Evaluation of Wound Healing and Anti-Inflammatory Activity of 80% Methanolic Extract of *Solanum incanum* Linnaeus (Solanaceae) Leaves in Mice

By

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A Thesis submitted to the department of pharmacology, school of medicine, college of health sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the degree of Master of Science in Pharmacology

Addis Ababa University

Addis Ababa, Ethiopia

October 2016

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by **Debesa Doyo** entitled " **Evaluation of wound healing and anti-inflammatory activity of 80% methanol extract of *Solanum incanum* L. leaves in mice"** and submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Chair of Department

Abstract

Evaluation of wound healing and anti-inflammatory activity of 80% methanol extract of *Solanum incanum* L. leaves in mice.

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Solanum incanum Linnaeus or Sodom/bitter apple (English), Embouy(Amharic), Hiddi(Afan Oromo) is a perennial, wild shrub like herb that grows up to 1.8 m in height that belongs to Solanaceae family which grows in many regions of Africa. In Africa, including Ethiopia the herb is used as a folklore remedy for different ailment such as skin problem like wound and inflammations. However, to date, no scientific report could be found in the literature concerning the in-vivo wound healing and anti-inflammatory activity. The purpose of this study is therefore to evaluate in- vivo wound healing and anti-inflammatory activity of 80% methanol extract of S. incanum L. leaves in mice.

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). The animals were randomly divided into four groups in wound healing models and five groups (each containing six mice) for anti-inflammatory activity test. Simple ointment base (negative control) and nitrofurazone 0.2%(w/w)ointment was used as standard for activity comparison. For the study of in- vivo anti-inflammatory activity, the 80% methanolic extract was dissolved in 2% Tween 80. Two models were used for wound healing activity in mice viz. excision and incision. Carrageenan induced hind paw edema model was used for anti-inflammatory study. Parameters such as wound contraction, and period of epithelialization were studied in case of the excision wound model, while incision wound model was evaluated by determining tensile strength. Leaves extract of

S. incanum was given orally at dose of 100, 200 and 400 mg/kg in carrageenan induced hind paw oedema model and oedema was evaluated by determining mean increase in paw volume and percentage edema inhibition. Indomethacin 10mg/kg was used as reference standard for the activity comparison.

Treatment of wound with ointment containing 5%(w/w) and 10% (w/w) 80% methanol extract exhibited significant($p<0.05$) increase in wound contraction rate, shorter epithelialization time and higher skin breaking strength in the two experimental model as compared to control.

S. incanum L. extract also shows dose-dependent significant reductions ($p < 0.05$) of inflammation as compared to control.

These results collectively demonstrate that the 80% methanol extract of S. incanum L. possesses wound healing and anti-inflammatory properties, and this justifies the use of the leaves of S. incanum L. for wound and inflammations as claimed in the folklore literature.

Key words: Solanum incanum L., wound healing activity, excision wound model, incision wound model, tensile strength, Anti-inflammatory, carrageenan

Acknowledgement

First of all, I would like to thank Almighty God for helping me to stand by myself.

I am deeply grateful to my advisor Getnet Yimer (MD, MSc, PhD.) for his untiring guidance and direction of this research work, for his consistent and immediate support and encouragement.

I would like to take the opportunity to thank School of Medicine, Department of Pharmacology and all of the people who have helped me during my study.

I would like thank school of pharmacy for material support.

My heartfelt thanks also go to my parents and friends for being beside me in all bad and good times in my life. My mom you are alive forever in my heart!

Lastly, I would like to acknowledge Arsi University for sponsoring the MSc. program and Addis Ababa University for financial assistance of this research.

Contents

List of Abbreviations

AAU - Addis Ababa University

AMR- Antimicrobial resistance

ANOVA-Analysis of variance

B.P- British Pharmacopoeia

ECM- Extracellular matrix

EPHI- Ethiopian Public Health Institution

FMHACA- Food, Medicine and Healthcare Administration and Control Authority

I.P- Intra peritoneal

I.V- Intra venous

ILAR- Institute for Laboratory Animal Research

MMP- Matrix Metalloprotienase

MRSA- Methicillin- resistant S. aureus

OECD- Organization for Economic Cooperation and Development

PII- Primary irritation index

SEM- Standard error of the mean

SPI- Score of primary irritation

SPSS- Statistical Package for Social Sciences

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1. Introduction

1.1.Overviews of skin and wound

The skin is the largest organ in our body, accounting for about 15% of the total adult body weight. It carried out many essential functions, including safeguards against external physical, chemical, and biological attackers, as well as prevention of excess water loss from the body and play role in thermoregulation (Kanitakis, 2002).

Wound may be described in different ways but the most common definition is a breakdown in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue (i.e. muscle, bone, nerves) following injury to the skin or underlying tissues/ organs(Nalwaya *et al*.,2009).

Wounds are inevitable events in everyday life and may be caused by physical, chemical, thermal, microbial or immunological (underlying pathological condition) insult to the tissue (Jaiswal *et al*., 2004). On the basis of physiology of wound healing, wound may be classified as chronic, such as the skin ulcers caused by diabetes mellitus, or acute, such as a gunshot wound or animal bite. On the bases of the underlying cause of wound creation, wounds can be open, in which the skin has been compromised and underlying tissues are exposed, or closed, in which the skin has not been compromised, but trauma to underlying structures has occurred (eg a bruised rib or cerebral contusion) (Mallefet and Dweck, 2008).

1.2.Physiology of wound healing

Wound healing is a dynamic process involving cellular, molecular, biochemical, and physiological phenomena that result in connective tissue repair and fibrous scar formation and lead to the restoration of the anatomical continuity and functional status of the skin(Velnar *et al*.,2009). The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution. These phases and their biophysiological functions must occur in the proper sequence, at a specific time and must continue for a specific duration at an optimal intensity (Gosain and DiPietro, 2004).

Hemostasis phase

The normal healing response begins the moment the tissue is injured. As the blood components spill into the site of injury, the platelets come into contact with exposed collagen and other elements of the extracellular matrix. This contact triggers the platelets to release clotting factors. Finally platelets also secrete cytokines such as *platelet-derived growth factor* (PDGF), and transforming growth factor beta (TGF-ß) which is recognized as one of the first factors secreted in initiating subsequent steps(Flanagan, 2000; Cheng,2011). Hemostasis occurs within minutes of the initial injury unless there are underlying clotting disorders (Diegelmann and Evans, 2004).

Inflammatory phase

Inflammation is a fundamental component of the normal adult wound healing response occurring even in the absence of infection (Röhl J *et al.,*2015). In skin repair, the infiltrating leukocytes are the principal cellular components of the inflammatory response (Eming *et al.,* 2007). The inflammatory phase of the wound healing cascade gets activated during the coagulation phase and can roughly be divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes into macrophages (Reinke and Sorg, 2012).

Within the first days after injury, neutrophil enter the wound site and begin the critical task of phagocytosis to remove foreign materials, bacteria and damaged tissue. They start their debridement by releasing highly active antimicrobial substances i.e. cationic peptides and eicosanoids, release of ROS and proteinases, i.e. elastase, cathepsin G, proteinase 3 and an urolinase-type plasminogen activator(Leoni *et al.,*2015).

Approximately 3 days after injury, macrophages enter the zone of injury and support the ongoing process by performing phagocytosis of pathogens and cell debris and by secreting growth factors, chemokines and cytokines. Macrophages have many functions including host defense, the promotion and resolution of inflammation, the removal of apoptotic cells and the support of cell proliferation and tissue restoration following injury. Beside their immunological functions as antigen-presenting cells and phagocytes during wound repair, macrophages supposedly play an integral role in a successful healing response through the synthesis of numerous potent growth factors, such as transforming growth factor (TGF-β, TGF-α, basic FGF, platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which promote cell proliferation (Koh and DiPietro, 2011).

Repair of wounds requires timely resolution of inflammation that is an active process (Leoni *et al*., 2015). Unless stimuli for neutrophil recruitment persist at the wound site, the neutrophil infiltration ceases after few days, and expended neutrophils are themselves phagocytosed by macrophages, which are present at the wound side within 2 days after injury (Eming *et al*.,2007). Removal of inflammatory cells, such as neutrophils, plays a role in the pathogenesis of non-healing wounds. A deficit in the capability of macrophages to effectively remove neutrophils has recently been reported to be a critical component of the impaired healing. Macrophages assist in the removal of neutrophils from sites of injuries in several ways. Macrophages respond to neutrophils and their products, and can induce apoptosis in neutrophils. Perhaps more importantly, macrophages recognize and actively ingest apoptotic neutrophils, thus helping to resolve wound inflammation (Koh and DiPietro, 2011).

Proliferative phase

In the phase of proliferation (approx. 3–10 days after wounding) the main focus of the healing process lies in covering the wound surface, the formation of granulation tissue and restoring the normal physiology of skin. During proliferation, the wound is rebuilt with new granulation tissue which is comprised of collagen and other extracellular matrix (ECM) and into which a new network of blood vessels develop, a process known as angiogenesis (Reinke and Sorg, 2012). Fibroblasts begin to form a collagen matrix in the wound known as granulation tissue. Collagen, a protein substance, is the chief constituent of connective tissue. Collagen fiber formation determines the tensile strength and pliability of the healing wound. Subsequently, the synthesis of collagen increases throughout the wound, while the proliferation of fibroblasts declines successively, adjusting a balance between synthesis and degradation of the ECM (Barker, 2011).

As healing progresses several other important biological responses are activated. The process of epithelialization is stimulated by the presence of EGF (epidermal growth factor) and TGFα (transforming growth factor alpha) that are produced by activated wound macrophages, platelets and keratinocytes. The re-epithelialization process is ensured by local keratinocytes at the wound edges and by epithelial stem cells from hair follicles or sweat glands. Epithelial cells finally resurface the wound, a process known as epithelialization (Werner and Grose, 2003).

At the end of this phase the number of maturing fibroblasts is reduced by myofibroblast differentiation and terminated by consecutive apoptosis. The high contractile force generated by myofibroblasts is beneficial for wound contraction and physiological tissue remodeling. Wound

contraction is a process that pulls the wound edges together for the purpose of closing the wound. In essence, it reduces the open area, and if successful, will result in a smaller wound with less need for repair by scar formation. Wound contraction can be very beneficial in the closure of wounds in areas such as the buttocks or trochanter but can be very harmful in areas such as the hand or around the neck and face, where it can cause disfigurement and excessive scarring (Hinz, 2007).

Remodeling phase (maturation stage)

Remodeling is the last phase of wound healing and occurs from day 21 to up to 1 year after injury. The formation of granulation tissue stops through apoptosis of the cells. A mature wound is, therefore, characterized as avascular as well as acellular (Campos *et al.*,2006). During the maturation of the wound the components of the ECM undergo certain changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I. This type of collagen is oriented in small parallel bundles and is, therefore, different from the basket-weave collagen in healthy dermis (Shankar *et al*., 2014). Later on the myofibroblasts cause wound contractions by their multiple attachment to collagen and help to decrease the surface of the developing scar. Furthermore, the angiogenic processes diminish, the wound blood flow declines, and the acute wound metabolic activity slows down and finally stops. During tissue repair all cell types undergo a rapid increase in number, perform their specific activities and later must fall to negligible amount once restoration is completed. The most likely mechanism involved in the resolution of the various phases of tissue repair is through apoptosis (Profyris *et al.,* 2012).

1.3. Pathophysiology of wound healing

Normal repair is the response where there is a re-established equilibrium between scar formation and scar remodeling. This is the typical response that most humans experience following injury. The pathological responses to tissue injury stand in sharp contrast to the normal repair response. In excessive healing there is too much deposition of connective tissue that results in altered structure and, thus, loss of function. Fibrosis, strictures, adhesions and contractures are examples of excessive healing. Keloids and hypertrophic scars in the skin are examples of fibrosis. Contraction is part of the normal process of healing but if excessive, it becomes pathologic and is known as a contracture. Deficient healing is the opposite of fibrosis; it exists when there is insufficient deposition of connective tissue matrix and the tissue is weakened to the point where

it can fall apart. Chronic non-healing ulcers are examples of deficient healing (Hochman *et al.*, 2012).

In pathologic conditions such as non-healing pressure ulcers, this efficient and orderly process is lost and the ulcers are locked into a state of chronic inflammation characterized by abundant neutrophil infiltration with associated reactive oxygen species and destructive enzymes such as collagenase (matrix metalloproteinase-8(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-ß. Healing proceeds only after the inflammation is controlled (Armstrong and Jude, 2002; Traversa and Sussman, 2001).

1.4. Factor affecting wound healing

There are many factors that can affect wound healing by interfering with one or more phases in wound healing process, thus causing improper or impaired tissue repair. Some of them are infection, tissue hypoxia, necrosis, exudate and excessive levels of inflammatory cytokines, delayed collagen synthesis, impaired epithelialization, increased apoptosis and reduced angiogenesis (Velnar, *et al*.,2009; Gosain and DiPietro,2004). Other patient related factor impair wound healing includes smoking, diabetic mellitus, nutritional deficiency of vitamin C, peripheral vascular disease, medications (steroids), alcoholism and advanced age (Sabale *et al*.,2012).

1.5.Wound Management

After injury, the objective of wound healing is to restore structure and function of an injured tissue in order to approximate pre-wound characteristics (Kore *et al*., 2011).The effective management of wounds will reduce the number of complications and allow rapid return to normal function. The way in which wounds are managed affect the rate of healing, the time to return to normal function, the final cosmetic appearance and hence the satisfaction of customers. Management of wounds depends on the stage of wound healing and can include irrigation, mechanical and chemical debridement, the use of antiseptics and antimicrobial and use of adherent and non-adherent dressing. The wound should be handled with an aseptic technique, thoroughly irrigated under adequate pressure and carefully debrided (Liptak, 1997).

1.5.1. Topical antimicrobials in wound management

The use of topical antimicrobials in wound management is controversial. The potential advantage of these agent overs antiseptics include selective bacterial toxicity, efficacy is not reduced in the presence of organic matter and combined efficacy with systemic antimicrobial therapy. They are proposed to promote normal healing by protecting the wound from superficial infection. Potential disadvantages include expense, reduced antimicrobial spectrum, potential for bacterial resistance, creation of super-infections. Some of common antimicrobial in use and their bacterial spectrum are Cephazolin(active against Gram-positive and some Gram-negative beacteria), Bacitracin-polymxin B- neomycin (active against Gram-positive and Gram-negative bacteria, not Pseudomona spp), Silver sulphadiazinine (active against Gram-negative and some Gram-positive bacteria and fungi), Gentamicin(active for Gram-negative bacteria, and Nitrofurazone (Gram-positive and Gram –negative bacteria, not Pseudomonas spp)(Liptak, 1997).

1.5.2. Medicinal plant for wound care

Many Ayurvedic plants have very important role in the process of wound healing. Various plant products have been used in treatment of wounds over the years. Plants are more potent healers because they promote the repair mechanisms in the natural way (Kumar B, 2007). Herbs which pose antiseptic, astringent, anti-inflammatory, antimicrobial, promote blood clotting and bio stimulatory property can also enhance the rate of wound healing. Medicinal plants are increasing the rate of tissue healing by providing essential substances, taken at various steps of regeneration and proliferation. The phyto-medicines for wound healing are not only cheap and affordable but are also purportedly safe as hyper sensitive reactions are rarely encountered with the use of these agents. Traditional healers used aqueous extract of some plants to cleanup and disinfect wounds. Flamed leaves of some plants are used to dress injured skin, promote wound healing and can be used to ward off infection (Agarwa, 2009; Alam *et al*., 2011).

1.6. Statement of the problem

The immense social and economic impact of wounds worldwide is a consequence of their high rate of occurrence in general and their increasing frequency in the ageing population in particular. In addition to a high number of acute wounds, there are also a large number of

chronic, hard-to-heal wounds associated with diseases and abnormalities that directly or indirectly culminate in damage of the cutaneous coverage, including arterial, venous, diabetic and pressure ulcers (Robson *et al.*, 2001).

The epidemiology and economic burden of chronic wound is well documented in the developed world. Each year in North America, between five and seven million chronic and/or complex wounds occur. A recent study in the UK showed a prevalence of patients with a wound was 3.55 per 1000 population. The majority of wounds were surgical/trauma (48%), leg/foot (28%) and pressure ulcers (21%). Prevalence of wounds among hospital inpatients was 30.7%. Wounds in Australia are a highly significant health issue: some estimates suggest that over 200,000 Australians have problem wounds at any one time. In India, a recent study estimated a prevalence rate of chronic wounds at 4.5 per 1000 population. The incidence of acute wounds was more than double at 10.5 per 1000 population. According to data from epidemiological studies, the incidence of chronic ulcers in surgical hospitalised patients in China is 1.5–20.3% (MacDonald, 2009).

The prevalence of these chronic wounds increases with age. For example, it has been estimated that chronic wounds affect 120 per 100 000 people aged between 45 and 65 years and rises to 800 per 100 000 people > 75 years of age. Furthermore, due to the complications that accompany acute wounds, when their healing does not progress in a timely and orderly manner, they can convert into chronic wounds, which are more difficult to manage (Robson *et al*.,2001).

Wounds cause pain, suffering, sepsis, infection, nausea, fatigue, depression, psychological disturbances, loss of function, loss of mobility, and personal financial cost. In many cases, wounds lead to amputation and even death (Hurd, 2013).

Economic and social impact of wound is high. In the UK, the attributable cost of wound care in 2006–2007 was 9.89 million pounds: 2.03 million pounds per 100,000 populations and 1.44% of the local health-care budget. Thus, the cost of wound care is significant. The most important components are the costs of wound-related hospitalization and nursing time. In addition to the preventable human suffering and disabilities, this burden encompasses the cost of caring for disabled men, women and children; lost earnings by the patients and sometimes family caregivers; and an ongoing cycle of poverty and deprivation for poor families and societies. Social interaction may be impeded due to odour and drainage seen in some wounds. The impact

of loss of self-esteem, continued pain, and possible depression is difficult to quantify, but is certainly real (MacDonald, 2009).

Numerous topical antimicrobials for wound care are available in different dosage form. Despite drugs are available, many wounds still fail to heal and remain a significant burden to patients and caregivers alike. The primary reason is antimicrobials resistance (AMR) to existing drugs with different levels of resistance (Meier and Nanney, 2006). Drug resistance is a global problem affecting both developing and developed countries (WHO, 2014).

The resistance pattern of common wound infecting pathogen such as *S. aureas* shows increments in Ethiopia. Resistance to methicillin increased from 87.5% in 2004 to 100% in 2008 and *E. coli* resistance to beta-lactam penicillin and tetracyclin increased from 60% in 2004 to 77% in 2008(FMHACA,2009).

According to WHO report, bacteria–antibacterial drug combinations resistance for *E. coli*/3rd generation cephalosporin, *E. coli*/fluoroquinolones, and methicillin-resistant *S. aureus* (MRSA) is 85%, 90% and 86% respectively. High rates of MRSA imply that treatment for suspected or verified severe *S. aureus* infections, such as common skin and wound infections must rely on second line drugs in many countries, and that standard prophylaxis with first-line drugs for orthopaedic and other surgical procedures will have limited effect in many settings. Second-line drugs for *S. aureus* are more expensive; also, they have severe side effects for which monitoring during treatment is advisable, increasing costs even further. High proportions of resistance to 3rd generation cephalosporins reported for *E. coli;* means that treatment of severe infections likely to be caused by these bacteria in many settings must rely on carbapenems, the last resort to treat severe community and hospital acquired infections. These antibacterials are more expensive, may not be available in resource-constrained settings, and are also likely to further accelerate development of resistance (WHO, 2014). 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).

1.7.Significance of the study

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. Many of the synthetic drugs currently used for the treatment of wounds are not

only expensive but also pose problems such as allergy, drug resistance and this situation have forced the scientists to seek alternative drugs (James and Victoria, 2010). Considering the principle drawbacks of conventional medicine, plants which are the gift from nature having traditional knowledge, provides excellent raw material for the treatment of various diseases and disorders (Sabale *et al*.,2012). Hence, herbal products are often promoted to the public as being "natural" and completely "safe" alternatives to conventional medicines (Adewunmi and Ojewole, 2004). Several herbs and medicinal plants proved to be a wound healers were identified and formulated for treatment and management of wounds. Various herbal products have been used in management and treatment of wounds over the years. Some of herbs and medicinal plants proved to be scientifically used for the treatment of cuts and wounds as a wound healer are *Gingko biloba, Centella asiatica, Nelumba nucifera, St. John wort(Hypericum mysorense), Ocimum sanctum,Eucalyptus globules(*Sabale *et al*.,2012*).* Several medicinal plants are used in the Ethiopian folklore medicine for wound management. One such plant is *Solanum incanum L.*

1.8.*Solanum incanum* Linnaeus

Solanum incanum Linnaeus or Sodom/bitter apple (English), Embouy(Amharic), Hiddi(Afan Oromo) is a perennial, wild shrub like herb that grows up to 1.8 m in height that belongs to Solanaceae family which grows in many regions of Africa, Middle East and Far East Asia. It is an erect or spreading perennial shrub with leaves and stem occasionally having small prickles. The fruits are small berries of 2-3 cm in diameter and yellowish orange or brown in color when ripe (Abdalla, 2015;Matu 2008). In Africa the whole part herb is used as a folklore remedy for sore throat, angina, stomach-ache, colic, and headache. Other uses include; relieve of painful menstruation, liver problems and pain caused by onchocerciasis, pleurisy, pneumonia and rheumatism. The plant parts are also widely used to alleviate skin problems, such as infections, whitlow, ringworm, burns, sores, rashes, wounds, warts, carbuncles, ulcers, inflammations and benign tumors. In West Africa, leaves are eaten or added to soup to improve flavor, while fruits are used as vegetables. Alternatively, the roots are chewed or its infusions applied externally on scarifications. Similarly, decoctions derived from leaves, roots and fruits are either gargled or drunk. Leaves parts are also used for washing painful areas, while in some cases they are burnt and the ash mixed with fat for use as an ointment. It is also used as an ingredient of arrow poison, as spice to improve flavor and as well as in curdling milk or in cheese making. In Ethiopia it is used in leather tanning and soap making (Matu 2008; Bussmann *et al*. 2006).

Fresh leaves juice traditionally was used in Ethiopia for their wound healing activity (Teklehaymanot, 2009).

Phytochemical studies indicate whole part of the herb contains substances such as steroidal alkaloids, glycol-alkaloids, antioxidants (flavonoids and chlorogenics), saponins and even carcinogenic substances (Mwonjoria *et al*., 2014). Phytochemical screening of methanol leaves extract reveals presence of alkaloids, flavonoids, saponins, triterpens, tannins and steroids as a major class of compounds (Manal *et al*., 2016).

Fruit extract of *solanum incanum* contain dimethylnitrosamine, a potent carcinogen that may be associated with high incidence of esophageal cancer in areas of Africa where the fruit sap is used to curdle milk. Similarly, extracts of the fruits has been found to cause skin cancer in animals. The unripe fruits of *S. incanum* were found to exhibited toxic effect in goats (Thaiya *et al.* 2010). However, oral administration of up to 15,000mg/kg doses of the extract to mice did not show signs of conventional toxicity (Assefa *et al.,* 2006).

Previous reports indicates that different extracts of *S. incanum* showed antimicrobial and antifungal activity(Manal *et al,* 2016), leaves extract showed antimicrobial activity against the Escherichia coli (Britto & Senthinkumar 2001),to *Streptococcus pyogenes, Staphylococcus aureus, and Pseudomonas aeuruginosa* (Taye *et al*.,2011), the juice obtained by chewing or squeezing *S. incanum* leaves significantly reduced the postprandial glucose surge in normoglycemic humans (Uchenna *et al.*,2009), the Diclomethane/methanol root extract of the *S. incanum* exhibited significant antinociceptive and antipyretic effect(Mwonjoria *et al.* 2011).

Another reports indicated that aqueous root extract shown anti-spasmolytic activity(Assefa *et al*. 2006), dichloromethane root extract exhibited significant anti-inflammatory and antinociceptive effect (Mwonjoria J. K. *et al.*,2014), Solamargine alkaloid isolated from *S. incanum* are under intensive clinical investigation for its anti-tumor activity (Yi-Hui Wu, *et al.* 2015).

However, to date, no scientific report could be found in the literature concerning the *in-vivo* wound healing activity of this plant. So this study tries to scientifically prove that the traditional plant claimed to be used for wound actually has the effect. In addition, the study tries to evaluate *in vivo* anti-inflammatory activity of *S. incanum* leaves extracts.

Furthermore, the study also calls for fractionation and isolation of the active constituents of the plant to identify the active compound that can be used as potential sources of drug.

Not only this, even before developing the plant content as a drug, the study is also provide evidence that encourages the traditional use of the plant especially by communities which reside in areas where modern health facilities did not reach due to geographic and socio-demographic challenges.

Fig. 1: photograph of *Solanum incanum* L. Family: Solanaceae, around Adama town

2. Objectives of the study

2.1.General objective

- ✓ The purpose of this study is to evaluate wound healing and anti-inflammatory activity of 80% methanolic extract of *Solanum incanum* L. leaves in mice.

2.2.Specific objectives

The specific objectives of the study are:

- ✓ To evaluate acute oral toxicity test in mice
- ✓ To study acute dermal toxicity test in rats
- ✓ To evaluate wound healing effect of 80% methanolic extract of *S. incanum* L. leaves using excision and incision wound model in mice
- ✓ To evaluate anti-inflammatory effect of 80% methanolic extract of *S. incanum* L. leaves using carrageenan induced paw edema model in mice

3. Materials and methods

3.1.Materials

3.1.1. Plant material

The leaves of *S. incanum* L. were collected from Adama town area, 100 km from Addis Ababa, Ethiopia. The plant was authenticated by a taxonomist at the Ethiopian Public Health Institute (EPHI) where a voucher specimen was deposited (collection no. DD001).

3.1.2. Drugs and Chemicals

The main chemicals that were used include, white soft paraffin(Anonchem limited, lot no.295-456-2),ketamine(Neon laboratories limited, India, batch no. 88409), normal saline(Euro-med laboratories phil.,INC,lot no. 081174), methanol(Carlo Erba Reagents S.A.S, batch no. V31637143L), 70% alcohol(Alpha laboratory Reagent, batch no. M120415), wool fat(BDH Chemicals ltd,England,prod. no 33069), hard paraffin(BDH Chemicals, England), cetostearyl alcohol(BDH Chemicals ltd,England, batch no. 26048), carrageenan, indomethacin(Cadila pharma plc, Ethiopia, batch no. D16005BX60), Tween 80(BDH Chemicals, England, prod.no. 560234h) and nitrofurazone USP 0.2% ointment(Galentic pharma pvt ltd, India, batch no.IE15006). All reagents used were of analytical grade.

3.1.3. Experimental animals

Healthy, adult white albino mice of both sex (25–35g, and 6–8 weeks of age) were obtained and maintained in the animal house facilities at department of pharmacology, School Medicine, AAU. Adult Wistar albino rats of both sex (150-250 g, aged 2-3 months) obtained from the animal house of the School of Pharmacy, AAU, were used for acute dermal toxicity test. They were individually housed in clean polypropylene cages under standard conditions (25 ± 2 °C, 55 ± 5 % relative humidity, and 12 h light and dark cycles) and provided with pellet diet and water *ad libitum*. Animal handling and care was carried out throughout the experiment following international laboratory animal use and care guidelines (ILAR, 2010). At the end of each experiment animals were sacrificed by cervical dislocation.

3.2. Method

3.2.1. Plant extraction

Leaves of *S. incanum* L. were collected, washed under running tap water, and dried for two weeks under shade. The dried leaves were crushed to course powder and 500g of the powder was

extracted by maceration with 80% methanol for 72 hours in conical flask with occasional stirring and shaking. The extract was then filtered by Whatman qualitative filter paper (What man No.1). The residue was further macerated with the 80% methanol twice for three more days to exhaustively extract the plant material and filtered. The filtrates were combined, concentrated by Rota vapor and placed under hot ovum to remove methanol. Concentrated extract was placed in lyophilizer (Operon,Korea) until dried. The resulting dry extract was weighed to calculate the percentage yield, which was 12.4% (w/w). The dried extract was stored in a refrigerator for the preparation of topical formulation (ointment).

3.2.2. Ointment formulation B.P

The drug formulations with different concentration of the extract was prepared, i.e. 5%(w/w) ointment, where 5g of extract was incorporated in 100g of simple ointment base and 10%(w/w) ointment where, 10g of extract was incorporated in 100g of simple ointment base B.P (British pharmacopoeia,1988).

Nitrofurazone ointment (0.2%w/w) was used as standard drug for comparing the wound healing potential of the extract in different animal model. Simple ointment B.P. was prepared using hard paraffin, cetostearly alcohol, white soft paraffin and wool fat. For anti-inflammatory activity study extracts and indomethacin powder were dissolved in 2% (v/v) of Tween 80.

3.2.3. Selection of dose

For the assessment of wound healing activity based on preliminary test result which was conducted on 1%, 5%, 10% ointment, two effective doses i.e. 5%w/w and 10%w/w were selected. For study of anti-inflammatory activity three dose levels of extract i.e. 100mg/kg, 200mg/kg and 400mg/kg were selected based on acute oral toxicity study result.

3.2.4. Grouping of animals

Animals were assigned in to four groups, each group consisting of 6 mice for both excision and incision model. Group I was given non-medicated simple ointment (negative control), group II was given standard drug i.e. nitrofurazone 0.2 %(w/w) ointment (positive control), groups III was received formulation of extract (5%w/w) and group IV was given (10%w/w) ointment of *S. incanum* L.. For evaluation of anti-inflammatory activity animals were assigned in to five groups (each consisting of 6 mice). Group I, II, III, IV and V received vehicle (2% Tween 80), Indomethacin 10mg/kg, 100mg/kg, 200mg/kg and 400mg/kg extracts respectively.

3.2.5. Acute dermal toxicity

Acute dermal toxicity test of 80% methanolic extract of *S. incanum* L. leaves was carried out using 3 adult male and 3 female albino rat weighing 150g-250g. They were kept carefully following an acclimation period of 7 days to ensure their suitability for the study. On both side 500mm^2 areas back of each rats was shaved prior to experiment, one for test substance and other for control. On respective site 10 %(w/w) extract and simple ointment were applied on shaved area. Both sites were covered by gauze and the back of rat was wrapped with a non-occlusive bandage. The animals were then returned to their cages kept individually. Animals were provided with food and water *ad libitum.* After 24 hours, the bandage and gauze were removed and test substance and simple ointment were washed out by distilled water. One hour later the sites were examined for skin irritation. Observations of sites was done at 24 hours after application, and repeated at 48 and 72hours thereafter. The reactions, defined as erythema and edema, were evaluated according to the scoring system for skin reactions explained by Draize JH *et al*.(Table 1) (Draize *et al*.,1994 ; More, *et al.,* 2013).

Table 1: Classification system for skin reaction

Reaction	Score
ERYTHEMA	
No erythema	0
Very slight erythema	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema(beet redness) to scare formation	4
EDEMA	
No edema	0
Very slight edema	1
Well defined edema(edges of the area well defined by define raising)	2
Moderate edema(raising approximately 1mm)	3
Severe edema (raising more than 1mm and extended beyond the area of exposure)	4
Total possible score for primary irritation	8

The score of primary irritation (SPI) was calculated for each rat as the following. Score for erythema and edema at 24, 48, and 72 hours were summed and divided by the number of the observations for the treated sites. The SPI for the control sites were calculated in the same fashion as test.

$$SPI\ for\ each\ rat = \frac{\sum erythema\ and\ edema\ grade\ at\ 24, 48, and\ 72\ hrs}{Number\ of\ observation}$$

The difference between the summation of SPI scores of six animals from the treated site and control site were calculated and were used for primary irritation index (PII) determination. The PII was calculated as the arithmetical mean of the SPI values of the six rats. The irritation degree was categorized as negligible, or slight, moderate or severe irritation based on the PII (table 2).

Table 2: Response categories of irritation in rat

Category	PII
Negligible	0-0.4
Slight irritation	0.5-1.9
Moderate irritation	2-4.9
Severe irritation	5-8

$$PII = \frac{\sum SPI(test) - \sum SPI(control)}{number\ of\ animals}$$

3.2.6. Acute oral toxicity test

Acute oral toxicity studies of the extracts were carried out as per the OECD guidelines, draft guidelines 423 (OECD, 2001). Six female mice (25–35 g) received 80% methanolic of *S. incanum* L. leave extract suspended in 2% Tween 80 at dose level of 2000 mg/kg orally by gavage. Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Observations included changes in skin and fur, eyes and mucous membranes, respiratory and behavior pattern. A special attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

The change in body weight, food and water intake was recorded at two days interval (Dash and Murthy, 2011).

3.2.7. Models for Wound Healing activity

In-vivo excision and incision wound models were used to evaluate the wound-healing activity of leaves extract of *S. incanum* L..

3.2.7.1. Excision wound model

Excision wound was used for the study of rate of wound contraction and epithelialization period (Subalakshmi *et al*. 2014). Animals were anesthetized prior to and during creation of the wounds with ketamine (80mg/kg, I.P) (Kumari *et al*., 2013). An impression was made on the dorsal thoracic region 1 cm away from vertebral column on the anaesthetized mouse. After wound area preparation with 70% alcohol, the dorsal fur of the animals was shaved with razor blade and the anticipated area of the wound to be created was outlined on the back of the animals with permanent parker. A full thickness circular excision wounds sized about 300 mm^2 was created along the markings using toothed forceps, scalpel and scissors (Dash, and Murthy, 2011). Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open (Subalakshmi *et al*. 2014). The mice were divided into four groups (6 mice per group) randomly and each mouse was placed in a separated cage. The treatment was done once daily topically in all the cases. 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 (Kokane *et al*., 2009).

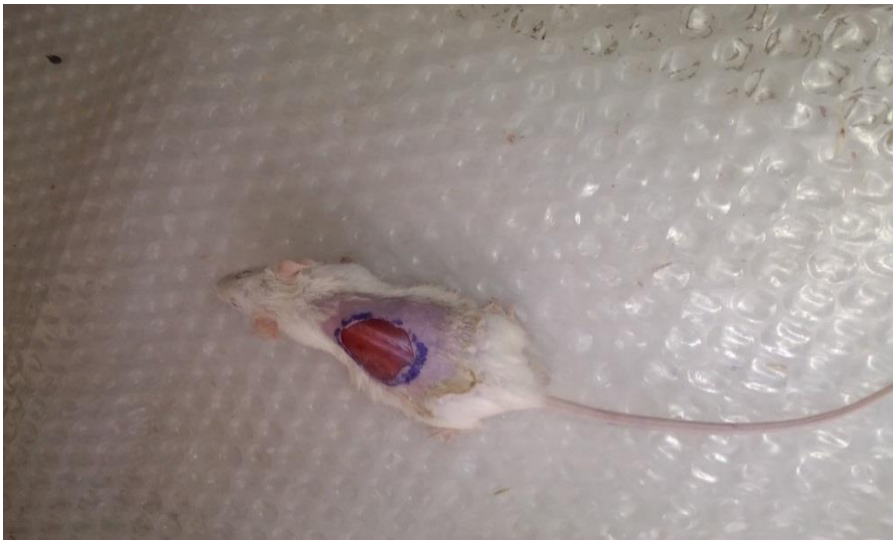

Fig.2: Excision wound on day 0

Measurement of wound contraction

The wound closure rate was assessed by tracing the wound on days 2, 4, 6, 8, 10, 12, 14 and 16 post wounding days using transparent paper and a permanent marker. The wound areas recorded were measured using $1mm^2$ scale of graph paper. Changes in wound area were evaluated, giving an indication of the rate of wound contraction and epithelialization period. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound as 100 % (Kumarasamyraja and Swamivelmanickam, 2015) as shown below:

$$\%wound\ contraction = \frac{initial\ wound\ size - nth\ days\ of\ wound\ size}{initial\ wound\ size} x\ 100$$

Epithelialization period measurement

Falling of scab leaving no raw wound behind was taken as end point of complete epithelialization and the days required for this was taken as period of epithelialization (Subalakshmi *et al.* 2014). The results of epithelialization period are tabulated in Table 4.

3.7.2.2. Incision wound model

Animals were anesthetized in the same manner described for excision wound model. The dorsal fur of each mouse then shaved and a 3 cm long longitudinal paravertebral incision 1cm away from vertebral column was made through the skin and subcutaneous tissue. The parted skin was then sutured 1 cm apart using a surgical thread (silk no. 00 round) as described by Ehrlich and Hunt with slight modification(Ehrlich and Hunt,1969). The continuous thread on both wound edges was tightened for good closure of the wounds.

After 24 h of wound creation (on 1st day), animals were treated as described under grouping section, with topical formulation of non-medicated simple ointment, extract or standard drug once daily for nine days. The suture was removed on day 8 post-incision and tensile strength was measured on the 10^{th} post-wounding day using continuous water flow technique (Shilpa *et al*.,2013).

Fig.3: incision wound on day 1

Measurement of tensile strength

Tensile strength (the force required to open the healing skin) was used to measure the extent of wound healing. The model used for this purpose consists of fixed shelves with a table. Two Allis forceps, one is fixed to the opposite side of shelve and another is tied with and hanged with rope that is attached to the empty IV bag on which the weights are placed. On the 10^{th} post-wounding day each mouse was anaesthetized using high dose of ketamine to secure animal to the table. The two forceps were firmly applied 1cm away from healed tissue on the incised part of the skin on to the line facing each other. Water is allowed to flow into bag from tap water through IV line. 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 an indirect measure of breaking strength in grams (Rajeev Kumar *et al*.,2011 Mulisa *et al*.,2015). The result presented in table 5:

Fig.4: Water flow technique: measurement of tensile strength

3.2.8. Anti-inflammatory activity

Carrageenan induced paw edema was used as model for acute inflammation. Swiss albino mice of either sex were used to determine the anti-inflammatory effects of 80% methanolic extracts of *S. incanum* L. leaves. 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). After determination of the basal volume, the animals were assigned into five groups (each containing 6 mouse) such that the mean volumes of the different groups were not significantly different. Swiss albino mice orally administered with the doses of the herb extracts i.e. 100mg/kg, 200mg/kg and 400mg/kg, Indomethacin 10mg/kg and the vehicle (2% Tween 80 in distilled water). The extract and the standard drug were dissolved in 2% Tween 80 to prepare suspension. One hour later the animals were injected with 0.05 ml of a solution of 1% carrageenan in 0.9% saline (w/v) in the sub-plantar region of the right hind paw to induce inflammation. The paw volume was measured 1, 2, 3, and 4h after the injection of the inflammatory stimulus (carrageenan) using plethysmometer. The difference between the paw edema after and before (basal volume) carrageenan injection was taken as the volume of edema and was determined for each mouse. The percentage of edema inhibition in treated animals was calculated in comparison to the control group (vehicle).The results obtained for doses of the extracts and Indomethacin were compared with the vehicle treated groups (Mwonjoria J. K. *et al*.,2014; Fernandes *et al*.,2010;Haque *et al*.,2014).

$$\%\ inhibition\ of\ edema = \frac{Co - Ct}{Co} x100$$

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 Indomethacin treated mice at the same time.

3.3.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 analyzed statistically by one-way ANOVA followed by Post Hoc Tukey test using SPSS version 20 software, to analyze the differences between different groups. The data were considered statistically significant at $p < 0.05$.

4. Results

4.1. Acute oral toxicity test

There was no mortality observed in animals through the 14-day period following single oral administration at dose level of 2000mg/kg of the 80% methanolic extract of *S. incanum* L. leaves. Morphological characteristics (fur, skin, eyes, and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy, or unusual behaviors such as self-mutilation walking backward and were observed; gait and posture, reactivity to handling or sensory stimuli, and grip strength were all normal.

4.2. Acute dermal toxicity test

Selection of topical base was important to prepare topical formulations with negligible risk of skin irritation, optimum flow, spreadability and release properties. All the developed ointments were stored in tightly closed containers and evaluated for acute dermal irritation test. Maximum concentration of hydro-alcohol ointment (10% w/w) applied using a limit dose of 2000mg/kg of body weight was found to be safe. After 72 h, the application site did not show any sign of inflammation and edema. Therefore, their PII values were zero implying the non- irritant nature of the test samples as per the dermal irritation scoring system of Draize. Hence *S. incanum* L. leaves extract formulation can be used safely as topical preparation to treat skin disease such as wound. There were no signs of toxicity seen when the animals were monitored for 14 days.

4.3. Wound healing (Excision model)

4.3.1. Wound contraction

Topical applications ointment of the 80% methanolic extracts of *S. incanum* L. leaves showed effect on the wound healing process on the mice. The progress of wound contraction induced by treatment of 5%(w/w) and 10% (w/w) *S. incanum* L. leaves of 80% methanolic extract, simple ointment base and nitrofurazone 0.2%(w/w) ointment is shown in Table 3.

The 10% (w/w) 80% methanolic extract ointment treated group showed significant ($p < 0.05$) wound contraction starting from the second day, and with highly significant ($p < 0.001$) difference were seen from 10^{th} day onward in comparison with the control group(simple ointment). As shown in Table 3, there was no significant difference in activity between the 10% (w/w) extract and 5%(w/w) extract. But, higher rate of wound closure was observed with 10 %

(w/w) ointment. Ten percent extract shows comparable efficacy with standard drug. The maximum rate of wound contraction was seen on the 10^{th}, 12^{th}, and 14^{th} day which was 95.67, 99.40, and 100%, respectively.

The animals treated with 5% (w/w) methanolic extract ointment showed significant ($p<0.05$) wound contraction from 8^{th} day onward as compared to control group. Significant ($p < 0.05$) wound contraction was also observed for nitrofurazone 0.2%(w/w) ointment treated group from 6^{th} day onward as compared to control group with highly significant($p<0.001$) wound contraction on 12^{th} ,14^{th} and 16^{th} days. The maximum rate of wound contraction for nitrofurazone 0.2%(w/w) ointment was seen on the 12^{th}, 14^{th} and 16^{th} day which was 97.73, 99.45%, and 100% respectively.

Table 3: Effect of topical application of the 80% methanolic extract of the leaves of *S. incanum L.* on wound contraction of excision wound model in mice.

Treatments	**Wound area (mm^2) post-wounding days**								
	0	2	4	6	8	10	12	14	16
Simple ointment	300.5±5.0 (00%)	263.8±12.3 (12.2%)	218.8±14.8 (27.17%)	178.3±20.2 (40.65%)	141.2±22.89 (53.02%)	98.00±16.88 (67.38%)	66.8333±11.83 (77.76%)	51.50±11.74 (82.86%)	35.16±9.5 (88.29%)
Nitrofurazone 0.2% ointment	308.16±2.95 (00%)	265.83±9.16 (13.73%)	177.50±4.58 (42.40%)	84.66±8.19** (72.525%)	49.50±8.1** (83.9%)	22.5000±3.10** (92.69%)	7.0000±1.69*** (97.72%)	1.67±1.08*** (99.45%)	.0000*** (100%)
5% Extract	300.33±5.78 (00%)	225.66±30.31 (24.84%)	175.00±26.89 (41.73%)	135.66±24.1 (54.82%)	76.5±16.5** (74.5%)	54.67±13.90* (81.79%)	23.00±7.50** (92.34%)	15.50±5.63** (94.83%)	8.0000±3.6** (97.33%)
10% Extract	304.33±6.10 (00%)	184.33±7.66* (39.43%)	128.33±7.49** (57.83%)	81.33±9.16** (73.27%)	41.167±6.25**(86.4%)	13.1667±3.49*** (95.67%)	2.00±1.0*** (99.34%)	.000*** (100%)	.000*** (100%)

Values are expressed as mean ± S.E.M (n = 6). $*P < 0.05$, $**P < 0.01$,***p<0.001 when compared to control group; one way ANOVA.

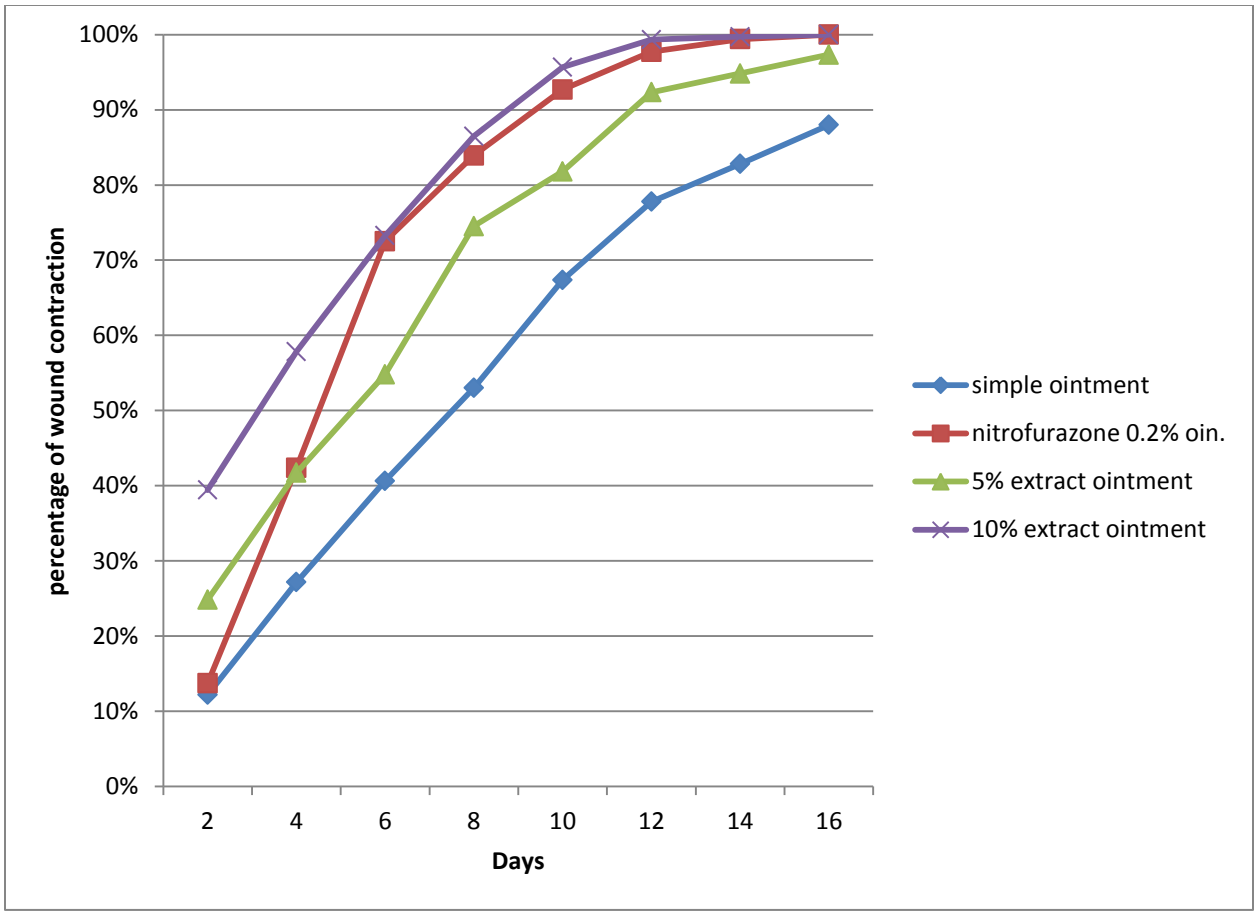

Fig. 5: Effects of the 80% methanolic extract of *S. incanum* L. *leaves* on the percentage wound closure of excision wound model.

4.3.2. Epithelialization period

The period of epithelialization was 17.83±1.11, 13.00±0.45, 14.50±0.96 and 12.16±0.40 for control group, standard drug, 5%(w/w) and 10%(w/w) extract ointment respectively. Ten percent extract ointment treated mice showed faster rate of epithelialization ($p<0.001$) compared to control group. There was no significant difference of epithelialization period between 5%(w/w), 10%(w/w) extract and standard drug. Both standard drug and 5%(w/w) ointment showed significant($p<0.05$) difference of epithelialization period as compared to control group (Table 4).

Table 4: Effect of topical application of the 80% methanolic extract of the leaves of *S. incanum* L. on Period of epithelialization (no. of days).

Groups	Period of epithelialization (no. of days) Mean ±SEM
Simple ointment base	17.83±1.11
Nitrofurazone 0.2%(w/w) ointment	13.00±.45**
5% (w/w) extract	14.50±.96*
10% (w/w) extract	12.16±.40***

Values are expressed as mean ± S.E.M (n = 6). $*P < 0.05$, **p<0.01, ***$P < 0.001$, when compared to control group; one way ANOVA.

4.4. Wound healing (incision model)

In incision wound model (Table 5), standard drug, 10%(w/w) , and 5%(w/w) extract treated animals showed significant increase in breaking strength (318.83±22.25, 288.75±19.59 and 269.17±7.70 respectively), when compared to the control group(simple ointment) (198.83±15.86). There was no significant difference in breaking strength between standard drug, 10%(w/w) , and 5%(w/w) extracts.

Table 5: Effect of topical application of the 80% methanolic extract of the leaves of

S. incanum L. on wound breaking strength (incision wound model).

Group	**Tensile strength (g) (mean± SEM)**
Simple ointment base	198.83±15.86
Nitrofurazone 0.2%(w/w) ointment	318.83±22.25***
5%(w/w) extract	269.17±7.70*
10% (w/w) extract	288.75±19.59**

Values are expressed as mean ± SEM (n = 6). $*P < 0.05$, **p<0.01, $***P < 0.001$, when compared to control group; one way ANOVA.

4.5. *In-vivo* anti-inflammatory activity

The anti-inflammatory activity of the 80% methanolic extract from leaves of *S. incanum* L. was evaluated by carrageenan-induced paw edema in mice. Two hour after administration of carrageenan, neither the extract nor the standard drug showed significant anti-inflammatory activity as compared to the control. The medium and high doses of *S. incanum* L. extract (200 and 400mg/kg) started suppression of oedema significantly ($p < 0.05$) after 3 h of carrageenan injection as compared to control showing 46.65% and 58.96% inhibition of oedema, respectively. Lower dose (100 mg/kg) also suppresses edema formation when compared to control but unable to reach significant level. The inhibitory values of oedema at 4 h post carrageenan injection were 40.19%, 62.14% and 70.48% for 100, 200 and 400 mg/kg of the extracts, respectively. The group which received the indomethacin showed significant ($p<0.01$) inhibition of inflammation starting from 3 h post carrageenan injection. The percentage inhibition of oedema by standard drug at 3 and 4 h post carrageenan injection were 59.39% and

77.09% respectively. The extracts showed dose dependent inhibition of paw edema in mice (table 6).

Table 6:Anti-inflammatory effects of orally administered 80% methanolic extracts of *S. incanum L.* leaves on carrageenan-induced mice paw oedema.

Groups	**Mean increase in paw eodema volume in ml(percentage of eodema inhibition) mean±S.E.M**				
	0h(basal)	1h	2h	3h	4h
Vehicle control	0.732±0.047	0.475±0.057	0.442±0.032	0.463±0.076	0.515±0.076
Indometh. 10mg/kg	0.628±0.060	0.345±0.054 (27.37%)	0.293±0.043 (33.71%)	0.188±0.029** (59.39%)	0.118±0.031*** (77.09%)
100mg/kg extract	0.690±0.013	0.448±0.069 (5.68%)	0.390±0.076 (11.76%)	0.355±0.058 (23.33%)	0.308±0.074 (40.19%)
200mg/kg extract	0.687±0.041	0.408±0.055 (14.10%)	0.335±0.039 (24.21%)	0.247±0.019* (46.65%)	0.195±0.018** (62.14%)
400mg/kg extract	0.620±0.026	0.365±0.046 (23.16%)	0.275±0.024 (37.78%)	0.190±0.020** (58.96%)	0.152±0.010*** (70.48%)

Values are expressed as mean ± SEM (n = 6). $*P < 0.05$, **p<0.01, ***$P < 0.001$ when compared to control group; one way ANOVA.

5. Discussion

Traditionally, medicinal plants have been used for many years as topical and internal preparations to promote wound repair. Medicinal plants have great potentials and have been shown to be very beneficial in wound care, promoting the rate of wound healing with minimal pain, discomfort, and scarring to the patient. Some of these plants owe their effects to direct effect on the wound healing processes and some to their anti-inflammatory effects. A combination of these properties is also possible in some of the medicinal plants used in wound care (Sabale *et al*., 2012).

Wound healing is a sequence of events which consists of coagulation, inflammation, collagenation, wound contraction and epithelialization (Murti and Kumar, 2012; Mittal *et al*., 2013).While the phase between coagulation to collagenation is intimately inter-linked, the phase of wound contraction and epithelialization are independent to each other and run concurrently (Bairy and Rao, 2001; Shirwaikar *et al*., 2003).

In order to evaluate wound healing activity *in-vitro* study and single model are not adequate to collectively represent the various components of the wound healing process as a whole. Hence, in the present study two different wound models were used to establish the *in-vivo* wound healing potential of 80% methanolic leaves extracts of *S. incanum* L. on various phases.

The results of wound healing effects of *S. incanum* L. showed significant promotion of wound healing activity with both 10%(w/w) and 5%(w/w) extracts in the excision and incision wound models as compared to control group. Although there was no significant difference between extracts, 10%(w/w) extract showed better healing activity than 5%(w/w) extract. Wound Healing effect of 10%(w/w) extract was comparable with standard drug(nitrofurazone).

Increased rate of wound contraction and decrease in period of epithelialization in the animals treated with the 80% methanolic extracts may be attributed to the presence of phytoconstituents like unsaturated sterols, triterpenoids, alkaloids, flavonoids, saponins, tannins and phenolic compound in the leaves extracts of *S .incanum* L.(Manal *et al*.,2016) which are known to promote the wound healing process mainly due to their antimicrobial property. Flavonoids and triterpenoids are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization (Bodenstein and Du Toit, 2005). Tannins are seen to be active

detoxifying agents and inhibit bacterial growth (Chirchir *et al.*,2014). *In-vitro* study on leave methanolic extract of *S. incanum L.* reveals activity against common wound pathogens such as *S. aureus*, *P. aeuruginosa* and *S. pyogens* which could support findings of this study (Taye *et al*., 2011).

During natural wound healing process, an infection mostly from *S. aureus, E. coli. P. auregenosa and Bacillus spp.* can delay healing by protracting the inflammatory phase, disrupting the normal clotting mechanism; hence, ultimately delaying angiogenesis (Arun *et al.*, 2016). Therefore, the presence of phytochemicals with antimicrobial activity in *S. incanum* L. may accelerate healing of wounds.

Increase in skin breaking strength in incision model indicated enhanced collagen maturation. Collagen, the major protein of extracellular matrix, is the component which gives strength, support and integrity to the wound matrix. Breakdown of collagen liberates free hydroxyproline and its peptides. A healing tissue synthesizes collagen, which is a constituent of growing cells (Arun *et al*., 2016).

Some phytoconstituents like flavonoids which are known to reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and improving vascularity may increase in the granulation tissue dry weight and hydroxyproline content. Flavonoids are a potent antioxidants and free radical scavengers which prevent oxidative cell damage (Arun *et al*, 2016). *S. incanum* L. also contains vitamin B2, C (a major water-soluble anti-oxidant in extracellular fluid) and vitamin E a major lipid soluble anti-oxidant that prevents lipid peroxidation (Auta *et al.*,2011; Auta and Ali, 2011). Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, by increasing the circulation, by preventing the cell damage and by promoting the DNA synthesis (Getie *et al*. 2002). Delaying inflammatory phase may also lead to generation of reactive oxygen species which, due to their damaging effects on cells and tissue, are noxious to wound healing process. The increase in free radical production and diminished antioxidant activity may worsen the condition and account for the delay in healing (Adly, 2010). The presence of reactive oxygen species and microbes at the wound site has synergetic effects causing delay in wound healing and hence antioxidant and antimicrobial properties of phyto- constituents will fasten wound healing process.

In the group treated with simple ointment base, very slow epithelial reorganization and wound closure was observed. But the *S. incanum* L. extracts 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 anti-inflammatory activity. The inflammatory process is the response to an injurious stimulus evoked by a wide variety of noxious agents (*e.g.*, infections, antibodies, or physical injuries). The ability to mount an inflammatory response is essential for survival in the face of environmental pathogens and injury. Inflammation is therefore 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. However, excessive inflammation is a major contributing factor to the persistence of chronic non-healing wounds, which are "stuck" in the inflammatory phase of healing and fail to re-epithelialise (Burke *et al*., 2006 ; Röhl *et al.*, 2015).

The present *in-vivo* study revealed that 80% methanolic extract of *S. incanum* L. leaves possess anti-inflammatory activity in carrageenan induced paw oedema model in mice. Carrageenan induced hind paw oedema model has been used widely for the discovery and evaluation of anti-inflammatory drugs, since the relative potency estimates obtained from most drugs tend to reflect clinical experience(Padilha *et al.*, 2010).

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 anti-inflammatory drugs, which have been frequently used to assess the antioedematous effect of natural products (Fernandes, *et al*.,2010;Marrassini *et al.*, 2010).

This investigation showed that the 80% methanolic extract of *S. incanum* L. had no significant anti-inflammatory activity at 1 and 2 h of post carrageenan injection but percent inhibition of inflammation was highest at 3 and 4 h after inflammation was induced. The suppression of local edema formation by the medium and high doses of 80% methanolic extracts were significant ($p<0.05$) after 3 h post carrageenan injection. Lower dose (100mg/kg) of extract also exhibit eodema suppression but unable to reach significant level this may be due to inadequate concentration of active constituent in lower dose of extract. The inhibition of edema observed was pronounced in the later phase of inflammation, which was similar to the effect of nonsteroidal anti-inflammatory drugs such as Indomethacin, indicating that the antiedematogenic activity is possibly mediated through a cyclooxygenase enzyme inhibitory pathway (Burke, *et al*., 2006). Finding of this study was in agreement with that of dichlomethane root extract of *solanum incanum* L. where significant edema reduction was observed at later phase of inflammation (Mwonjoria J. K. *et al*.,2014).Previous studies have shown that Phenolic compounds such as flavonoids possess anti-inflammatory activity (da Costa, 2015). Flavonoids are known to prevent the synthesis of prostaglandins (Sowemimo *et al*., 2013). The polyphenols present in this plant may be responsible for the observed anti-inflammatory activities (Auta, *et al*.,2011; Manal *et al*,2016 **)**.Previous reports indicate that a number of plants with anti-inflammatory 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 neutrophil-derived matrix metalloproteinases (MMPs), a family of proteases that can degrade the ECM and decreased levels of tissue inhibitor of metalloproteinase 1 (TIMP-1) (Armstrong and Jude, 2002). 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 anti-inflammatory agents were used for wound treatment, they are well known to inhibit wound repair *via* global anti-inflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis (Guo and Dipietro, 2010).

6. Conclusion and Recommendation

6.1. Conclusion

In this study, the different phases of wound repair, including wound contraction, epithelialization and collagen synthesis as measured by tensile strength were improved by ointments prepared from the 80% methanolic extract of the leaves of *S. incanum* L. as compared to the control group. This study also showed significant reduction of paw edema induced by carrageenan indicating *S. incanum* L. possesses anti-inflammatory activity. These results collectively demonstrate that the 80% methanolic extract of *S. incanum* L. possesses wound healing and anti-inflammatory properties, and this justifies the use of the leaves of *S. incanum* L. for wound and inflammations as claimed in the folklore literature.

6.2. Recommendations

- ✓ Chronic toxicity studies should be performed.
- ✓ It is necessary to make activity guided fractionate and isolate the possible active components focusing on polar components to identify which fractions of the plant extract responsible for wound healing and anti-inflammatory activity.
- ✓ As chronic wounds such as diabetic wounds, infected wound are major global burden, it is worthwhile to study the activity of the plant on chronic wounds.

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