Evaluation of Wound Healing Activity of 80% Methanol Leaf Extract of *Vernonia leopoldi* (Sch. Bip. Ex Walp.) Vatke (*Asteraceae*) in Mice

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This is to certify that the thesis prepared by Aklilu Asmelash, entitled "Evaluation of Wound Healing Activity of 80% Methanol Leaf Extract of *Vernonia leopoldi* (Sch. Bip. Ex Walp.) Vatke (*Asteraceae)* 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 Wound Healing Activity of 80% Methanol Leaf Extract of *Vernonia leopoldi* (Sch. Bip. Ex Walp.) Vatke (*Asteraceae*) in Mice

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Addis Ababa University, 2019

Vernonia leopoldi (Sch. Bip. Ex Walp.) Vatke locally known as "Nechilo" is a shrub that is endemic to Ethiopia. Traditionally, it is used for the treatment of wounds. However, there is no scientific evidence that justifies the traditional claims. Thus, the present study assessed the wound healing potential of 80% methanol leaf extract of *V. leopoldi* in mice.

Extraction of the leaf was carried out by maceration using 80% methanol. The extract was studied for wound healing activity by formulating a topical ointment using two concentrations (5% and 10% w/w). Simple ointment base (negative control) and nitrofurazone 0.2% (w/w) ointment (positive control) were used for comparison of wound healing activity in mice. Two wound models were used i.e. excision and incision. For anti-inflammatory study, carrageenan-induced hind paw edema model was used. The extract of *V. leopoldi* was given orally at doses of 100, 200 and 400 mg/kg. The anti-bacterial activity of the extract was determined using disk diffusion technique against both gram positive and gram negative bacteria that are associated with wound at a concentration of 50-500 µg/µl. Treatment of wound with ointment containing 5% and 10% (w/w) extract exhibited significantly increased ($p<0.05$) wound contraction rate, shorter epithelialization time, and higher skin breaking strength as well as increased collagen deposition, fibroblast proliferation and vascularization as evidenced by histopathological analysis. The 10% (w/w) extract ointment showed better wound healing activity than the 5% (w/w) ointment and its effect was comparable to that of nitofurazone. *V. leopoldi* extract also produced dose-related significant reductions ($p<0.01$-0.001) of inflammation as compared to the control. The extract also displayed better antibacterial activity on gram positive than gram negative organisms.

From the results obtained, it can be concluded that the 80% methanol extract of *V. leopoldi* is endowed with wound healing activity most probably via its anti-inflammatory and, anti-bacterial

activity as well as collagen deposition, supporting the traditional use of this plant as wound healing agent.

Key words: *V. leopoldi*, Excision model, Incision model, Anti-inflammatory. Anti-bacterial, Histopathological analysis, Wound healing activity.

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LIST OF ACRONYMS

ATCC	American Type Cell Culture
BP	British Pharmacopoeia
C3a	Complement 3a
C5a	Complement 5a
CFU	Colony Forming Units
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EPHI	Ethiopian Public Health Institute
FGF	Fibroblast Growth Factor
IL-1	Interleukin-1
MBC	Minimum Bactericidal Concentration
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
OECD	Organization for Economic Cooperation and Development
PDGF	Platelet Derived Growth Factor
SEM	Standard Error of the Mean
SPSS	Statistical Package for Social Sciences
TGF-β	Transforming Growth Factor-Beta
TNF-α	Tumor Necrosis Factor Alpha
WHO	World Health Organization

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1. INTRODUCTION

1.1. Overview of Skin and Wound

Skin is a vital organ which performs several functions such as thermoregulation, homeostasis, metabolic, neurosensory and immunologic functions. It is also a physical barrier against infections; thus, when it is injured, pathogens can have a direct access to the deep tissues (Singer, 1988).

Wound is simply defined as the disruption of the cellular and anatomic continuity of a tissue (Bennet, 1988). It is a collective form of conditions in which there is interruption or damage to the structural integrity of the skin or underlying tissue (Bhora *et al*., 1995). It can also be described as a break in the continuity of tissue from violence and trauma. Physical, chemical, thermal, microbial, or immunological insults to the tissue are among the factors mentioned in wound production (Majumdar, 2005; Hussain *et al*., 2014). The processes involved in wound healing are well organized biochemical and cellular events leading to the growth and regeneration of damaged tissues in specific manner. Healing of wounds is an important biological process involving the activity of an intricate network of blood cells, cytokines, and growth factors, which ultimately leads to the restoration to normal condition of the injured skin or tissue (Bowler *et al*., 2001: Esimone *et al*., 2009).

Wounds can be broadly classified as acute or chronic depending on physiology or the time it takes to heal. Without complications, most wounds are acute wounds and tend to heal within few weeks. Chronic wounds, in contrast, require prolonged time to heal, do not heal, or recur frequently. These wounds tend to occur when the normal wound healing process has been compromised due to microbial infection, metabolic disturbances, or an underlying disease (Agyepong *et al*., 2015).

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

Chronic wounds, rarely seen in healthy individuals and usually associated with diseases like diabetes and obesity, and are defined as wounds, which have failed to progress through an orderly and timely reparative process of healing and therefore enter a state of pathologic inflammation. As a result, the healing process is delayed, incomplete, and does not proceed in a coordinated manner, subsequently resulting in poor anatomic and functional integrity over a period of 3 months (Menke *et al*., 2007; Trostrup *et al*., 2013). Identifying and treating the underlying aetiology of a chronic wound such as venous insufficiency, arterial perfusion, diabetes, or unrelieved pressure as well as systemic factors such as nutritional status, immunosuppression, and infection that may contribute to poor wound healing are key to successful wound treatment (Werdin *et al*., 2009).

The epidemiology and economic burden of chronic wound is well documented in the developed world (Mac Donald, 2009). Loss of 2-4% of the total health care expenses for the clinical management of chronic wounds in Scandinavian countries is a proof of the reality (Sen *et al*., 2009). Non-healing wounds affect about 3 to 6 million people in the United States, with persons 65 years and older accounting for 85% of these events. Non-healing wounds result in enormous health care expenditures, with the total cost estimated at more than $3 billion per year (Mathieu *et al*., 2006; Menke *et al*., 2007).

Wound is a major problem in developing countries, often having severe complications and involving high costs of therapy (Shenoy *et al*., 2011). An estimated 1 % to 2 % of the populace in developing countries, such as Sub-Saharan African and South Asian countries, experiences a chronic wound during their lifetime. The prevalence of chronic wounds in the community was reported to be 4.5 per 1000 population, whereas that of acute wounds was nearly double at 10.5 per 1,000 populations. These wounds predominantly affect patients aged older than 60 years. The poor hygienic condition in some third world countries is the main cause of this problem (Sasidharan *et al*., 2010; Siddiqui and Bernstein, 2010).

There are no much data concerning the current status of wound problem in Ethiopia. Majority of the study focuses on microbial infection of the skin and others (Adane, 2018; Sinatayehu, 2017; Walker *et al*., 2017; Woldeamanuel *et al*., 2005). However, Ethiopia is one of the poor Sub-Saharan African countries whose population faces chronic wound at least once in their life time.

1.2. Wound Healing Cascade

Cutaneous wound healing is an essential physiological process consisting of the collaboration of many cell strains and their products (Shaw, 2009). An attempt to restore the lesion induced by a local aggression begins very early on in the inflammatory stage. In the end, they result in repair, which consists of the substitution of specialized structures brought about by the deposition of collagen, and regeneration, which corresponds to the process of cell proliferation and posterior differentiation through preexisting cells in the tissue and/or stem cells (Eming, 2007). These mechanisms do not mutually exclude themselves, that is, after a skin lesion in the same tissue regeneration and repair can occur, depending on the cell strains compromised by the injury.

Cell and biochemical events in wound repair can be divided into the following stages: inflammatory reaction, cell proliferation and synthesis of the elements which make up the extracellular matrix, and the posterior period, called remodeling. These stages are not mutually exclusive, but rather overlap over time (Nayak, 2009).

1.2.1. *Hemostasis*

As soon as injury occurred, an important step of initiation and continuation of the healing process called hemostasis appears. It is characterized by vasoconstriction, platelet degranulation and aggregation, and fibrin deposition leading to formation of a clot and bleeding cessation (Pakyari *et al*., 2013). The clot and surrounding wound tissue then release pro inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, inflammatory cells migrate into the wound (chemo-taxis) and promote the inflammatory phase (Gosain and DiPietro, 2004; Broughton *et al*., 2006; Campos *et al*., 2008).

1.2.2. *Inflammation*

Inflammation is characterized by erythema, edema, heat, and pain as first described by Hunter in 1794. The inflammatory process is the response to an injurious stimulus evoked by a wide variety of noxious agents (e.g., infections or physical injuries). Although inflammation is a biological response as a defense mechanism to avoid harmful stimuli followed by healing process, uncontrolled inflammation could lead to serious illnesses which cause great impact on public health and economy (Devasvaran and Yong, 2016). 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.

At the tissue level, increased vascular permeability and the sequential migration of leukocytes into the extravascular space characterize inflammation. One of the primary functions of inflammation is to bring inflammatory cells to the injured area. These cells then destroy bacteria and eliminate debris from dying cells and damaged matrix so that the repair can proceed (Majno *et al*., 1969). There are early and late inflammatory phases in wound healing process. Early inflammatory phase (starts during the late phase of coagulation) activates the complement cascade and initiates molecular events, leading to infiltration of the wound site by neutrophils, whose main function is to prevent infection (Broughton *et al*., 2006). These cells are attracted to the wound site by various chemo-attractive agents, including TGF-β, complement components such as C3a and C5a, and formylmethionyl peptides produced by bacteria and platelet products (Robson, 2001). Once in the wound environment, neutrophils phagocytose foreign material and bacteria, destroying them by releasing proteolytic enzymes and oxygen-derived free radical species (Flangan, 2000; Richardson, 2004; Broughton *et al*., 2006). Upon completing the task, the neutrophils must be eliminated from the wound prior to progression to the next phase of healing. Redundant cells are disposed of by extrusion to the wound surface as slough and by apoptosis, allowing elimination of the entire neutrophil population without tissue damage or potentiating the inflammatory response (Hunt *et al*., 2000; Hart, 2002). The cell remnants and apoptotic bodies are then phagocytosed by macrophages (Roboson *et al*., 2001).

In the late stages of the inflammatory phase (48-72 h after injury), blood monocytes undergo phenotypic changes on arrival to become tissue macrophages. They act as key regulatory cells and providing an abundant reservoir of potent tissue growth factors, particularly TGF-β, as well

as other mediators (TGF-α, heparin binding epidermal growth factor, fibroblast growth factor [FGF], collagenase), activating keratinocytes, fibroblasts and endothelial cells (Ramasastry, 2005). Thus, they are essential for the transition from the inflammatory to the repair phase because of their essential role in wound (Williamson and Harding, 2004). The last cell to enter the wound site in the late inflammatory phase (>72 h after injury) are lymphocytes. They play an important role in collagenase regulation, which is later needed for collagen remodeling, production of extracellular matrix components and their degradation (Hart, 2002).

1.2.3. *Proliferation and repair*

The proliferative phase lasts approximately from days 3-14 and in the absence of significant infection or contamination, the inflammatory phase is short, and after the wound has been successfully cleared of devitalized and unwanted material, it gives away to the proliferative phase of healing (Majumdar, 2005). This phase comprised of events such as angiogenesis, fibroblasia and granulation tissue formation, collagen deposition, epithelialization and contraction that overlap each other (Guo and Dipietro, 2010; Ayuk, 2012). Fibroblasts migrate inwards from the wound margins stimulated by many chemical activators and messengers, mostly released by macrophages, which dominate towards the end of the inflammatory phase. Fibroblasts themselves secrete a variety of cytokines, allowing other vital cells to proliferate and aid the healing process. Such cells include endothelial cells and angiocytes. Expansion of these cell numbers contributes to a process known as angiogenesis, the generation of new blood vessels (Nigam *et al.*, 2010). Hypoxia and acidosis stimulate angiogenesis (Guo and Dipietro, 2010). Fibroblasts begin to form a collagen matrix in the wound known as granulation 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 (extracellular matrix) ECM (Barker, 2011). As healing progresses, several other important biological responses are activated. The process of epithelialization is stimulated by the presence of EGF and TGFα 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 cell from hair follicles or sweat glands. Epithelial cells finally resurface the wound, a process known as epithelialization (Werner and Grose, 2003). Fibroblasts

also participate in the process of wound contraction after differentiation into myofibroblasts (Strodtbeck, 2001). The 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 wound 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 closure of wounds in areas such as the hand or around the neck and face, where it can cause disfigurement and excessive scarring (Hinz, 2007).

1.2.4. *Remodeling*

The final stage of wound healing process, which start from day 7 and lasts up to 2 years thereafter. After the main steps of the proliferative phase are fulfilled, the density of cells, such as macrophages, keratinocytes, fibroblasts and myofibroblasts is reduced by apoptosis (Orsted *et al*., 2004; Sinno and Prakash, 2013). Collagen III, which was produced in the proliferative phase, is now replaced by 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).

1.3. Factors Affecting Wound Healing

Multiple factors can lead to impaired wound healing. In general terms, the factors that influence repair can be categorized into local and systemic. Local factors are those that directly influence the characteristics of the wound itself, while systemic factors are the overall health or disease state of the individual that affect his or her ability to heal (Guo and DiPietro, 2010). Local factors that can lead to impaired wound healing are mainly oxygenation, infection, foreign body and venous insufficiency, while systemic factors includes age and gender, sex hormones, stress, ischemia, diseases (diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia), obesity, medications (glucocorticoid steroids, non-steroidal anti-inflammatory drugs), alcoholism and smoking, immunocompromised conditions (cancer, radiation therapy, AIDS) and nutrition (Guo and DiPietro, 2010; Ayuk, 2012). Wounds will heal in a short period of time if the factors that impaired wound healing are correctly identified and managed. Some of the factors are:

Wound hypoxia: Oxygen is important for cell metabolism, especially energy production by means of ATP, and is critical for nearly all wound-healing processes. It prevents wounds from infection, induces angiogenesis, increases keratinocyte differentiation, migration, and re-epithelialization, enhances fibroblast proliferation and collagen synthesis, and promotes wound contraction (Bishop, 2008; Rodriguez *et al*., 2008).

In addition, the level of superoxide production (a key factor for oxidative killing pathogens) by polymorphonuclear leukocytes is critically dependent on oxygen levels (Guo and DiPietro, 2010). In wounds where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing (Bishop, 2008; Rodriguez *et al*., 2008).

Infections: Wounds are known to be easy portals for infections and provide suitable medium for the proliferation of microbial organism (Odimegwu *et al*., 2008). In the absence of effective decontamination, however, inflammation may be prolonged, since microbial clearance is incomplete. Both bacteria and endotoxins can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal (Edwards and Harding, 2004; Menke *et al*., 2007).

1.4. Wound Management

The aim of wound care is to restore structure and function of an injured tissue in order to approximate pre-wound characteristics (Kore et al., 2011). Assessment of the wound and the patient starts with a diagnosis of the wound's aetiology and continues with optimizing the patient's medical condition, particularly blood flow to the wound area is considered to be the first stage in wound management. The wound needs to be debrided and dressed correctly. The next important stage in wound management is the lavage of micro-organisms, dead tissues and foreign bodies, which decrease tissue bacterial count using bacitracin or normal saline solution (Velnar *et al*., 2009).

The wound should be handled with an aseptic technique, thoroughly irrigated under adequate pressure and carefully debrided (Liptak, 1997). However, since wounds harbor microbes, techniques to rid the wound of microbes should be used. These include the use of expensive

silver-containing dressings, the application of topical antibiotics or antiseptics or even the routine use of long-term systemic antibiotics (Esimone *et al.*, 2005; Joseph, 2011).

Topical antimicrobial therapy is one of the most important methods of wound care (Odimegwu *et al.,* 2008). Antibiotics are proposed to promote normal healing by protecting the wound from superficial infection (Liptak, 1997). They are chosen based on their ability to destroy or inhibit the growth of pathogenic organisms, while the tissue is left unharmed (Thakare *et al.,* 2011).

Numerous antibiotics are available for topical use. Some of common antibiotics in use include amikacin (in gel or cream), bacitracin, chloramphenicol, clindamycin (cream, lotion, and foam), gentamicin **(**available in form of ointment or cream), nitrofurazone (available as a 0.2% cream, solution, or soluble dressing) and polymyxin B (Elmetti, 2008; Sasidharan *et al*., 2010).

Currently, novel techniques such as topical growth factor application and incisional priming with PDGF or IL-1 can optimize both the cellular and molecular environment, thus decreasing healing time by modifying inflammation and accelerating the proliferative phase. Electrical field stimulation may also optimize the remodeling phase by promoting more efficient fibroblast recruitment and collagen deposition (Velnar *et al*., 2009).

1.5. Medicinal Plants Used in the Management of Wound

The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India, and the Near east, but it is doubtless to be an art as old as mankind (Mohsenzadeh, 2007). Natural products perform various functions and many of them have interesting and useful biological activities. There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose (Philip *et al*., 2009). As estimated by the World Health Organization, 80% of the populations of Asia, Africa and Latin America use traditional medicine to meet their primary health care needs (WHO-AFRO, 2010).

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

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 which 95% of traditional medicinal preparations are of plant origin (Getaneh and Girma, 2014) due to the cultural acceptability, relatively low cost and limited access to modern health facilities (Kassaye *et al*., 2006). It has about 800 species of plants that are used in the traditional health care system to treat nearly 300 mental and physical disorders (Teklehaymanot *et al*., 2007). Several reports indicate that skin disorders are very common in Ethiopia (Tadeg *et al*., 2005).

There are many plants which are traditionally used for wound healing activities in Ethiopia. These include *Gallium thunbergianum* (Abiyu *et al*., 2014), *Rhusvulgaris ficuscaricus, Acacia abysinica* (Gebeyehu *et al*., 2014) and many others are being used in the treatment of wounds and other diseases in the traditional health care system of the country.

Several studies in Ethiopia show that various medicinal plants have wound healing property. Plants like *Achyranthes aspera* (Fikru *et al*., 2012), *Allophylus abyssinicus* (Yesuf and Asres 2013), *Kalanchoe petitiana A. Rich* (Mekonnen *et al*., 2013), *Rumexa abyssinicus* (Mulisa *et al*., 2015), *Vernonia amygdalina Del* (Gebeyehu *et al*., 2014) etc are scientifically proved to exhibit wound healing activity.

1.6. The Experimental Plant

The genus Vernonia contains about 500 species found mainly in tropical and warmer parts of North and South America, tropical Africa, Madagascar and South East Asia (Bremer, 1994). Traditionally *Vernonia amygdalina*, known as bitter leaf, is used for the treatment of diabetes, fever reduction, headache, venereal diseases, wounds, hepatitis and gastrointestinal problems (Igile *et al*., 1994; Akah *et al*., 1995; Akinpelu, 1999; Moundipa *et al*., 2000). Members of the genus Vernonia are good sources of sesquiterpene lactones such as vernolide, vernolepin, vernodalin and hydroxyvernolide known for their cytotoxic and antitumor activity (Kuo *et al*., 2003). The structure of triterpene 24-hydroxytaraxer-14-ene and other triterpenes namely, β-amyrin acetate, β-amyrin benzoate, lupeol and its acetate, β-sitosterol, sigmasterol and α-spinasterol were also isolated from *V. cinerea*. Vernolides are the major sesquiterpenoids and are reported as the active principle in this plant (Kuo *et al*., 2003).

Vernonia leopoldi (Sch. Bip. Ex Walp.) Vatke (Figure 1) belongs to the family Asteraceae. This species is endemic to Ethiopia and is found mainly in shoa and Gojjam but is occasional in Wellega, Keffa, Gonder and Tigray. It is locally known as Nechillo in Oromifa. It is a shrub or rarely wood herb that grows 0.5-2.5 m high. It is relatively fast growing and prefers altitude ranging from 1901 to 2940 m above sea level. The leaves of *V. leopoldi* are alternate, petiolate and decrease in size acropetally. They are dorsiventrally flatted, simple, symmetrical and ovate to ovate lanceolate. The apex is usually acuminate, sometimes attenuate, while the base is cuneate to decurrent (Stella, 1990; Mesfin, 2004).

In Ethiopia, the leaf of the plant is used for healing wounds by rubbing around the injured areas after crushing the fresh leaf with little water (Abiyu *et al*., 2014). Although the leaf of the plant is claimed to have wound healing activity when it is extracted using distilled water, the pilot study showed fast rate of wound contraction with 80% methanol extract in the excision wound models as compared to aqueous extract. In addition, plant of the same genus *Vernonia auriculifera Hiern* was investigated for anti-bacterial activity and tannins, flavonoids, terpenoids and saponins were isolated from methanol leaves extract, whereas anthraquinones, steroids and alkaloids were absent (Albejo *et al*., 2015).

According to the ethnobotanical (Abiyu *et al*., 2014) and traditional claims, *V. leopoldi* is a potential plant for the treatment of wounds. However, no scientific report could be found in the literature concerning the wound healing effects of the plant. It was, therefore, important to conduct scientific experiments to check whether the plant extract has biological activity for wound healing or not.

Figure 1: Photograph of *Vernonia leopoldi* at time of flowering from Gulele plant center, Addis Ababa

1.7. Rationale for the Study

Wounds remain a challenging clinical problem with early and late complications presenting a frequent cause of morbidity and mortality (Alonso *et al*., 1996; Natarajan *et al*., 2000).

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 (Roboson *et al*., 2001).

Furthermore, due to the complications that accompany acute wounds, when their healing does not progress in a timely and orderly manner, they can convert in-to chronic wounds, which are more difficult to manage (Natarajan *et al*., 2000).

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 (Mac Donald, 2009).

Increase in drug resistance of the microorganisms that cause infected wound like *P. aeruginosa, S. aureus, Streptococcus faecalis, E. coli, Clostridium perfringens, Clostridium tetani,* coliform bacilli, and enterococcus are a big problem that compromises the effectiveness of many antibiotics almost as quickly as scientists discover (Emele *et al*., 1999; Bowler *et al*., 2001; Radyowijati & Haak, 2002).

The use of traditional medicinal plants for primary health care has steadily increased worldwide in recent years (Samie *et al*., 2010). Because of this, 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 sever complications (Iyyam *et al*., 2010).

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 these situations have forced the scientists to seek alternatives drugs (James and Victoria, 2010). Considering the drawbacks of conventional medicine, medicinal plants provide excellent raw materials (biologically active ingredients) for the treatment of various diseases and disorders (Sabale *et al*., 2012).

There are many plants in Ethiopia and throughout the world which are traditionally used for treatment and management of wounds, among which *V. leopoldi* is one of them. Therefore, this study attempted to validate the traditional use of this medicinal plant and for further assurance of the crude extract has wound healing activity. Also, the study aimed to provide a clue about the qualitative phytochemical constituents of the plant so as to get insight into the nature of photochemical responsible for its action. The finding of this research could be used as an input in searching of new agent for wound healing, anti-inflammatory and antibacterial activities that might solve problems associated with the conventional drugs.

2. OBJECTIVES

2.1. General Objective

The aim of this study was to evaluate wound healing activity of 80% methanol leaf extract of *V. leopoldi* in mice.

2.2. Specific Objectives

The specific objectives of the study were:

- To investigate oral and dermal acute toxicity of 80% methanol crude leaf extract.
- To assess wound healing activity of crude leaf extract using excision and incision models
- To evaluate anti-inflammatory activity of 80% methanol crude leaf extract using carrageenan induced paw edema model in mice
- To determine antibacterial activity including its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).
- To perform preliminary phytochemical screening of secondary metabolites

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 *Chemicals, Reagents and Drugs*

The following chemicals and reagents were used. Methanol (Blulux, India, Purchased from ZAF pharmaceuticals Pvt.Ltd.Co), nitrofurazone 0.2% (Shanghai General Pharmaceuticals Co, Ltd, China), carrageenan (Sigma-Aldrich Steinheim, Germany), hard paraffin, white soft paraffin (BDH Laboratory Supplies Poole, England), cetosearyl alcohol, wool fat, hydrochloric acid, sulphuric acid, ketamine hydrochloride (Rotex Medica, Germany), indomethacin (Ajanta pharma Ltd, India), Muller Hinton agar (Himedia laboratories Pvt. Ltd, India), nutrient agar (Himedia laboratories Pvt. Ltd, India), nutrient broth (Oxoid Ltd, Basingstoke, Hampshire, England), Ciprofloxacin (Becton, Dickinson and Company), Cefoxitin, amoxicillin, ampicillin, tween 80 (Atlas Chemical Industries Inc, USA).

3.1.2. *Plant material*

The leaves of *V. leopoldi* were collected in July 2017 from Gulele plant center, Gulele sub-city, Addis Ababa, Ethiopia. Botanical identification and authentication were done by Mr. Melaku Wondafrash at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University and a voucher specimen (number AA-001) was deposited for future reference.

3.1.3. *Experimental animals*

Healthy adult Swiss albino mice of either sex (25-30 g, and 6-8 weeks of age) were procured from animal house of the Ethiopian Public Health Institute (EPHI). The animals were maintained with free access to food and water and kept under 12-hour light/ dark cycle. The animals were allowed to acclimatize to the laboratory condition for a week before the starting of the experiment. At the end of the experiment, the animals were sacrificed under high dose of diethyl ether.

3.1.4. *Bacterial test organisms*

The standard American Type Cell Culture (ATCC) gram positive bacterial species such as *Staphylococcus aureus* (ATCC29737) and *Streptococcus pyogenes* (ATCC19615) as well as

gram negative bacteria such as *Escherchia coli* (ATCC10536), *Klebsiella pneumoniae* (ATCC700609) and *Salmonella typhimurium* (ATCC14028) were obtained from Aklilu Lemma Institute of Pathobiology laboratory.

3.2. Methods

3.2.1. *Plant Extraction*

After collection, the fresh leaves were initially washed using running tap water to remove debris or dust particles and dried under shade for three weeks. The leaves were then coarsely powdered using a grinder. The powdered material (500 g) was macerated with 80% methanol (5000 ml) at solid to solvent ratio of 1:10 (w/v) for three days in a conical flask with occasional stirring and shaking using mini orbital shaker (Bibby Scientific Limited Stone Staffo Reshire, UK). The entire mixture was first filtered through a funnel plunged with muslin cloth two times and then the filtrate was passed through whatman filter paper (No 1) (Maidstone, Uk) under vacuum. After filtration the remaining residue or marc was re-macerated twice to obtain maximum yield. The resulting filtrate after successive filtration was evaporated using a rotary evaporator (Buchii model R-200, Switzerland) set at 40^0C to remove methanol. Finally, the concentrated aqueous solution was placed in deep freezer set at -20 ºC to solidify and dried in a lyophilizer (Operan, Korea vacuum limited, Korea). The resulting dry extract which was not gummy weighed to calculate the percentage yield, which was 18.1% (w/w). The extract was stored in a refrigerator for ointment preparation.

3. 2 .2 *Ointment formulation*

Simple ointment base for the 80 % methanol plant extract was prepared using the formula (Table 1) described in the British Pharmacopoeia (BP, 1988).

Table 1: Formula used for preparation of simple ointment

Ingredients	MF	RF
Wool fat	50 g	10 g
Hard paraffin	50 g	10 g
Cetostearyl alcohol	50 g	10g
White soft paraffin	850 g	170 g
Total	1000 g	200 g

MF, Master formula: RF, Reduced formula

Three ointment preparations (each 200 g), with (5 % w/w and 10%w/w of 80% methanol extract) and without (simple ointment only and served as a control) the extract were formulated using the reduced formula from the master formula (Table 1). To prepare the simple ointment base, the different ingredients were melted in a beaker over a water bath as per their descending order of melting point with constant stirring until they became homogeneous. The mixture was removed from the water bath and stirred until cold (Ansel, 1985).

To prepare 5% and 10% medicated ointment, first the extract was powdered in mortal and pestle. Then, 10 g and 20 g of the 80 % methanol extract was mixed with 190 g and 180 g of the ointment base, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture. Finally, the extract ointment was transferred to a clean container for topical application during the experiment (Ansel, 1985).

3.2.3. *Acute oral toxicity test*

Acute toxicity test was carried out using female mice based on the limit test recommendations of OECD 420 guideline (OECD 420, 2001). A single female mouse was used as sighting study for the extract and fasted for 4 h prior to the experiment and 2 h after the experiment. The mouse was administered with a single dose (2000 mg/kg) of extract dissolved in 2% tween 80 orally using oral gavage. The mouse was then observed for physical or behavioral changes within 24 h strictly, with special attention during the first 4 h. Since no death was observed within 24 h, additional four mice were administered with the same dose followed by similar strict observation. The observation was done for 4 h with 30 min interval during the experiment and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity, food and water intake and mortality.

3.2.4. *Acute dermal toxicity test*

Acute dermal toxicity was performed as per the OECD 402 guideline (OECD, 1987). Two groups of five female albino mice were randomly chosen and assigned to each group. Approximately, 10% of the body surface area hair was shaved from the dorsal part prior to the extract ointment application. The extract ointments (5% and 10%) were applied thinly and uniformly to the entire shaved site for a period of 24 h. The site was covered by gauze and

secured with non-irritating tape so as to keep contact with the skin. After 24 h, the gauze was removed and observed for inflammation. Additional observation for changes on the skin was also made for the following 14 days.

3.2.5. *Animal grouping and dosing*

For excision model, four groups of mice, each containing six mice were used. The first group was treated with simple ointment, and served as a negative control. The second and third groups were treated with 5% and 10% of 80% methanol leaf extract ointments, respectively. The fourth group was treated with nitrofurazone (0.2 %) and served as a positive control.

For incision model, five groups of mice, containing six mice per group were used. The animals of Group one up to four were treated in a similar fashion with excision wound model, but animals in the fifth group were left untreated and served as untreated negative control.

For assessment of anti-inflammatory activity, five groups of mice each containing six animals were used. Group I was given the vehicle (2% Tween 80) and served as a negative control. Groups II, III and IV received 100 mg/kg, 200 mg/kg and 400 mg/kg crude extracts of the leaves. Group V was treated with indomethacin (10 mg/kg) and served as positive control. Dose levels were chosen based on acute oral toxicity results as 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. All administrations were performed orally using gavage with a maximum volume of 1ml/100g. Extracts as well as standards were dissolved using 2% Tween 80.

3.2.6. *Wound healing test*

The effect of 80% methanol leaf extract was evaluated on excision and incision wound model in mice. The wound healing activity of the extract was assessed by the period of epithelialization and rate of wound contraction (Heidari *et al*., 2018) and by the extent of breaking strength (Nalwaya *et al*., 2009).

A. Excision wound model

On wounding day, animals were anesthetized using subcutaneous injection of ketamine (1 ml/kg) and diazepam (1 ml/kg). The dorsal fur of the animals was shaved with shaving machine and the anticipated area of the wound to be created was outlined on the back of the animals on the dorsal thoracic region 1 cm away from vertebral column. Excision wound was inflicted by cutting away a 300 mm^2 full thickness of skin from a predetermined area; the wound was left undressed to the open environment. The mice were then randomly divided into four groups as described earlier (6 mice per group) and each mouse was placed in a separated cage. The wounding day was considered as day 0. The standard drug, extract, and simple ointment were applied topically to the respective groups till the wound was completely healed (Kokane *et al*., 2009; Kodanti *et al*., 2011). In this model, wound contraction and epithelialization period were monitored. Wound contraction was measured as percent contraction every 2 days after wound formation (Lodhi *et al.*, 2006).

Measurement of wound contraction

Contractions, which contribute for wound closure in the first two weeks, were studied by tracing the wound on a transparent paper initially. An impression was then taken on a millimeter scale graph paper (Rout, 2015). The wounds were monitored and the area of wound was measured on 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 post wounding day. The wound healing effect was calculated (taking the initial size of wound i.e. 300 mm^2 as 100 %) by using the following formula (Sharma *et al*., 2011).

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

Epithelialization time measurement

The epithelialization period was determined by considering the number of days required for falling off of the scab without any residual raw wound (Shenoy *et al*., 2011).

Histopathological analysis

For histopathological examination; animals were anaesthetized before taking skin samples using diethyl ether (Londi *et al*., 2016). Samples of healed skin tissue were taken from the animals of control, standard and treated groups of excision wound models (Shenoy *et al*., 2011). The skin specimens from each group were collected at 14 and 19 days after beginning of the experiment to evaluate the histopathological alterations. The analysis was performed by a blinded Dermatopathologist Dr. Adane Ayele from Addis Ababa University. Samples were fixed in 10% buffered formalin, processed and blocked with paraffin. Samples were then sectioned into 5 μm-thick sections and stained with hematoxylin and eosin (HE). The tissues were examined by light microscope (Olympus BX51 attached DP70 Digital Camera System) and graded subjectively as mild (+), moderate (++) and severe (+++) for epidermal or dermal remodeling, re-epithelialization; fibroblast proliferation, mononuclear and/or polymorphonuclear cells and collagen depositions in dermis were analyzed to score the epidermal or dermal remodeling (Fujita *et al*., 2003; Sumitra *et al*., 2009; Isabela *et al*., 2013).

B. Incision wound model

Animals were anesthetized in the same manner as described for excision wound model. The dorsal fur of each mouse was then shaved and a three cm long longitudinal paravertebral incision was made through the skin and subcutaneous tissue. The parted skin was then sutured one cm apart using a surgical thread (no. 000) with curved needle (Figure 6A). The continuous thread on both wound edges was tightened for good closure of the wounds. After 24 h of wound creation (on day 1), animals were treated as described under grouping and dosing section, with topical formulation of vehicle, extract or standard daily for nine days, leaving out the last group without applying any of the interventions. The sutures were removed on day 8 post-incision and treatment was continued up to 9^{th} day (Rajeev Kumar *et al*., 2011; Wang *et al*., 2011). Tensile strength was measured on the 10^{th} post-wounding day using continuous water flow technique (Lee, 1968) (Figure 2).

Measurement of tensile strength

The force required to open the healing skin (tensile strength) was used to measure the extent of wound healing. On the 10^{th} day after wounding, each mouse was anesthetized using diethyl ether to secure animal to the table. The two forceps were firmly applied 1 cm away from healed tissue on the incised part of the skin onto the line facing each other. One of the forceps was supported firmly; whereas the other was connected to a freely suspended light weight plastic bag container through a string run over to a pulley. 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 was determined and noted as an indirect measure of breaking strength in grams (Rajeev *et al*., 2011; Mulisa *et al*., 2015). Tensile strength was measured using the formula given below (Akkol *et al*., 2011).

$$\%\ \text{Tensile of strength (TS) extract} = \frac{\text{TS extract- TS s.o}}{\text{TS s.o}} \times 100$$

$$\%\ \text{Tensile of strength (TS) of reference} = \frac{\text{Ts reference-TS s.o}}{\text{TSs.o}} \times 100$$

$$\%\text{Tensile strength (TS) of s.o} = \frac{\text{TSs.o-TSl.u}}{\text{TSl.u}} \times 100$$

Where Ts is tensile strength, s.o is simple ointment and lu is left untreated

Figure 2: Water flow technique for measurement of tensile strength

3.2.7. *Antibacterial activity assay*

The anti-bacterial activity of the plant extract was assessed by agar well diffusion and micro-dilution methods. The agar well diffusion method was used for determination of zone of inhibition of the plant extract against test organisms, while the micro-dilution method was used for determination of MIC and MBC.

Inoculum preparation

The inocula preparation was carried out by growth methods. The test organisms were grown in a nutrient agar medium to get a pure colony at 37 ^{o}C for 24 h. Three to five pure colonies were then selected and transferred in to a sterile test tube containing 5 ml of sterile nutrient broth and solutions were mixed by vortex mixer. The broth culture was incubated at 37 ^{o}C until it reached a turbidity of 10^{5}-10^{6} CFU/mL, which was equivalent to 0.5 Mc Farland standards (usually about 8 h) and turbidity (organism suspension) was adjusted visually by comparing the test with the standard (NCCLS, 2000).

Agar well diffusion

Anti-bacterial activity of the extract was analyzed using agar well diffusion assay according to the technique described elsewhere (Sahm and Washington 1990; Lino and Deogracious, 2006). Bacterial broth cultures were prepared to a density of 10^{8} cells ml^{-1} as described in the inoculums preparation section. The aliquot was spread evenly onto Muller Hinton agar plates by sterile cotton swab. On each plate, six equidistant wells were made with a 6 mm diameter sterilized cork borer, 2 mm from the edge of the plate. Five of the holes were aseptically filled with 50 µl of different concentrations of the plant extract (50 µg/µl, 100 µg/µl, 200 µg/µl, 400 µg/µl and 500 µg/µl) sequentially. Ciprofloxacin, amoxicillin, ampicillin and cefoxitin drugs discs were also placed on the culture to be used as positive controls. The vehicle, distill water, was used as negative control. Petridishes were placed for 2 h to allow diffusion of the extract in to the agar and then turned upside down. The wells were labeled with a marker and then incubated at 37 ^{o}C for 24 h. At the end, the inhibition zones formed were measured to the nearest millimeters. The experiment was performed in triplicates and average values were taken as the diameter of the inhibition zone.

Determination of minimum inhibitory concentration

Resazurin based Microtitre Dilution Assay was used to determine the minimum MIC of the plant extracts using 96 well microtitration. The first row of microtiter plate was filled with 100 µl of test materials in 2.5% Dimethyl Sulfoxide (DMSO) using sterile automatic pipette (Medical diagnostic *in vitro* device, Italy). The solvent DMSO (2.5%) that would not inhibit growth of the microorganisms (Zgoda and Porter, 2001) was used as the negative control for all the experiments. All the wells of microtitre plates were filled with 100 µl of Muller Hinton broth. A twofold serial dilution was done by mixing the contents in each well of the first row and transferring 100 µl test material to the second well of the same column and the same was done up to the last well of the same column. At the end, the last 100 µl from the last well was discarded so as each wells had 100 µl. Finally, a volume of 10 µl bacterial suspension was added to each well to achieve a final concentration of 5×10^6 CFU/ml. To avoid dehydration of the bacterial culture, each plate was wrapped loosely with cling film. Each microtitre plate had a set of two controls: (a) a column with all solutions with the exception of the test extract, and (b) a column with all solutions except bacterial solution replaced by 10 µl of Muller Hinton broth. The results were observed after 24 h incubation at 37 ^{o}C, followed by the addition of 25 µl Resazurin solution in each well as indicator after a further incubation of 4h at 37 ^{o}C. The colour change in the well was then observed visually. Any colour change observed from purple to pink or colourless was taken as positive. The lowest concentration of plant leaf extract at which no colour change occurred was recorded as the MIC value. All the experiments were performed in triplicates. The average values were taken for the MIC of test material (NCCLS, 1999; Ramalivhana *et al*., 2014).

Determination of minimum bactericidal concentration

The MBC of plant extracts were determined by taking a loop full of samples from wells having no visible growth in MIC assay and sub-cultured into antibiotic free agar medium (Chukwudi and Mohammed, 2013; Mathur, 2013). In this procedure, the loop was dipped into each wells having a concentration of plant extract greater than or equal to MIC and inoculated to agar medium All test bacterial species were then incubated at 37 $^{\circ}$C for 24 h. The least concentration of the plant extract which inhibited colony formation on solid agar medium was considered as

MBC. The entire test was done in triplicate for each bacterial species and MBC was the average of three triplicates.

3.2.8. *Anti-inflammatory activity test*

The acute anti-inflammatory activity of the extract was determined by carrageenan induced paw edema model (Winter *et al*., 1962; Adeyemi *et al*., 2002). Following overnight fasting with free access to water, the basal volume of the right hind paw of each mouse was determined before administration of any drug using plethysmometer (Ugo Basile , Italy) (Padilha *et al*., 2010). After determination of the basal volume, the animals were divided into five groups such that the mean volumes of the different groups were similar. Extract (100 mg/kg, 200 mg/kg and 400 mg/kg), standard (Indomethacin, 10 mg/kg) and vehicle (2% tween 80) administered orally by gavage. One hour later the animals were injected with 0.05 ml of solution of 1 % carrageenan in 0.9 % saline (w/v) in the sub plantar region of the right hind paw to induce inflammation (Rahman *et al*., 2011). The paw volume was measured 1, 2, 3 and 4 h after injection of the carrageenan. The difference between the paw edema after and before (basal volume) carrageenan injection was taken as the volume of edema and determined for each mouse (Recio *et al*., 1995; Marrassini *et al*., 2010). The percentage inhibition of oedema for each group was calculated using the following formula described by Mahomed and Ojewole, (2004) and Owoyele *et al*. (2009).

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

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

3.2.9. *Preliminary phytochemical screening*

The qualitative phytochemical investigations of 80% methanol extract of *V. leopoldi* leaves were performed using standard tests (Harborne, 1998; Sheel *et al*., 2014; shetty *et al*., 2016; Sahalie *et al*., 2018) as illustrated below.

Test for terpenoids (Salkowski test)

0.25 g of the extract was added to 2 mL of chloroform. Concentrated H_2SO_4 (1.5 mL) was added carefully. A reddish-brown coloration of the interface indicates the presence of terpenoids.

Test for saponins (frothing test)

0.5 g of extract was added to 5 mL of distilled water in a test tube. The solution then shaken vigorously and observed for a stable persistent froth.

Test for tannins (ferric chloride test)

About 0.25 g of the extract was boiled in 10 ml of water in a test tube and then filtered. Three drops of 0.1% ferric chloride were added to the filtrate. Presence of tannins was confirmed by the formation of brown greenish or blue-black color.

Test for steroids (Liebermann Burchard reaction)

1 g of extract were added to 10 mL of chloroform and then filtered. 2 mL of acetic anhydride and concentrated H_2SO_4 was added to the 2 mL of the extract. Formation of blue, greenish coloration indicates the presence of steroids.

Test for alkaloids

The methanolic plant extract was warmed with 2% H_2SO_4 for two minutes. It is filtered and few drops of reagents were added separately. A creamy-white colored precipitation (Mayer's reagent) appeared giving a positive result or a reddish-brown precipitate appeared (Wagner's reagent) which also confirms the presence of alkaloids in the extract.

Test for flavonoids

Ten ml of ethyl acetate was added into a test tube having 0.25 g of 80% methanol extract and each solvent fraction, and heated on a water bath for 3 minutes. The mixture was cooled and filtered. Then, 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonial layer indicated the presence of flavonoids.

Test for Phenols

About 0.25 g of 80% methanol extract and each solvent fraction was treated with few drops of 5% neutral ferric chloride solution; the appearance of a greenish color indicated the presence of phenols.

3.3. Statistical analysis

The raw data obtained from the experiments were expressed as mean ± SEM (standard error of the mean). The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Post Hoc Tukey test using SPSS version 20 software and data were considered significantly different at $p < 0.05$.

4. RESULTS

4.1. Acute Oral Toxicity Test

The 80% methanol extract of *Vernonia leopoldi* was found to be safe up to 2000 mg/kg body weight by oral route. None of the animals died and there were no observed signs of toxicity till the end of the 14th day. Morphological characteristics (skin, fur and eye) appeared normal. No tremors, convulsion, diarrhea salivation, diarrhoea were observed. Therefore, the LD_{50} of the plant is greater than 2000 mg/kg.

4.2. Acute Dermal Toxicity Test

The purpose of this study was to assess the skin irritation potential of the extract from a single topical application. During the entire period of experimentation, no animal showed any sign of inflammation, edema and other behavioral changes (Figure 3).

A B C

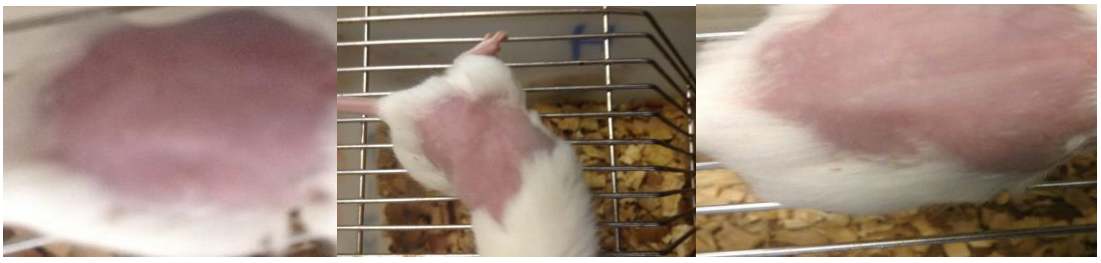

Figure 3: Photograph of acute dermal toxicity result: Simple ointment (A), 5% extract (B) and 10% extract (C)

4.3. Wound Healing Study

4.3.1. *Excision wounds*

Wound contraction

Topical applications of ointments of the 80% methanol extracts of *V. leopoldi* leaves showed significant effect on wound healing process in mice (Figure 4). The progress of wound contraction induced by treatment of 5% (w/w) and 10% (w/w) ointment of 80% methanol extract, simple ointment base, and nitrofurazone 0.2% (w/w) ointment were shown in Table 2.

The 10 % (w/w) crude extract ointment treated group showed significant ($P < 0.05$) wound contraction starting from the second day. This effect was highly significant ($P < 0.001$) from 6th day onward in comparison with the control group (simple ointment). As shown in Table 2, there was no significant difference in wound healing activity between the 10% (w/w) and 5% (w/w) extracts, but higher rate of wound closure was observed with 10% (w/w) ointment.

The animals treated with 5% (w/w) crude extract ointment also showed significant wound contraction from 6th day onward as compared to control group ($P < 0.05$).

Significant ($p<0.05$) wound contraction was observed for nitrofurazone 0.2% (w/w) ointment treated group from the 4th day. This effect was highly significant ($p<0.001$) from 6th day onwards as compared to the control group.

A B

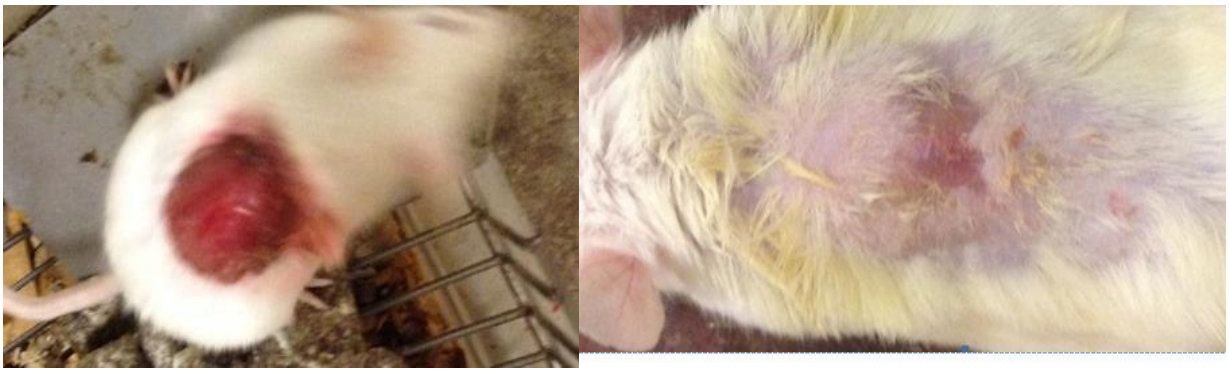

Figure 4: Photograph of Excision wound test result for 10 % extract: Wound day 0 (A) and Day 13 (B)

Table 2: Effect of topical application of 80% methanol *V. leopoldi* leaf extract ointment on excision wound model in mice

Group	Wound area (mm^2) post- wounding days								
	2	4	6	8	10	12	14	16	18
Simple ointment	271.25±6.87 (9.58%)	219.91±12.26 (26.69%)	176.29±14.86 (41.23%)	146.27±13.25 (51.24%)	104.87±7.14 (65.04%)	72.85±9.01 (75.71%)	44.60±8.84 (85.13%)	19.16±4.90 (93.61%)	7.08±3.67 (97.64%)
Nitrofurazone 0.2% ointment	255.09±9.96 (14.97%)	176.59±8.14* (41.13%)	88.98±6.94*** (70.34%)	65.49±6.43*** (78.17%)	41.40±4.41*** (86.20%)	19.27±3.42*** (93.57%)	4.87±2.74*** (98.37%)	0 100%	0
5% extract	263.37±10.72 (12.21%)	182.32±13.21 (39.22%)	100.56±12.07** (66.48%)	79.85±15.49** (73.38%)	52.85±12.89** (82.38%)	34.83±9.46** (88.39%)	16.58±5.67** (94.47%)	3.75±1.79** (98.75%)	0 100%
10% extract	227.29±9.26* (24.23%)	148.40±7.06** (50.53%)	85.39±9.25*** (71.53%)	50.44±6.61*** (83.18%)	28.88±4.42*** (90.37%)	15.53±4.90*** (94.82%)	3.95±1.89*** (98.68%)	0 100%	0

Values are expressed as mean ± SEM (n=6 animals in each group) and analyzed by one way ANOVA followed by tuckey post hoc test; *p<0.05, **p<0.01, ***p<0.001 when compared to negative control group.

The maximum percentages of wound contraction were observed in animals treated with 10% extract ointment on the 10^{th}, 12^{th} and 14^{th} day, which were 90.37%, 94.82% and 98.68%, respectively. Comparable percentages of wound contraction (86.20%, 93.57% and 98.37%) were observed in animals treated with the standard drug on the 10^{th}, 12^{th} and 14^{th} day. However, there was no significant difference in wound healing activity between 5% and 10% extracts and the standard drug. Furthermore, complete wound closure was observed in 10% (w/w) extract and standard ointment treated groups on day 16.

Although the 10% extract treated group showed relatively maximum percentage of wound contraction on the 10^{th}, 12^{th} and 14^{th} day as compared to nitrofurazone treated group, the overall study result (incision wound model and histopathological analysis of healed wound) showed as the standard drug was more effective for wound healing activity.

Epithelialization Period

The time for complete epithelialization was short in extract ointment and nitrofurazone treated groups as compared to control (simple ointment treated group). The period of epithelialization was 18.50, 14.83, 16.50, and 14.33 for control group, standard drug, and 5% (w/w) and 10% (w/w) extract ointment, respectively. Both standard drug and 10% (w/w) ointment showed significant ($p<0.01$) difference of epithelialization period as compared to the control group. Moreover, they showed faster rate of epithelialization compared to 5% (w/w) extract but the difference failed to reach statistical significance. Likewise, 5% (w/w) extract treated group failed to produce a statistically significant difference as compared to the control group. There was no significant difference of epithelialization period between 5% (w/w), 10% (w/w) extract and standard drug (Table 3).

Table 3: Effect of topical application of 80% methanol *V. leopoldi* leaf extract ointment on period of epithelialization

Group	Period of epithelialization (days) Mean ± SEM
Simple ointment	18.50±0.670
0.2 w/v Nitrofurazone	14.83±0.752**
5% (w/w) extract	16.50±0.718
10% (w/w) extract	14.33±0.718**

Values are expressed as mean ± SEM (n = 6), **p<0.01, compared to control group; one way ANOVA followed by tuckey post hoc test.

Histopathological analysis

The histological profiles of granulation tissue of control and treated animals are shown in Table 4 and Figure 5. Granulation tissue of healed wound in the extract and standard drug treated groups exhibited relatively moderate number of inflammatory cells and moderate epithelialization on day 13, whereas, reduction of the inflammatory cells was observed on day 18. In contrast, negative control showed persistence of inflammatory cells.

Higher collagen fibers were observed on day 13 for both extract treatment group and positive control group, while negative control group showed less collagen fiber formation. On day 18, the amount of collagen and fibroblasts increased for both extract groups (5% and 10% w/w extract) and positive control group, while the negative control group (simple ointment group) showed small amount of collagen formation. Standard drug treated wound exhibited higher collagen fibers as compared to 10% extract on day 18. On day 18, 10% extract exhibited collagen deposition in normal proportion and organization as compared to the standard drug.

Table 4: Qualitative histological determination of wound healing process of 80% methanol *V. leopoldi* leaf extract at different time interval

Group	CD		FB		MNC		PMC		NV	
	day 13	day 18	day 13	day 18	day 13	day 18	day 13	day 18	day 13	day 18
Simple ointment	+	+	+	+	+++	++	+++	++	+	+
0.2% w/v nitrofurazone	++	+++	++	+++	++	+	++	+	+	++
5% w/w extract	+	++	+	++	++	+	++	+	+	+++
10% w/w extract	++	+++	++	+++	++	+	++	+	+	++

+ (mild), ++ (moderate), +++ (severe) for epidermal or dermal remodeling, FB (Fibroblast), CD (Collagen deposition), MNC (mononuclear cells), PMN (polymorphonuclear cells), NV (neovascularization).

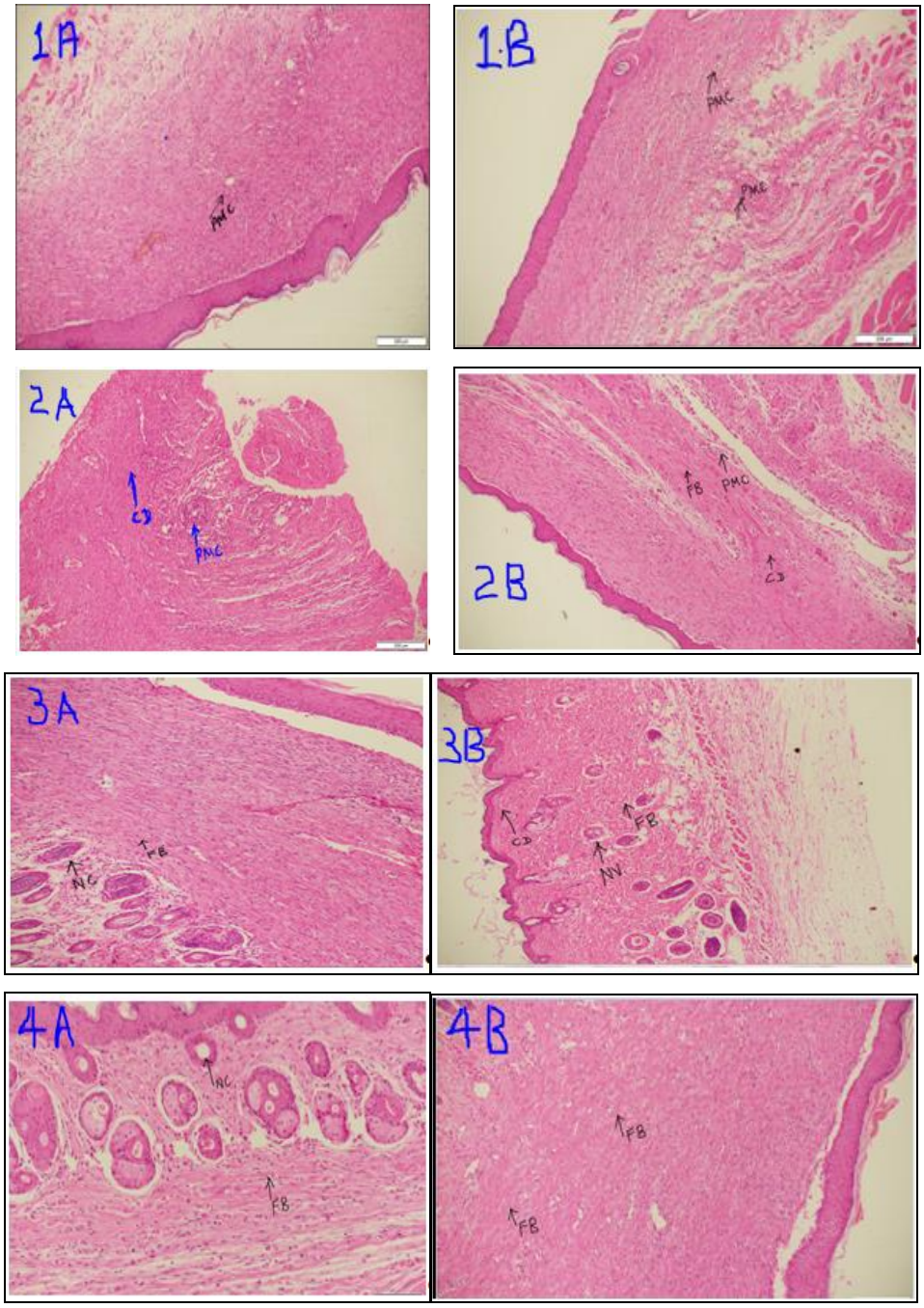

Figure 5: Haematoxylin and eosin stained sections of the granulation tissue from excision wound model in mice at different time intervals.

Simple ointment (1); 5% extract (2); 10% extract (3); Nitrofurazone (4); A and B represent staining obtained on day 13 and 18, respectively; PMC, polymorphonuclear cells; CD, collagen deposition; FB, Fibroblast; NV, neovascularization.

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As depicted in Figure 5, histopathological changes revealed polymorphonuclear cell infiltration on day 13 and day 18 excision wound of the control group (Figure 5. 1A, B), whereas polymorphonuclear cell infiltration observed on day 13 granulation tissue from 5% extract treated group (Figure 5. 2A). Granulation tissue of healed wound in simple ointment treated group exhibited less collagen deposition, fibroblast and neovascularization on both day13 and 18 (Figure 5. 1A, B). Whereas, 10% extract treated exhibited high amount of collagen deposition, fibroblast and fewer neovascularization as compared to 5% extract treated group on day 13 and 18, which exhibited less collagen deposition, fibroblast and higher neovascularization. Well organized bands of collagen observed on day 18 excision wound of 10% treated group (Figure 5. 3A, B). Higher collagen fiber, fibroblast and lesser blood capillaries were observed in standard treated group as compared to 5% extract treated group (Figure 5. 4A, 4B). In extract treated group, healing was advanced compared to control group as evidenced by fibroblast proliferation and collagen deposition (Figure 5. 2A, 2B, 3A, 3B).

4.3.2. *Incision wounds*

The standard drug, 10%(w/w) extract, and 5% (w/w) extract treated groups showed significant increase in breaking strength by 74.82 ($p<0.001$), 55.51 ($p<0.01$), and 30.72% ($p<0.05$), respectively, when compared to the negative control (Table 5 and Figure 6). In this finding, the increase in tensile strength was found to be higher in nitrofurazone as compared to 10% and 5% extract ointment treated groups, although it failed to reach statistical significance with 10% of the extract. By contrast, tensile strength in group treated with 10% extract was significantly higher ($p<0.05$) than 5% extract (Table 5).

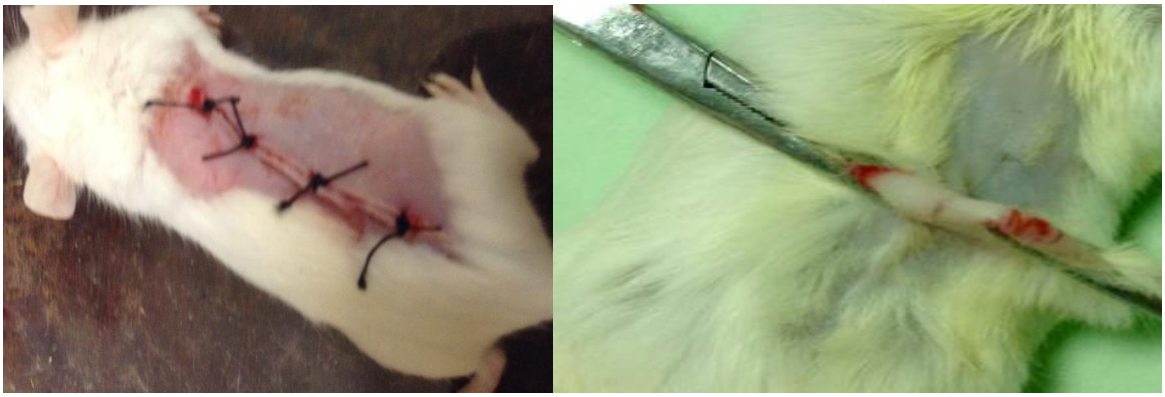

Figure 6: Incision wound: Wound day 0 (A) and Healed wound (B)

Table 5: Effect of topical application of the 80% methanol leaf extract of *V. leopoldi* on tensile strength in incision wound model

Group	Tensile strength in gram (mean±SEM)	% Tensile strength
Untreated group	209.00±10.592	-
Simple ointment	255.50±12.503	22.24%
0.2% w/v Nitrofurazone	446.67±12.219 [a3b3]	74.82%
5%w/w extract	334.00±16.141[a2]	30.72%
10% w/w extract	397.33±8.492[a3b1]	55.51%

Values are expressed as mean ±SEM (n=6), one way ANOVA [a] against control group treated with simple ointment base, [b] against 5% (w/w) extract, [1]p<0.05, [2]p<0.01, [3]p<0.001

4.4. Antibacterial Activity

i. Zone of inhibition

The antibacterial activity of various concentrations of the extract is depicted in Table 6 and Table 7. Activity appeared to be concentration dependent and gram positive organisms seemed to be more susceptible than gram negative organisms at all extract doses (Figure 7). *S. aureus* (50-500 µg/µl) and *E. coli* (at 400 µg/µl and 500 µg/µl) were the most susceptible gram positive and gram negative organisms, respectively. The extract with increasing concentration produced a significant increase in zone of inhibition compared to the standard in gram positive organisms, but the situations was reversed in gram negative organisms.

As depicted in Table 6, the most susceptible gram positive organism at all extract doses was standard strains of *S. aureus*. However, the strains of *S. pyogen* were relatively less susceptible compared to the standard strains *S. pyogen* at comparable concentration of the crude extract.

Among the gram negative organisms, standard strains of *E. coli* was most susceptible followed by *S. thyphimurium* at 400 µg/µl and 500 µg/µl of the crude extract. However, the strains of *K. pneumoniae* were totally not susceptible compared to the other bacterial species by comparable concentration of the crude extract.

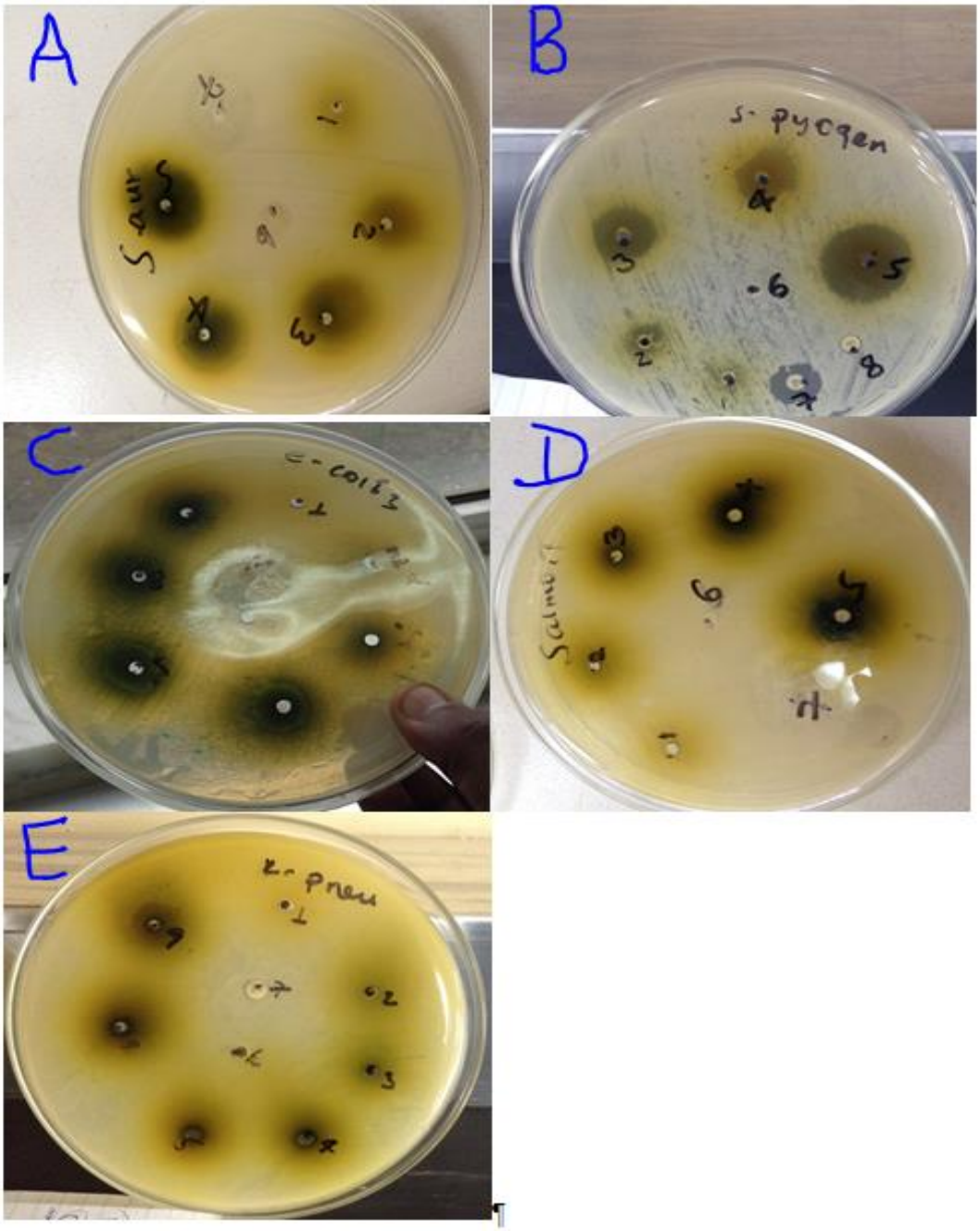

Figure 7: Zone of inhibition of 80% methanol leaf extract of *V. leopoldi* against five different bacteria strains.

S. aureus (A); *S.pyogen* (B); *E.coli* (C); *S.thyphimurium* (D); *K. peumoniae* (E); 1, 2, 3, 4 and 5 represent inhibition of zones exhibited by the extract at 50 µg/µl, 100 µg/µl, 200 µg/µl, 400 µg/µl and 500 µg/µl concentration respectively.

Table 6: Antibacterial activities of 80% methanol leaf extract of *V. leopoldi* against gram positive bacteria

Test bacteria	Extract & control	Concentration of extract					
		50 µg/µl	100 µg/µl	200 µg/µl	400 µg/µl	500ug/µl	(+) control
S. aureus	80% methanol	10.33±1.20^{adef3c2}	14.67±0.88^{adef3b2}	21.33±0.67^{a1bcef3}	26.50±0.50^{bcd3}	28.33±0.33^{a1bcd3}	
	Cefoxitin (30 µg)						24.67±0.67
S. pyogenes	80% methanol	7.33±0.67^{a2def3}	12.67±0.88^{e2f3}	18.33±1.45^{b3f2}	22.00±1.52^{ac1b3}	26.83±2.45^{abc3d2}	
	Amoxicillin (30 µg)						15.67±0.88
	Ampicillin (10 µg)						00±0.00

Values are expressed as mean ±SEM (n=3), one way ANOVA followed by tuckey post hoc test [a] compared with control value, [b] to 50 µg/µl, [c] to100 µg/µl, [d] to 200 µg/µl, [e] to 400 µg/µl, [f] to 500 ug/µl g; [1]p<0.05, [2]p<0.01, [3]p<0.001. 0= no activity

Table 7: Antibacterial activities of 80% methanol leaf extract of *V. leopoldi* against gram negative bacteria

Test bacteria	Extract & control	Concentration of extract					
		50 µg/µl	100 µg/µl	200 µg/µl	400 µg/µl	500ug/µl	(+) control
E. coli	80% methanol	0	0	0	13.33±0.88[a3]	15.67±1.20[a3]	
	Ciprofloxacin (5 µg)						28.00±0.57
S. thyphimurium	80%methaol	0	0	0	7.67±0.33[a3f2]	9±0.57[a3e2]	
	Ciprofloxacin (5 µg)						33.00±0.0
K. pneumoniae	80% methanol	0	0	0	0	0	
	Cefoxitin (30 µg)						8.00±0.00

Values are expressed as mean ±SEM (n=3), one way ANOVA followed by tuckey post hoc test [a] compared with control value, [e] to 400 µg/µl, [f] to 500 ug/µl g; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$. 0= no activity, 0 =no activity

ii. Minimum inhibitory concentration and Minimum bactericidal concentration

MIC assay was also employed to evaluate effectiveness of the extracts to inhibit the growth of the bacterial test organisms. The more susceptible is the bacterium, the lower is the concentration of the extract required for growth inhibition in most of the test bacteria. The lowest MIC (1.04 µg/µl) was exhibited by methanol leaf extract against *S. aureus*, whereas the highest MIC (233.33 µg/µl) was exhibited by *S. thyphimurium* (Table 8). MBC value of extract, which was determined by sub-culturing the contents of wells having concentration of extract greater than or equal to MIC in prepared agar plate, was presented in Table 7 and 2.08 µg/µl (*S.aureus*) was recorded as a minimum value for 80% methanol leaf extract. Based on the MIC index determination method (Table 8) the crude extract showed bactericidal activity against all the test bacterial strains.

In general, the plant extract was more potent and killed gram positive than gram negative bacteria at lower concentration.

Table 8: Minimum inhibitory concentrations and minimum bactericidal concentration of 80 % methanol leaf extract of *V. leopoldi*

Bacterial strains.	MIC (µg/µl)	MBC (µg/µl)	MIC index
S. aureus	1.04	2.08	2
S. pyogen	4.16	8.33	2
E. coli	116.67	233.33	1.99
S. typhmurium	233.33	311.11	1.33

MIC= Minimum inhibitory concentrations, MBC= Minimum bactericidal concentration

MBC/MIC ≤ 4: Bactericidal effect, > 4 MBC/MIC < 32: Bacteriostatic effect

4.5. In-vivo Anti-Inflammatory Activity

On the first and second hour after administration of carrageenan, only the higher dose (400 mg/kg) of the extract and the standard drugs treated animals showed significant reduction of edema as compared to the negative control (Table 9). On the last consecutive two hours, the standard drugs were showen to reduce edema ($p<0.001$) compared to negative control, ($p<0.01$) to the smallest dose and ($p<0.01$) to the middle dose (200 mg/kg) of the extract. The higher dose (400 mg/kg) of the extract also reduced edema significantly at the third ($p<0.01$) and the fourth hour ($p<0.001$) compared to negative control. Lower dose (100 mg/kg) also suppresses edema formation when compared to control but failed to reach significant level. The middle dose showed reduction of edema ($p<0.01$) at the 3rd and the 4th hour compared to the negative control. There were significant differences between the middle dose and the standard drug at 1and 2 h ($p<0.05$) but failed to reach statistical significance with the higher dose (400 mg/kg) at all-time points.

Table 9: Anti-inflammatory activity of the 80% methanol leaf extract of *V. leopoldi* using carrageenan induced paw edema

	Mean increase in paw edema volume in ml				
Groups	Basal	1h	2h	3h	4h
Control	0.66±0.03	0.68±0.01	0.71±0.06	0.61±0.06	0.50±0.06
Indomethacin	0.65±0.02	0.41±0.05^{a3b3c1} (39.71%)	0.30±0.03^{a3b2c1} (57.75%)	0.20±0.02^{a3b2} (67.21%)	0.10±0.03^{a3b2} (80%)
V.leopoldi, 100 mg/kg	0.633±0.05	0.66±0.02 (2.94%)	0.58±0.07 (18.30%)	0.5±0.08 (18.03%)	0.35±0.06 (30%)
V.leopoldi, 200 mg/kg	0.617±0.01	0.56±0.03 (17.64%)	0.53±0.04 (25.35%)	0.30±0.02^{a2} (50.82%)	0.18±0.04^{a2} (64%)
V.leopoldi, 400 mg/kg	0.61±0.03	0.51±0.01 ^{a2b1} (25%)	0.38±0.05 ^{a2} (46.48%)	0.26±0.03 ^{a2b1} (57.67%)	0.15±0.02^{a3b1} (70%)

Values are expressed as mean ±SEM (n=6), one way ANOVA [a] against control, [b] against 100 mg/kg extract, c against 200 mg/kg [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$

4.6. Preliminary Phytochemical Screening

Qualitative phytochemical screening of 80% methanol leaf extract of *V. leopoldi* showed the presence of saponons, tannins, terpenoids, flavonoid, phenols and steroids as shown in table 10; however, the extract was found to be negative for alkaloids and steroids.

Table 10: Preliminary phytochemical screening of 80% methanol leaf extract of *vernonia leopoldi*

Secondary metabolite	Result
Alkaloids	-
Tannins	+
Saponins	+
Terpenoids	+
Flavonoid	+
Phenols	+
Steroids	-

(+= present, - =absent)

5. DISCUSSION

Optimal topical wound treatments must be biocompatible, nontoxic, and safe. Medicinal plant derived compounds for enhancing cutaneous wound healing are attractive treatment options because they are both cost-effective and safe. People used a number of plants and/or their derivatives as wound healing agents assuming that they are effective and safe without scientific validation of their pharmacological parameters (Sabale *et al*., 2012). The objective of this study was to evaluate the wound healing, anti-inflammatory and antibacterial activities of the leaves of *Vernonia leopoldi*.

Since a single model may not be sufficient to collectively present all the components of wound healing processes, two different animal models (excision and incision) were used to evaluate the wound healing activities of 80% methanol leaf extract ointment of *V. leopoldi* in the present study. Even though a large effort has been made to study *in vitro* wound healing activity, *in vivo* studies still remain indispensable for wound healing activity investigation as wound healing is a complex and dynamic process of restoring cellular structure and tissue layers in damaged tissue as closely as possible to its normal state (Abdulla *et al*., 2010).

Wound contraction indicates the rate of reduction of the unhealed area during the healing process. Thus, a fast rate of wound contraction indicates better efficacy of medication. Wound contraction plays a crucial role as it minimizes the dimension of the wound and hence shortens the healing time. Moreover, contraction reduces the amount of extracellular matrix needed to repair the defect and assists re-epithelialization by shortening the distance migrating keratinocytes travel (Prasad and Dorle, 2006; Mulisa *et al*., 2015). In the present study, treatment with 10% extract and the standard drug significantly enhanced the rate of wound contraction and epithelialization, and provided strength to the regenerated tissue as evidenced by the increased levels of collagen (Figure 5: 3B and 4B). The histopathological evaluation strongly supported the biochemical results which indicated a significant increase in collagen and fibroblastic deposition in standard and 10% treated animals as compared to control group. The results of wound healing effects of *V. leopoldi* showed significant enhancement of wound healing activity with both 10% (w/w) and 5% (w/w) extract 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. The higher wound contraction rate of the 10% (w/w) extract ointment may be due to its higher anti-inflammatory effect than 5% (w/w) extract ointment.

Epithelialization which is an essential component of wound healing is used as a defining parameter of successful wound closure ability (Pastar *et al*., 2013). As epithelialization proceeds, contractile property of myofibroblasts is enhanced, epithelial cells are proliferated and crawl across the wound bed to cover it, where these proliferation and migration processes along with contractile property of myofibroblasts are attributed to the significant effect of the extracts on the epithelialization period (Samanta *et al*., 2016). The epithelialization time was significantly reduced from 18 days (control) to 14 days for 10% extract (Table 3). The occurrence of enhanced epithelialization and wound contraction could be due to the ability of the extracts to enhance collagen synthesis as evidenced by histopathological analysis (Figure 5). The changes in the 10% extract might be attributed to the potential of the test extracts or its constituents to promote epithelialization either by facilitating proliferation or by increasing the viability of epithelial cells (Mukherjee *et al*., 2013)

Enhanced healing activity has been attributed to increased collagen formation, angiogenesis and fibroblast proliferation (Shukla *et al*., 1999; Habibipour *et al*., 2003). Collagen imparts strength and elasticity to healed skin. As the wound heals, collagen molecules are synthesized and laid down at the wound site. These molecules become cross-linked to form fibers (Pather *et al*., 2011). In this study, the strength of the repaired wound tissue might be the result of the remodeling of collagen and the formation of stable intra- and inter-molecular cross linking which is necessary for maturation of collagen as described elsewhere (Pather *et al*., 2011; Abraham *et al*., 2012) using different plant extracts. Accordingly, these results may suggest that the extracts could increase collagen synthesis (Figure 5: 3A and 3B). Angiogenesis in granulation tissues on the other hand improves circulation to the wound site thereby providing oxygen and nutrients necessary for healing process that include re-epithelialization (Szabo *et al*., 1995).

The wound healing potential of the crude extract is further evidenced by the results of the incision wound model where an increased wound tensile strength was seen on the 10^{th} post wounding day. The breaking strength was measured in incision wound model as a parameter

which shows how much the repaired tissue resists to breaking under tension. The percentage of increase in breaking strength of animal wounds treated by 5% (w/w), 10% (w/w) extract, and 0.2% nitrofurazone ointments were 30.72%, 55.51%, and 74.82%, respectively. However, the percentage of the breaking strength of simple ointment treated group was 22.24%, which was twofold less than the percentage of 10% (w/w) extract ointment. The effect could be due to the presence of secondary metabolites such as flavonoids, tanins, saponins, which are responsible for enhancement of collagen maturation which gives strength and integrity to the wound matrix (Umadevi *et al*., 2006). Furthermore, the increment in tensile strength may be associated with the promotion of collagen synthesis, angiogenesis, and stabilization of fibers and hence the overall effect improves circulation for oxygen and nutrients supply that are vital for wound healing cascade (Arun *et al*., 2016).

Both microbes and endotoxins can lead to a prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. This can cause a chronic inflammatory state that promotes the development of matrix metalloproteases (MMPs), thus inhibiting wound healing. (Guo and Dipietro., 2010; Joseph, 2011). Thus, the antimicrobial activity of the extract on these wounds may partly contribute to the wound‑healing effect by eliminating infection and thus allowing initiation of natural tissue repair processes.

The antibacterial activity of the extract was more pronounced on the gram-positive bacteria than the gram-negative bacteria. The reason for the difference in sensitivity between gram positive and gram-negative bacteria might be ascribed to the differences in morphological constitutions between these microorganisms, gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances. The gram-positive bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of gram negative organisms are more complex in lay out than the gram positive ones acting as a diffusion barrier and making them less susceptible to the antimicrobial agents than are gram positive bacteria (Bland *et al*., 2001). The reason for the difference in sensitivity between gram positive and gram negative bacteria are concordant with other studies described elsewhere (Tadeg *et al*., 2005).

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-epithelialize (Burke *et al*., 2006; Röhl *et al*., 2015). Previous reports indicate that a number of plants with anti-inflammatory activity do also possess wound healing effect. These includes Memecylon endule Roxb (Nualkaew *et al*., 2009), *Calophyllum inophyllum Linn* (Van *et al*., 2017) and. *Aloe megalacantha Baker* (Leake *et al*., 2018).

Carrageenan induced hind paw edema model has been used widely for the discovery and evaluation of anti-inflammatory drugs. This model is based upon the ability of such agents to inhibit the edema produced in the hind paw of the mice after injection of a phlogistic agent. One of the most commonly used *in vivo* animal assays is the one that measures the ability of anti-inflammatory agents to inhibit edema induced in mice paw by carrageenan (Padilha *et al*., 2010).

The present *in vivo* study revealed anti-inflammatory activity of the 80% methanol leaf extract of *V. leopoldi* in carrageenan induced paw oedema model in mice. This study showed that unlike the higher dose, the smallest dose and the middle dose had no significant anti-inflammatory activity during the first and second hour but inhibition of edema formation was highest on the last consecutive two hours (3^{rd} and 4^{th} h) after carrageenan injection (Table 9). The reason could be the smallest and middle dose might be able to achieve maximum plasma concentration at 3 h for third phase inhibition. Finding of this study was in agreement with that of dichlomethane root extract of *Solanum incanum L.* where significant edema reduction was observed at the late phase of inflammation (Mwonjoria *et al*., 2014).

The percent inhibition of edema of the extract was higher in the late phase (after 2 h) demonstrating that the active ingredients have potently inhibited the release of prostaglandin like substance involved in the inflammatory response. Finding of this activity was supported by *in vitro* anti-inflammatory study described elsewhere (Getie *et al*., 2003).

The preliminary phytochemical analysis of *V. leopoldi* extract revealed the presence of flavonoids, triterpenoids, saponins, tannins and phenolic compounds. These metabolites are usually responsible for the pharmacological activities of medicinal plants (Nair *et al*., 2005). Saponins and flavonoids have been reported to possess wound-healing activity (Jian, 2010). Terpenoids are known to promote wound-healing process, mainly due to their astringent and antimicrobial activities which seem to be responsible for wound contraction and increased rate of epithelialization (Scortichini, 1991). Tannins are seen to be active detoxifying agents and inhibit bacterial growth (Chirchir *et al*., 2014).

Although this study was able to show the potential wound healing, anti-inflammatory and antibacterial effects of the *V. leopoldi* extract, it does not conclude which metabolites are responsible for the observed effects. Thus, wound healing property of *extract* may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect. It is, therefore, expected that subsequent studies on the leaves of *V. leopoldi* be directed at fractionating, isolating and characterizing the active ingredients and proposing their mechanisms of action.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

In this study, the different phases of wound repair, including wound contraction, epithelialization and collagen synthesis (observed on histo-pathological analysis) were improved by 80% methanol extract of *V. leopoldi* leaves as compared to control group. It was observed also that the extract has anti-inflammatory and antibacterial activity. Therefore, these activities were the possible mechanisms for wound healing effect exerted by *V. leopoldi*. The observed results justify the use of the leaves of *V. leopoldi* for wound as claimed in ethnobotonical study and traditionally.

6.2. Recommendations

Based on the findings of this study, the following works are suggested for further investigation on the plant in-depth.

- ✓ Performing wound healing and anti-inflammatory activity tests with various solvent fractions
- ✓ The results of the present study should be corroborated with hydroxyproline assay
- ✓ Chronic toxicity studies should be performed
- ✓ As chronic wounds such as diabetic wounds are major global burden, it is worthwhile to study the activity of the plant on chronic wounds.
- ✓ Conducting quantitative phytochemical study to clearly quantify the active components against wound and inflammation from the plant

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