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Evaluation of *in vivo* wound healing activity of 80% methanol crude extracts of the bulbs of *Crinum abyssinicum* in Mice

By

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Addis Ababa, Ethiopia

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A Thesis submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology

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School of Graduate Studies

This is to certify that the thesis prepared by Abdi Hussein, entitled "Evaluation of *in vivo* wound healing activity of 80% methanol crude extracts of the bulbs of *Crinum abyssinicum* 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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ABSTRACT

Evaluation of *in vivo* wound healing activity of 80%methanol crude extracts of the bulbs of *Crinum abyssinicum* in mice

Abdi Hussein

Addis Ababa University, 2019

Crinum abyssinicum is a species of bulbous plant that belongs to the family *Amaryllidaceae.* It is believed that the plant is native to Ethiopia, and known by the local name yejibb shinkurt (in Amharic). This plant is traditionally used for treatment of wound. No scientific evidence which substantiates its traditional claim is, however, available to date. The objective of this study is therefore to evaluate the wound healing activity of bulbs extracts of *C. abyssinicum* in mice. Mice were used for acute toxicity, wound healing and anti-inflammatory studies. For wound healing study, the 80% methanol extract of *C. abyssinicum* bulbs were formulated for topical application by incorporating it in simple ointment base B.P. in a concentration of 5% (w/w) and 10% (w/w). The simple ointment and nitrofurazone (0.2%) skin ointment were used as negative and positive controls, respectively. Toxicity of the formulated ointments was studied by skin irritation test on Swiss albino mice. For anti-inflammatory study, carrageenan-induced hind paw edema model was used to evaluate the anti-inflammatory effect of the extract at concentrations of 100, 200 and 400 mg/kg. Mice treated with Indomethacin (10mg/kg) and 2% Tween 80 served as positive and negative controls, respectively. The antimicrobial activity of the extract at concentrations of 50, 100 and 200 mg/ml was studied using agar well diffusion assay. Level of wound contraction, period of epithelialization, skin breaking strength, edema and zone of inhibition were determined. The ointment formulation of the extract was found to be non-irritant at 5% and 10% concentrations. Treatment of wound with ointment containing 5% (w/w) and 10% (w/w) of 80% methanol extract of the bulbs exhibited significant increase in wound contraction rate, shorter epithelialization time and higher skin breaking strength in the excision and incision wound model used as compared to control. The plant extract also showed dose-dependent significant reductions ($p < 0.05$) of inflammation compared to control. The wound healing potentials, anti-inflammatory and antimicrobial effect of the 80% methanol extracts of *C. abyssinicum* bulb observed in this study is consistent with the traditional claim of this plant for wound healing activity, and hence it is

recommended to perform fractionation of crude extract in order to identify which fraction/s of the plant extract is/are responsible for wound healing.

Key words: *Crinum abyssinicum*, wound healing, excision, incision, anti-inflammatory, antimicrobial

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ABBREVATIONS AND ACRONYMS

AFRO	Africa Regional Office
ANOVA	Analysis of Variance
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
IL-1	Interleukin One
ILAR	Institute for Laboratory Animal Research
LD_{50}	Median Lethal Dose
OECD	Organization for Economic Cooperation and Development
PMNLs	Polymorph Nuclear Leucocytes
SEM	Standard Error of the Mean
SPSS	Statistical Package for the Social Sciences
TGF-β	Transfer Growth Factor b
TNF-α	Tumor Necrosis Factor alpha
TS	Tensile Strength
WHO	WHO World Health Organization

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1 INTRODUCTION

1.1 Overview About Wound

Wounds are injuries that interrupt the tissue wholeness and bring about an inability to keep protective function of the skin, cellular and anatomical or functional continuity of living tissues. Skin wound may range from simple break specified to superficial epithelial to the deeper underlined structures (Lordani *et al*., 2018; Ozay *et al.*, 2018). Wounds are unescapable events of life, which may arise due to physical, chemical, thermal, radial, microbial, immunological, surgical, toxic, or other underlying pathological insults to the tissue. Irrespective of the cause and type, wound causes damage of tissue as well as disruption of the vicinity cells. Therefore, proper restoration of the integrity of a damaged tissue needs an orderly intricate process to resume their ordinary anatomical structure and physiological function called wound healing (Udegbunam *et al*.., 2015; Muniandy *et al*., 2018).

The classification of wound is based on various criteria or biological processes. Based on the cause, wound is classified into open and closed wound. In an open wound, blood escapes the body, and bleeding is clearly visible. This type of wound may include: Incised wound, laceration, superficial wound, puncture wound, gunshot wound, etc. In closed wound, on the other hand, blood escapes in the circulatory system but remains in the body. It includes: contusion or bruises, heamatoma or blood tumor, Crush injury etc. (Alam *et al*., 2011; Goorani *et al*., 2018).

Furthermore, wound may be classified into acute and chronic according to time frame of healing (Agyepong *et al*., 2015). Acute wound is a tissue injury that normally precedes through an orderly and timely reparative process which results in sustained restoration of tissue integrity. It is usually caused by cuts or surgical incisions that end healing within the expected time frame (Velnar *et al*., 2009). Conversely, chronic wound is injury that failed to proceed through an orderly and timely reparative process to bring tissue integrity. This situation happens when the normal wound healing process is compromised due to numerous phenomenon such as microbial infection, metabolic disturbances, systemic problems such as diabetes mellitus, malnutrition, immunodeficiency (Järbrink *et al*., 2017).

As mentioned above, specially wound contaminating organisms prolong the duration of wound healing process by producing toxins that may further destroy the wounded tissues and/or degrade the biochemical substances that enhance wound healing (Dubey *et al*., 2017). Therefore, drugs that may be applied topically, orally or systemically are often used to shorten the duration of wound healing by overcoming microbial wound contamination and to achieve optimum healing. Many synthetic drugs are available for treatment of wounds, but these drugs are usually costly and are associated with several adverse effects. Because of these reasons alternative treatment approaches are needed for wound healing (Farahpour and Habibi, 2012; Ozay *et al.,* 2018).

Medicinal plants have been used for long time to treat a lot of ailments worldwide and still play a crucial role in the health systems in many developed and developing countries. Emergence of resistance to herbal medicines has been relatively rare. They are also more available, assessable and affordable compared to their synthetic counterparts. Looking for medicinal plants as alternative treatment for wound healing is, therefore, crucial (Barreto *et al*., 2014; Dubey *et al*., 2017).

1.2 Wound Healing Sequence

Normal wound healing is a dynamic and complex process involving a series of coordinated events such as cellular, molecular, biochemical, and physiological phenomena that bring about proper restoration of disrupted anatomical and functional status of tissue, which commences following the damage or wounding of tissue as normal biological process. The mechanisms underlying the processes of wound healing sequence consists of highly integrated and overlapping phases such as: hemostasis, inflammation, proliferation, and tissue remodeling (Kasuya and Tokura, 2014; Frykberg and Banks., 2015; Oryan *et al*., 2018). These phases of wound healing are discussed in the subsequent subsections.

1.2.1 Hemostasis

Immediately after the injury, blood components are released into the wound site activating the clotting cascade. Thereby clot induces hemostasis that releases chemotactic cytokines and provides a matrix for the influx of inflammatory cells. At this stage, narrowing of the damaged vessels occur resulting from the activity of vasoconstriction factors, such as serotonin, thromboxane A2, or adrenaline (Olczyk e*t al*., 2014; Pastar *et al*., 2018).

Furthermore, activated platelets cause vasodilation and increase capillary permeability possibly by releasing histamine. This enhances serum rich in proteins such as fibronectin, fibrinogen and fibrin to leak into the interstitial space, where these combine with the clot to produce a fibrin plug that temporarily closes the wound (Gopalakrishnan and Rajameena, 2012; Sahib *et al*., 2014). It also protects the structural integrity of vessels and provides a provisional matrix or scaffolding which guide and supports the influx of fibroblast and keratinocytes. This provisional matrix is composed of fibronectin that participates in wound healing in a variety of ways. It serves as a chemoattractant for the migration of wound repair cells and as a template for the deposition of collagen fibers. It also participates in wound debridement and activation of the phagocyte of macrophages (Diegelmann and Evans, 2004; Jimi *et al.*, 2017).

1.2.2 Inflammation

Inflammation phase of the healing process develops during 24 hours starting the occurrence of injury up to 48 hours on average. The primary goal of this phase of wound is to prepare the wound bed for healing by removing necrotic tissue, debris, and bacterial contaminates and to localize damaged area. It is characterized by leukocyte migration and arrival to the site of injury and cardinal signs such as redness, body heat, swelling, pain, and loss of function (Diegelmann and Evans, 2004; Wild *et al.*, 2010).

The important inflammatory cells are polymorphonuclear leukocytes (PMNLs) mostly referred to neutrophils from granulocytes and activated monocytes or macrophages from agranulocytes. Furthermore, these cells besides keeping the wound aseptic by active phagocytosis and debridement, they simultaneously release a large number of active mediators like cytokines and growth factors (Menke and Diegelmann, 2006; Mosser and Edwards, 2008). At the same time, many different cell types respond to initial inflammatory signals and migrate to the wound site, including keratinocytes, endothelial cells, and both circulating and local progenitor cells that indicate the initiation of the next phase of the healing process (Li *et al*., 2007; Guo and DiPietro, 2010; Bodnar, 2014).

1.2.3 Proliferation and repair

After hemostasis and inflammatory phases are completed, the process of rebuilding the damaged tissue is intensified. The specific mechanisms of this stage are to ensure the wound surface coverage with new skin (re-epithelization), restoring vascular integrity to the region

(neovascularization), and repairing the structural integrity of the tissue defect by filling it with new connective tissue (granulation). In addition, tensile strength of the wound begins to develop during this stage. Crucial cells for this purpose are the fibroblasts and keratinocytes as well as endothelial cells (Eaglstein, 2001; Schreml *et al.*, 2010; Stechmiller, 2010; Ayuk *et al.*, 2012). These mechanisms are discussed below.

i. Neovascularization

For healing cascade of wound, the availability of a steady supply of nutrients and an intact delivery system is crucial. The process of restoring the vascular network is called neovascularization or angiogenesis. This process restores blood circulation in the place of damage and prevents the development of ischemic necrosis simultaneously stimulating the tissue repair process (Tonnesen *et al*., 2000; Strodtbeck, 2001). It is stimulated by microenvironment local factors (i.e. low oxygen tension, low pH, or high lactic acid concentration) and many soluble mediators secreted by epithelial cells, fibroblasts, endothelial cells, and macrophages (i.e. bFGF, TGF-β, TNF-α, VEGF, angiogenin, and angiotropin). Furthermore, lactic acidosis and hypoxia stimulates angiogenesis. However, angiogenesis process is hindered by the angiostatin and thrombospondin (Li *et al*., 2003; Flanagan, 2000). Another essential requirement of neovascularization is the formation of an appropriate extracellular matrix with an adequate supply of oxygen. Thus, endothelial cells migrate to the temporary matrix of the wound and join independent "budding" branches that creates a structure which gives the beginning for a new blood vessel loop (Strodtbeck, 2001; Olczyk *et al*., 2014).

ii. Re-epithelization

This process occurs after the entire skin defect is covered by keratinocytes. Keratinocytes proliferate and start migration across the wound bed after stimulated by locally released growth factors within 12 to 24 hours after injury. The leading edge of the migrator keratinocyte attaches to a new spot in the wound bed. The cell then contracts, pulling itself forward across the wound surface. This process is repeated again and again until the migrating cells from opposite sides of injured area touch each other (Schultz *et al*., 2003; Gurtner *et al*., 2008). After the opposite side cells contact each other the migration is halted and called contact inhibition. Once migration is completed, the keratinocytes stabilize themselves by forming firm attachments to each other and

the new basement membrane close the wound (Pastar *et al*., 2014). Hence, epithelial cells, participating in closing the wound surface, originate both from the wound edges and epithelial appendages, such as hair follicles, sweat glands, or sebaceous glands (Flanagan, 2000).

iii. Granulation

Granulation is process developed after fibrin clot scaffold is replaced with new tissue rich in hyaluronic acid, fibronectin, and other extra cellular matrix (ECM) compounds. Granulated tissue is highly vascular, very active metabolically and supports the proliferation of a variety of cells and proteins. The predominant cell type found in this process is the fibroblast that produces collagen and numerous other substances that comprise the ECM (Campos *et al*., 2008; Palpandi *et al*., 2010).

Extracellular matrix is composed of substances that have different roles in wound healing cascade such as fibronectin which promote adhesion and migration, glycosaminoglycans that promote tissue hydration, proteoglycans or matrix proteins that are involved in the regulation, migration, storage, and coagulation proteins (chondroitin sulfate); and glycoproteins that provide tissue strength and resiliency (collagens, elastin). As the wound closes, the immature fibrin matrix and granulation tissue are replaced by collagen and scar. However, wound healing as a process does not end at wound closure (Flanagan, 2000; Gurtner *et al*., 2008; Guo and DiPietro, 2010).

1.2.4 Remodeling

The final stage of wound healing is remodeling or maturation of the granulation tissue into mature connective tissue and/or scar. During this process, there is reduction of capillary amounts, i.e. aggregating into bigger blood vessels, the contents of glycosaminoglycans and proteoglycans together with that of water. The important cells involved in remodeling are macrophages and fibroblasts. Mechanisms used in remodeling are cell maturation, programed cell death or apoptosis and ECM reshaping by cross-linking collagens (Sabol *et al*., 2012; Jimi *et al.*, 2017).

There is ongoing collagen synthesis and breakdown as the ECM is continually remodeled, equilibrating to a steady state. Remodeling is thus a balance between synthesis of new collagen and degradation of the old one. The initial type III collagen is replaced by type I collagen until a type I: type II ratio of 4:1 is reached, which is equal to normal skin. The density of cells such as macrophages, keratinocytes, fibroblasts, and myofibroblasts, is reduced by apoptosis.

Keratinocytes are the first cells to undergo programmed cell death; myofibroblasts are the second (Ferguson and O'Kane, 2004; Guo and DiPietro, 2010). However, the mechanism triggering apoptosis in this process is not clearly known (Desmoulière *et al*., 1995). Hence, when the wound healing sequence is switched off, the new connective tissue matures and changes from pinkish-red to a white color (Davis and Senger, 2005).

1.3 Factors Affecting Wound Healing

There are numerous factors which can impair wound healing process. These factors can be generally categorized into local and systemic. Local factors are those that directly influence the characteristics of the wound itself. Some of these factors may include oxygenation, infection, foreign body, venous insufficiencies, etc. Systemic factors on the other hand are the overall health or disease state of the individual that affect his or her ability to heal wound. Some of the systemic factors are age, gender, sex, hormones, stress, ischemia, diseases like diabetes, uremia and obesity; medications like glucocorticoids, non-steroidal anti-inflammatory drugs, alcoholism and smoking; immune-compromised conditions like cancer, radiation therapy, acquired immune deficiency syndrome; and malnutrition (Odimegwu *et al.*, 2008; Guo and DiPietro, 2010; Vowden, 2011).

1.4 Management of Wound

The primary target of wound treatment is to promote wound healing in the short time as soon as possible, with minimum pain, discomfort, and scarring to the patient. Besides, it must occur in physiologic environment conducive for repair and regeneration (Mohanty *et al.*, 2010). Almost all wounds harbor microbes that require techniques to get rid of them. This technique may include use of expensive silver-containing dressing system, application of topical and/or systemic antimicrobials (Joseph, 2011). Application of topical antimicrobial therapy is one of the most important methods of wound care. The aim of this therapy in wound care is to control microbial colonization that may hinder subsequent proliferation and thus promoting the healing of the wounds (Odimegwu *et al.*, 2008; Sasidharan *et al.*, 2010).

There are plenty of topically applicable antibiotics in use prepared in the form of lotions, creams, ointments, powders, gels, foam and sprays. The advantages of topical antimicrobials over systemic antimicrobials are low risk of systemic adverse effects, advantage of concentrating on the affected area, low quantity of drug usage, and none interference with intestinal flora. Some examples of

topical antimicrobials are bacitracin, clindamycin (cream, lotion, and foam), gentamicin **(**ointment or cream), nitrofurazone (0.2% cream, solution, or soluble dressing (Elmetti, 2008; Sasidharan *et al*., 2010). Furthermore, negative pressure wound therapy and advanced silver-containing dressing products that support the body to achieve the ideal moist and warm state, skin substitutes, growth factors, biologic wound products and hyperbaric oxygen are among the modern treatments (Han and Ceilley, 2017).

1.5 Traditional Medicinal Plants Used for Wound Healing

Medicinal plants have been traditionally used since time immemorial for treatment of wounds especially cut wound, superficial wounds, puncture wounds, burns etc. (Kokane *et al.*, 2009). The World Health Organization estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare. According to WHO, currently around 21,000 plant species have the potential for being used as medicinal plants (Kumar *et al*., 2017). Besides, 80% of the people living in developing countries of Asia, Africa and Latin America use traditional medicine to meet their primary health care needs (WHO-AFRO, 2010). In Ethiopia, it has been estimated that traditional remedies are the most important and sometimes the only source of therapeutics for nearly 80% of the population of whom 95% of traditional medicinal preparations are of plant origin (Getaneh and Girma, 2014).

There are many plants which are traditionally claimed and used for wound healing activity in Ethiopia. Among those plants which have been tested for their wound healing activities include *Rumex abyssinicus (*Mulisa *et al*., 2015*),* A*llophylus abyssinicus* (Yesuf and Asres, 2013), *Brucea antidysentrica, Datura stramonium, Croton macrostachyus, Acokanthera schimperi (*Taye *et al*, 2011*), Acalyphavolkensii Pax, Amorphophallus gallaensi (*Gidey *et al*., 2009*), Brucea antidysentrica* (Tessema *et al*, 2018). Hence, there are several medicinal plants used in Ethiopia folklore medicine for wound healing awaiting scientific proof and *Crinum abyssinicum* is one of such plants traditionally used for wound healing in Ethiopia which needs to be evaluated scientifically.

1.6 Crinum Abyssinicum

Crinum abyssinicum is a species of bulbous plant that belongs to the family of the *Amaryllidaceae* (Figure 1). This plant prefers a sunny condition on fresh to moist soil to grow well. Bulbs up to 15 cm in diam., with a considerable neck, often propagating vegetative to form dense clusters. Leaves glaucous to greyish green, erect, linear to narrowly lanceolate, mostly with intact apices, 40 x 1–3.5(–5) cm, with a distinct midrib. Scape 40–80 cm long, produced with the leaves or somewhat earlier; bracts erect for a while, papery, greyish. Flowers 2–6, sessile or almost so, heavily scented, red to pink in bud, fading to pure white, sometimes tinged pink, during anthesis, only rarely with a pink dorsal streak; tube curved, 6–10 cm long; segments broadly lanceolate, 8–10 x c. 2 cm, forming a bell, with reflexed outer parts during anthesis. Filaments white, 4–6 cm long, of unequal length; anthers 6–10 mm long, curved, black to brownish (Nordal, 1995). It is believed to be native to Ethiopia and recognized in Ethiopia by the local name Yejib Shinkurt (in Amharic) (Abebe, 2003). As a traditional medicinal plant, the bulbs of *C. abyssinicum* have been reported to be used in Ethiopia as a remedy for wound, chancroid, hypertension, diabetes, hemorrhage, hepatitis B, Skin infection and some forms of tumor (Abebe, 2003; Teklehaymanot and Giday, 2007; Yineger *et al*., 2008). The fresh root *C. abyssinicum* or dried prepared by Concocted, crushed, mixed with water or butter that applied topically or oral. Although no scientific report could be found in the literature concerning the wound healing effects of the plant, it is claimed for its wound healing properties. Therefore, it is considered worthy to conduct scientific experiments to prove whether extracts of the plant have genuine wound healing activity or not.

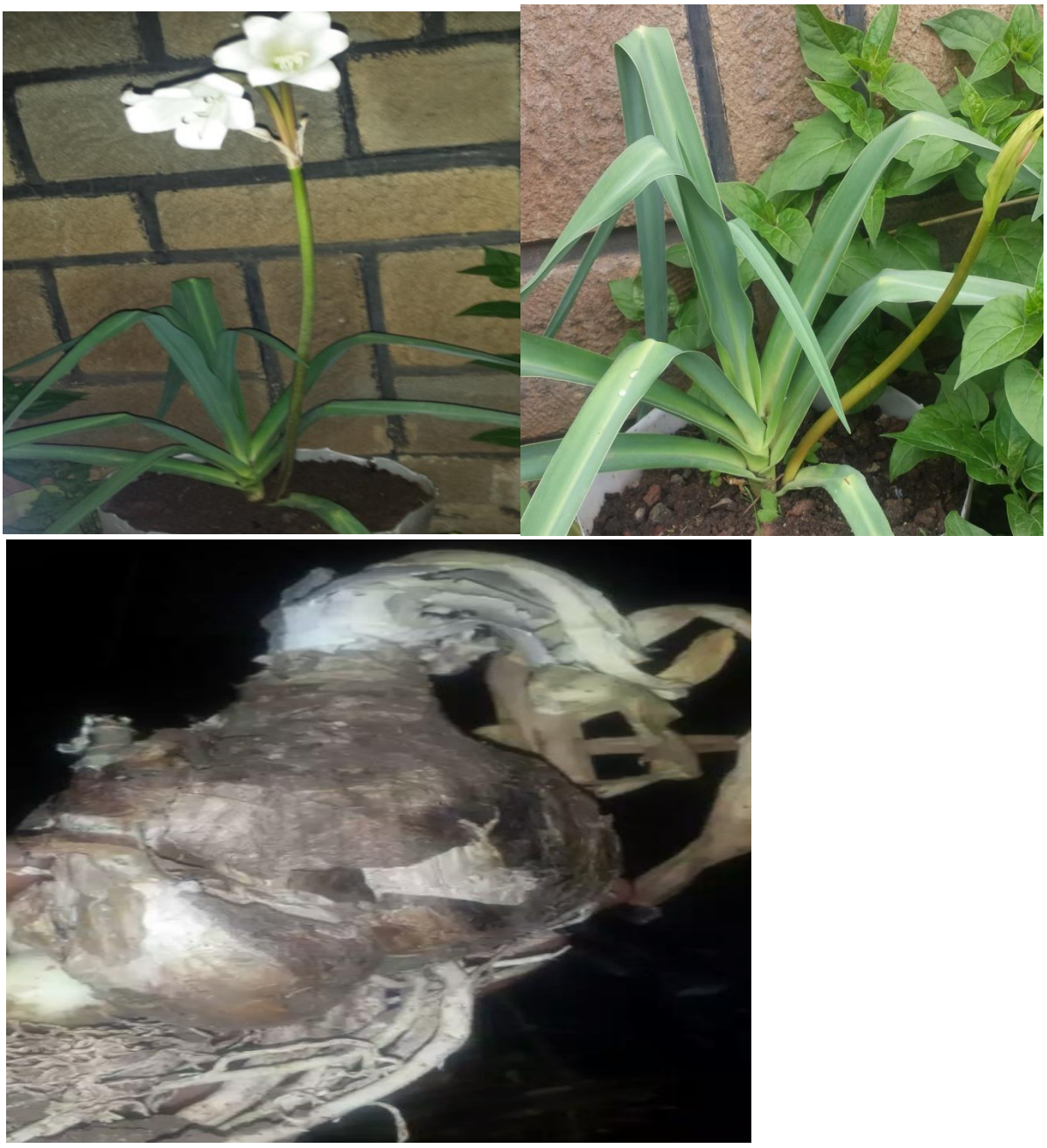


Figure 1: *C. abyssinicum* flower, leave and bulb

1.7 Statement of the Problems

Recent studies have shown that for every million wound patients, at least proximately 10,000 die from wound due to complication associated with microbial infections and other factors. In addition, nearly 6 million people suffer from wounds worldwide. Therefore, Wounds do have multi-dimensional impacts on different sectors that cause significant morbidity, disability, socioeconomic crisis and mortality worldwide (MacDonald, 2009; Kokane *et al*., 2009; Wong *et al*., 2015**)**.

Economies and social impact of wounds are immense. For instance, the global advanced wound management market was valued at $20 billion in 2015 and was expected to grow at a combined annual growth rate of 7% for the next four years to reach approximately $26 billion by 2018 (Brocair, 2015). Furthermore, Management of wound healing is a complicated and expensive program and thus research on drugs that increase wound healing is a developing area in modern biomedical science (Sengupta, 2017). Therefore, the world health organization (WHO) urged researchers to examine traditional medicines and/ or promoting traditional medicine as a source of less expensive, comprehensive medical care, especially in developing countries (Shafiuddin *et al*., 2009; Gopalakrishnan and Rajameena, 2012). Because, many of the conventional wound healing drugs currently used for treating wounds are not only expensive but also pose numerous side effects such as toxic effects, allergy and drug resistance (Prasad and Dorle, 2006). Hence the need for cheaper, safer and easily available plant products that promote the repair mechanisms of the wound in the natural way should be the concerned issue. Currently, researchers consider medicinal plants as a novel source for the development of wound healing agents.

2 OBJECTIVES

2.1 General Objective

- The aim of this study is to evaluate the wound healing effect of 80% methanol crude extracts of the bulbs of *Crinum abyssinicum* in experimental animals

2.2 Specific Objectives

- To study acute toxicity of the 80% methanol extract of *C. abyssinicum* bulbs in experimental animals
- To evaluate the anti-inflammatory effect of the 80% methanol extract of *C. abyssinicum* bulbs on carrageenan induced hind paw edema model in mice
- To evaluate the wound healing activity of 80% methanol extract of the *C. abyssinicum* bulbs on excision wound models in mice.
- To evaluate the wound healing activity of 80% methanol extract of the *C. abyssinicum* bulbs on incision wound models in mice.
- To evaluate the anti-microbial effect of the 80% methanol extract of *C. abyssinicum* bulbs
- To screen the secondary metabolites found in *C. abyssinicum* bulbs

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, reagents and drugs

Tween 80 (Atlas Chemical Industries Inc, USA), Mueller Hinton agar (Oxoid Ltd, Basingstoke, Hampshire, England), Muller Hinton Broth (Oxoid Ltd, Basingstoke, Hampshire, England), nutrient agar (Himedia Laboratories Pvt. Ltd, India), Sabouraud's Dextrose broth and agar (Oxoid Ltd, Basingstoke, Hampshire, England), methanol (Carlo Erba Reagents S.A.S), wool fat (BDH Chemicals ltd, England), hard paraffin (BDH Chemicals, England), cetostearyl alcohol (BDH Chemicals ltd,England), ketamine hydrochloride (Rotex Medica, Germany), carrageenan (Sigma-Aldrich Steinheim, Germany), nitrofurazone ointment (Shanghai General Pharmaceuticals Co., Ltd, China), normal saline (Euro-med laboratories phil.,INC), indomethacin (Greenfield Pharmaceuticals, China), Gentamycin (Oxoid Ltd, Basingstoke, Hampshire, England) were used in this study. Chemicals and reagents used were of analytical grade.

3.1.2 Instruments and apparatus

The apparatus and instruments used for this research work were sensitive digital weighing balance (Mettler Toledo, Switzerland), mini orbital shaker (Bibby Scientific Limited Stone Staffo Reshire, UK), Whatman filter paper (Nº1) (Maidstone, UK), Plethysmometer (Ugo Basil 7140, Italy), Erlenmeyer conical flask, rotary evaporator (Buchii model R-200, Switzerland), lyophilizer (Operan, Korea vacuum limited, Korea), permanent marker, graph paper, cotton swab, water bath, beaker, ointment slab, gauze and elastic bandage, syringe with needles, gloves, mortar and pestle, petridish, Incubator, sterilizer, gel puncher.

3.1.3 Plant materials

Fresh bulbs of *Crinum abyssinicum* were collected from its natural habitat in and around Gursum Woreda, eastern Hararge, eastern part of Ethiopia, which is about 615 km away from Addis Ababa in the month of January, 2018. This area selected in order to appreciate whether the habitat ecotype of this plant has showed pronounce wound healing effect or not. The plant was identified at the National Herbarium, College of Natural Sciences, Addis Ababa University where a voucher specimen was deposited (collection Nº. AH001).

3.1.4 Experimental animals

Healthy adult Swiss albino mice of either sex (25-30 g, and 6-8 weeks of age) procured from the animal house of the Ethiopian Health and Nutrition Research Institute (EHNRI) were used for the study. Animals were housed individually to prevent fighting among themselves with free access food and water and kept under 12hour light dark cycle throughout the experiment. The animals were allowed to acclimatize to the laboratory condition for a week before the experiment done and handled according to international laboratory animal use and care guidelines throughout the experiment (ILAR, 1996). At the end of the experiment the animals were sacrificed using high dose of diethyl ether

3.2 Methods

3.2.1 Extraction of plant materials

C. abyssinicum bulbs were first washed with water using brush to remove soil attached to them and sliced into smaller pieces to enhance drying under shade. The dried bulbs were crushed to course powder, and 600g of the powder was macerated with 80% methanol for three days in conical flask with occasional stirring and shaking. The extract was then filtered with Whatman Nº. 1 filter paper. The residue was further macerated for three more days in similar manner to exhaustively extract the plant materials and filtered. The filtrates were mixed and concentrated by Rota vapor to remove methanol and the concentrated extract was placed in lyophilizer until dried. The dried products of the extracts were stored in tight containers and stored in a refrigerator at 4ºC until used for formulation of ointments.

3.2.2 Ointment formulation

Simple ointment of the plant extract was prepared following the formula described in the 1988 British Pharmacopoeia (Table 1). Three ointment preparations (each 200 g), with (5 % and 10 % w/w) of the extract and simple ointment only (without plant extract) which served as a control were formulated using the reduced formula. All the ingredients were melted over a water bath with constant stirring until they became homogeneous and then stirred until cooled. For preparing medication ointment, 10 g and 20 g of the extract were mixed with 190 g and 180 g of the ointment base to prepare 5% and 10% of extracts ointments, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture. In preparing the

control ointment, 200 g of the base was taken and treated in the same manner to formulate ointment without an active ingredient. Finally, the simple ointment base and extract ointment was transferred to a clean container for topical application during the experiment (Ansel, 1985; Langley and Belcher, 2008; Deshmukh *et al.*, 2009).

Table 1:Formula used for preparation of simple ointment base and extract ointments

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

3.2.3 Acute toxicity studies

3.2.3.1 Acute oral toxicity study

Acute oral toxicity test was performed according to the Organization of Economic Corporation and Development (OECD) 420 guideline (2001) for crude leaf extracts. The experiment was conducted on healthy Swiss albino female mice. The mice were fasted three to four hours with free access to water before used for this study. The experiment started with single female mouse which was given 2000 mg/kg of the extract (1 ml/100 g) as a single dose by oral gavage. After observation for any sign of toxicity i.e. behavioral change, body fur erecting and/or locomotory abnormality within 24 hr., another four female mice were given the same amount of extract. Then clinical sign of toxicity was assessed daily for the next 14 days. Since there were no signs of toxicity observed, the dose was scaled up to 5000 mg/kg following the same procedure.

3.2.3.2 Acute dermal toxicity study

Acute dermal toxicity study was performed according to (Kokane *et al*., 2009; More, *et al.,* 2013) with some modification. For this study, five male and five female mice were randomly chosen and assigned to two groups. The animals were acclimatized to the laboratory condition for 7 days before experimentation. The hair of about 10% of the body surface area was shaved from the back trunks of the mice 24 hr. prior to extract ointment application. Then extract ointments (5% and 10%) were applied uniformly to the entire shaved site and covered with porous gauze that secured

with nonirritating tape in order to keep contact with the skin. The mice were then returned to their respective cages and provided food and water *ad libitum.* After 24 hr. of the exposure to extract ointments, the bandage and gauze were gently removed. Finally, mice were observed for the next 14 days for development of inflammation or any adverse skin reactions.

3.2.4 Grouping and dosing of experimental animals

For excision model, four groups of mice, each containing six mice were used. Animals in Group I were treated with simple ointment. Group II and III received 5% (w/w) and 10% (w/w) 80% methanol extract ointments, respectively, and the animals in group IV were treated with nitrofurazone (0.2%) ointment. Simple ointment and nitrofurazone (0.2%) ointment served as negative and positive controls, respectively. For incision model, five groups of mice, containing six mice per group were used. Groups I-IV were treated in a similar manner to that of excision wound model, but animals in Group V did not get any treatment.

For assessment of anti-inflammatory activity, five groups of mice containing six animals per group were used. Dose of the extract was chosen based on acute oral toxicity results described in OECD (2001). A middle dose, which is one-tenth of the maximum dose obtained during acute toxicity study, a low dose, which is half of the middle dose, and a high dose which is twice of the middle dose. Group I was treated with a 10 ml/kg of 2% tween 80. Groups II-IV were treated with 10 ml/kg of three different doses of the extract (100 mg/kg, 200 mg/kg and 400 mg/kg) and Group V was treated with indomethacin. Vehicle (2% tween 80) and indomethacin served as a negative and positive controls, respectively.

3.2.5 Models for wound healing activity

Wound healing activity was evaluated by measuring wound parameters of excision model such as rate of wound contraction, period of epithelization and skin tensile strength for incision model.

3.2.5.1 Excision wound model

The mice were anaesthetized prior to creation of the wounds, with 1 ml/kg of ketamine hydrochloride intramuscularly. The skin of the dorsolateral flank area was shaved with a shaving machine. After the dorsal flanks were depilated, mice were placed in ventral decubitus and fixed for the surgical procedure. The depilated areas were cleaned with 70% ethanol and circular area of full thickness of skin approximately 314 $mm^2$was marked with thin permanent marker. Then

demarcated area was carefully excised using sharp sterilized scissors and forceps. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The wound was left undressed to the open environment.

The mice were divided into four groups (6 mice per group) randomly, and each mouse was placed in a separate cage. The inflicting day was considered day 0 as shown in Figure 4a. The simple ointment, extract and standard were applied topically once a day to the respective groups till the wound was completely healed as mentioned under grouping and dosing. For this model, wound contraction and epithelization period were monitored. Wound contraction was expressed as percent contraction every 2 days after wounding day (Lodhi *et al.,* 2006; Shetty *et al.,* 2006; Pereira *et al*., 2018).

a) Measurement of wound contraction

The wound healing progress for this parameter was assessed by measuring wound margin area using a transparent paper sheet and a permanent marker. The tracing paper was placed on a 1 mm^2 graph sheet and traced out. The area was measured on days 2, 4, 6, 8, 10, 12, 14, 16 and 18^{th} post wounding (Kokane *et al*., 2009; Tessema *et al*., 2018). The traced out wound area was used to calculate the percentage of wound contraction, taking initial size of the wound as 100% using the following formula (Shivhare *et al*., 2010; Tessema *et al*., 2018).

$$\%\ \text{Wound contraction} = \frac{\text{Wound area on day 0} - \text{ Wound area on day n}}{\text{Wound area on day 0}} \times 100$$

Where n=the days where measurement was taken; 2^{nd}, 4^{th}, 6^{th} ……....18^{th} day

b) Epithelization time

Epithelization time was assessed by recording the number of days required to complete healing of wounds without any residual of raw wound behind (Shrimanker *et al*., 2013).

3.2.5.2 Incision wound model

Anesthesia was done in the similar fashion as described above in an excision wound model. After the mice was anesthetized and dorsal fur was shaved, a straight line was marked at a distance of 1 cm from the paravertebral region. An incision wound of 3 cm long and of full skin thickness, longitudinal to the paravertebral region, was made with a sterile scalpel. Then, the incised area

was closed by interrupted suture with the interval of 1 cm apart using a surgical thread (No. 000) and curved needle (No. 11) (Figure 2a). The mice were randomly divided into five groups (6 mice per group) and the experimental mice were treated in similar manner to the excision wound model except the fifth group which was left without any treatment. The sutures were removed on day 8 post-incision, but treatment continued up to the 9th day, and the tensile strength of the wound was measured on the 10th day (Kumar *et al*., 2013; Zeng *et al*., 2016).

Measurement of tensile strength of the wound

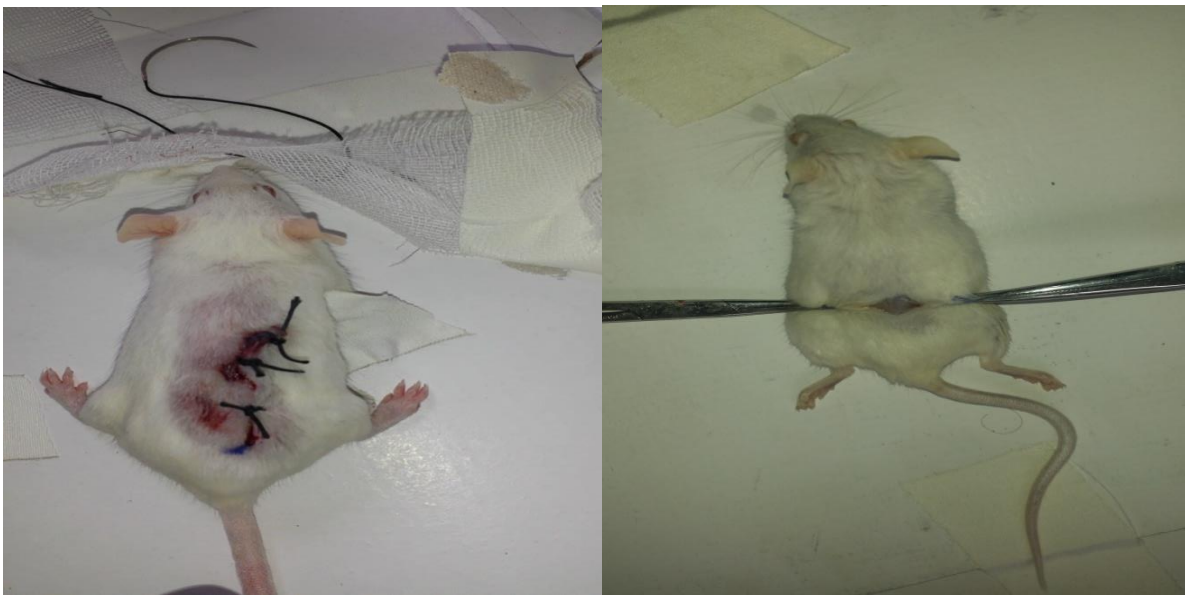
On the 10th day of post-wounding, each mouse was anaesthetized with diethyl ether. Then animals were secured to the working table. Two forceps were firmly applied 3 mm away from the edge of wound facing each other on opposite side of the wound edge. One of the forceps was fixed on stands, while the other was connected to a freely suspended light weight plastic through a string run over to a pulley in which water was allowed to flow continuously from the reservoir slowly and steadily into the container. As soon as wound opening appeared as shown in Figure 2b, water flow was ceased, and the volume of water collected in the container was determined and taken as a measure of breaking strength in grams. Finally, percentage of tensile strength for extracts and controls were calculated using the following formula (Ilango and Chitra, 2010; Akkol *et al.*, 2011).

$$Tensile\ strength(TS)ofExtract(\%) = \frac{TSextract - TSvehicle \times 100}{TSvehicle}$$

$$Tensilestrength(TS)ofReference(\%) = \frac{TSreference - TSvehicle \times 100}{TSvehicle}$$

$$Tensilestrength(TS)ofVehicle(\%) = \frac{TSvehicle - TSle.u \times 100}{TSle.u}$$

Where "*le.u"* is stands for left untreated



a. Sutured wound b. Opened wound

Figure 1:Photograph of Incision wound model.

3.2.6 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 activity of 80% methanolic extracts of *C. abyssinicum* bulbs. Following overnight fasting with free access to water, the basal volume of the right hind paw of each mouse was determined before any treatment using plethysmometer (Ugo Basile, Italy) (Padilha *et al*., 2010; Hassan *et al*., 2013).

After determination of basal volume, the mice were assigned into five groups such that the mean volume of the different groups were equivalent. Vehicles, plant extract as well as the standard drug were administered orally to their respective groups one hour prior to carrageenan injection. Paw swelling of mouse was induced by sub-plantar injection of 0.05 ml of a solution of 1% carrageenan in 0.9% saline (w/v) into the right hind paw. Then, the inflammation was quantified by measuring the volume displaced by the paw at 1^{st}, 2^{nd}, 3^{rd} and 4^{th} hr. after carrageenan injection (Hassan *et al*., 2013; Kai *et al*, 2018). Results were expressed as the paw volume (ml) variation with respect to the basal values. Hence, the anti- inflammatory activity (percentage inhibition of edema) was calculated according to the following formula (Emran *et al.*, 2015; Sathyaraj and Indumathy, 2018).

Anti-inflammatory activity (%) = $\frac{Ac-Bt}{Ac} \times 100$

Where A_c is the average inflammation of the control group at a given time and B_t is the average inflammation of the test sample or standard drug treated mice at the same time.

3.2.7 Antimicrobial activity

3.2.7.1 Microorganisms used

The pure cultures of test organisms that were used in this study includes: gram positive bacteria such as *Staphylococcus aureus* ATCC 29737, *Streptococcus pyogens* ATCC19615, gram negative bacteria such as *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* ATCC 700609, *Salmonella Typhimurium* ATCC 14028 and *Candida albicans* ATCC 53324 fungus were used.

3.2.7.2 Culture media and inoculum

The medium used for the activation of the bacteria and fungus were a nutrient agar medium and Sabouraud's Dextrose, respectively. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi that were incubated after agitation for 3-6 hr. at 37°C and 25°C, respectively. Then it was incubated till it reached the turbidity equal to that of the 0.5 McFarland standard solution which is equivalent to approximately 3.0 $X10^8$ CFU/ml (Selvamohan *et al.*, 2012; Sahraei *et al*., 2014).

3.2.7.3 Agar diffusion assay

The modified agar well diffusion method was employed (Joung *et al*. 2010). The 60 ml of sterilized Muller Hinton Agar was poured into sterile petriplate. After the agar solidified, fresh culture of test organisms of bacteria was swabbed uniformly over the surface of agar plates using sterile cotton swab. The plated medium was then allowed to dry at room temperature for 30 min. In each petridish equidistant bores were made with six millimeters diameter using sterile gel puncher. Each bore was then filled with 50 µL of the plant extract and negative control which is distilled water. Likewise, the same procedure was followed for the fungi using Sabouraud dextrose agar. Simultaneously, Gentamycin (10µg/disc) for the bacteria and nystatin (1.0 µg/ml) for *C. albicans* were used as positive controls. The test was carried out in triplicate. The plates were incubated at

37°C for 24hr., except for *C. albicans* which was incubated at 25°C. The antimicrobial activity was determined by applying the following formula (Mathur *et al*., 2010).

$$\% \text{ RIZD } = \frac{(\text{IZD Sample} - \text{ IZD negative control})}{\text{IZD antibiotic standard}} \times 100$$

Where RIZD is the percentage of relative inhibition zone diameter and IZD is the inhibition zone diameter (mm). The resulting IZD of the samples were either higher than or equal to the IZD of the negative control. Therefore, the obtained percentages were positives when IZD of the samples were higher than IZD of the negative control. The test was considered negative when the IZD of the sample was equal to the IZD of the negative control.

3.2.8 Preliminary phytochemicals screening

The presence of major phytochemicals (secondary metabolites) such as alkaloids, flavonoids, saponins, tannins, terpenoids, glycosides, phenol and steroid were evaluated in the crude *C. abyssinicum* bulb using standard testing methods with little modification.

1. **Test for alkaloids (Wagner's test)**

Ten mg of the crude extract was dissolved in 1ml of distilled water. Then three drops of Wagner's reagent (i.e. a solution prepared by dissolving 2 gm of Iodine and 6 gm of potassium iodide in 100ml of distill water) was added. Finally, presence of alkaloids was confirmed by the formation of reddish-brown colored solution (Geetha and Geetha, 2014).

2. **Test for terpenoids**

About 250 mg of crude extract was dissolved in 2ml of chloroform. Then 3ml of concentrated sulfuric acid was carefully added that form a layer. A reddish-brown coloration of interface indicates the presences of terpenoids (Mohammed *et al.*, 2016).

3. **Test for flavonoids (Alkaline reagent or NaOH test)**

About 300mg of the crude extract was dissolved into 2ml of distilled water. To this solution, three drops of 20% sodium hydroxide solution was added that produce an intense yellow color. Then upon addition of three drops of 20% hydrochloric acid turned to colorless indicated the presence of flavonoids. Likewise, lead acetate test was performed but what used in place of 20% sodium

hydroxide and 20% hydrochloric acid was 3 drops of 10% lead acetate and formation of yellow precipitate indicates the presence of flavonoids (Jones and Kinghorn, 2012; Onwukaeme *et al*. 2007).

4. **Test for saponins**

About 0.5mg of crude extracts was dissolved in 20 ml of distilled water. Then after vigorous shaking in graduated cylinder for 15 minute the formation of foam to length of 1cm was observed. This was taken as an indicative for the presence of saponins and steroids (Geetha and Geetha, 2014).

5. **Test for steroids**

About 250mg of the extract was placed in a test tube and dissolved by 2 ml of acetic anhydride. Then 4 drops of chloroform were added. Following this, two drops of concentrated sulphuric acid was added at the side of the test tube. Finally, development of a brownish ring at the interface of the two liquids and the appearance of violet color in the supernatant layer was used to confirm the presence of steroids (Njoku and Obi, 2009).

6. **Test for phenols and tannins**

Lead acetate test: Ten mg of extract was taken and 0.5 ml of 1% lead acetate solution was added. Finally, development of precipitate indicates the presence of tannins and phenolic compounds.

Ferric chloride test: Five mg of extract was taken and 0.5 ml of 5% ferric chloride was added and hence the development of dark bluish or black color confirms the presence of tannins (Ugochukwu *et al*., 2013; Geetha and Geetha, 2014).

7. Test for anthraquinone

About 0.5 gm of the extract was kept in dry test tube and 5 ml of chloroform was added and shaken for 5 minutes. Then extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. The presence of a pink violet or red color in the lower layer confirmed the presence of anthraquinone (Harborne, 1998; Sasidharan *et al*., 2010).

3.3 Statistical Analysis

The raw data obtained from the experiments were expressed as mean ± standard error of the mean (SEM), and analysis was done using SPSS version 20. Test of statistical significance was carried out by employing one-way analysis of variance (ANOVA) followed by Tukey's post Hoc test and data were considered as statistically significant at $p < 0.05$.

4 RESULTS

4.1 Acute Oral Toxicity Test

Acute toxicity study results of a single oral administration of *Crinum* bulbs extract both at 2000 mg/kg and 5000 mg/kg doses showed no sign of toxicity i.e., there was no locomotory change, loss of appetite, physical change or strange behaviors in the first 24 hour as well as in the following 14 days of observation. The lethal dose (LD_{50}) of the extract was greater than 5000 mg/kg.

4.2 Acute Dermal Toxicity

Application of both 5% and 10% extracts ointment formulations did not show any sign of inflammation, irritation, redness, or edema at the site of application after 24hr of application. No sign of toxicity was also observed 2 days after application. Moreover, no signs of toxicity as well as mortality was noted during the following 14 days of observation (Figure 3).

A. Male mice B. female mice

Figure 2: Photograph of acute dermal toxicity test result.

4.3 Effect of the Extract on Wound Healing

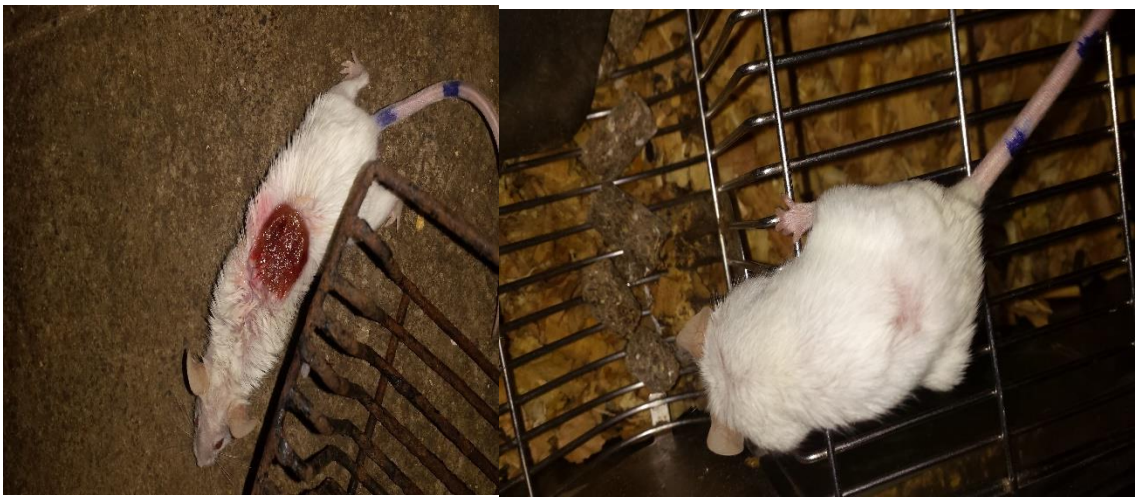
4.3.1 Excision wound model

4.3.1.1 Effect on wound contraction

The topical application of the 80% methanol extract of the *C. abyssinicum* bulb formulated in simple ointment base showed to have wound healing activity on the mice. Wound contraction induced by treatment of 5% and 10% (w/w) bulb extract ointment, simple ointment base and standard drug are illustrated in Table 2 and Figure 5.

The 10% plant extract and standard drug facilitated wound contraction significantly starting from the second day of administration ($p<0.01$ and $p<0.05$, respectively) and highly significant ($p< 0.001$) difference was seen on both from day 4 onwards ($p<0.001$) compared to control. On the other hand, the animals treated with 5% (w/w) extract ointment showed no significant wound contraction on the second, sixth, eighth, 10^{th} and 12^{th} days. However, this ointment showed significant difference on the 4^{th}, 14^{th} and 16^{th} days ($p<0.01$, $p<0.05$ and $p<0.01$, respectively) compared to control. Furthermore, the 10% of extract and standard drug treated groups showed significant difference starting the 6^{th} day of treatment but failed to reach significant level on the second and fourth day compared to the 5% (w/w) extract ointment as shown in Table 2.

The 10% (w/w) extract ointment and standard drug treated groups showed high percentage of wound closure. The 5% (w/w) extract ointment treated group showed maximum percentage of wound contraction on days 14^{th} and 16^{th} which were 96.61 and 99.26, respectively as shown in figure 5. The healing process with complete wound closure was observed with standard, 10% (w/w) extract ointment and 5% (w/w) extract ointment treated groups within 13, 14 (Figure 4b), and 16 days, respectively, while it took about 18 days for complete wound closure in the control group.



a. Wounding day (day 0) b. Healed day (day 14)

Figure 3: Photograph of excision wound model of 10% w/w *C. abyssinicum* bulb extract

Table 2: Effect of topical application of the 80% methanol extract of *C. abyssinicum* bulb extract on wound contraction of excision wound model in mice.

Treatment group		Simple ointment	0.2%NF	5% w/w extract	10% w/w extract
Wound area (mm^2)	Day2	288.83±4.06	$263.17±6.4^{c1}$	278±6.71	$256.17±5.71^{c2}$
	Day4	205.33±5.32	$151.33±8.84^{c3}$	$172.5±4.79^{c2}$	$151.17±4.53^{c3}$
	Day6	165±4.37	$76±6.85^{c3e3}$	149.17±5.36	$77.33±4.26^{c3e3}$
	Day8	126.17±5.35	$37.67±4.76^{c3e3}$	126.67±8.83	$41.17±3.34^{c3e3}$
	Day10	79.17±12.67	$16.5±3.73^{c3e3}$	70.83±5.87	$20±2.5^{c3e3}$
	Day12	48.83±9.11	$6.83±3.66^{c3e1}$	31.83±5.24	$9±2.02^{c3e1}$
	Day14	25±4.93	$1.67±1.1^{c3}$	$10.67±2.5^{c1}$	$1.33±0.99^{c3}$
	Day16	10.67±2.23	0^{c3}	$2.33±1.57^{c2}$	0^{c3}

n= 6 Swiss albino mice per group; Values are expressed as mean ± SEM, one-way ANOVA). ^{c} against control, ^{e} against 5% (w/w) extract, $^{1}p<0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

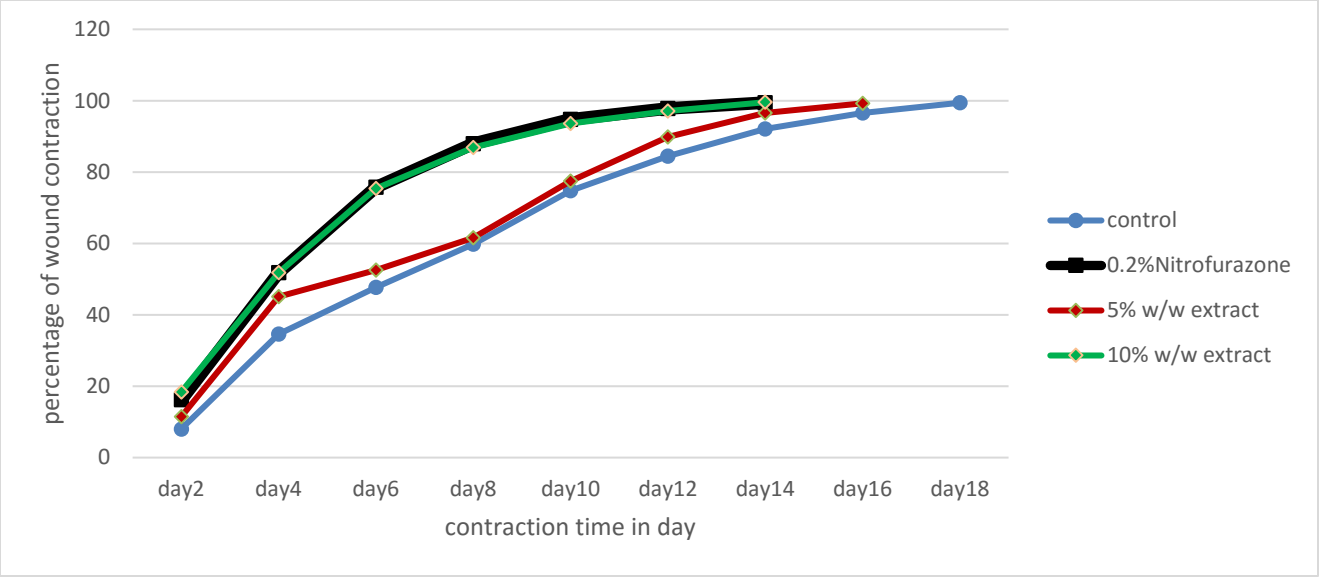


Figure 4: Effect of topical application of the 80% methanol extract of *C. abyssinicum* bulb on the percentage wound closure of excision wound model in mice.

4.3.1.2 Effect on epithelization period

Ten percent (w/w) extract and 0.2% (w/v) nitrofurazone ointments treated group had a significantly shorter ($p< 0.001$) epithelization period compared to the control group. Even though, 5% (w/w) extract ointment treated group showed shorter epithelization period (15.83) compared to control (17.67), it failed to reach significant level. Furthermore, standard treated group showed significant difference compared to 5% (w/w) extract ointment. However, there was no significant difference in the epithelization period between the two doses of extract (Table 3).

Table 3: Effect of topical application of the 80% methanol extract of *C. abyssinicum* bulb on wound epithelization period of excision wound model in mice

Groups	Epithelization period (days)
Control	17.67±0.33
0.2% w/v Nitrofurazone	13 ± 0.68^{c3e2}
0.5% w/w extract	15.83±0.60
10% w/w extract	13.83 ± 0.40^{c3}

n = 6 mice in each group; Values are expressed as mean ± SEM, one-way ANOVA). ^{c} against control, ^{e} against 5% (w/w) of the extract, $^{1}p<0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

4.3.2 Incision wound model

Tensile strength of incision wound model

The result showed effectiveness of *C. abyssinicum* bulb extract in increasing the breaking strength of the wound healing as shown in Table 4. The group treated with standard drug, 5% and 10% ointment preparations of the crude extracts showed statistically significant ($p< 0.001$) increment in tensile strength compared to both untreated control group as well as simple ointment control group. In addition, simple ointment showed significant increment compared to untreated group. Furthermore, the percentage of tensile strength of 10% of extract and standard drug was significantly increased by about 82.24% ($p < 0.001$) and 82.64% ($p < 0.001$) respectively as compared to simple ointment (control) group. However, there was no significant difference in tensile strength between the reference drug and 10% of the extract treated group.

Table 4: Effect of topical application of 80% methanol extract of *C. abyssinicum* bulb on tensile strength of incision wound model.

group	Tensile strength (g)	% Tensile strength
Untreated control	180.5±4.76	-
Control	208.33±4.96	15.42
0.2% w/v Nitrofurazone	380.5±7.24^{u3c3e3}	82.64
0.5% w/w extract	326.67±5.26 ^{u3c3}	56.80
10% w/w extract	379.67±6.30 ^{u3c3e3}	82.24

n = 6 mice in each group; Values are expressed as mean ± SEM, one-way ANOVA). ^{c} against control (simple ointment), ^{u} against Untreated control, ^{e} against 5% (w/w) of the extract, $^{1}p<0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

4.4 *In Vivo* anti-Inflammatory effect

The anti-inflammatory activity of 80% methanol extract of *C. abyssinicum* bulbs in carrageenan-induced hind paw edema model demonstrated that the extract capability in inhibiting edema in mice as shown in Table 5.

After administration of carrageenan, animals treated with the lower dose (100mg/kg) of extract showed significant reduction of edema ($p <0.001$) compared to the control at the 3rd and 4th hour. Middle dose (200 mg/Kg) and higher dose (400 mg/Kg) of the extract as well as standard drugs

treated animals showed significant suppression of edema at all-time points compared to controls. Furthermore, the study revealed 400 mg/Kg of the extract and standard drug treated animals showed significant difference at all-time points compared to 100mg/Kg, except 400mg/kg of extract that failed to bring significant change at the 3rd hour. It was also observed that the standard drug showed significant difference compared to 200 mg/kg at 4th hr. ($p < 0.05$), while the difference was not significant compared to 100 mg/kg and 400 mg/kg doses.

Table 5: Anti-inflammatory activity of 80% methanol extract of *C. abyssinicum* bulb on carrageenan-induced mouse paws oedema.

Group	Mean increase in the paw volume (ml)				
	Basal vol.	1hr	2hr	3hr	4hr
control	0.82±0.07	1.55±0.1	1.52±0.11	1.42±0.11	1.43±0.09
C. abyssinicum (100 mg/kg)	0.82±0.07	1.38±0.08 (10.97%)	1.22±0.08 (19.74%)	$0.78\pm0.05^{\alpha 3}$ (45.07%)	$0.57\pm0.05^{\alpha 3}$ (60.14%)
C. abyssinicum (200 mg/kg)	0.83±0.03	$1.12\pm0.09^{\alpha 1}$ (27.74%)	$0.93\pm0.07^{\alpha 3}$ (38.82%)	$0.68\pm0.07^{\alpha 3}$ (52.11%)	$0.42\pm0.03^{\alpha 3}$ (70.63%)
C. abyssinicum (400 mg/kg)	0.82±0.08	$0.98\pm0.08^{\alpha 2\beta 1}$ (36.77%)	$0.72\pm0.06^{\alpha 3\beta 2}$ (52.63%)	$0.57\pm0.05^{\alpha 3}$ (59.86%)	$0.32\pm0.03^{\alpha 3\beta 1}$ (77.62%)
Indomethacin (10 mg/kg)	0.80±0.03	$0.95\pm0.06^{\alpha 3\beta 2}$ (38.71%)	$0.67\pm0.05^{\alpha 3\beta 3}$ (55.92%)	$0.37\pm0.02^{\alpha 3\beta 2}$ (73.94%)	$0.28\pm0.03^{\alpha 3\beta 2\gamma 1}$ (80.42%)

Data were expressed as mean ± SEM with % inhibition depicted in the parenthesis, n = 6 Swiss albino mice per group; $^{\alpha}$ against control, $^{\beta}$ against 100 mg/kg, $^{\gamma}$ against 200 mg/kg, $^{1}p < 0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$.

4.5 Antimicrobial Activity

Table 6 shows the antimicrobial activity of 80% methanol extract of *C. abyssinicum* bulb against six bacterial strains and one fungus. According to the agar well diffusion test, the growth of the bacterial strains such as *Salmonella Typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pyogens* were inhibited by the tested concentrations of the crude extract of the plant in a concentration dependent manner. On the contrary, *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Candida albicans* were not affected by all concentrations of plant extracts tested.

In this study, maximum percentage of relative inhibition zone diameter (%RIZD) of crude extract at 50 mg/ml was observed against *Staphylococcus aureus* (25%) followed by *Streptococcus pyogens* (16.7%), *Salmonella typhimurium* (15%) and *Escherichia coli* (11%). When the concentrations of the crude extract increased to 200mg/ml the maximum antimicrobial activity against *Escherichia coli* (826%) was observed followed by *Staphylococcus aureus* (79.2%), *Streptococcus pyogens* (79.2%) and *Salmonella typhimurium* (68.2%) as shown in Table 6.

Table 6: Antimicrobial activity of the methanol extract of *C. abyssinicum* bulb

Plant extract	Microorganisms (% RIZD)						
	Salm typ	*S. aureus*	*E. coli*	*Str. pygo*	*K. pneum*	*p. aurog*	*C. alb*
50 mg/ml of extract	15	25	11	16.7	NA	NA	NA
100mg/ml of extract	19.7	37.5	36.2	37.5	NA	NA	NA
200mg/ml of extract	68.2	79.2	82.6	75	NA	NA	NA

Salm typ=salmonella typhimurium, S. aures= Staphylococcus aureus, E. coli= Escherichia coli, Str. pygon=Streptococcus Pyogens, K. pneum= Klebsiella pneumonia, p. aurog= Pseudomonas aeruginosa, C. alb=Candida albicans, NA= No activity

4.6 Preliminary Phytochemical Screening

The results of phytochemical screening of the 80% methanol extract of bulbs of *C. abyssinicum* indicated the presence of different secondary metabolites with variable degrees of color intensity as shown in Table 7.

Table 7: Preliminary phytochemical screening of 80% methanol extracts of *C. abyssinicum* bulb.

Phytochemicals	Test used	Test results from 80% methanol extracts of *C. abyssinicum bulb*
Flavonoids	NaOH test	+++
Phenols	Lead acetate test	++
Tannins	Ferric chloride test and Lead acetate test	+++
Terpenoids	Terpenoids test	++
Anthraquinone	Anthraquinone test	+
Steroids	Steroids test	+++
Alkaloids	Wagner's test	+
Saponins	Frothing test	++

Symbol indicates intensity of color produced by phytochemicals which are (+) Trace amount, (++) Moderate amount and (+++) Appreciable amount.

5 DISCUSION

In the present study, wound contraction and percentage of wound contraction as well as time of complete healing following creation of excision wounds were determined in order to ascertain the rate of healing. Wound contraction plays crucial role by decreasing the margin of the wound, the amount of extracellular matrix needed to repair the defect leading to shortening the healing time (Tessema *et al*., 2018). *Crinum abyssinicum* bulb at 10% (w/w) concentration showed equivalent wound healing effect to that of the reference drug (Nitrofurazone) used in this study. The ointments at 10% concentration also showed the highest percentage of wound contraction and short period of wound re-epithelialization compared to 5% and negative control groups. The wound contraction ability of *C. abyssinicum* bulb observed in this study might suggest a definite pro-healing activity of the plant as 88% of the healing of wound occurs due to contraction, and the other 12% occur due to scar formation (Ejaz *et al*., 2009).

Another distinguishing quality of natural products with better wound healing activity is the short epithelization period they may bring about (Süntar *et al*., 2010). In the present study, 10% concentration of the ointment significantly reduced the epithelization time from 18 days in negative control group to 14 days. This might be attributed to the enhancement of collagen deposition by the extract which occurred better at a higher concentration (Udegbunam *et al*., 2015) that facilitated proliferation and migration of epithelial cells and/or increased viability of epithelial cells (Sanwal and Chaudhary, 2011).

Efficacy of the crude extract in wound healing process might also be measured by the tensile strength in incision wounds. The tensile strength of a wound indicates how much the repaired tissue resists to breaking under tension and might also indicate the quality of repaired tissue (Shivhare *et al.,* 2010). Continuous accumulation of collagen and proliferation of fibroblast confirms the existence of more tensile strength of the skin (Sharma *et al.,* 2011). The tensile strength of both 5% and 10% ointments of *C. abyssinicum* bulb in this study showed significantly higher tensile strength compared to the untreated negative control group. This might be due to the increased collagen synthesis per cell resulting in facilitating the cross-linking of the proteins (Agarwal *et al*., 2009; Wang *et al.*, 2011).

Carrageenan induces a biphasic acute inflammation in carrageenan induced paw oedema model in mice. The first phase which lasts up to 2 hrs., is attributed to the release of serotonin, histamine,

bradykinin and substance-P. The second phase that lasts from 3–5hrs, on the other hand, is mainly due to the neutrophil's infiltration into the inflammatory site and the production of large amounts of pro-inflammatory mediators such as prostaglandin E2 and various cytokines such as IL-1β, IL-6, and TNF-α (Kuedo *et al*.,2016; Tessema *et al*., 2018). In the present study, 80% methanolic extract of *C. abyssinicum* bulbs showed anti-inflammatory activity at all points of time after carrageenan injection. This indicates that the anti-inflammatory activity of the extract could be by inhibiting effects mediated by histamine, bradykinin and prostaglandin (Guo and Dipietro, 2010). This finding is in agreement with that of the ethanol leaf extract of *Becium grandiflorum* where significant suppression of edema was observed at all-time points (Beshir *et al*., 2016).

The 80% methanolic extract of *C. abyssinicum* bulbs displayed relatively higher activity against Gram-positive bacteria that may complicate the wound healing by disrupting the normal clotting mechanisms, promoting disordered leukocyte function and ultimately delaying angiogenesis (Annan and Houghton, 2008; Deshmukh *et al*., 2009). On the other hand, the extract showed no activity against Gram-negative bacteria and fungus. The resistance capability of Gram-negative bacteria to extracts may related to phospholipid membrane coverage, carrying the structural lipopolysaccharide component that makes their cell wall impermeable to antimicrobial substances (Patrone and Stein, 2007).

Furthermore, the antimicrobial and anti-inflammatory activity of extracts of *C. abyssinicum* bulbs support wound healing that agree with Previous studies of various plants which have shown antimicrobial and anti-inflammatory activity and support wound healing (Deshmukh *et al.*, 2009; Pereira *et al*., 2018). Some of those plants that have anti-inflammatory activity and support wound healing include *Curcuma aromatic* (Kumar *et al.*, 2009), *Rumex abyssinicus* (Mulisa *et al*., 2015) and *Brucea antidysenterica* (Tessema *et al*., 2018) and those having antimicrobial effect include *Tanacetum chiliophyllum* (Salamc *et al*., 2007), *Bowdichia virgilioides* (Agra *et al*., 2013) and *Tanacetum densum* subsp. *Sivasicum* (Özbilgin *et al*., 2018).

Qualitative phytochemical screening done with the 80% methanol extracts of *C. abyssinicum* bulbs showed presence of phytochemical constituents with variable intensity of color which are trace, moderate and appreciable amount. In recent studies, various plant extracts proved to possess wound healing effect attributed to their different phytochemical constituents (Mulisa *et al*., 2015).

The extracts of *Crinum* bulbs have shown appreciable amount of tannins, steroids, and flavonoids, moderate amount of saponins, terpenoids and phenol as well as trace amounts of alkaloids and anthraquinone. The anti-inflammatory, antimicrobial and wound healing activity of *Crinum* bulbs are may be due to the existence of tannins, steroids, flavonoids, saponins etc. (Bedi and Shenefelt, 2002; Getie *et al*., 2002; Mukherjee, 2002; Okach, 2013)

For treatment of wound healing, drugs should fulfill the criteria such as rapid contraction, reduced epithelization time and increased tensile strength (Kanti *et al.*, 2001), and hence plant-based remedy should have effect on at least two different processes of wound healing before it can be said to have wound healing activity (Houghton *et al*., 2005). Therefore, the present study provided evidence for the wound healing potential of *C. abyssinicum* bulbs.

6 CONCLUSION AND RECOMMENDATIONS

CONCLUSION

The results of this study showed that the *C. abyssinicum* bulbs potentiates wound healing by increasing the tensile strength of the repaired tissue, wound contraction and rapid epithelialization. The wound healing potentials, anti-inflammatory and antimicrobial effect of the extract may be attributed to the presence of biologically active secondary metabolites such as tannins, saponins, flavonoids, steroids, anthraquinone, terpenoids, and alkaloids in the extract. These secondary metabolites bring about the overall effect by acting either individually or synergistically. The result of this study is consistent with the traditional claim of *C. abyssinicum* bulb for wound healing activity.

RECOMMENDATIONS

From this study, it is recommended to

- Perform chronic toxicity studies.
- perform *in vivo* tests for the antimicrobial activity of the crude extract and its fractions.
- investigate the possible mechanism/s of action at cellular and macromolecular levels.
- perform fractionation of crude extract in order to identify which fraction/s of the plant extract is/are responsible for wound healing, anti-inflammatory and antimicrobial activities.

7 REFERENCES

Abebe D (2003). Medicinal plants and other useful plants of Ethiopia. Ethiopian Health and Nutrition Institution, Addis Abeba. Pp 205-218.

Agarwal P, Singh A, Gaurav K, Goel S, Khanna H, and Goel R (2009). Evaluation of wound healing activity of extracts of *Plantain banana* (*Musa sapientum*var. *paradisiaca*) in rats. *Ind J of Exper. Biol.,* **47** (1): 32–40.

Agra IK, Pires LL, Carvalho PS, Filho EA, Smaniotto S, and Barreto E (2013). Evaluation of wound healing and antimicrobial properties of aqueousextract from *Bowdichia virgilioides* stem barks in mice. *An. Acad. Bras. Cienc.*, **85** (3): 946-954

Agyepong N, Agyare CC, and SampeneOssie PP (2015). Antioxidant and *in vivo* Wound Healing Activities of *Clausena anisata. Eur. J of Med. Pl.,* **10** *(2)*: *1-8.*

Akkol EK, Süntar I, Orhan IE, Keles H, Kanc A, and Çoksari G (2011). Assessment of dermal wound healing and in vitro antioxidant properties of *Avena sativa* L. *J of Cereal Sci.,* **53** (3): 285–90.

Alam G, Singh MP, and Singh A (2011). Wound healing potential of some medicinal plants. *Int. J of Pharmac. Sci. Revi. and Res.,* **9** (1): 136- 145.

Annan K, and Houghton J (2008). Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. And *Gossypium arboretum* L., wound healing plants of Ghana. *J of Ethnopharm.*, **119** (1): 141-144.

Ansel HC (1985). Introduction to Pharmaceutical Dosage Forms, 4th ed., Lea and Febiger, Philadelphia, pp. 299-301.

Ayuk MS, Tech B, Houreld NN, Tech D, and Abrahamse H (2012). Collagen Production in Diabetic Wounded Fibroblasts in Response to Low-Intensity Laser Irradiation at 660nm. *Diab. Techn. & therap.,* **14** (12): 1110-1117.

Barreto RS, Júnior RC, Araújo AA, Almeida JR, Santos MR, Barreto AS, DeSantana JM, Lima PS, Quintans JS, and Júnior LJ (2014). A Systematic Review of the Wound-Healing Effects of Monoterpenes and Iridoid Derivatives. *J mole.,* **19** (1): 846-862.

Bedi MK, and Shenefelt PD (2002). Herbal therapy in dermatology. *Arch Derm*., **138**: 232-42.

Beshir K, Shibeshi W, Ejigu A, and Engidawork E (2016). In Vivo Wound Healing Activity of 70% Ethanol leaf extract of Becium grandiflorum Lam. (Lamiaceae) in mice, *Ethio Pharmaceut J*, **32**: 117–130.

Bodnar RJ (2014). Chemokine regulation of angiogenesis during wound healing. *Adv. in wo. Care*, **00** (00): 1-10.

Brocair P (2015). Analysis of the Wound Care Market. Market analysis. NewYork. Retrieved from http://www.brocair.com/pdfs/WoundCare_Market_Report_Apr_2018.pdf

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

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

Deshmukh T, Fernandes J, Atul A, and Toppoa E (2009). Wound healing activity of *Calotropis gigantea* root bark in rats. *J of Ethnopharm.,* **125** (1): 178–181.

Desmoulière A, Redard M, Darby I, and Gabbiani G (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am. J of Patho.,* **146** (1): 56–66.

Diegelmann R F, and Evans M C (2004). Wound Healing: An Overview of Acute, Fibrotic and Delayed Healing. *J Front. in Biosci.,* **9**: 283-289.

Dubey G, Prasad S, Ghatuary SK, Sarathe A, Prajapati K, and Sheride R (2017). Evaluation of Wound Healing Activity of Aerial Parts of *Ocimum Basilicum* Linn and Bark of *Ficus Benghalensis* Linn and Their Polyherbal Formulation in Wound Healing Models. *J of Pharm. and Bio. Sci.,* **12** *(4)*: *50-56*.

Eaglstein WH (2001). Moist wound healing with occlusive dressings: a clinical focus. *Derm. Surg.*, **27** (2): 175–182.

Ejaz S, Chekarova I, Cho JW, Lee SY, Ashraf S, and Lim SW (2009). Effect of aged garlic extract on wound healing: a new frontier in wound management. *Drug Chem Tox.* **32**: 191–203.

Elmetti C (2008). Local antibiotics in dermatology. *Derm. Thera.*, **21**: 187-195.

Emran TB, Nasir Uddin MM, Rahman A, Uddin Z, and Islam M (2015). Phytochemical, Antimicrobial, Cytotoxic, Analgesic and Anti-Inflammatory Properties of *Azadirachta Indica*: A Therapeutic Study. *J Bio. anal Biomed.*, **12**: 1-7.

Farahpour MR, and Habibi M (2012). Evaluation of the wound healing activity of an ethanolic extract of *Ceylon cinnamon* in mice. *Vet. Med.*, **57** (1): 53-57.

Ferguson MW, and O'Kane S (2004). Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Phil. Trans. of Roy. Socie. Bio. Sci.*, **359** (1445): 839–850.

Flanagan M (2000). The physiology of wound healing. *J of Wo. Care,* **9** (6): 299-300.

Frykberg GR, and Banks J (2015). Challenges in the Treatment of Chronic Wounds. *Adv. in `wo. care,* **4** (9): 560-582.

Geetha TS, and Geetha N (2014). Phytochemical screening, quantitative analysis of primary and secondary metabolites of *Cymbopogan citratus* (DC) Stap f. Leaves from Kodaikanal hills, Tamilnadu. *Int. J Pharm. Tech. Res*., **6**: 521-9

Getaneh S, and Girma Z, (2014). An Ethnobotanical study of medicinal plants in Debre LibanosWereda,central Ethiopia. *Afr. J of Pl. Sci.* **8** (7): 366-379.

Getie M, Gebre-Mariam T, Rietz R, and Neubert RH (2002). Evaluation of the release profiles of flavonoids from topical formulations of the crude extract of the leaves of *Dodonea viscosa* (Sapindaceae). *Pharmazie,* **57**: 320-322.

Gidey M, Asfaw Z, and Woldu Z (2009). Medicinal plants of the Meinit ethnic group of Ethiopia: An Ethnobotanical study. *J of Ethnopharm.*, **124**: 513–521.

Goorani S, Zangeneh MM, Zangeneh A, Poorshamohammad C, Abiari M, Moradi R, Najafi F, and Tahvilian R (2018). Study of Wound Healing Potential of *Stevia rebaudiana*Ethanol Extract in Male Rats. *Res. J Pharmaco.*, **5** (1): 23-30.

Gopalakrishnan S, and Rajameena R (2012). Evaluation of Ethanolic Extract of *Desmodium Gyrans* DC Leaves on Wound Healing Activity in Rats. *Pharmaceu. Anal. Acta.* **3** (7): 169.

Guo S, and DiPietro LA (2010). Factors Affecting Wound Healing. *J Dent. Res.*, **89** (3): 219-229.

Gurtner GC, Werner S, Barrandon Y, and Longaker MT (2008). Wound repair and regeneration. *J Nat.*, **453** (7193): 314–321.

Han G, and Ceilley R (2017). Chronic Wound Healing: A Review of Current Management and Treatments. *Adv Ther*.,. DOI 10.1007/s12325-017-0478-y: 1-12

Harborne JB (1998). *Phytochemical methods: A guide to modern technique of plant analysis*. 3rd ed. Champman and Hall: London. (pp. 40–235).

Hassan MM, Khan SA, Shaikat AH, Hossain ME, Hoque MA, Ullah MH, and Islam S (2013). Analgesic and anti-inflammatory effects of ethanol extracted leaves of selected medicinal plants in animal model, *Vet World*, **6** (2): 68-71.

Houghton P, Hylands P, Mensah A, Hensel A, and Deters A (2005). *In vitro* tests and ethnopharmacological investigations: Wound healing as an example. *J of Ethnopharm.*, **100** (12): 100–107.

Ilango K, and Chitra V (2010). Wound healing and antioxidant activities of the Fruit Pulp of LimoniaAcidissima Linn. (Rutaceae) in rats. *Trop. J of Pharmaceu. Res.*, **9** *(*3): 223–230.

Institute for Laboratory Animal Research (ILAR) (1996). Guide for the care and use of laboratory animals. National Academy Press, Washington D.C.

Järbrink K, Ni G, Sönnergren H, Schmidtchen A, Pang C, Bajpai R, and Car1 J (2017). The humanistic and economic burden of chronic wounds: a protocol for a systematic review. *J Syst. Rev.*, **6** (15): 2-7.

Jimi S, Kimura M, De Francesco F, Riccio M, Hara S, and Ohjimi H (2017). Acceleration Mechanisms of Skin Wound Healing by Autologous Micrograft in Mice. *Int. J. Mol. Sci.*, **18**: 1675.

Jones WP, and Kinghorn AD (2012). Extraction of Plant Secondary Metabolites BT - Natural Products Isolation. In S.D. Sarker & L. Nahar (Eds.), *Methods in Molecular Biology (Methods and Protocols)*. Totowa, NJ: Humana Press. pp. 341–366

Joseph WS (2011). The use of topical antimicrobials and antibiotics in wound care. *Adv. Ance. Wo. Care*,**2**: 219-224.

Joung H, Kwon DY, Choi JP, Shin DY, Chun SS, YU YB, and Shin DW (2010). Antibacterial and synergistic effects of *Smallanthus sonchifolius* leaf extracts against methicillin-resistant *Staphylococcus aureus* under light intensity. *J Nat Med*., **64**: 212-215.

Kai KL, Shan SS, Xiangyi D, Hoi TS, Wing SS, Ping CL, Chun HK, and Bao HC (2018). Dihydrofisetin exerts its anti-inflammatory effects associated with suppressing ERK/p38 MAPK and Heme Oxygenase-1 activation in lipopolysaccharide stimulated RAW 264.7 macrophages and carrageenan induced mice paw edema. *Int. Immunopharm.*, **54**: 366–374.

Kanti T, Biswajit A, and Nitai P (2001). Wound healing activity of human placental extracts in rats. *Acta. Pharm. Sinica.*, **22** (12): 1113–1116.

Kasuya A, and Tokura Y (2014). Attempts to accelerate wound healing, *J. Derm.. Sci.*, **76**: 169-172.

Kokane DD, More RY, Kale MB, Nehete MN, Mehendale PC, and Gadgoli CH (2009). Evaluation of wound healing activity of root of *Mimosa pudica*. *J of Ethnopharm.*, **124** (2): 311–315.

Kuedo Z, Sangsuriyawong A, Klaypradit W, Tipmanee V, and Chonpathompikunlert P (2016). Effects of astaxanthin from Litopenaeus vannamei on carrageenan-induced edema and pain behavior in mice. *J Mole.*, **21** (3): 382.

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

Kumar PR, Priyadarsini S, Thirumal M, and Musenda TJ (2017). Anticancer potential of indian medicinal plants–a review. *Int J Pharm Bio Sci.,* **7** (4): 128-132.

Kumar V, Ahmed A, and Nagarajan K (2013). Animal models for the evaluation of wound healing activity. *Int. Bull. of Drug Res.*, **3** (5): 93-107.

Langley C, and Belcher D (2008). Pharmaceutical Compounding and Dispensing. Pharmaceutical Press, London, pp. 107-113.

Li J, Chen J, and Kirsener R (2007). Pathophysiology of acute wound healing. *Cli. Derm.,* **25** (1): 9–18.

Li J, Zhang YP, and Kirsner RS (2003). Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. *Micr. Res. and Techni.,* **60** (1): 107-114.

Lodhi S, Pawar R, Jain A, and Singhai AK (2006). Wound healing potential of *Tephrosia purpurea* (Linn.) Pers. in rats. *J of Ethnopharm.,* **108**: 204-210.

Lordani TV, de Lara CE, Ferreira FB, Monich MS, da Silva CM, Lordani CR, Bueno FG, Teixeira JJ, and Lonardon MV (2018). Therapeutic Effects of Medicinal Plants on Cutaneous Wound Healing in Humans: A Systematic Review. *J Medi. of Infl.,* Article ID 7354250: 1- 12.

MacDonald J (2009). Global Initiative for Wound and Lymphoedema Care (GIWLC). *J of Lymphoedema*, **4** (2): 92-94.

Mathur A, Bhat R, Prasad GB, DuaVK, Verma SK, and Agarwal PK (2010). Antimicrobial activity of plants traditionally used as medicines against some pathogens. *RASĀYAN J. Chem*. **3** (4): 615-620.

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

Mohammed N, Mohammedberhan A, and Faiz M (2016). Antimalarial Activity of Crude Extract of *Buddleja Polystachya* Fresen (Buddlejacea) Against *P. Berghei* in Mice. *IOSR-JPBS,* **11** (5): 27-35.

Mohanty A, Sahu PK, and Mohanty CDA (2010). Wound healing activity of methanolic extract of *Cissus quadrangularis*on albino rats: A research. *Int. J of Drug Form. and Res.,* **1** (2): 176–184.

More BH, Sakharwade SN, Tembhurne SV, and Sakarkar DM (2013). Evaluation for skin irritancy testing of developed formulations containing extract of *Butea monosperma* for its topical application. *Int. J of Tox. & Appl. Pharm.,* **3** (1): 10-13.

Mosser DM, and Edwards JP (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.,* **8**: 958-969.

Mukherjee PK (2002). Quality control herbal drugs: An approach to evaluation of botanicals. New Delhi*: Business Horizons*; Pp: 546-9.

Mulisa E, Asres A, and Engidawork E (2015). Evaluation of wound healing and antiinflammatory activity of the rhizomes of *Rumex abyssinicus* J. (Polygonaceae) in mice. *BMC Compl.. and Altern Med.,* **15**: 341.

Muniandy K, Gothai S, Tan WS, Kumar SS, Esa NM, Chandramohan G, Al-Numair KS, and Arulselvan P (2018). In Vitro Wound Healing Potential of Stem Extract of *Alternanthera sessilis*. *Evid.-Based Compl. and Altern. Med.,* Article ID 3142073: 1-14.

Njoku V, and Obi C (2009). Phytochemical constituents of some selected medicinal plants. *Afr. J of Pure and Appl Chem.,* **3** (11): 228–233.

Nordal I (1995). Crinum abyssinicum in global plants on JSTOR [website] retrieved from https://plants.jstor.org/compilation/crinum.abyssinicum.

Odimegwu DC, Ibezim EC, Esimone CO, Nworu CS, and Okoye FB (2008). Wound healing and antibacterial activities of the extract of Dissotis theifolia

(Melastomataceae) stem formulated in a simple ointment base, *J of Medi. Pl. Res.*, **2**(1): 11–16.

OECD (2001). Guideline for testing of chemicals: Acute oral toxicity-Fixed dose procedure (420). pp. 1-14. Retrieved from http://www.oecd-ilibrary.org

Okach DO, Nyunja AR, and Opande G (2013). Phytochemical screening of some wild plants from Lamiaceae and their role in traditional medicine in Uriri District Kenya. *Int. J of Herb Medi.*, **1** (5): 135–143.

Olczyk P, Mencner A, and Vassev K (2014). The Role of the Extracellular Matrix Components in Cutaneous Wound Healing. *Bio Med Res Int.*, Article ID 747584, Doi.org/10.1155/2014/747584: 1-8.

Onwukaeme DN, Ikuegbvweha TB, and Asonye CC (2007). Evaluation of phytochemical constituents, antibacterial activities and effect of exudates of *Pycanthus angolensis* Weld Warb (Myristicaceae) on corneal ulcers in rabbits. *Trop J of Pharmaceu. Res.*, **6**: 725–730.

Oryan A, Alemzadeh E, and Moshiri A (2018). Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed. & Pharmacother.*, **98** (1): 469–483.

Ozay Y, Guzel S, Halil I, Yildirim Z, Pehlivanoglu B, Aydın B, and Darcan S (2018). Evaluation of the Wound Healing Properties of Luteolin Ointments on Excision and Incision Wound Models in Diabetic and Non-Diabetic Rats. *Rec. Nat. Prod*., **12** (4): 350-366.

Özbilgin S, Akkol EK, Öz BE, Ilhan M, Saltan G, Acıkara ÖB, Tekin M, Keleş H, and Süntar I (2018). In vivoactivity assessment of some Tanacetum species used as traditional wound healer along with identification of the phytochemical profile by a new validated HPLC method. *Iran J Basic Med Sci.*, **21**: 145-152.

Padilha MM, Vilela FC, and Rocha CQ (2010). Antiinflammatory properties of *Morus nigra* leaves. *Phytother. Res.*, **24**: 1496-1500.

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

Pastar I, Ojeh N, Glinos GD, Stojadinovic O, and Tomic-Canic M (2018). Physiology and Pathophysiology of Wound Healing in Diabetes. *The Diabetic Foot, Contemporary Diabetes*, Doi.org/10.1007/978-3-319-89869-8_7: 109-130

Pastar I, Stojadinovic O, Yin NC, Ramirez H, Nusbaum AG, and Sawaya A, *et al*. (2014). Epithelialization in wound healing: a comprehensive review. *Adv Wo. Care (New Rochelle),* **3** (7): 445–64.

Patrone JB, and Stein DC (2007). Effect of gonococcal lipooligosaccharide variation on human monocytic cytokine profile. BMC Microbiol. **7**: 1-7.

Pereira LO, Vilegas W, Tangerina MM, Arunachalam K, Balogun SO, Matos PE, and Martins DT (2018). *Lafoensia pacari*A. St.-Hil: wound healing activity and mechanism of action of standardized hydroethanolic leaves extract, *J of Ethnopharmac.*, Doi.org/10.1016/j.jep.2018.02.038: 1-45.

Prasad V, and Dorle K (2006). Evaluation of ghee-based formulation for wound healing activity. *J of Ethnopharmac.*, **107** (1): 38–47.

Sabol F, Dancakova L, Gal P, Vasilenko T, Novotny M, Smetana K, and Lenhardt L (2012). Immunohistological changes in skin wounds during the early periods of healing in a rat model. *Vet Med-Czech*, **57** (2): 77–82.

Sahib HB, Ouda MH, Abaas IA, Abduljalil AI, Khadum EA, and Khadum MK (2014). The Wound Healing Activity of (Rue) *Ruta graveolens* L. Methanolic Extract in Rats. *Int. J. Pharm. Sci. Rev. Res*., **29** (2): 263-266.

Sahraei S, Mohkami Z, Golshani F, Javadian F, Saeidi S, and Sohil Baig G (2014). Antibacterial activity of five medicinal plant extracts against some human bacteria. *Euro.* **J**. *Exp. Bio.*, **4** (3): 194-196.

Salamc E, Kordal S, Kotan R, Cakır A, and Kaya Y (2007). Chemical compositions, antimicrobial

and herbicidal effects of essential oils isolated from Turkish *Tanacetum aucherianum*and *Tanacetum chiliophyllum* var. chiliophyllum. *Biochem Syst Ecol*., **35**: 569-581.

Sanwal R, and Chaudhary A (2011). Wound healing and antimicrobial potential of *Carissa spinarum* Linn. in albino mice. *J of Ethnopharm.,* **135** (3): 792–796.

Sasidharan S, Nilawatyi R, Xavier R, Latha LY, and Amala R (2010). Wound healing potential of *Elaeis guineensis* Jacq leaves in an infected albino rat model. *J Mole.,* **15**: 3186- 3199.

Sathyaraj K, and Indumathy R (2018). Evaluation of *in vivo* anti-inflammatory activity of whole plant of *Trianthema triquetra* rottler ex wild.*World J of Pharm and Pharmaceu. Sci.,***7** (6): 835-846.

Schreml S, Szeimies RM, Prant L, Karrer S, Landthaler M, and Babilas P (2010). Oxygen in acute and chronic wound healing. *Br. J of Derm.,* **163**: 257-268.

Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, Romanelli M, Stacey MC, Teot L, and Vanscheidt W (2003). Wound bed preparation: a systematic approach to wound management. *Wo. Repair Reg*., **11** (1): 1–28.

Selvamohan T, Ramadas V, and Kishor S (2012). Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. *Adv. Appl. Sci. Res.,* **3** (5): 3374-3381.

Sengupta R (2017). Combined wound healing activity of *Calendula officinalis* and Basil leaves. *J of Pharmacogn. and Phytochem*., **6** (1): 173-176.

Shafiuddin M, Abdullah K, and Sadath A (2009). Wound healing activity of traditional herbal formulation. *Int J Chem. Sci.,* **7** (2): 639-643.

Sharma GN, Dubey SK, Sati N, and Sanadya J (2011). Evaluation of wound healing activity of *Aegle marmelos* seed. *Pharm. online,* **2**: 171-178.

Shetty BS, Udupa SL, Udupa AL, and Somayaji SN (2006). Effect of *Centella asiatica* L (Umbelliferae) on normal and dexamethasone-suppressed wound healing in wistar albino rats. *The Int. J of Low. Extr. Wo.,* **5** (3): 137-143.

Shivhare Y, Singour PK, Patil UK, and Pawar RS (2010). Wound healing potential of methanolic extract of *Trichosanthes dioica* Roxb (fruits) in rats. *J of Ethnopharm.,* **127**: 614-619.

Shrimanker M, Patel N, Modi H, and Dave R (2013). A Review: Screening Models for Wound Healing Activity in Animals. *Am. J. Pharm. Tech. Res.*, **3**: 1-15.

Stechmiller JK (2010). Understanding the role of nutrition and wound healing. *Nutr. In Cli. Pract.,* **25** (1): 61-68.

Strodtbeck F (2001). Physiology of wound healing. *Newb. and Infa. Nurs. Rev.,* **1** (1): 43-52.

Süntar IP, Akkol EK, Yilmazer D, Baykal T, Kirmizibekmez H, Alper M, and Yes-ilada E (2010). Investigations on the in vivo wound healing potential of *Hypericum perforatum* L. *J of Ethnopharm.,* **127** (2): 468–477.

Taye B, Giday M, Animut A, and Seid J (2011). Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia. *Asi. Pacif. J of Trop. Biomed.,* **1** (5): 370–375.

Teklehaymanot T, and Giday M (2007). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *J Ethnobio. Ethnomed.,* **3**: 3-12.

Tessema Z, Makonnen E, Debella A, and Molla Y (2018). Evaluation of *in vivo* wound healing and anti-inflammatory activity of Crude extract of the fruits of *Brucea antidysentrica* in mice, *Wo. Med.*, Doi.org/10.1016/j.wndm.2018.05.005: 1-23

Tonnesen MG, Feng X, and Clark RA (2000). Angiogenesis in wound healing. *J of Invest. Derm. Sympo. Proc.*, **5** (1): 40–46.

Udegbunam SO, Kene RO, Anika SM, Udegbunam RI, Nnaji TO, and Anyanwu MU (2015). Evaluation of wound healing potential of methanolic *Crinum jagus* bulb extract. *J Inter. Ethnopharm.,* **4** (3): 194-201.

Ugochukwu SC, Uche IA, and Ifeanyi O (2013). Preliminary Phytochemical screening of different solvent extracts of stem bark and roots of *Dennetia tripetala* G. Baker. *Asian J of Pl. Sci. and Res.,* **3** (3): 10-13.

Velnar T, Bailey T, and Smrkolj V (2009). The Wound Healing Process: An Overview of the Cellular and Molecular Mechanisms. *J of Int. Med. Res.,* **37**: 1528-1542.

Vowden P (2011). Hard-to-heal wounds: made easy. *Wo. Int..*, **2** (4): 1-6.

Wang J, Ruana J, Caia Y, Luob Q, Xuc H, and Wua Y (2011). *In vitro* and *in vivo* evaluation of the wound healing properties of *Siegesbeckia pubescens*. *J of Ethnopharm.*, **134** (3): 1033–1038.

WHO-AFRO (2010). World Health Organization Regional Office for Africa. African traditional medicine day, 31 august.

Wild T, Rahbarnia A, Kellner M, Sobotka L, and Eberlein T (2010). Basics in nutrition and wound healing. *J Nutri.*, **26**: 862-866.

Wong SY, Manikam R, and Muniandy S (2015). Prevalence and antibiotic susceptibility of bacteria from acute and chronic wounds in Malaysian subjects. *J of Inf. in Dev. Count.*, **9** (09): 936-944.

Yesuf A, and Asres K (2013). Wound healing and antiinflammatory properties of *Allophylus abyssinicus* (Hochst.) Radlk. *Phytopharm.*, **4** (2): 442–453.

Yineger H, Kelbessa E, Bekele T, and Lulekal E (2008). Plants used in traditional management of human ailments at Bale Mountains National Park, Southeastern, Ethiopia. *J. Med. Pl. Res.*, **2** (6): 132-153.

Zeng Q, Xie H, Song H, Nie F, Wang J, Chen D, and Wang F (2016). In Vivo Wound Healing Activity of *Abrus cantoniensis* Extract. *Evid.-Based Compl. and Altern. Med.*, Article ID 6568528: 1- 7.