vessel growth by the sprouting of preexisting vessels adjacent to the wound (Li *et al*., 2007). Due to the high metabolic activity at the wound, there is an increasing demand for oxygen and nutrients. To address this demand the process of angiogenesis (neovascularization) is stimulated by vascular endothelial growth factor (VEGF), bFGF, and TGF-β produced by epidermal cells, fibroblasts, macrophages and vascular endothelial cells (Diegelmann and Evans, 2004). In addition, low oxygen tension, lactic acid and biogenic amines produced in the wound stimulate angiogenesis (Li *et al*., 2007). It is interesting to note a signaling pathway that involves the role of low oxygen tension where the low oxygen tension stimulates the expression of a nuclear transcription factor termed "hypoxia-inducible Factor" (HIF) by vascular endothelial cells. The HIF binds to specific sequences of the DNA that regulate the expression of VEGF thus stimulating angiogenesis. When new blood vessels enter the wound repair site and oxygen tension returns to a normal level, oxygen binds to HIF and blocks its activity leading to a decreased synthesis of VEGF (Tonnesen *et al*., 2000; Diegelmann and Evans, 2004). Angiogenesis is dependent on not only on the cells and cytokines present but also the production and organization of ECM components particularly laminins. According to recent developments, it is shown that laminins are one of the major ECM proteins important in wound angiogenesis (Li *et al*., 2007).

Fibroplasia describes a process of fibroblast proliferation, migration into wound fibrin clot and production of new collagen and other matrix proteins such as proteoglycans and elastin (Li *et al*., 2007). At least 23 individual types of collagen have been identified to date of which type I is predominant in the scar tissue of skin. After transcription and processing of the collagen mRNA, it is attached to polyribosomes on the endoplasmic reticulum where the new collagen chains are produced. During this process, there is an important step involving hydroxylation of proline and lysine residues. The collagen molecule begins to form its characteristic triple helical structure and the nascent chains undergo further modification by the process of glycosylation. The procollagen molecule is then secreted into the extracellular spaces where it is further processed. Hydroxyproline in collagen is important because it gives the molecule its stable helical conformation. Fully hydroxylated collagen has a higher melting temperature. When hydroxyproline is not present, for example in collagen produced under anaerobic or Vitamin C-deficient conditions (scurvy), the collagen has an altered structure and can undergo denaturation much more rapidly and at a lower temperature. Finally, the collagen released into the extracellular space undergoes further processing by cleavage of the procollagen N and C-terminal peptides. In the extra-cellular spaces an important enzyme, lysyl oxidase, acts on the collagen to form stable cross-links. As the collagen matures and becomes older, more and more of these intramolecular and intermolecular cross-links are placed in the molecules. This important cross-linking step gives collagen its strength and stability over time (Diegelmann and Evans, 2004). After the fibroblasts migrate into the wound, their major function becomes protein synthesis and they also change their phenotype into myofibroblasts and participate in wound contraction (Li *et al*., 2007).

1.2.4. The remodeling phase

Remodeling consists of the deposition of the matrix and its subsequent changes over time. It occurs throughout the entire repair process as fibrin clot formed in the early inflammatory phase is replaced by the granulation tissue that is rich in type III collagen and blood vessels. During remodeling, the granulation tissue is subsequently replaced by a collagenous scar predominantly of type I collagen with much less blood vessels. Type III collagen first appears after 48 to 72 h and is maximally secreted between 5 and 7 days. The total amount of collagen increases early in

repair, reaching a maximum between 2 and 3 weeks after injury. Over the period of 1 year or longer, the dermis gradually returns to the stable preinjury phenotype, consisting largely of type I collagen. Tensile strength increases to 40% of strength before the injury at 1 month and may continue to increase for 1 year, reaching up to 70% of its preinjury strength. This process is accomplished through a tightly controlled synthesis of new collagen and lysis of old collagen, mainly carried out by the actions of MMPs. The catalytic activity of MMPs is controlled in part by a family of TIMPs. (Li *et al*., 2007).

Specific collagenase enzymes in fibroblasts, neutrophils and macrophages clip the molecule at a specific site through all three chains, and break it down to characteristic three-quarter and one-quarter pieces. These collagen fragments undergo further denaturation and digestion by other proteases (Diegelmann and Evans, 2004).

1.3. Abnormal wound healing

The normal healing cascade begins with an orderly process of hemostasis and fibrin deposition, which leads to an inflammatory cell cascade, characterized by neutrophils, macrophages and lymphocytes within the tissue. This is followed by attraction and proliferation of fibroblasts and collagen deposition, and finally remodeling by collagen cross-linking and scar maturation. Despite this orderly sequence of events responsible for normal wound healing, pathologic responses leading to fibrosis or chronic ulcers may occur if any part of the healing sequence is altered (Diegelmann and Evans, 2004).

A chronic wound does not follow the orderly process of wound healing, and may take a considerably longer time to heal or may not heal at all. At molecular level, chronic wounds differ from acute wounds in that there is a difference in the expression of growth factors, cytokines and other proteins that help regulate and control the wound healing process. In diabetic wounds, the lack of reepithelization is linked to decreased expression of IGF-1 as it is shown to be decreased in both human diabetic skin and in diabetic mice. It has also been shown that an increased levels of proteases such as MMP-2, MMP-8 and MMP-9 stimulated by prolonged inflammatory response and reduced levels of MMP inhibitors prolongs the healing process because of the

destruction of proteins and growth factors that are required for normal healing (Rafehi *et al*., 2011).

It is the complexity of the reparative response that makes the process of wound healing prone to several abnormalities. Abnormalities in the inflammatory stage can lead to a delayed or deficient wound healing process. In these abnormalities, chronic inflammation persists as widely seen in diabetic foot ulcers and chronic venous leg ulcers. Healing of these wounds can only proceed after inflammation has been controlled (Kapoor and Appleton, 2005).

1.3.1. Fibrosis

Fibrosis can be defined as the replacement of the normal structural elements of the tissue by distorted, non-functional and excessive accumulation of scar tissue such as keloids and hypertrophic scars in the skin.

Keloids are characterized by fibroblasts that produce about 2 to 3 times more collagen compared to fibroblasts isolated from normal skin in the same patients due probably to increased expression of TGF-β and also an up-regulation of receptors for TGF-β. Hypertrophic scars are also characterized by excessive accumulation of scar collagen and are frequently misdiagnosed as keloids. There is one very significant biological marker that distinguishes keloids from hypertrophic scars and that is the absence of myofibroblasts in keloids and an abundance of these contractile cells in hypertrophic scars. It is also interesting to note that most conditions of fibrosis are characterized by an increased density of mast cells that contain specialized enzymes capable of processing procollagen and it has been suggested that abnormal peptides are produced that can actually stimulate collagen synthesis thus producing fibrosis (Diegelmann and Evans, 2004). A keloid scar extends beyond the margins of the original injury while a hypertrophic scar does not. Furthermore, the normal healing cycle of inflammation, proliferation and maturation is prolonged in the hypertrophic scar while a keloid scar does not follow this process (Chalmers, 2011).

1.3.2. Chronic ulcers

Chronic non-healing dermal ulcers such as pressure ulcers contribute significantly to the morbidity and even mortality of many patients. Pressure ulcers are a serious and frequent occurrence among the immobile and debilitated patients. Spinal cord injury patients are particularly vulnerable to pressure ulcer formation.

Excessive infiltration of these ulcers by neutrophils appears to be a significant biological marker. The over-abundant neutrophil infiltration is responsible for the chronic inflammation characteristic of non-healing pressure ulcers. The neutrophils release significant amounts of enzymes such as collagenase (MMP-8) that is responsible for destruction of the connective tissue matrix. In addition, the neutrophils release an enzyme called elastase that is capable of destroying important healing factors such as PDGF and TGF-β. Another marker of these chronic ulcers is an environment containing excessive reactive oxygen species that further damage the cells and healing tissues. These chronic ulcers will not heal until the chronic inflammation is reduced (Diegelmann and Evans, 2004).

1.4. Wound healing experiments

Medicinal plants have been used since time immemorial for treatment of various skin disorders such as cuts, wounds and burns. Indian system of classical medicine is shown to employ a large number of medicinal plants used to treat skin disease including cuts, wounds and burns (Kumar *et al*., 2007).

Wound healing disorders present a serious clinical problem and are likely to increase since they are associated with diseases such as diabetes, hypertension, and obesity. Additionally, increasing life expectancies will cause more people to face such disorders and further aggravate this medical problem. Thus, several animal models have been established to serve as an experimental basis to determine molecular and cellular mechanisms underlying and controlling an undisturbed healing process. (Dipietro and Burns, 2003). Such animal models are used for evaluation of both conventional new drugs as well as traditional medicines of claimed value in wound healing.

The cost burden inflicted upon the society as a result of clinical care for chronic wound patients has recently turned the attention to the investigation of cost effective, accessible alternative therapeutic strategies. Traditional forms of medicine practiced for centuries in Africa and Asia are being scientifically investigated for their potential in the treatment of wounds and related disorders. The majority of these investigations have taken the form of *in vitro* models of the various phases as represented by fibroblasts, keratinocytes and endothelial cells. A recent view of such studies is that, while the *in vitro* approach has merit, the pharmacological validation of herbal medicines is ultimately dependent on demonstrable effects in animals and humans. *Aloe vera* is the only traditional wound healing herbal medicine which has been subjected to a variety of cell culture-based, animal and human based studies (Krishnan, 2006).

Although a number of researches are being undertaken now a days to utilize drugs of herbal origin for wound healing, only few have succeeded to be used in a clinical setting. Among the currently used products for wound healing, Moist Exposed Burn Ointment (MEBO) developed by Julphar Gulf Pharmaceutical Industries, UAE and patented in the USA in 1995 is one. It is composed of six herbal extracts having a β-sitosterol as an active ingredient in a base of beeswax and sesame oil and is shown to have an effect on mast cells and several wound healing cytokines thereby promoting wound healing (Jurjus *et al*., 2007).

1.4.1. *In vitro* studies

As a result of their role during the various stages of wound healing, fibroblasts, keratinocytes, and endothelial cells are the most common targets in cell culture-based studies of the healing process (Krishnan, 2006).

The last 25 years have evidenced the fact that ethnopharmacological studies have increasingly included *in vitro* bioassays replacing experiments using tissues or whole animals not only due to the ethical and commercial problems of using animals, but the unsuitability of animal

experiments for bioassay guided fractionation of the compounds observed to be active (Houghton *et al*., 2005).

These *in vitro* procedures can be based on cultured cells, enzyme studies, receptor-ligand binding experiments and gene expression arrays (Houghton *et al*., 2005).

Apart from cell culture-based studies, enzyme based studies have also led to a conclusion that the enzymes nitric oxide synthase and arginase are two of the possible targets in wound healing research. These two enzymes are shown to regulate wound healing by being involved in the metabolism of L-arginine where L-arginine and its metabolic products are involved in several mechanisms relevant to wound healing such as angiogenesis, cell proliferation, collagen synthesis and re-epithelization (Kapoor and Appleton, 2005).

Although used widely, *in vitro* assays are not without deficiencies and it is important to indicate some of the observed shortcomings of the approach here. It is very unusual for one *in vitro* assay alone to represent a disease state and therefore, a battery of relevant tests should be preferred, since most disease states are complex and several mechanisms are involved. Even with a variety of relevant tests, it is generally acknowledged that *in vitro* tests are too reductionist to extrapolate their results to provide evidence for clinical efficacy. Thus, this eventually makes animal testing and clinical trials necessary to be performed (Houghton *et al*., 2005).

1.4.2. Animal models

Animal studies of wound healing focus on a variety of small animals such as mice, rats or guinea pigs as the mammalian target. Specific wounds are inflicted and this generally involves the application of mechanical or thermal trauma. Among the animal models that are used in wound healing experiments, excision, incision, dead-space and burn wound models are the most widely used.

Excision wound model where a circular wound is inflicted on the shaved back of the animal is used to monitor wound contraction, closure time and reduction of wound size, usually in terms of

area, thus enabling the determination of the rate of wound healing. Furthermore, direct biochemical analysis of excised tissue can be performed to determine biochemical markers of wound healing particularly hydroxyproline. The actual wound depth, as in partial-thickness wounds, is very dependent on species; the mouse has the thinnest skin, while the pig and other large domestic animals have a dermis that is as thick as or thicker than man.

The incision model of wound healing where a linear incision wound is inflicted on the back of the animal, on the other hand, is used to determine the tensile (breaking) strength of a healing wound to have an insight whether a wound healing agent causes wound healing by promoting the synthesis and cross-linking of collagen in the wound site. As a consequence, this type of wound is excellent for biomechanical analysis of wound strength. 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 epithelialization.

Dead-space wound model works by employing porous subcutaneous implants where the connective tissue (collagen) formed in the wound space is used for biochemical assessment and thereby to evaluate the wound healing pattern. Limitations of the implant 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.

Burn wound model, where a partial skin thickness burn lesions are inflicted on appropriate animal model, is used to evaluate wound contraction, epithelization period and scar formation in burn wounds. (DiPietro and Burns, 2003; Krishnan, 2005; Kokane *et al*., 2009; Suntar *et. al*., 2011).

However, in many parts of the world, the use of animals in experiments is being severely curtailed for financial and ethical reasons giving a way for *in vitro* models to be used widely (Houghton *et al*., 2005).

1.5. Dovyalis abyssinica

Dovyalis abyssinica A. Rich. is a shrub that belongs to family Salicaceae. It is native to and common in the forests of Eastern Africa countries including Ethiopia, Kenya and Uganda (Cavalcante and Martins, 2006). Vollesen also described it as shrubs or trees up to 8m, usually with spiny branches with leaves having 2-7mm long petioles and containing 9x4.5cm blades. The male flowers are usually solitary or in 2-3 flowered fascicles with pedicles that are 5-13mm long and sepals 4-5 or 5-6 mm long and colored pale green. The female flowers are also solitary with pedicles 4-12mmlong and sepals slightly enlarged in fruits. Fruits are round berries that are 1.5-2cm in diameter and yellow in color. The seeds are few in number and appear to be appressed hairy (Fig. 1) (Vollesen, 2000).

The ripe fruits of *D. abyssinica* which contain sweet-sour flesh around the seeds, are widely eaten raw, mainly by children and herdsmen, as supplementary diet and also during food shortage seasons in most parts of Ethiopia, Kenya and Tanzania. The roots boiled in soup are consumed not only as a food but also as medicine in Kenya (Teketay *et al*., 2010).

A B

Fig. 1 *Dovyalis abyssinica* plant being used as a fence (A) and fruits of *Dovyalis abyssinica* (B)

Apart from its use as a source of food in these areas, it is traditionally used for the treatment of ailments such as hemorrhoids, ulcers and swelling of the throat (Asres *et al*., 2001). Toothbrush

sticks of *D. abyssinica* have been used traditionally for maintaining oral hygiene and are shown to possess antimicrobial activity in an experiment carried out to establish the scientific basis of the use of the toothbrush sticks (van Vuuren and viljoen, 2006). In addition to its use as a source of food and for the treatment of the above mentioned ailments, personal communications with some local traditional practitioners has revealed that the fruit of *D. abyssinica* are used for wound healing purposes.

Preliminary phytochemical screening has shown that the 80% methanol extract of *D. abyssinica* fruits contains alkaloids, flavonoids, saponis, anthraquinones and polyphenols. It has also been shown that the same extract exhibits antimicrobial activities against the bacterial strains of *Escherichia coli, Pseudomonas aerugenosa, Streptococcus pyogen* and *Staphylococcus aureus* and on the fungal strain of *Candida albicans* but failed to possess antifungal activity against *A. niger*. It has also been demonstrated by *in vitro* DPPH assay that the 80% methanol extract of *D. abyssinica* fruits has an antioxidant activity (Asmare, 2009).

Although preliminary phytochemical screening, antimicrobial and DPPH radical scavenging activities have been undertaken for the crude extract of the fruits of *D. abyssinica*, no literature could be found on the traditionally claimed wound healing effects of the plant. It is, therefore, the objective of this study to explore the potential wound healing activity of the fruits of *D. abyssinica* and find out if the traditional claims are supported with scientific findings.

2. OBJECTIVES

2.1. General objectives

- To evaluate the anti-inflammatory and wound healing effects of the 80% methanol extract of the fruits of *D. abyssinica* in mice.

2.2. Specific objectives

- To undertake acute oral toxicity studies of the 80% methanol extract of the fruits of *D. abyssinica*
- To evaluate the wound healing effects of the hydroalcoholic extract of the fruits of *D. abyssinica* on excision and incision wound models
- To estimate a biochemical marker for wound healing
- To evaluate the anti-inflammatory effects of the fruit of *D. abyssinica* on carrageenan-induced hind paw edema

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and solvents

Methanol and hydrochloric acid were purchased from Aston Fields Road Whitehouse Industrial Estate, USA. Chloroform was purchased from Research-Lab Fine Chem. Industries, Mumbai/India. Copper sulfate, hydroxyproline, and paradimethylminobenzaldehyde were purchased from Merck Specialties Private Limited, Mumbai/India. Diazepam was purchased from Intas Pharmaceuticals, India, ketamine hydrochloride from Rotex Medica, Germany, and nitrofurazone 0.2% ointment from Shanghai General pharmaceuticals Co., Ltd., China. Wool fat, hard paraffin, white soft paraffin and Cetostearyl alcohol were obtained from the department of Pharmaceutics, School of Pharmacy, Addis Ababa University. Carrageenan, and indomethacin standard were also obtained from the department of Pharmacognosy and Pharmaceutical Chemistry, School of Pharmacy, Addis Ababa University.

3.1.2. Plant material

Fresh fruits of *D. abyssinica* were collected from Shashemene town, some 250 km South of Addis Ababa. The plant material was identified by Ato Melaku Wondafrash of the National Herbarium, College of Natural Sciences, Addis Ababa University. A specimen was deposited at the National Herbarium with a collection number 001-HDA.

3.1.3. Experimental animals

Healthy Swiss albino mice of either sex (30-40 g) and age (8-10 weeks old) were obtained from the animal house of School of Pharmacy, Addis Ababa University. The animals were kept in an air-conditioned room and were allowed to acclimatize for 72 h before the procedure. All animals were individually housed in a cage provided with paper-based bedding so that their wound conditions would not be aggravated by biting and the bedding material. The animals were kept at

room temperature and were exposed to a 12-h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996). The animals were provided with water and food pellets *ad libtum* before and till the end of the experiment period after which all were sacrificed by cervical dislocation.

3.2. Methods

3.2.1. Extraction

One kg of the ripe fruits of *D. abyssinica* was macerated with 80% methanol three times for 72 h, 48 h and 24 h with frequent agitation. The extracts were filtered using filter paper (Whatman No 3, Whatman Ltd., England) and the filtrates were combined and evaporated in an oven at 40^{o}C to a thick solid residue.

3.2.2. Acute oral toxicity studies

Acute oral toxicity study was performed according to OECD Guideline 420 (Deshmukh *et. al*., 2009). A dose of and 2000 mg/kg body weight of the *D. abyssinica* extract solution in saline was orally given to 6 mice each of 3 males and 3 females in the group. The female mice used were nulliparous and non-pregnant. The mice were then observed every 30 min for the first 4 h and then every day for 14 days for any signs of toxicity like diarrhea, seizure, weight reduction etc.
The result was used as a basis for dose selection of the extract solutions in saline for oral administration while evaluating the incision wound model and the carrageenan induced hind paw edema model. Thus, three dose levels were chosen in such a way that the middle dose was one-tenth of the maximum dose during acute toxicity study (200 mg/kg) and low dose was 50% of the one-tenth dose (100 mg/kg) and a high dose was twice that of one-tenth dose (400 mg/kg) (Owoyele *et al*., 2009).

3.2.3. Formulation of ointments

Simple ointment of the 80% methanol extract was prepared by using the following formula as described in British Pharmacopoeia (BP, 1988).

Ingredients	**MF**	**RF**
Wool fat……..	50 g	15 g
Hard paraffin……...	50 g	15 g
White soft paraffin……..850 g		255 g
Cetostearyl alcohol……	50 g	15 g
	1000 g	300 g

A total of 300 g of the simple ointment base, BP was prepared using the reduced formula. Out of this 100 g was used as a control and the rest was used to prepare 100 g each of 5% and 10% ointments of the *D. abyssinica* fruit extract. First, all the ingredients of the simple ointment base were mixed, heated gently with stirring until homogenous and stirred until cool. Then, in the case of the two test preparations, 5 g and 10 g of the methanolic extracts were added to 95 g and 90 g of the base to prepare the *D. abyssinica* extract ointments in the strengths of 5% and 10%, respectively mixing with the simple ointment (base) by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture (Ansel, 1985).

3.2.4. Grouping and dosing of experimental animals

The mice used for evaluation of wound healing and anti-inflammatory activity of the 80% methanol extract of the fruits of *D. abyssinica* were grouped in such a way that there were six mice in each group. For excision and incision wound models where the extract ointments, simple ointment and standard were applied topically, there were four groups of six mice each. Group I was control (simple ointment), group II was 5% extract ointment, group III was 10% extract ointment and group IV was standard (nitofurazone ointment). The preparations were applied

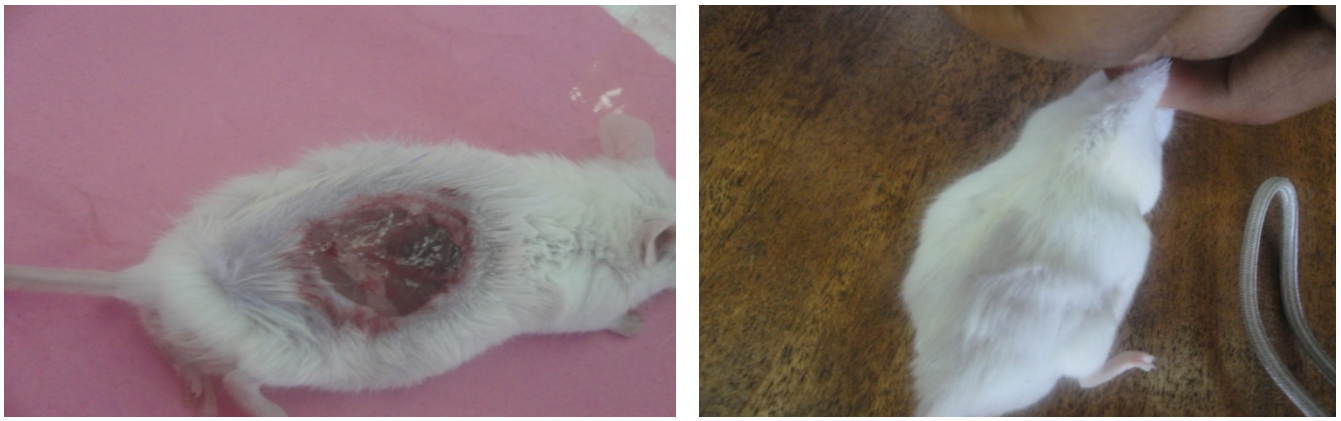
topically in sufficient amounts to cover the wound. Each mouse was treated topically once daily until complete healing for the excision wound model and until the ninth day for the incision wound model.

For evaluation of anti-inflammatory activity by oral administration of extract solutions, there were five groups of six mice each. Group I received control (saline solution), group II received 100 mg/kg body weight dose of the extract solution in saline, group III received 200 mg/kg body weight dose of the extract solution in saline, group IV received 400 mg/kg body weight dose of the extract solution in saline and group V received 5 mg/kg body weight dose of the standard indomethacin in saline solution. Each animal in this model received 0.5 ml of vehicle, extract or standard orally 1 h before carrageenan injection.

3.2.5. Excision wound model

After the mice were left for 72 h to acclimatize the laboratory condition, each of the mice was anesthetized with subcutaneous injection of ketamine (10 mg/kg) and diazepam (5 mg/kg) and the dorsal fur was removed by shaving with an electric shaver. About 312 mm^2 area on the back of each animal was marked with thin permanent marker. Then, the full thickness of the marked area was carefully excised by using sharp and small sterilized scissors to create about the same wound area in all excised mice (Fig. 2) considering the wounding day as Day 0. The animals were then left until complete recovery from the anesthesia and homeostasis. After recovery the mice were classified into four groups of 6 mice each (Morton and Malone, 1972).

The topical ointments were applied to the wounds inflicted on each mouse starting from Day 1 until complete healing. Wound area was measured on days 4, 8, 12 and 16 using a transparency sheet and a permanent marker. Then, the traced area for each mouse was calculated by using scaled paper (graph paper), each small square representing 1 mm^2 (Mrityunjoy *et al.*, 2007).



A B

Fig. 2. Excision wound immediately after wounding (A) and a completely healed excision wound after application of the 80% methanol extract of *Dovyalis abyssinica* (5% ointment) for 17 days (B)

Then, the wound healing rate given by % of wound closure was calculated using the following formula:

$$\%\ \text{of wound closure} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \text{x100}$$

where *n* = number of days 4th, 8th, 12th, and 16th day (Kokane *et al*., 2009)

The period of epithelization was also calculated as the number of days required for falling off of the dead tissue remnants without any residual raw wound.

The same procedure as above was used to inflict excisional wound for measuring hyroxyproline content. The wounds were then treated with topical application of ointments as described above for 10 days. The scabs were removed on 11th day and dried in oven at 110 °C. The hydroxyproline content in dried scab was determined by extracting hydroxyproline from scab using concentrated hydrochloric acid followed by reaction between the amino groups of hydroxyproline and p-dimetylaminobenzaldeyde to develop red colour. The red colour was then measured on spectrophotomete at a wavelength of 572 nm (Kokane *et al*., 2009).

Determination of hydroxyproline

The Neuman and Logan (1950) technique was used for the determination of hydroxyproline in protein hydrolysate, and the procedure 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*-dimethyaminobenzaldehyde by heating in the presence of dilute sulphuric acid to produce a pink colour, the intensity of which was compared with a standard (Leach, 1960). Then, the hydroxyproline was estimated at 572 nm using a spectrophotometer. Standard hydroxyproline was used to produce a calibration curve (Fig. 3) and hence to determine the hydroxyproline content of the scabs harvested from the mice.

Preparation of hydroxyproline standard

Hydroxyproline (0.05 g) was dissolved in water and diluted to about 400 ml with water. Twenty ml of conc. HCl was added and the solution was made up to 500 ml with water. The 100 µg /ml solution was diluted to give 5, 10 and 15 µg/ml of hydroxyproline. Triplicate solution of each of these concentrations and blank solution were prepared. One ml of 0.05 M $CuSO_4$ was placed into each tube, followed by 1 ml of 2.5 N NaOH, and the tube contents were each mixed by gentle swirling of the liquid. The tubes were placed in a water bath at 40ºC for about 3-5 min., 1 ml of 6% hydrogen peroxide was added, which was immediately mixed by swirling the contents of a tube before the additionwas made to the next tube. 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 3N-H_2SO_4 and 2 ml of 5% *p*-dimethylaminobenzaldehyde solution were added, the contents of the tubes mixed by swirling after each addition. Caps were placed on the tubes, which were kept in a water bath at 70ºC 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 calibration curve produced (Fig. 3) for the standard was used for the determination of hydroxyproline content in the tissue harvested from the mice in this study.

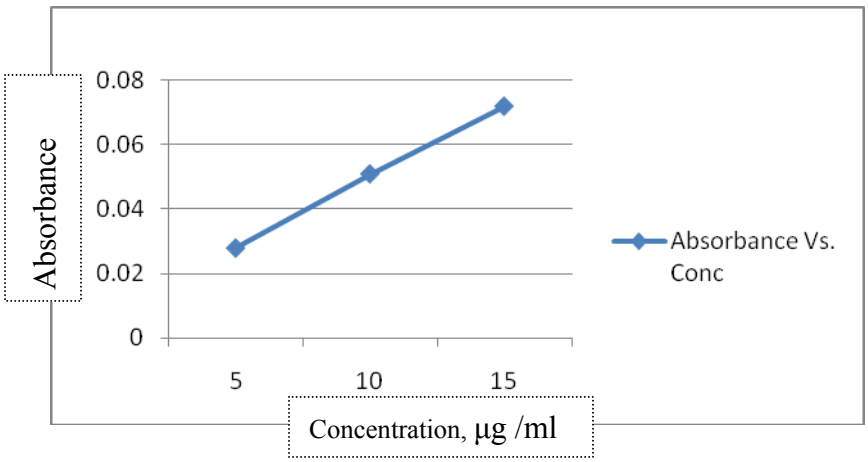


Fig. 3. Calibration curve for standard hydroxyproline.

Determination of hydroxyproline in the scab

1 ml of the supernatant solution taken from each of the acid hydrolysate of the animal tissue (there were totally 18 tissue preparations) was treated in the same manner to that of the standard hydroxyproline and their absorbance determined at wave length of 572 nm in 1 cm cell (Leach, 1960). The concentration of each solution was then calculated by deriving an equation which was obtained from the calibration curve of the standard hydroxyproline.

Calculation of hydroxyproline in the scab

The above calibration curve was expressed in terms of straight line equation:

$$y - mx + b$$

where m is the slope of the line and b the y-intercept. The values m and b were calculated from the above values of the 5, 10 and 15 µg/ml standard concentrations of hydroxyproline and their absorbance obtained from the spectrophotometric readings.

Thus, the value of m was found to be 0.0044 and that of b 0.006. Hence the above equation was expressed as follows:

$$y = 0.0044x + 0.006$$

Since y denotes the absorbance (A) and x the concentration (C), the above equation was converted to:

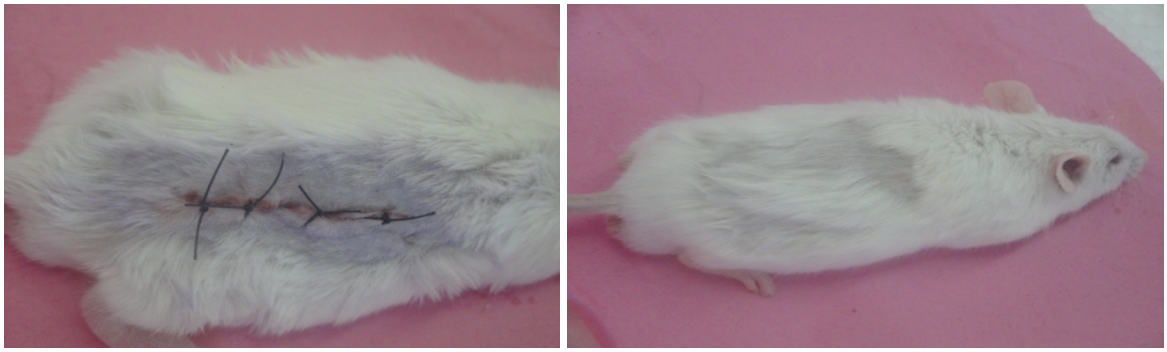
$$A = 0.0044C + 0.006$$

Since the absorbance (A) was obtained from the spectrophotometric reading of the sample solutions, the above equation was rearranged to calculate the concentration (C) as follows:

$$C = 227.273A - 1.364$$

3.2.6. Incision wound model

After the mice were left for 72 h to acclimatize the laboratory condition, each of the mice was anesthetized with subcutaneous injection of ketamine (10 mg/kg) and diazepam (5 mg/kg) and the dorsal fur was debilitated by shaving with an electric shaver as for excision wound model. A site of 3 cm length parallel to the vertebral column of the animal was marked with permanent marker and incised with sharp surgical blade. Then, the parted skin was closed with interrupted suture within 1cm, equidistant position (Ehrich and Hunk, 1969) (Fig. 4). The animals were then left until complete recovery from the anesthesia and homeostasis. After recovery the mice were classified into four groups of 6 mice each. The animals in different groups were treated topically as per the grouping and dosing under section 3.2.4 above for a period of 9 days considering the wounding day as day 0. After the wounds were cured, the sutures were removed on the 8th post-wounding day and the tensile strength of the wound was measured on the 10th day by a continuous water flow method. The same procedure was followed for the oral administration of the extract solutions (Kokane *et al*., 2009).



A B

Fig. 4. Incision wound immediately after wounding (A) and a completely healed incision wound after application of the 80% methanol extract of *Dovyalis abyssinica* (5% ointment) for 15 days (B)

Determination of tensile strength

The tensiometer used in this study consists of a 6-12 inch wooden board with one arm of 4 inch long, fixed on each side of the possible longest distance of the board. The board was placed at the edge of a table. A pulley was mounted on the top of one arm. A forceps was tied on the tip of the other arm by a string in such a way that the forceps could reach the middle of the board. Another forceps was tied on a longer string with a polyethylene bottle on the other end. Two days before performing the experiment (measurement of tensile strength) the sutures were removed from the stitched wounds of mice after recovery and tensile strength was measured as follows.

On the 8^{th} day after wounding the sutures were removed and the tensile strength was measured on 10^{th} day. For measuring the tensile strength the mice were again anesthetized and each mouse was placed on the middle of the board. It was made sure that the wound was on the same level as the tips of the arms. The forceps were then carefully clamped on the skin of the opposite edges of the wound at a distance of 0.5 cm away from the wound. The longer pieces of the string were placed on the pulley and finally to the polyethylene bottle. The position of the board was adjusted so that the bottle receives a rapid and constant rate of water from a tap, until the wound began to open. The amount of water in the polyethylene bag was measured and equated as the tensile strength of the wound. The tensile strength values induced by the extract and by

nitrofurazone ointment treated wounds were compared with the control (Mukherjee *et. al*., 2000; Kokane *et al*., 2009).

3.2.7. Carrageenan-induced hind paw edema model

In this model, pedal inflammation was produced by injecting 0.1 ml of 1% carrageenan in to the right hind foot of each mouse. The animals for this study were divided into five groups as described in animal grouping and dosing above and were treated accordingly. The extract solutions in saline, saline solution or indomethacin were administered orally to the animals after 12 h of fasting and 1 h before carrageenan injection. Paw sizes were measured immediately before and 1-5 h following carrageenan injection by wrapping a piece of cotton thread round the paw. The length of the thread corresponding to the paw circumference was determined and was used for determination of activity which is expressed as percentage inhibition by the formula given below (Owoyele *et al.*, 2009):

$$\%\ Inhibition = \frac{(Ct - Co)control - (Ct - Co)treated}{(Ct - Co)control}\ x\ 100$$

where Co is paw circumference before carrageenan injection and Ct paw circumference at time t after carrageenan injection.

3.2.8. Statistical analysis

Wound healing data in this study were expressed as mean ± S.E.M and was evaluated by one-way ANOVA followed by Tukey multiple comparison test (comparing all pairs), 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 GraphPad Instat®.

4. RESULTS

4.1. Extraction

Out of the 1 kg of fruits of *D. abyssinica* macerated in 80% methanol, 124.80 g residue was obtained making the % yield to be 12.48%. The residue was then kept in a refrigerator until use.

4.2. Acute oral toxicity study

Acute toxicity study results showed that the plant extract appeared to be safe at least up to 2000 mg/kg dose. None of the mice died and there was no observed sign of toxicity till the end of the 14^{th} day. Therefore, the LD50 of the plant is greater than 2000 mg/kg.

4.3. Wound healing activity tests

4.3.1. Excision wound model

The 80% methanol extract of the fruits of *D. abyssinica* formulated in simple ointment was found to be highly active on excision wounds (Fig. 2). This was shown by the % of wound closure and epithelialization period that shows the rate at which wound healing progresses as well as hydroxyproline content that shows whether a substance supports wound healing or not.

The results obtained from the excision wound experiments are summarized in Table 1.

Table 1. Effect of topical application of *Dovyalis abyssinica* extract ointment on excision wound model in mice

Group	% of Wound Contraction				Period of Epithelialization, Days	Hydroxyproline Content, µg
	Day 4	Day 8	Day 12	Day 16		
I	23.96±1.77	39.02±0.44	69.02±0.42	79.99±0.64	20.75±0.44	3.62±0.20
II	36.01±0.30^{a1}	57.98±0.39$^{a}{}_{1}$	81.01±0.17$^{a}{}_{1}$	90.99±0.19$^{a}{}_{1}$,$^{b}{}_{1}$	17.92±0.30$^{a}{}_{1}$	5.75±0.14$^{a}{}_{1}$
III	38.99±1.23^{a1}	64.02±0.74$^{a}{}_{1}$,$^{b}{}_{1}$,$^{c}{}_{1}$	86.00±0.75$^{a}{}_{1}$,$^{b}{}_{1}$,$^{c}{}_{1}$	93.99±0.38$^{a}{}_{1}$,$^{b}{}_{1}$,$^{c}{}_{1}$	17.75±0.21$^{a}{}_{1}$	6.59±0.13$^{a}{}_{1}$,$^{b}{}_{2}$,$^{c}{}_{2}$
IV	34.86±1.00^{a2}	57.37±0.75$^{a}{}_{1}$	79.54±0.61$^{a}{}_{1}$	87.34±0.49$^{a}{}_{1}$	18.50±0.18$^{a}{}_{1}$	5.59±0.10$^{a}{}_{1}$

a: compared against control. a_1: $p < 0.001$; a_2: $p < 0.01$; b: compared against standard. b_1: $p < 0.001$; b_2: $p < 0.01$; c: compared against 5% extract ointment. c_1: $p < 0.001$; c_2: $p < 0.01$; I, simple ointment base (control); II, 5% extract ointment; III, 10% extract ointment; IV, nitrofurazone (standard); n = 6 mice in each group; All values are expressed as Mean ± SEM

At day 4, both the 5% and 10% extract preparations exhibited a significant wound contraction ($p < 0.001$) compared with control. During the same day, the standard nitrofurazone ointment also resulted in a comparable wound contraction compared with control ($p < 0.01$). Although the 10% extract ointment showed greater effect than the 5% extract preparation and both the extract preparations have shown greater effect than the standard, the differences were not significant.

At days 8 and 12 both the extract preparations and the standard exhibited significant wound healing effect compared with the control ($p < 0.001$). At the same time, the 10% extract ointment was observed to be significantly effective compared with both the 5% extract ointment and the standard ($p < 0.001$). It also appears that the effect of the 5% extract ointment was not significantly different from that of the standard.

At day 16, all the effects that have been exhibited at days 8 and 12 were maintained with the exception that the effect of the 5% extract ointment was seen to be significantly higher than that of the standard ($p < 0.001$).

Looking at the effects on epithelization period, both the extract ointments and the standard had shown to result in a significantly shorter epithelization period than the control ($p < 0.001$). However, the difference in their effect on epithelization period was not significant although it seems that the extract ointments have resulted in a shorter epithelization period than the standard (Fig. 5).

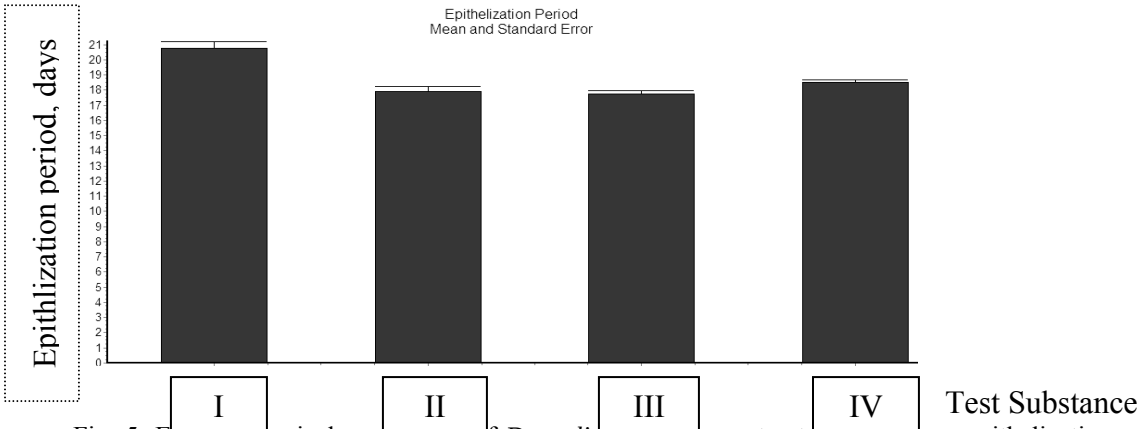


Fig. 5. Effect of topical application of *Dovyalis abyssinica* extract ointments on epithelization period of excision wound in mice; I: simple ointment; II: 5% extract ointment; III: 10% extract ointment; IV: nitrofurazone ointment (from data in Table 1)

Another wound healing parameter that can be extracted from excision wound is estimation of the hydroxyproline content in the scab with the method described above. Thus, both the extract ointments and the standard have resulted in significantly higher hydroxyproline content than the control ($p < 0.001$). At the same time, the effect of the 10% extract ointment in bringing about more hydroxyproline was seen to be higher than both the 5% extract ointment and the standard ($p < 0.01$) while the effects of the 5% extract ointment and the standard on the same parameter were not found to be significantly different (Fig. 6).

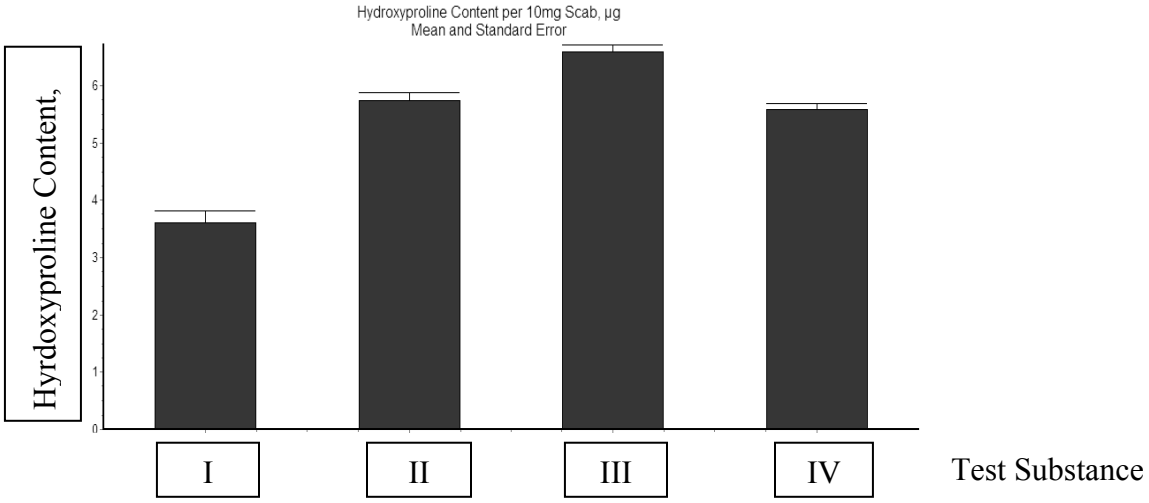


Fig. 6. Effect of topical application of *Dovyalis abyssinica* extract ointments on hydroxiproline content of scab in excision wound in mice; I: simple ointment; II: 5% extract ointment; III: 10% extract ointment; IV: nitrofurazone ointment (from data in Table 1)

4.3.2. Incision wound model

The effect of the 80% methanol extract of the fruits of *D. abyssinica* on incision wound model tested by topical application of the extract formulated in simple ointment was shown to be effective in increasing the tensile strength of the healing wound as discussed below (Fig. 7).

The results obtained from the experiments are also shown in Fig. 4.

The effects of both 5% and 10% extract ointments on the tensile strength of the healing wound was shown significantly higher compared against control ($p < 0.001$). The standard also resulted in a comparable tensile strength compared against control ($p < 0.01$). However, the effects of the extract ointments and the standard on the same parameter were not shown to be significantly different among each other.

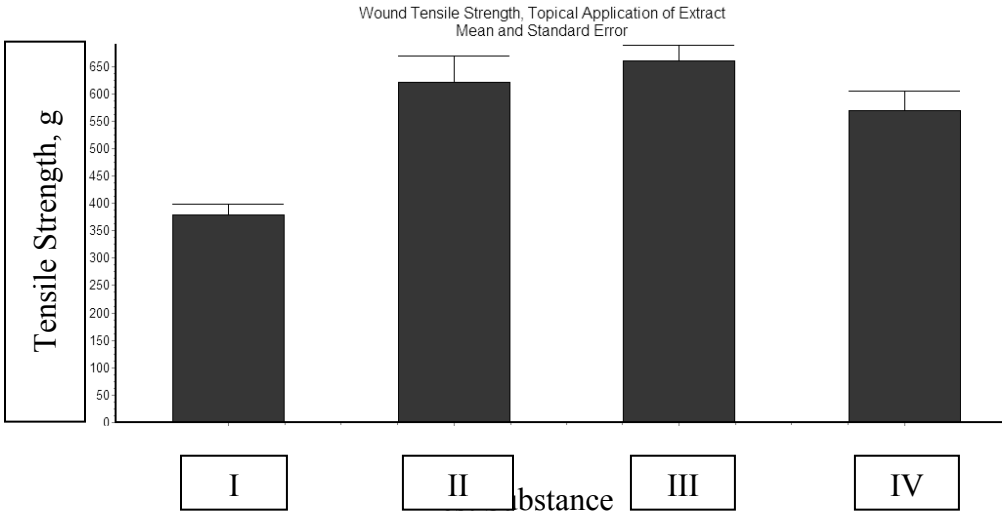


Fig. 7. Effect of topical application of *Dovyalis abyssinica* extract ointments on wound tensile strength in incision wound in mice; I: simple ointment; II: 5% extract ointment; III: 10% extract ointment; IV: nitrofurazone ointment

The oral administration of the extract solutions in saline have demonstrated that only the higher dose (400 mg/kg body weight) had shown a significant effect ($P < 0.01$) compared with the control while the middle dose (200 mg/kg body weight) resulted in a lesser significant effect ($P < 0.05$). The comparisons between control and the lower dose (100 mg/kg body weight) and among the three dose levels have also shown effects that were not significantly different (Fig. 8).

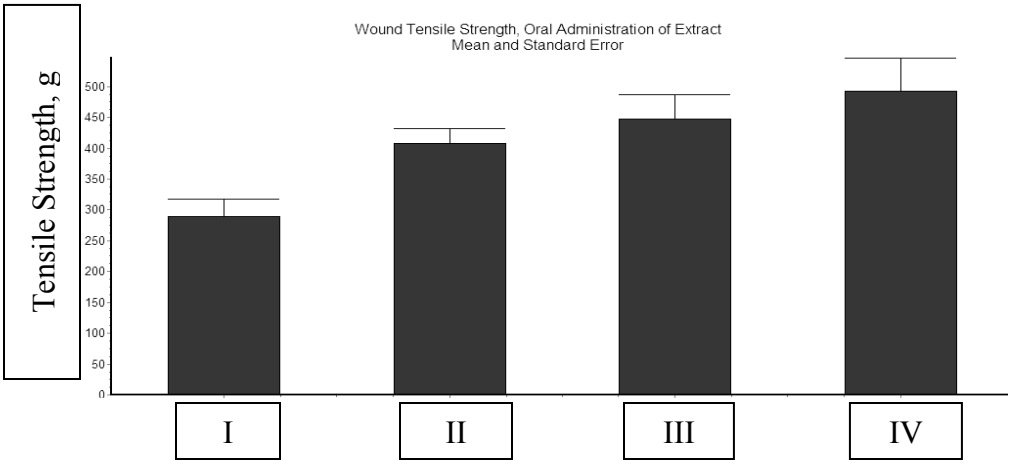


Fig. 8. Effect of oral administration of *Dovyalis abyssinica* extract solutions on wound tensile strength in incision wound in mice; I: saline solution; II: 100 mg/kg body weight dose of extract; III: 200 mg/kg body weight dose of extract; IV: 400 mg/kg body weight dose of extract

4.4. Carrageenan-induced hind paw edema model

Experiments to evaluate the anti-inflammatory activity of the 80% methanol extracts of *D. abyssinica* in carageenen-induced hind paw edema model had demonstrated that the extract was highly effective in inhibiting the edema in mice (Table 2).

Table 2. Effect of oral administration of *Dovyalis abyssinica* extract solution on carageenen induced hind paw edema in mice

Groups	1h	2h	3h	4h	5h
Increase in Paw Size, mm, Mean ± SEM					
I	3.08±2.57	3.08±2.57	3.08±2.57	3.08±2.57	3.08±2.57
II	2.91±0.15	2.50±0.18	$2.08\pm0.20^{a}{}_{2}$	$1.83\pm0.21^{a}{}_{1}$	$1.66\pm0.25^{a}{}_{1}$
III	2.75±0.25	$2.33\pm0.11^{a}{}_{3}$	$1.83\pm0.17^{a}{}_{1}$	$1.58\pm0.08^{a}{}_{1}$	$1.25\pm0.21^{a}{}_{1}$
IV	2.75±0.28	$2.25\pm0.17^{a}{}_{2}$	$1.33\pm0.67^{a}{}_{1},^{b}{}_{3}$	$1.00\pm0.13^{a}{}_{1},^{b}{}_{2}$	$0.83\pm0.11^{a}{}_{1},^{b}{}_{3}$
V	2.50±0.13	$1.75\pm0.11^{a}{}_{1}$	$1.17\pm0.11^{a}{}_{1},^{b}{}_{2}$	$0.67\pm0.11^{a}{}_{1},^{b}{}_{1},^{c}{}_{1}$	$0.50\pm0.00^{a}{}_{1},^{b}{}_{1},^{c}{}_{2}$

a: compared against control. a1: $p < 0.001$; a2: $p < 0.01$; a3: $p < 0.05$; b: compared against 100 mg/kg BW dose. b1: $p < 0.001$; b2: $p < 0.01$; b3: $P < 0.05$; c: compared against 200 mg/kg BW dose. c1: $p < 0.01$; c2: $p < 0.05$; I, saline solution (control); II, 100mg/Kg extract solution; III, 200mg/Kg extract solution; IV, 400mg/Kg extract solution; V, indomethacin solution (standard); n = 6 mice in each group; All values are expressed as Mean ± SEM

After the administration of control, standard and extract solutions and 1 h after carageenen injection, no significant edema inhibitory activity was observed with any of the administered substances. After 2 h, however, the higher dose (400 mg/kg body weight) and the standard indomethacin exhibited a significant edema inhibitory activity compared with the control ($p < 0.01$) while the middle dose (200 mg/kg body weight) showed a comparable effect ($p < 0.05$) and the rest showed no significant effect (Fig. 9). After 3 h, the middle and the higher extract doses and the standard compared with the control exhibited a significant edema inhibition ($p < 0.001$) while the lower dose (100 mg/kg body weight) in comparison with the control showed a comparable edema inhibitory effect ($p < 0.01$). During the same time, the standard showed a comparable edema inhibitory effect ($p < 0.01$).At the same time, the higher extract dose compared with the middle dose exhibited a slightly significant effect ($p < 0.05$) while comparisons of results between the lower and middle extract doses, the middle and higher extract doses, as well as the higher dose with the standard gave no significant edema inhibitory results.

4 h after carageenan administration, all extract solutions and the standard compared with the control and the standard compared with the lower extract dose exhibited a very significant edema inhibition ($p < 0.001$). Furthermore, the effect of the 400 mg/kg dose compared with the 100 mg/kg dose of the extract, and the standard compared with the middle dose were found to be significantly active ($p < 0.01$). However, the effects of the middle dose compared with the lower and the higher dose compared with the middle as well as the standard compared with the higher dose were found to be insignificantly different.

While the effects of extract preparations and the standard compared with the control and the standard compared with the lower extract dose have comparably significant effects 5 h after carageenen injection as after the 4^{th} h ($p < 0.001$), the effects of the higher extract dose compared with the lower, and the standard compared with the middle dose have shown a slight significant difference ($p < 0.05$). The non-significant effects after 5 h remained the same as those of after 4 h.

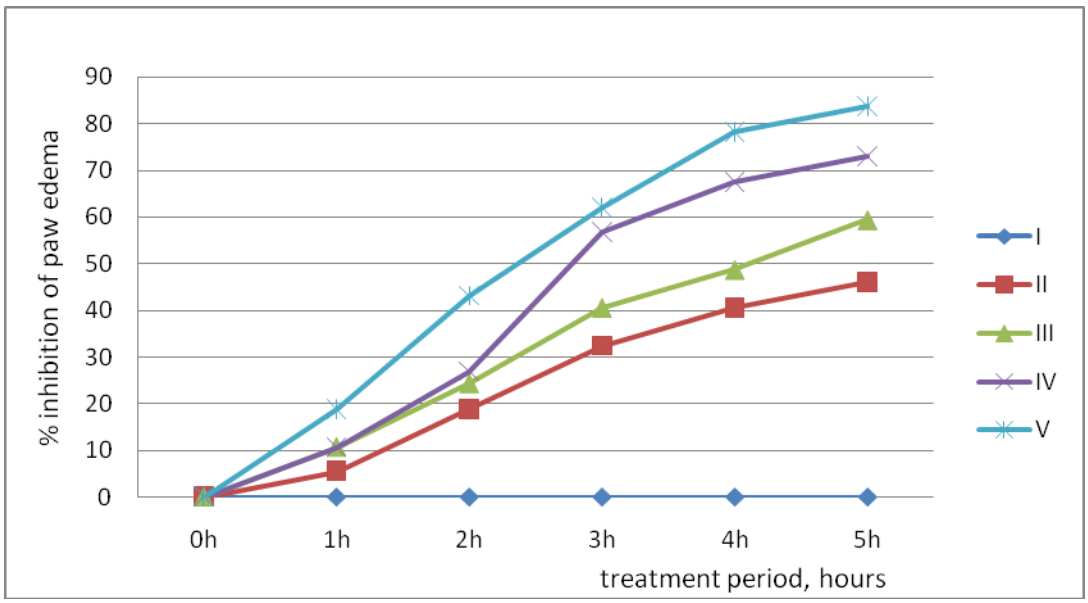


Fig. 9. Effect of oral administration of *Dovyalis abyssinica* extract solution on carrageenan induced paw edema in mice; I: Control; II: 100 mg/kg body weight dose of the extract solution; III: 200 mg/kg body weight dose of the extract solution; IV: 400 mg/kg body weight dose of the extract solution; V: Indomethacin in saline

5. DISCUSSION

The aim of the present study was to evaluate the wound healing and anti-inflammatory activities of the fruits of *D. abyssinica* and, therefore, scientifically validate the traditional claim that the fruits possess wound healing effect. This study is the first of its kind to report the wound healing effects of *D. abyssinica*.

The 80% methanol fruit extract of *D. abyssinica* was tested using excision and incision wound and carrageenan-induced hind paw edema models in mice to evaluate the wound healing and anti-inflammatory activities, respectively. Complete wound healing of the extract preparations were studied by counting the number of days and evaluating the hydroxyproline content of the scab for the excision wound model as presented in Table 1. The tensile strength of the wounds was measured for the incision wounds for both topical application and oral administration of the extract preparations and the results are presented in Tables 2 and 3. For the anti-inflammatory activity tests, increase in paw size were measured and % inhibition of edema was calculated as in Table 4. The results demonstrate that the fruits of *D. abyssinica* possess a significant wound healing and anti-inflammatory activities.

The effects of the fruit extract on the excision wound model were demonstrated by the three parameters, namely, % of wound contraction, epithelization period and hydroxyproline content of the scab tissue. Percent of wound contraction was evaluated on days 4, 8, 12 and 16 post wounding and the results demonstrate that both the 5% and 10% extract ointments resulted in a significant increase in the rate of wound contraction compared with the control ($p < 0.001$) throughout the treatment period. It is interesting to note here that this effect was very intense particularly at days 4, 8 and 12 demonstrating a fact that the extracts have promoted the wound healing processes such as proliferation of epithelial cells, endothelial cells and fibroblasts and collagen synthesis during which period these processes are at their peak. This is also evidenced by a shorter period of epithelization by the extract ointments compared with the control ($p < 0.001$). It was further demonstrated that the 10% extract ointment showed even a higher wound contraction on days 8, 12 and 16 compared with both the 5% extract and the reference nitrofurazone ointments ($p < 0.001$). This shows that wound contraction effected by the

secondary plant metabolites responsible for the effect might be affected in a dose dependent manner.

Another parameter measured during this study was the hydroxyproline content of the scab. Hydroxyproline is one of the biomarkers that indicate whether wound healing is promoted to progresses normally. Thus it was shown that both the 5% and 10% extract ointments have resulted in a higher hydroxyproline content compared to the control ($p < 0.001$). It was further demonstrated that the 10% extract ointment has resulted in even a higher hydroxyproline content compared with both the 5% extract ointment and the reference nitrofurazone ointment ($p < 0.01$). Once again, we see here that hydroxyproline content has increased with an increase in the dose of the extract applied to the wound in direct correlation with the effect observed on the rate of wound contraction. The increase in the hydroxyproline content also strengthens our argument that the fruit extract of *D. abyssinica* enhances the proliferation of cells that are involved in wound healing in that hydroxyproline content increases as a result of aerobic conditions that is favored by angiogenesis. And for angiogenesis to be promoted, proliferation and activation of endothelial cells in the wound site might have been facilitated by the secondary metabolites contained in the extract.

The wound healing potential of the fruits of *D. abyssinica* is further evidenced by the results of the incision wound model where an increased wound tensile strength was seen on the 10^{th} post wounding day. For the incision wound model the extract was not only applied topically in a simple ointment base but also administered orally as a solution dissolved in saline. In both cases, the extract preparations exhibited a wound healing activity and resulted in an enhanced tensile strength.

In the case of the topical application of the extract ointment on the incisional wounds, the tensile strength of the skin of extract treated mice was significantly higher compared with the control ($p < 0.001$), whereas the standard nitrofurazone ointment resulted in a lesser tensile strength compared with same ($p < 0.01$). Although the extract ointments resulted in a higher tensile strength than the standard nitrofurazone, the comparison between the standard and extracts was not found to be statistically significant. However, the higher tensile strength produced by the

extract ointments gives an insight to the fact that the extract possesses other effects that promote wound healing like collagen synthesis other than antimicrobial effect.

With regard to the oral administration of the extract, the results showed that the 80% methanol fruit extract of *D. abyssinica* also enhances healing of incision wounds when administered orally as evidenced by an increase in the tensile strength of the healing skin. Not only do the extracts exhibit an increase in wound tensile strength but also they do this in a dose dependent manner. This was evidenced by the fact that the 400 mg/kg body weight dose resulted in a higher tensile strength ($P < 0.01$) than the 200 mg/kg body weight dose ($P < 0.05$) while the lower dose (100 mg/kg body weight) did not have a considerable effect all compared with the control saline solution. This might be due to the fact that the extract constituents that may be responsible for collagen synthesis and hence enhance the incision wound healing were not available at the wound site in sufficient quantity to exert their effect for the lower dose. This was also shown by the fact that the tensile strength of the incision wounds has increased progressively as the dose of the extract was increased from 200 to 400 mg/kg body weight.

Thus, the topical application of the 5% and 10% *D. abyssinica* extract ointments have increased the percentage of wound contraction and completed wound healing by 17^{th} day with increased hydroxyproline content in the scab. This indicates rapid epithelization and collagenization and sufficient angiogenesis. Increased tensile strength of the incision wounds also indicates increase in collagen and obviously facilitation of wound healing. There is rapid biosynthetic activity in topically treated mice with *D. abyssinica* during initial phase of granulation and in remodeling phase. Maturation of collagen has also taken place by the formation of inter and intra-molecular cross links, hence the tensile strength was increased.

Another potential activity tested for the 80% methanol fruit extract of *D. abyssinica* was anti-inflammatory activity and the results obtained were presented in Table 2.

Inflammation is a normal and essential response to any noxious stimulus that threatens the host and may vary from a localized response to a generalized response (Borne, 2002). Inflammation has different phases: the first phase is caused by an increase of vascular permeability resulting in

exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes from the blood into the tissues and the third one by granuloma formation. Accordingly, anti-inflammatory tests have to be divided into those measuring acute inflammation, subacute inflammation and chronic repair processes (Vogel, 2002).

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent (Vogel, 2002). One of the most commonly used *in vivo* animal assays is the one that measures the ability of anti-inflammatory agents to inhibit edema induced in mice paw by carrageenan (Borne, 2002). This method has also been described as a model of acute inflammation (Owoyele et. al., 2009).

The development of edema in the paw of the rat, after the injection of carrageenan has been described as a biphasic event. The initial phase, observed during the 1st h, is attributed to the release of histamine and serotonin; the second one is due to the release of prostaglandin-like substances (Krishnaveni et. al., 1997).

The paw edema method has been used by many investigators and has been proven to be suitable for screening purposes as well as for more in depth evaluations. Dependent on the irritant steroidal and nonsteroidal anti-inflammatory drugs, antihistamines and also, to a lesser degree, serotonin antagonists are active in the paw edema tests. Since so many different irritants have been used by the various investigators the results are often difficult to compare (Vogel, 2002).

After the administration of control, standard and extract solutions and 1 h after carageenen injection, only a slight change that was not statistically significant edema inhibitory activity was observed with any of the administered substances. During the first hour of treatment by the extract solutions, the % inhibition of edema produced was 5.52% for 100 mg/kg body weight dose and 10.71% inhibition for the middle and higher doses giving an insight to the fact that the active ingredients produce higher effect at higher dose. It is also expected that, with the isolation, characterization and dose adjustment of the active ingredients, a better effect can be produced at the first hour of inflammation. We can also see that the paw edema was significantly reduced

after 3 h demonstrating that the active ingredients have potently inhibited the release of prostaglandin like substances involved in the inflammatory response. It is also demonstrated that the higher dose have produced the higher edema inhibitory effect (56.82%) followed by the middle dose (40.58%) and the least effect being produced by the lower dose (32.47%) showing further that the anti-inflammatory effects are dose dependent. Thus, evaluation of the anti-inflammatory activity of the extract solution disclosed that the fruits of *D. abyssinica* are endowed with a strong anti-inflammatory effect.

A number of secondary plant metabolites are attributed to wound healing and anti-inflammatory effects. The presence of saponins, flavonoids and other phenolics could contribute to wound healing because of their detergent ability to remove grease, dirt and bacteria from the wound site and act as antimicrobials thereby assisting the natural body mechanisms of wound healing. Flavonoids have also been reported to possess antiinflammatory and antioxidant properties, two of the activities that are known to be beneficial to promote wound healing (Houghton *et. al*., 2005). Flavonoids are also known to promote the wound healing process mainly due to their astringent and antimicrobial properties (Tsuchiya *et. al*., 1996)

Herbal remedies that have a direct wound healing effect also possess anti-inflammatory activity. As the reduction of inflammation shortens the inflammatory period, plant materials with anti-inflammatory effect may accelerate wound healing. In the present study, it has been noted that the antiinflammatory activity appears to be one of the wound healing mechanism responsible for shorter epithelization period showing rapid wound healing. The antiinflammatory and antioxidant activities are also of great value while treating chronic and non-healing wounds where the chronic inflammation and ROS have to be controlled before the wound healing progresses normally. In addition to the antiinflammatory and antioxidant effects, antimicrobial effect of herbal remedies might also shorten the healing period of wounds by preventing infection (Csupor *et. al*., 2010). The healing process may also be hampered by the presence of ROS or microbial infections. Thus, extracts with antioxidant and antimicrobial activities accelerate wound healing (Ozgen et. al., 2006).

The fruits of *D. abyssinica* are known to contain such secondary plant metabolites as alkaloids, flavonoids, saponins, anthraquinones and polyphenols (Asmare, 2009). Although this study was able to disclose the potential wound healing and antiinflammatory effects of the fruits, it does not conclude which metabolites are responsible for the observed wound healing and anti-inflammatory effects. The observed effects might be attributed to a single active ingredient or may be due to the combined and synergistic activity of various active ingredients. It is, therefore, expected that subsequent studies on the fruits of *D. abyssinica* be directed at fractionating, isolating and characterizing the active ingredients and proposing their mechanisms of action.

6. CONCLUSION

The 80% methanol extract of the fruits of *D. abyssinica* possesses wound healing activity proving the traditional claim that the fruit has. The topical application of the extract ointments on excision as well as incision models of wound healing both demonstrated a higher wound healing effect than the standard nitrofurazone ointment showing that the extract might contain active metabolite/s that enhance/s wound healing other than antimicrobial ingredients. The oral administration of the extract solution in the incision wound model has also demonstrated that the higher dose (400 mg/kg body weight) exhibited a significant wound healing activity. It was observed also that the extract has a significant antiinflammatory activity as shown by a reduction of edema in carrageenan-induced hind paw edema model in mice. It appears that anti-inflammatory activity was one of the possible mechanisms for the wound healing effect exerted by *D. abyssinica*.

7. SUGGESTIONS FOR FURTHER WORK

1. Performing wound healing and anti-inflammatory activity tests with various solvent fractions and other animal models.
2. Employing *in vitro* test methods to further test the wound healing and antiinflammatory activity of the fractions.
3. Isolation of active ingredients of the solvent fraction with the best activity.
4. Characterization of the active ingredient/s responsible for wound healing and anti-inflammatory activities.
5. Elucidation of the possible mechanism of action for the observed activities.

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