Evaluation of Wound Healing Activity of 70% Ethanol Leaf Extract of *Becium grandiflorum* Lam. (Lamiaceae) in Mice

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This is to certify that the thesis prepared by Kald Beshir, entitled "Evaluation of Wound Healing Activity of 70% Ethanol Leaf Extract of *Becium grandiflorum* Lam. (Lamiaceae) in Mice" and submitted in partial fulfillment for the requirements of 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 70% Ethanol Leaf Extract of *Becium grandiflorum* Lam. (Lamiaceae) in Mice

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Based on traditional claims, leaves of *Becium grandiflorum* were investigated for their potential wound healing activity using excision and incision wound models. In addition histological analysis, antiinflammatory and antibacterial activity of the leaves was performed. Wound healing activity was studied by topical application of simple ointment of the leaf extract at a concentration of 5% and 10% (w/w) after it was extracted by maceration using 70% ethanol. Toxicity of the formulated ointments was studied by skin irritation test on Swiss albino mice. For antiinflammatory study, carrageenan-induced hind paw edema model was used at a concentration of 100, 200 and 400 mg/kg. The antibacterial activity of the extract was examined using disk diffusion technique against several pathogenic bacterial strains that are commonly occurred in wound at a concentration of 50, 100 and 200 µg/µl. The ointment formulation of the extract was found to be non-irritant at 5% concentration. However, continuous application of 10% showed skin irritation. Treatment of wound with the ointments exhibited significant ($p< 0.001$) increase in wound contraction rate, shorter epithelization time, higher skin tensile strength that was supported by considerable deposition of collagen, fibroblast proliferation and vascularization form histological analysis. The plant extract also showed significant ($p<0.001$) inhibition of inflammation and antibacterial activities, that could be a mechanism of wound healing. From the results obtained, it can be concluded that the 70% ethanol extract of *B. grandiflorum* enhance

wound healing activity most probably via its higher collagen deposition, antiinflammatory and antibacterial effects, supporting the traditional use of this plant as wound healing agent.
Key words: Wound healing, Excision model, Incision model, Histophatological analysis, Antiinflammatory, Antibacterial, *B. grandiflorum*.

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ANOVA	Analysis of Variance
BFGF	Basic Fibroblast Growth Factor
B. grandiflorum	*Becium grandiflorum*
BP	British Pharmacopoeia
C5a	Complement 5a
CAGR	Combined Annual Growth Rate
CFU	Colony Forming Units
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
EWMA	European Wound Management Association
GAGs	Glycosaminoglycans
HA	Hyaluronic acid
IL	Interleukin
K. pneumonia	*Klebsiella pneumonia*
LPO	Lipid Peroxidation
MBC	Minimum Bactericidal Concentrations
MRSA	Multi Drug Resistant *Staphylococcus aureus*
MIC	Minimum Inhibitory Concentration
MMP	Matrix Metalloproteinase
OECD	Organization of Economic Corporation and Development
P. aeruginosa	*Pseudomonas aeruginosa*

P. mirabilis	*Proteus mirabilis*
PDGF	Platelet-derived Growth Factor
PMNLs	Polymorph Nuclear Leucocytes
S. aureus	*Staphylococcus aureus*
S. pnuemonea	*Streptococcus pnuemonea*
S. pyogens	*Streptococcus pyogens*
SEM	Standard error of the mean
TGF-β	Transforming Growth Factor-beta
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF-α	Tumor Necrosis Factor alpha
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WHO	World Health Organization
WHS	Wound Healing Society

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1. INTRODUCTION

1.1 Overview of Wound

In normal skin, the epidermis and dermis form a protective barrier against the external environment. Once this barrier is broken, a wound will be inflicted (Nagori and Solanki, 2011). Wound is a rupture in the epithelial integrity of the skin which arises due to physical or chemical injuries or microbial infections (Farahpour and Habibi, 2012).

On the basis of physiology of wound healing, wounds can be classified as acute or chronic. Acute wounds are tissue injuries that heal through an orderly sequence of physiological events which results in sustained restoration of anatomic and functional integrity, and have generally less than 8 weeks. In this type of wound *Staphylococcus aureus* is the most important pathogen (Bowler and Davies, 1999). On the other hand, chronic wounds are wounds that have failed to proceed through an orderly and timely process to produce anatomic and functional integrity even after 3 months (Lazarus *et al.*, 1994; Bowler, 2002). Wound Healing Society (WHS) classifies chronic wounds into four categories based on their etiologies: pressure ulcer, diabetic ulcer, venous ulcer and arterial insufficiency ulcer (Graves and Zheng, 2014). The latent microorganisms of both aerobic and with higher prevalence of anaerobic bacteria (e.g. *Peptostreptococcus* spp., *Bacteroides* spp., *Porphyromonas* spp. and *Prevotellas*) are common in chronic wounds (Bowler and Davies, 1999).

In most chronic wounds, the healing process is thought to be 'stuck' in the inflammatory or proliferative phases (Enoch and Price, 2004). In addition, oxidative damage by free radicals or condition-specific factors such as neuropathy in diabetes or ischemia in peripheral

vascular disease may lead to the non-healing nature of chronic wounds (Fadeev and Nemtseva, 2009).

Every year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring (Meier and Nanney, 2006). The prevalence and costs of chronic wounds is increasing globally. Venous leg ulcers alone typically consume 1–3% of healthcare budgets. The number of diabetic foot ulcers is expected to reach some 380 million by 2025, representing 7.1% of the adult population worldwide (Lantis and Price, 2011).

In developing countries, such as sub-Saharan African and South Asian countries, about 1% to 2% of the population experiences a chronic wound during their lifetime. The prevalence of chronic wounds was reported as 4.5 per 1,000 populations, whereas that of acute wounds was nearly double at 10.5 per 1,000 populations (Sasidharan *et al.*, 2010; Siddiqui and Bernstein, 2010).

In Africa the rate of surgical site infection varied from 2.5% to 30.9% following various types of surgical procedure (Bagheri *et al.*, 2011). In Ethiopia, Hawassa University Referral Teaching Hospital, study indicated that *S. aureus*, *Klebsiella*, *Escherichia coli*, and *Pseudomonas aeruginosa* were the most important bacteria responsible for post-operative wound infection in the hospital and high rates of drug resistance to some commonly used antibiotics were observed (Guta *et al.*, 2014). Other study from Armed Force Referral and Teaching Hospital, Ethiopia, show that the multi-drug resistance (MDR) of gram positive and gram negative bacteria isolates were 73.6% and 67.6%, respectively, out of which 79.2% of *S. aureus* were resistant to three or more antibiotics (Ayalew, 2014).

1.2. Wound healing cascade

Wound healing is the process of repairing the injured part of skin and other soft tissues (Nayak *et al.*, 2007). Healing begins the instant the wound is made (Molnar, 2007). All tissues in the body are capable of healing by one of two mechanisms: regeneration or repair. Regeneration is the replacement of damaged tissues by identical cells and is more limited than repair. In humans, it occurs in a limited number of cells for example, epithelial, liver and nerve cells. The main healing mechanism is repair where damaged tissue is replaced by connective tissue which then forms a scar (Diegelmann and Evans, 2004).

Wound healing occurs as a sequential cascade of four overlapping but well-defined phases; hemostasis, inflammation, proliferation and remodeling (Figure 1) (Clark, 1996; Deore *et al.*, 2014).

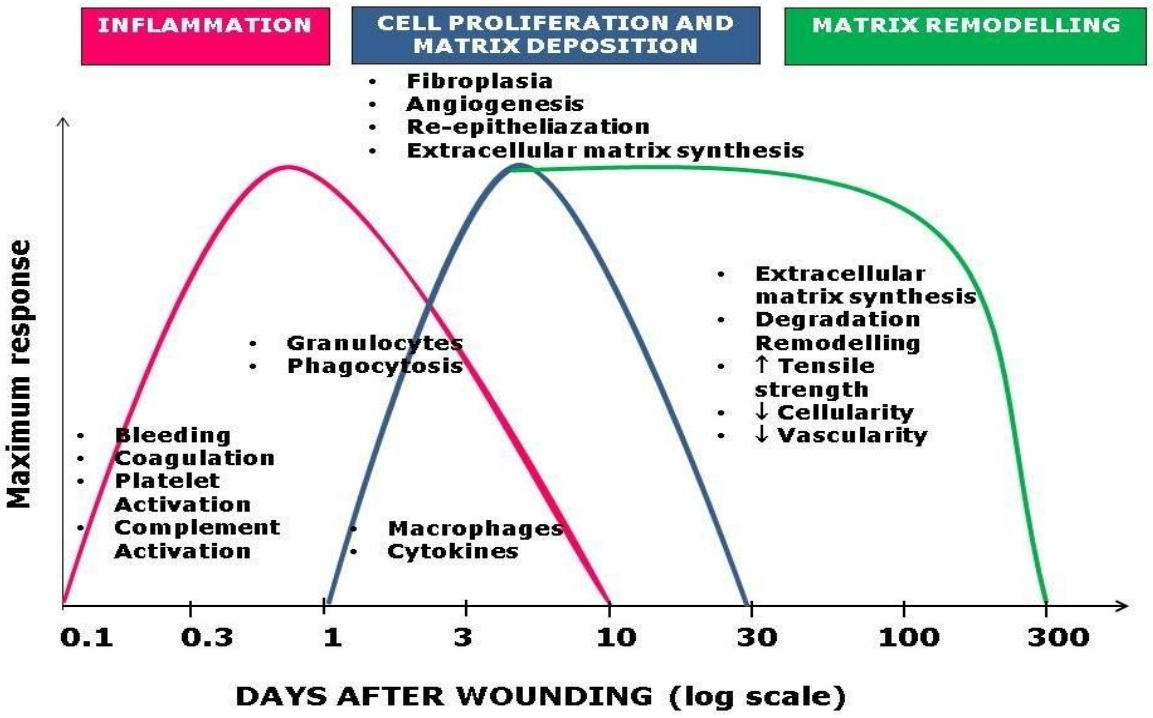


Figure 1: Wound repair phases in acute wound

1.2.1. Hemostasis

Injured vessels constrict rapidly and the coagulation cascade is activated via Hageman Factor XII to limit blood loss (Grinnell *et al.*, 1981; Williamson and Harding, 2004). Blood fibrinogen quickly converts to fibrin, which, along with platelets, helps to form a temporary protective barrier called scab. Platelets contain dense bodies that store vasoactive amines such as serotonin that increase micro-vascular permeability. This leads to the exudation of fluid into the extravascular space and results in tissue edema (Enoch and Price 2004). The leaked fluid or exudate is rich in cytokines that stimulate new tissue growth (Rodriguez *et al.*, 2008).

1.2.2. Inflammation

The inflammatory phase begins as early as two hours after injury. The crucial inflammatory cells are polymorphonuclear leukocytes (PMNLs) mostly referred to neutrophils from granulocytes and activated monocytes or macrophages from agranulocytes. They are responsible for bound wound bed preparation of the healing wound (Menke and Diegelmann, 2006). **Early stage of inflammation** begins with the activation of complement and infiltration of the wound with granulocytes or PMNLs. These cells are attracted to the wound site by complement 5a (C5a), platelets, formyl-methionyl peptide products from bacteria and Transforming Growth Factor-beta (TGF-β) (Enoch and Price 2004). Neutrophils release a battery of proteolytic enzymes, such as elastase and matrix metalloproteinase (MMP)-8, to assist in their movement through the tissue and needed for the removal of damaged extracellular matrix (ECM). Once in the wound environment, they begin the debridement of devitalized tissue and phagocytize bacteria and other foreign particles **(**Li *et al.*, 2007). On the other hand, other type of PMNL, mast cell granules are

filled with enzymes such as chymase, tryptase, histamine and other active amines which cause the classic signs of inflammation: rubor (redness), calor (heat), tumor (swelling), and dolor (pain) when released (Diegelmann and Evans, 2004).

In the **later stages of the inflammatory process,** blood monocytes undergo a phenotypic change on arrival at the wound site to become tissue macrophages. Appear to act as the key regulatory cells for repair. They release cytokines and growth factors into the wound, recruiting fibroblasts, keratinocytes and endothelial cells to repair the damaged blood vessels (Leibovich and Ross, 1975). Redundant cells are cleared away from the wound by extrusion to the wound surface as slough or phagocytosis by macrophages (Diegelmann and Evans, 2004). The lymphocytes, the other agranulocyte cells, are the last cell type to enter the wound during the inflammatory phase (>72 h after wounding) and play a key role in regulation of collagenase, indicating that the lymphocyte may be involved in collagen and ECM remodeling (Enoch and Price, 2004).

1.2.3. Proliferative phase

The proliferative phase starts at about day three and lasts for two weeks after wounding. The proliferative phase includes; neoangiogenesis, formation of granulation tissue and ECM, and re-epithelialization (Schreml *et al.*, 2009). Key cells for these processes are the fibroblasts and keratinocytes (Eaglstein, 2001).

i. Fibroblast migration

Fibroblasts are dermal cells that produce hyaluronan (glycosaminoglycans), fibronectin, proteoglycans, collagen and numerous other substances that comprise the ECM (Lee *et al.*, 2004; Ghosh *et al.*, 2006). Wound fibroblasts tend to be involved in the process of wound

contraction by the help of actin after differentiation into myofibroblasts (Hinz and Gabbiani, 2003; Junkera *et al.*, 2008). Contraction decreases healing time because it decreases the size of the wound and reduces the amount of ECM needed to repair the defect (Lorenz and Longaker, 2008) and shorten re-epithelization by the distance migrating keratinocytes must travel (Satish and Kathju, 2010).

ii. Collagen synthesis

Collagen is synthesized by fibroblasts and provides strength and integrity for all tissues in the body. Collagen is first released in precursor form as a triple helix protein called procollagen. Procollagen is then formed into fibers that are arranged in parallel fashion and cross-linked to form thicker and stronger strands. This process converts the loose granulation tissue matrix into a stable ECM (Kjær, 2004).

iii. Angiogenesis

The process of restoring the vascular network is called neovascularization or angiogenesis. It is stimulated by growth factors and tissue hypoxia (Van der Veera *et al.*, 2009). TGF-β and Platelet-derived growth factor (PDGF) attract macrophages and granulocytes and promote angiogenesis. Macrophages in particular play a key role in angiogenesis by releasing other angiogenic substances including Tumor Necrosis Factor alpha (TNF-α) and basic fibroblast growth factor (bFGF). Angiogenic capillary sprouts invade the fibrin/fibronectin-rich wound clot and organize into a microvascular network throughout the granulation tissue within a few days. As collagen accumulates in the granulation tissue to produce scar tissue the density of blood vessels diminishes (Tonnesen *et al.*, 2000).

vi. Granulation tissue formation

Granulation occurs as the fibrin clot scaffold is replaced with new tissue rich in hyaluronan, fibronectin, and other ECM components. The predominant cell type found in granulation tissue is the fibroblas (Strodtbeck, 2001). ECM is composed of substances that promote adhesion and migration (fibronectin); glycoaminoglycans that promote tissue hydration (hyaluronan); proteoglycans involved in the regulation, migration, storage and expression of growth factors, enzymes, and coagulation proteins (chondroitin sulfate); and glycoproteins that provide tissue strength and resiliency (collagens, elastin) (Gurtner *et al*., 2008). The structure and composition of granulation tissue undergoes constant change in collagen composition as it matures (Palpandi *et al*., 2010).

v. Re-epithelization

Re-epithelization is the process of covering of the skin defect by largest group of epithelial cells within the epidermis called keratinocytes (Ziegler *et al*., 1997). Because the site of proliferation is usually proximal to the injury, the new keratinocytes must migrate to the repair site. Migration requires a fluid environment in the absence of a fluid surface, the keratinocyte secretes proteolytic enzymes that enable it to burrow downward to find the necessary moisture for migration (Schultz *et al*., 2003). Keratinocytes also participate in shaping the ECM by expressing surface markers that enhance migration across the matrix. Once migration is complete, the keratinocytes stabilize themselves by forming firm attachments to each other and the new basement membrane (Gurtner *et al*., 2008). When the skin surface is completely covered with new epidermal cells, the wound is considered closed. Early closure of an open wound with a viable epidermis is essential because it induces remodeling of the underlying tissue (Strodtbeck, 2001).

1.2.4. Remodeling phase

Matrix synthesis and the remodeling phase are initiated concurrently with the development of granulation tissue and continue over prolonged periods of time up to two years (Clark *et al.*, 1995). The wound develops its final strength during this stage of wound healing. However, a maximum of 80 % unwounded skin strength can be achieved (Levenson *et al.*, 1965). The key cells for remodeling are macrophages and fibroblasts. ECM reshaping by cross-linking collagens, cell maturation, and program cell death or apoptosis are the mechanisms used in wound remodeling (Strodtbeck, 2001). There is ongoing collagen synthesis and breakdown as the ECM is continually remodeled, equilibrating to a steady state about 21 days after wounding. This breakdown continues for 6 months to 1 year after injury. 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 (Ferguson and O'Kane, 2004). Collagen degradation is achieved by specific MMPs that are produced by fibroblasts, granulocytes and macrophages. As remodeling of the wound continue MMP activity decreases and tissue inhibitors of metalloproteinases (TIMPs) activity increases. TGF-β plays an important role in mediating this, as it promotes matrix accumulation (Enoch and Price 2004).

With time, the density of cells is reduced by apoptosis triggered by unknown sources (Desmoulière *et al.*, 1995). Keratinocytes are the first cells to undergo programmed cell death; myofibroblasts are the second. It has been suggested that apoptosis may be signaled by the withdrawal of cytokines as the wound heals, other theories exist myofibroblast differentiation itself signaling apoptosis (Jürgensmeier *et al.*, 1994). Remodeling is thus a balance between the synthesis of new collagen and the degradation of

old (Robson *et al.*, 2001). As the wound healing process 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

A number of factors can lead to impaired wound healing. These factors can be generally 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; Robbins and Cotran, 2014). Oxygenation, infection, foreign body, venous insufficiencies are some of the local factors affecting wound healing. Some of the systemic factors are age, gender, sex hormones, stress, ischemia; diseases like diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia and obesity; medications like glucocorticoids, non-steroidal antiinflammatory drugs, chemotherapy; alcoholism and smoking; immune-compromised conditions like cancer, radiation therapy, acquired immune deficiency syndrome (AIDS); and nutrition (Stadelmann *et al.*, 1998; Guo and DiPietro, 2010; Nagori and Solanki, 2011; Vowden, 2011).

Infection may prolong the inflammatory phase of the wound and thus leads to the failure of wound healing (Abo *et al.*, 2004). The supposed mechanism by which wound infection occurs is believed to be liberation of bacterial enzymes and MMPs that may degrade fibrin as well as wound growth factors (Stadelmann *et al.*, 1998). During the prolonged inflammatory phase, neutrophils produce free radicals, which will result in oxidative stress leading to lipid peroxidation (LPO), DNA breakage and enzyme inactivation (Douglas and Alan, 2003; Kumar *et al.*, 2007).

Nutritional deficiencies can impede wound healing. Vitamin A is required for epithelial and bone formation, cellular differentiation, and immune function. Vitamin C is necessary for collagen formation, proper immune function, and as a tissue antioxidant. Vitamin E is the major lipid-soluble antioxidant in the skin. Glucosamine appears to be the rate-limiting substrate for hyaluronic acid (HA) production in the wound. Tissue levels of the amino acids arginine and glutamine may influence wound repair (Douglas and Alan, 2003).

1.4. Wound management

The main objective of holistic wound management is to facilitate healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to repair and regeneration (Mohanty *et al.,* 2010).

The overall treatment of wound depends on the type, cause, and depth of the wound. Treatment of recent lacerations involves examining, cleaning, and closing the wound. However, since chronic wounds harbor microbes, antibiotics are chosen based on their ability to inhibit the growth of pathogenic organisms (Lipsky and Hoey, 2009). Some examples of the preparations include amikacin (in gel or cream), bacitracin, chloramphenicol, clindamycin (cream, lotion, and foam), gentamicin (in ointment or cream), nitrofurazone (solution, or soluble dressing) and polymyxin B **(**Lipsky and Hoey, 2009; Sasidharan *et al*., 2010).

Further, negative pressure wound device and advanced silver-containing dressing products help the body achieve the ideal moist and warm state. In addition, protected wound healing environment, skin substitutes for burn wounds, growth factors and biologic wound products and hyperbaric oxygen are among the modern treatments (Esimone *et al.,* 2005).

1.5. Medicinal plants used in the management of wound

Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns (Kokane *et al.*, 2009). Between 70% and 95% of citizens in the majority of developing countries, and 70% to 90% of populations in some industrialized nations use traditional medicine as primary health care to address their health-care needs and concerns. Populations using traditional medicine for primary care in African countries accounts for 90% in Ethiopia, 75% in Mali, 70% in Rwanda, 60% in Tanzania, and 60% in Uganda (Robinson and Zhang, 2011).

In Ethiopia, the practice of traditional medicine in general and herbal medicines in particular has been the main stay management for majority of the rural population since long time ago. In Ethiopia there are about 800 species of plants that are used in the traditional health care system to treat nearly 300 mental and physical disorders; and many studies show that skin disorders including wounds are very common in the country (Teklehaymanot *et al.*, 2007). The wide spread use of traditional medicine among both rural and urban population could be due to their cultural acceptability, physical accessibility and economic affordability as compared to modern medicine (Prasad *et al.*, 2012).

Herbal preparations and their products are considered an imperative and main source of modern medicine in the globe (Garg and Sardana, 2016). As a result of this, a huge number of drugs are produced from such preparations. Studies estimate that at least 25% of all modern medicines are derived from medicinal plants and approximately one-third of all traditional medicines in use are applied for the treatment of wounds and skin disorders (Mantle *et al.*, 2001). However, the availability of modern drugs used for the treatment of wound accounts only for 1-3% of the total drugs. Many of these drugs are not only

expensive but also pose problems such as allergy and drug resistance (Prasad and Dorle, 2006).

Several studies from different parts of the world show that various medicinal plants have wound healing property. Plants like *Alternanthera sessilis, Carica papaya, Catharanthus roseus, Cecropia peltata, Clerodendrum serratum, Euphorbia hirta, Euphorbia nerrifolia, Ginkgo biloba, Lycopodium serratum, Morinda citrifolia, Ocimum sanctum, Pterocarpus santalinus, Sesame indicum, Trigonella foenum-graecum* etc. are scientifically proved to exhibit wound healing activity which affect various processes of the wound healing, such as coagulation, inflammation, epithelization, collagenation and wound contraction (Asif *et al.*, 2007; Nagori and Solanki, 2011).

In Ethiopia, plant extracts are tested for their wound healing activities. For example, crude extracts and various fractions of *Rumex abyssinicus* (Mulisa *et al*, 2015), 70% ethanol extract and the different solvent fractions of *Allophylus abyssinicus* (Yesuf and Asres, 2013), crude extract and various fractions of *Kalanchoe petitiana* (Mekonnen *et al*., 2013) were tested for their wound healing, antiinflammatory and antibacterial activities.

1.6. The experimental plant

B. grandiflorum Lam. (Lamiaceae) (Figure 2) is a drought tolerant species endemic to Ethiopia and Eretria (Paton, 1995; Bein *et al*., 1996). It is locally known as Tebeb (Tigrigna), and Mentesie or Matosh (Amharic). It is medium sized, perennial aromatic woody shrub, which grows in highlands and mid-altitude areas ranging from1600 m to 3100 m above sea level. The species grows on eroded soils, particularly in rocky slopes and sandy soil, in mountain bush land and pastures (Fichtl and Admassu, 1997).

B. grandiflorum is valued for various purposes. The numerous showy pale pink flowers, together with their violet veins and fragrance, are very attractive to honeybees. It is one of the major honey plants of Tigray region (Fichtl and Admassu, 1997).

According to ethnobotanical studies and traditional claims, *B. grandiflorum* Lam. is a potential plant for the treatment of many ailments. Besides, plant of the same species *B. grandiflorum* var. *obovatum* was investigated for medicinal activity in Natal, South Africa. Two new triterpenoid saponins, beciumecine 1 and 2, were isolated from the root bark of *B. grandiflorum* var. obovatum, eliciting the typical response in cancer cells. It was found that dilution of a hot water extract of the powdered root bark of this plant induces the formation of numerous intra-cytoplasmic vesicles in the cancer cells within 48 h. These vesicles tended to increase in size and eventually inhibited cell division and cell growth (Burger *et al*., 1998). Other plant of the same genus *Becium obovatum* used traditionally for gastrointestinal infections, anthelminthic, swellings/warts, genital stimulant/ depressant (Okach, 2013).

Traditionally, farmers in the Tigray region use the branches of *B. grandiflorum* for making "*shintar*" (one-fingered fork) that is used for eating the traditional food made from barley flour called "*Tihlo*'' (Haftom, 2012). The species is also used for fuel wood, broom, roofing, human food (the whole flower is plucked from a branch and eaten fresh), food flavouring, soil and water conservation. *B. grandiflorum* is also used as a traditional medicine against malaria (Nurya, 2010; Haftom, 2012), "*Mich*" (Teklehaymanot *et al*., 2007) and for treatment of Black spider bite that ends with wound (Teklay *et al*., 2013).

To assess other traditional uses, an interview was conducted with traditional medicine practitioners practicing in Adishuhu and Machew, Tigray, and the practitioners confirmed the use of the plant for wound healing, respiratory depression, for swellings and influenza. Traditionally, the fresh leaves are crushed and applied directly to the wound or it is also applied to wound after raw leaves are chopped and mixed with butter, other mechanism is directly rubbing fresh leaves on inflamed wounds.

Figure 2: Photograph of matured *Becuim grandiflorum* at time of flowering

1.7. Rationale for the study

Wound is a clinical entity and is as old as mankind. The incidence of wound is common worldwide and usually occurs while people perform their daily activity. When acute wound healing does not progress in an orderly and timely manner, or inappropriate treatment of wound occur, incisions can dehisce; hernias can form; anastomoses can leak; and fistula can develop (Robson *et al.*, 2001). Chronic or non-healing wounds are the most important medical problems which represent a significant cause of morbidity, disability, socio-economic crisis and mortality for a large portion of the population (Etufugh *et al.*, 2007; Kokane *et al.*, 2009**)**.

Wounds do not have a one-dimensional impact but rather can impact under three domains; that is, to the individual, the health service and to society. Psychosocial damage incurred by patients is incalculable. They also face disability that results in lost wages, decreased productivity and a diminished quality of life. Furthermore, chronic wounds have the potential for cellulitis, abscess formation, osteomyelitis, gangrene, sepsis and even malignant transformation (i.e., Marjolin's ulcer) (Menke *et al.*, 2007).

The emergence of antibiotic-resistant strains of bacteria, mostly those wound causing bacteria is a public health crisis globally. Methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA) and multi-drug resistant *Pseudomonas* and *Acinetobacter* species are some examples (Sharpe and Concannon, 2012). As a result, wound infection is persisting as one of the most serious influencing factors for the existence of non-healing wounds and remains a significant burden for patients and caregivers alike (EWMA, 2013).

Efforts are being made all over the world to discover agents that can promote healing and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications (Iyyam *et al.*, 2010). However, the holistic management and current medicine for treating of chronic wound is very expensive. In England, the National Health Service (NHS) described that the cost incurred for wound treatment is between £2.5 billion and £3.1billion per year, accounting for 3% to 4% of the healthcare budget (Vowden and Vowden, 2016). The resulting costs of wound care are staggering. The global advanced wound management market valued at $20 billion and is expected to grow at a combined annual growth rate (CAGR) of 7% for the next four years to reach approximately $26 billion by 2018 (Brocair, 2015).

Many of the available drugs for wound management are not only expensive but also pose problems such as allergy and drug resistance (Prasad and Dorle, 2006). In addition it is only 1 to 3% of drugs listed in Western Pharmacopoeia are intended for use on the skin and on wounds; but at least one third of herbal remedies are for such uses (Sasidharan *et al.*, 2010). So, phytomedicines for wound healing are required to tackle the cost, adverse effects and antibiotic resistance. However, there is a need for scientific validation, standardization and safety evaluation of plants used in traditional medicine before these could be recommended for healing of wounds (Raina *et al.*, 2008). The present plant *B. grandiflorum* is traditionally claimed for having wound healing activity, which requires scientific investigation. The finding of this study may serve as baseline information for scientific community for further investigation, and it may initiate advanced studies on identification of the chemical compound responsible for the wound healing, antiinflammatory and antibacterial activities.

2. OBJECTIVES

2.1. General objective

The general objective of this study was to evaluate wound healing activity of crude extract of *B. grandiflorum* leaves *in vivo*.

2.2. Specific objectives

- To investigate oral and dermal acute toxicity of 70% ethanol crude leaf extracts.
- To assess wound healing activity of crude leaf extract using excision and incision models,
- To evaluate antiinflammatory activity of the extract
- To determine antibacterial activity including its MIC and MBC
- To perform preliminary phytochemical analysis of secondary metabolites

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Reagents and Drugs

Distilled Water (Tigray regional laboratory, Mekelle, Ethiopia), ethanol (Carlo Erba Reagents, Italy), Mueller Hinton agar (Oxoid Ltd, Basingstoke, Hampshire, England), Muller Hinton Broth (Oxoid Ltd, Basingstoke, Hampshire, England), nutrient agar (Himedia Laboratories Pvt. Ltd, India), ciprofloxacin (Becton, Dickinson and Company, Sparks, USA), ceftriaxone, amoxicillin, ampicillin (Oxoid Ltd, Basingstoke, Hampshire, England), nitrofurazone ointment (Shanghai General Pharmaceuticals Co., Ltd, China), diclofenac, indomethacin (Ajanta pharma Ltd, India), resazurin sodium salt (Serva Feinbiochemica, Heidelberg, New York), Dimethyl Sulfoxide (DMSO) (Riedel-de Haen, Honeywell, Germany), tween 80 (Atlas Chemical Industries Inc, USA), carrageenan (Sigma-Aldrich Steinheim, Germany) obtained from their respective vendors were used. Chemicals and reagents used were of analytical grade.

3.1.2. Plant material

The leaves of *B. grandiflorum* were collected from Adishuhu, Tigray about 687 km towards north from Addis Ababa at the end of November 2015. Identification and authentication of the plant specimens was done by a taxonomist Dr. Yemane Gberezgabiher Department of Biology, Mekelle University, and also at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University where a plant sample was deposited with specimen number KBTMU-001 for future reference.

3.1.3. Experimental animals

Healthy adult Swiss albino mice of either sex weighing 20-30 g obtained from Department of Pharmacology, Mekelle University and Department of Pharmacology and Clinical Pharmacy, Addis Ababa University were used for this study. Animals were kept under 12 h. light: 12 h. dark cycle in animal house. The animals were acclimatized to laboratory condition for one week prior to the experiments, and were fed with standard pellet diet and water. Each group was kept in a separate cage. Their cages were cleaned daily, food and water changed daily.

3.1.4. Test of microorganisms

The antibacterial activity of the crude extracts and the standard drugs was tested against a set of microorganisms of either clinical isolates (From Ayder Comprehensive Specialized Hospital) or standard source (From Tigray regional laboratory, Mekelle and Ethiopian Public Health Institute, Addis Ababa). Gram-positive bacteria such as *S. aureus* (ATCC 25923), *Streptococcus pnuemonea* (ATCC 49619) and *Streptococcus pyogens* (ATCC 19615), and the Gram-negative bacteria such as *E. coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700609), *Proteus mirabilis* (ATCC 35659), *P. aeruginosa* (ATCC 28753) and *Enterococcus* were used. The antibacterial drugs and the bacteria were selected based on antibacterial spectrum and also by considering the availability of these agents in the study area and their existence on wound, respectively.

3.2. Methods

3.2.1. Plant extraction

The leaves were sorted out to obtain only fresh leaves and washed with distilled water without squeezing to remove debris and dust particle and then dried at room temperature under shade for three weeks. The dried plant was pulverized using grinder. Then 450 g of the powder was macerated using 2500 ml of 70% ethanol for 72 h (72h) with constant occasional stirring. Then the macerate was filtered using filter paper (Whatman No. 1) under vacuum. The residue was re-macerated three times to obtain maximum yield. To reduce concentration of the filtrate, the ethanol was evaporated using Rota vapor. The combined aqueous filtrate was then evaporated in a ventilated oven at 40 °C until dried (Mekonnen *et al*., 2013; Mulisa *et al*, 2015).

Out of 450 g powder, the resulting dry extract was weighed and provided a percentage yield of 29.7%. The dried extract was stored in a refrigerator at 4^{o}C for further experimental use.

3.2.2. Ointment formulation

Simple ointment of the plant extract was prepared following the formula (Table 1) described in the British Pharmacopoeia (BP, 1988). Three ointment preparations (each 200 g), with (5 % and 10 % w/w) of the extract and without simple ointment only which served as a control were formulated using the reduced formula. All ingredients of the ointment base were mixed and heated gently, with stirring until homogenous and then stirred until cooled. For preparing medicated ointment, 10 g and 20 g of the extract was mixed with 190 g and 180 g of the ointment base, to prepare 5% and 10% medicated ointments, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and

smooth texture (Ansel, 1985). 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.

Table 1: Master formula and reduced formula used for simple ointment preparation

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

3.2.3. Acute oral toxicity study

Acute toxicity test was performed according to the Organization of Economic Corporation and Development (OECD) 420 guideline (2001) for crude leaf extracts. Three to four h fasted female mice were used for the toxicity study. First, a sighting study was performed to determine the starting dose. For this, a single female mouse was given 2000 mg/kg of the extract (1 ml/100 g) as a single dose by oral gavage. After observation for mortality or any sign of toxicity (body weight, body texture, activity or locomotion) within 24 h, another 4 female mice were given the same dose and was observed for onset, duration, and severity of toxic signs in the next 14 days. Then the dose was scaled up to 5000 mg/kg, the same procedure as above.

3.2.4. Acute dermal toxicity study

Acute dermal toxicity was tested according to Mulisa *et al.* (2015) and Kokane *et al.* (2009) with some modification. For dermal toxicity, two groups a total of 5 female albino mice in each group were used. Animals showing normal skin texture were housed individually in a

cage and acclimatized to the laboratory condition for five days prior to the test. Then around 10 % of the body surface area fur was shaved from the dorsal part 24 h before the study. Then extract ointments (5% and 10%) were applied on the shaved area. At the end of the exposure period (24 h), the residual test substance was removed and the animals were observed for 24 h and for the next 14 days for development of any adverse skin reactions like inflammation, irritation or redness. In addition, skin toxicity of extract ointments was observed when the ointment was applied for several days (> 10 days).

3.2.5. Grouping and dosing of animals

For excision model four groups of mice and for incision models five groups of mice, each containing six animals was 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 the extract ointment, respectively. The fourth group was treated with nitrofurazone and served as a positive control, and the fifth was left untreated with any agents and served as untreated controls (only for incision model). For assessment of antiinflammatory activity, five groups of mice containing six animals per group were used. Group I was treated with a 10 ml/kg of vehicle (2% tween 80) and served as a negative control. 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), Group V was treated with indomethacin. Dose levels were 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.

3.2.6. Wound healing activity test

Wound healing activity was evaluated by excision and incision models. This was achieved by observing the rate of wound contraction, decrease in the period of epithelization and skin tensile strength.

i. Excision wound model

On wounding day, animals were anesthetized using subcutaneous injection of ketamine (1 ml/kg) and diazepam (1 ml/kg). The back hair of the animals was depilated by shaving. About 300 mm^2 circular areas were then marked and the full thickness of the marked area was carefully excised by using sharp sterilized scissors. After 24 h of wound creation, the ointments were applied gently once daily, according to the respective grouping as described under grouping and dosing section, to cover the wounded area until complete healing was achieved. Wound contraction and epithelization period were monitored. Wound contraction was measured as percent contraction every 3 days until complete wound closure was achieved (Kokane *et al.*, 2009; Mekonnen *et al.*, 2013).

Measurement of wound contraction

The wound healing progress was evaluated by measuring wound areas using a transparency sheet and a permanent marker. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound (300 mm^2) as 100 % (Shivhare *et al.*, 2010) as shown below:

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

Epithelization time measurement

The period of epithelization was calculated as the number of days required for falling off of the dead tissue remnants without any residual raw wound (Wesley *et al.*, 2009).

Histopathological Analysis

In order to confirm the experimental results, histopathological analysis was also performed at 13th day of post-wounding (Al-Henhena *et al.*, 2011). The analysis was blindly done by a senior pathologist Dr. Binyam Tsegaye from Pathology Department, Mekelle University. The skin specimens from each group were collected at the end of the experiment after the mice were sacrificed by overdose of Ketamine and diazepam (four times the anesthetic dose intraperitoneally) (IACUC, 2015). Samples were fixed in 10% buffered formalin, processed, and blocked with paraffin and then were sectioned into 5 micrometer sections and stained with hematoxylin and eosin (HE) stains. The Phases in wound healing processes (inflammation, proliferation, angiogenesis and remodeling) were examined by light microscope (Olympus CX41 attached Camera Digital Image Analyze System) and were graded as mild (+), moderate (++), and severe (+++) for epidermal or dermal remodeling. Mononuclear and/or PMNLs, neovascularization, and collagen depositions in dermis were analyzed to score the epidermal or dermal remodeling (Tumen *et al.*, 2012).

ii. Incision wound model

On wounding day (Figure 3A), animals were anesthetized in the same manner described for excision wound model. The dorsal fur of each mouse was then shaved and a 3 cm long longitudinal paravertebral incision was made through the skin and subcutaneous tissue. The parted skin was then sutured (Figure 3B) 1 cm apart using a surgical thread (no. 000) and

curved needle (no. 11). The continuous thread on both wound edges was tightened for good closure of the wounds. After 24 h of wound creation (on 1st day), animals were treated as described under grouping and dosing section, with topical formulation of vehicle, extract or standard daily for nine days, leaving out the last group which did not receive any of the interventions (Kokane *et al.*, 2009). The sutures were removed on day 8 post-incision and tensile strength was measured on the 10th post-wounding day using continuous water flow technique (Figure 3C) (Wang *et al.*, 2011) and according to the formulas shown below (Akkol *et al.*, 2011).

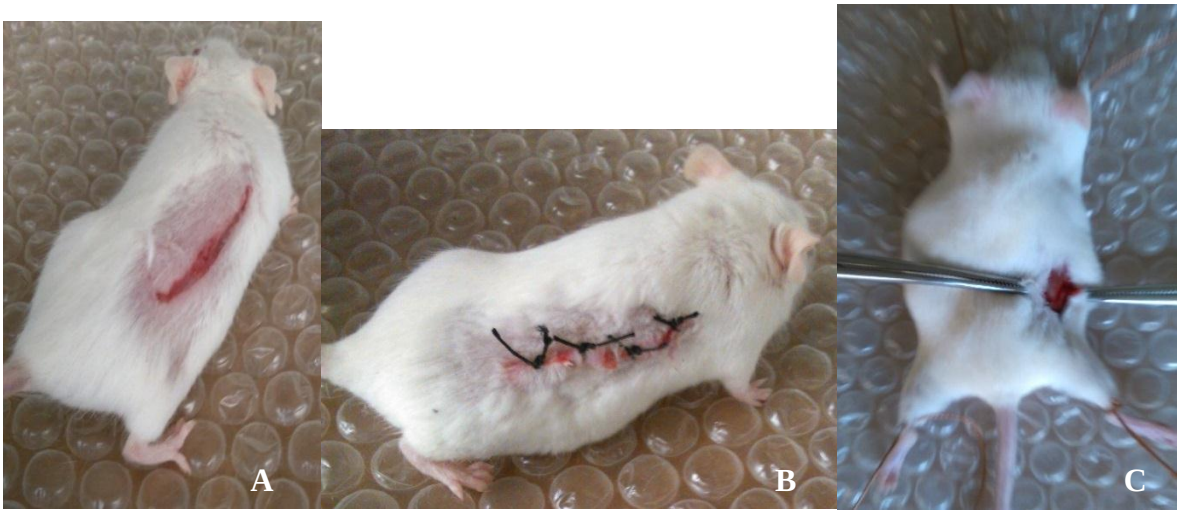


Figure 3: Photograph of Incision wound model

Measurement of tensile strength

Mice were anesthetized and secured to the operating table. Two forceps were firmly applied 3 mm away from the edge of wound facing each other on opposite side of the incision wound. One of the forceps was fixed on stands, while the other was connected to a freely suspended lightweight plastic of volume 1000 ml through a string run over to a pulley. Water was allowed to flow continuously from the reservoir slowly and steadily into the container. The moment the wound just opened up (Figure 3C), the water flow was arrested

and the volume of water collected in the container (approximately equal to its weight) was noted as tensile strength (Ilango and Chitra, 2010).

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

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

$$\%\,\text{Tensile strength (TS) of s.o} = \frac{\text{TS s.o - TS l.u}}{\text{TS l.u}} X\ 100$$

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

3.2.7. Antibacterial activity assay

i. Inoculum preparation

Preparation and standardization of the inoculum was carried out according to the procedure described by Merawie *et al.* (2013)**.** The test organisms were grown in a nutrient agar medium and three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. Each isolate was grown in 100 ml broth in Erlenmeyer flask. Inoculum preparation was standardized with 0.5 McFarland turbidity checker machine.

ii. Agar well diffusion

The antibacterial agar well diffusion method was conducted following the method described by Taye *et al.* (2011) and Andualem *et al.* (2014). Bacterial broth culture was prepared to a density of 10^8 cells/ml of 0.5 McFarland standard. The aliquot was then spread evenly onto Muller Hinton agar by sterile cotton swab. The plated medium was then allowed to dry at room temperature for 30 min. On each plate, equidistant sterilized filter papers of 6 mm diameter were placed. The plant extract was dissolved in dimethylsulfoxide (DMSO) which

was found to be inactive at the concentration used (20% DMSO). An appropriate concentration of plant extract (50 µg/µl, 100 µg/µl and 200 µg/µl) was then aseptically spilled on a respective filter paper. Ciprofloxacin, amoxicillin, ampicillin and ceftriaxone drug discs were also placed on the culture to be used as positive controls. The vehicle DMSO was used as negative control. This was followed by leaving the agar plate on the bench for 40 min for pre-diffusion. The plates were then incubated at 37°C for 24 h. At the end of the incubation period, the diameter of the inhibition zones were measured and recorded. All the experiments were performed in triplicates. The average values were taken as the diameter of the inhibition zone. Finally the results were compared with the standard drugs and percentages of inhibition were calculated.

iii. Determination of minimum inhibitory concentration

Under aseptic conditions, 96 well microtitre plates were used for Resazurin based Microtitre Dilution Assay. The first row of microtiter plate was filled with 100 µl (600 µg/µl) of test materials in DMSO. All the wells of microtitre plates were then filled with 100 µl of Muller Hinton broth. Two fold serial dilution (throughout the column) was carried out by starting transferring 100 µl test material from first row to the subsequent wells in the next row of the same column so that each well had 100 µl of test material in serially descending concentrations. Twenty five µl of resazurin solution, as indicator, was added in each well. 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 which was used as sterility control; and (b) a column with all solutions except bacterial solution replaced by 10 µl of

Muller Hinton broth which was used as color reference. The plates were incubated in temperature controlled incubator at 37°C for 24 h. The color change in the well was then observed visually. Any color change observed from purple to pink or colorless was taken as positive. The lowest concentration of plant leaf extract at which no color 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, 2012).

iv. Determination of minimum bactericidal concentration

The MBC is defined as the lowest concentration where no bacterial growth is observed (bactericidal concentration). In this technique, the contents of all well resulting MIC and all concentrations above MIC were streaked using a sterile wire loop on agar plate and incubated at 37ºC for 24 h (Sahgal *et al.*, 2009). The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

3.2.8. Antiinflammatory activity test

i. Carrageenan induced mouse paw edema method

Antiinflammatory activity of the leaf extract of *B. grandiflorum* was determined using mouse paw edema model. Following 3 to 4 h 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. The mice were then treated as described under grouping and dosing section 1 h before carrageenan injection. Paw swelling 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. The inflammation was quantified by measuring the

volume displaced by the paw 1, 2, 3 and 4 h after carrageenan injection. Results were expressed as the paw volume (ml) variation of each mouse with respect to its basal values and the average were calculated. The percentage inhibition of edema for each group was calculated using the following formula (Mahomed *et al.*, 2004).

$$\text{Percentage inhibition of edema} = \frac{\text{Cc} - \text{Ct}}{\text{Cc}} \text{ X } 100$$

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

3.2.8. Phytochemical screening

The presence of phytochemicals like alkaloids, flavonoids, saponins, tannins, terpenoids, glycosides, phytosterols, cumarin and anthraquinones was evaluated in the crude leaf extract using standard testing methods (Harborne, 1998; Debella, 2002; Sasidharan *et al.*, 2010).

i. Test for anthraquinones

One hundred mg of the extract was shaken vigorously with 10 ml of benzene and the extract was filtered. The filtrate was treated with 5 ml 10% ammonia solution and shaken. The formation of pink, violet or red color in the ammonia phase was considered positive for free anthraquinones.

ii. Test for phenolic compounds

To 100 mg of the extract dissolved in methanol or water, three drops of a mixture (prepared immediately before the reaction of one ml 1% $FeCl_3$ and one ml 1% $K_4Fe(CN)_6$) were added and the formation of green blue color was inspected.

iii. Test for flavonoids

Shinoda reduction test

One hundred mg of the extract dissolved in five ml of 50% methanol was divided into two test tubes and to one of the test tube metallic magnesium and to the other zinc was added then five drops of concentrated HCl were added to each test tubes and the formation of an orange or red color was taken as positive for the presence of flavonoids.

Lead acetate test

To 2 ml of the extract in the methanol five drops of 2% lead acetate solution were added and the development of yellow or orange precipitate was inspected.

iv. Ammonia test for coumarins

To 100 mg of the extract dissolved in 5 ml of ethanol, 2 ml of 10% ammonia was added and the occurrence of an intensive fluorescence under UV light was inspected. Comparison was made by taking another 5 ml of the extract in ethanol without 10% ammonia as a reference.

v. Test for saponins

Five hundred mg of the extract in 10 ml of distilled water was shaken in a test tube and the formation of honeycomb froth that persists for half an hour was considered as positive for saponins.

vi. Test for tannins

One g of the extract was heated in a test tube with 10 ml of distilled water for five minutes. After cooling, the solution was filtered through filter paper and 5 ml of 2% NaCl was added to the clear filtrate. Then 5 ml of 1% gelatin was added and the formation of precipitate was inspected.

vii. Test for steroids

One g of the extract was macerated with petroleum ether, filtered and the filtrate was concentrated. The residue was dissolved in chloroform and to it 5 drops of concentrated H_2SO_4 were added carefully and the production of a red or violet color was regarded as positive for the presence of steroidal compounds.

viii. Test for terpenoids

Five (5) ml extract was mixed in 2 ml chloroform. Then 3 ml concentrated sulphuric acid is carefully added to observe a reddish brown coloration between upper and lower layer.

ix. Test for alkaloids

Five hundred mg of the extract was treated in a test tube with 10 ml of 1% HCl for 30 minutes in a water bath and then filtered through cotton in to a test tube. Small portion of the extract was transferred into two test tubes and to one of the test tubes, five drops of Mayer's reagent and to the second five drops of Wagner's reagent were added and the formation of whitish opalescence (Mayer's reagent) or reddish brown precipitate (Wagner's reagent) was inspected.

3.3. Statistical analysis

The experimental results are 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. $P < 0.05$ was considered as statistically significant.

4. RESULTS

4.1. Acute oral toxicity test

Acute toxicity study results of the plant at both 2000 mg/kg and 5000 mg/kg doses showed no sign of toxicity (loss in body weight, change in body texture, decrease in activity or locomotion or mortality) in the first 24 h as well as in the following 14 days cage side observation. Therefore, the dose causing 50% death of the animals (LD_{50}) of the plant is greater than 5000 mg/kg.

4.2. Acute dermal toxicity

After 24 h of application of both 5% and 10% ointment formulations, the site did not show any sign of inflammation, irritation or redness. There were also no overt signs and symptoms observed when the animals were monitored for 48 h. Moreover, no signs of toxicity as well as no mortality were noted during the 14 days cage side observation. But, the 10% extract ointment showed skin irritation and redness during successive application. However, the 5% extract did not show any irritation and redness even after several days of application (Figure 4).

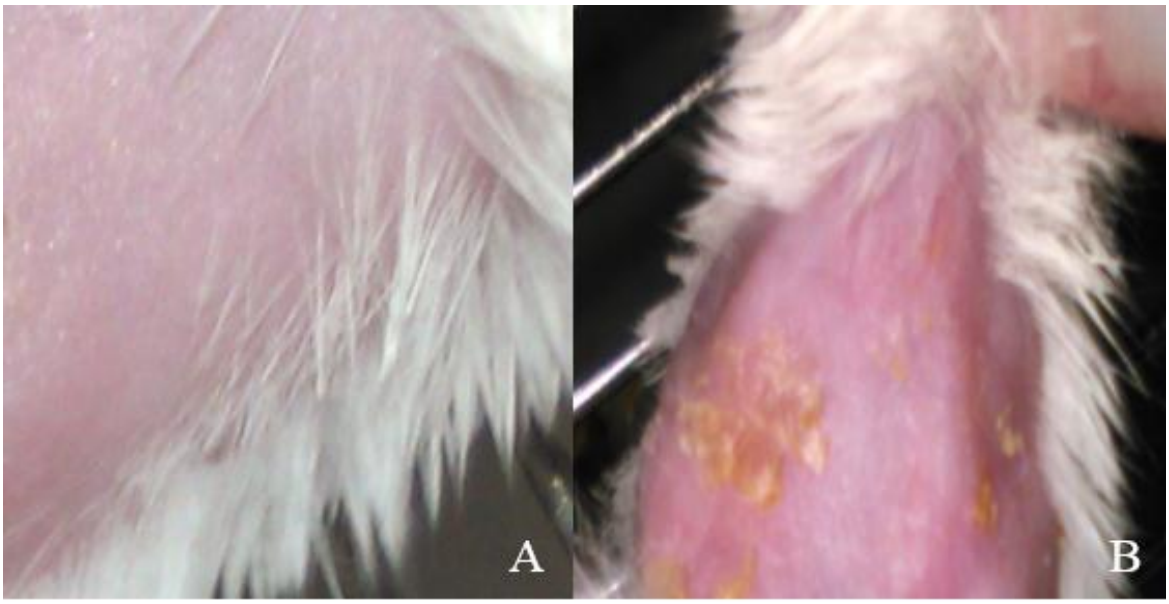


Figure 4: Photograph of acute dermal toxicity test result. A: 5% w/w ointment application B: 10% w/w ointment application

4.3. Wound healing activity

i. Excision wound model

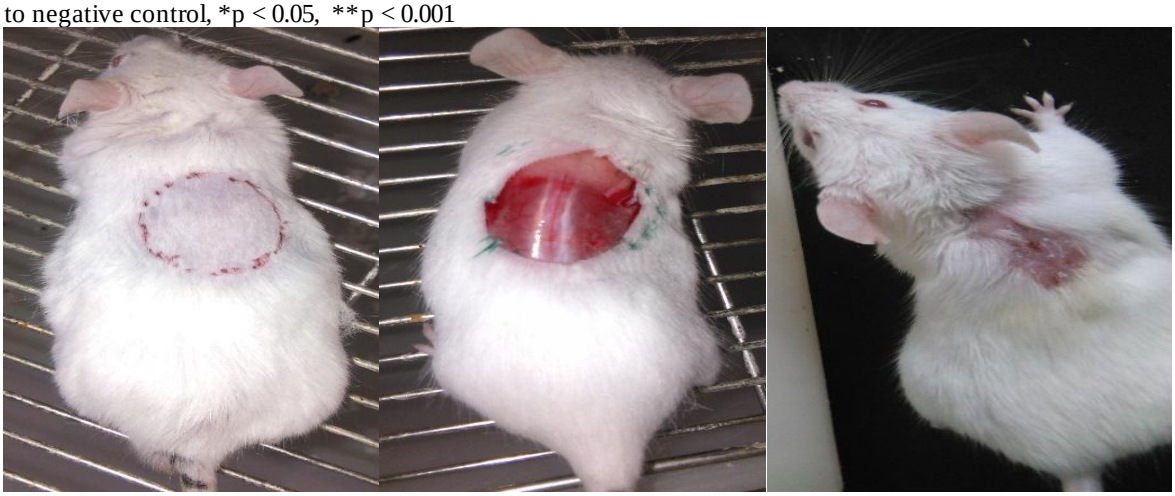
Wound contraction

The effect of the extract on wound contraction is shown in Table 2. Wound area decreased with time and a significant difference ($p<0.001$) was observed between treatment and controls at all-time points with 5% extract ointment and standard. However, although the pattern observed with 10% of the extract ointment was similar with that of the 5% and standard in most of the cases, it failed to produce significant difference at Day 14. It is the 5% (w/w) containing ointment treated group that showed better wound contraction than those treated with 10% with complete contraction on day 13 (Figure 5).

Table 2: Effect of topical application of ointments of 70% ethanol leaf extract of *B. grandiflorum* on wound area in mm^2

Group	Day 4	Day 8	Day 12	Day 14	Day16
Simple ointment	269.13±6.56	173.18±7.19	84.50±11.76	31.94±3.19	5.76±0.83
0.2% w/v Nitrofurazone	237.19±15.97[a]**	140.32±8.98[a]**	20.68±3.76[a]**	0[a]**	-
5% w/w extract	242.92±18.37[a]**	137.25±14.35[a]**	18.61±2.77[a]**	0[a]**	-
10% w/w extract	237.97±19.65[a]**	142.03±23.42[a]*	16.01±16.01[a]**	19.24±8.60	0

n = 6 Swiss albino mice in each group; Values are expressed as mean ± SEM, one way ANOVA. [a] as compared to negative control, *p < 0.05, **p < 0.001



Marked (Day 0)→→→→→→→→→→Wounded (Day 1)→→→ →→→→→Healed (Day 13)

Figure 5: Photograph of excision wound model of 5% w/w *B. grandiflorum* extract

As presented in Figure 6, the 70% ethanol extract showed significant increase in percentage closure of excision wounds. The percentage contraction was significantly higher ($p< 0.001$) for 5% and nitrofurazone as compared with the negative control in all days of contraction measurement. No significant difference in activity and rate of wound closure was observed between the 5% extract and the standard drug. The wound closure achieved by 5% extract on 12^{th} post wounding day was almost the same to the contraction value of reference drug (95%) (Figure 6).

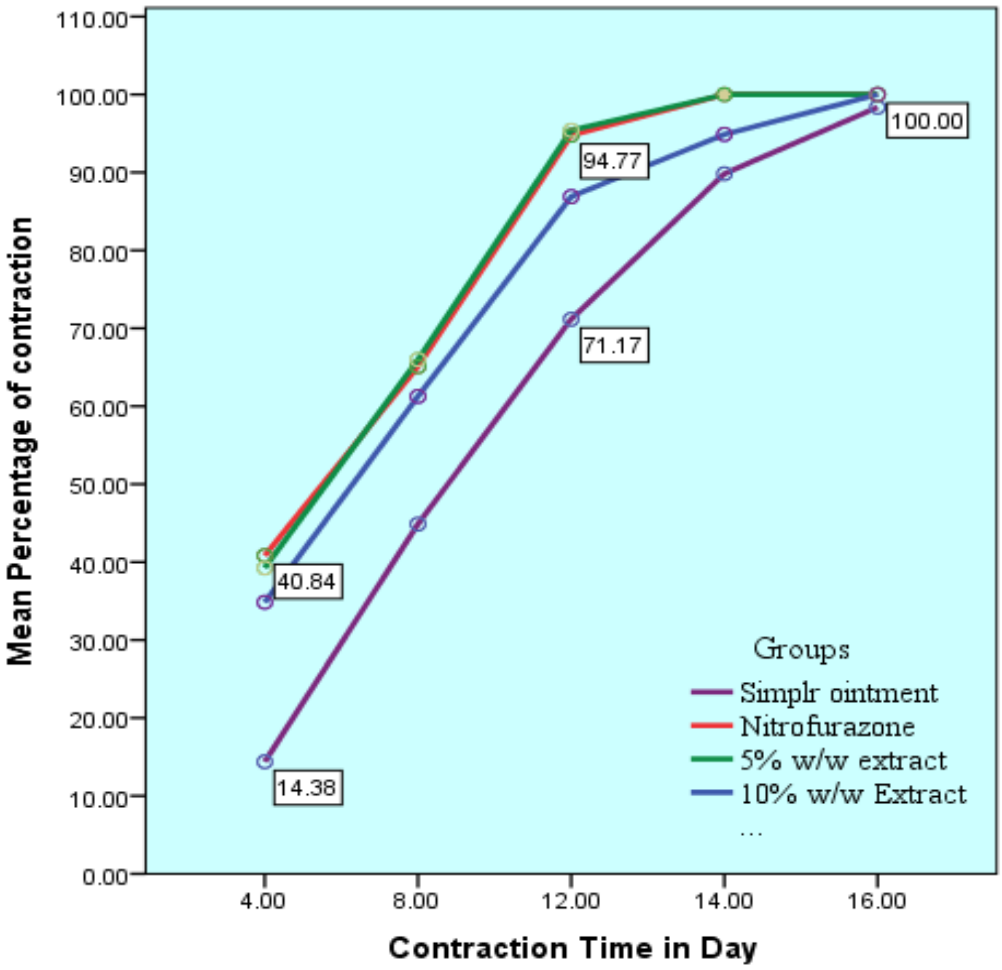


Figure 6: Effects of the 70% ethanol extract of *B. grandiflorum* on percentage wound closure in excision wound model.

Period of epithelization

Time for complete epithelization was short in extract ointment and nitrofurazone treated groups as compared to control (Table 3). Although animals treated with 10% (w/w) of the extract ointment failed to produce a statistically significant difference in period of epithelization, those treated with 5% (w/w) had a significantly shorter ($p < 0.001$) epithelization period as compared to the control group. However, there was no significant difference between extract and standard as well as among the different doses of the extract.

Table 3: Effect of topical application of ointments of 70% ethanol leaf extract of *B. grandiflorum* on wound epithelization period in excision wound

Group	Period of epithelization (day)
Simple ointment	16.17 ± 0.477
0.2 w/v Nitrofurazone	13.67 ± 0.667 [a]**
5% w/w extract	13.17 ± 0.477[a]**
10% w/w extract	14.67 ± 0.667

n= 6 Swiss albino mice per group; values represents mean ± SEM, one way ANOVA. [a] as compared to negative control, ** $p < 0.001$.

Histopathological analysis

Granulation tissue of healed wound in standard and 5% w/w extract treated groups showed few concentration of inflammatory cells as well as more collagen fiber, fibroblasts and proliferating blood capillaries (angiogenesis) compared with control treated group. Control groups had less collagen fibers, fibroblasts and blood capillaries, and more inflammatory cells, and thus showed delayed wound healing processes (Table 4, Figure 7).

Table 4: Histological qualitative determination of wound healing processes and healing phases of 70% ethanol extract of *B. grandiflorum*

Group	FP	CD	MNC	PMN	NV
Simple ointment	++	+	+++	+++	+
0.2% w/v Nitrofurazone	+++	+++	+	+	+
5% w/w extract	+++	+++	+	+	++
10% w/w extract	++	++	++	++	++

Low concentration (+), moderate concentration (++), and high concentration (+++) for epidermal and/or dermal remodeling. FP: fibroblast proliferation, CD: collagen depositions, MNC: mononuclear cells, PMN: polymorphonuclear cells, NV: neovascularization.

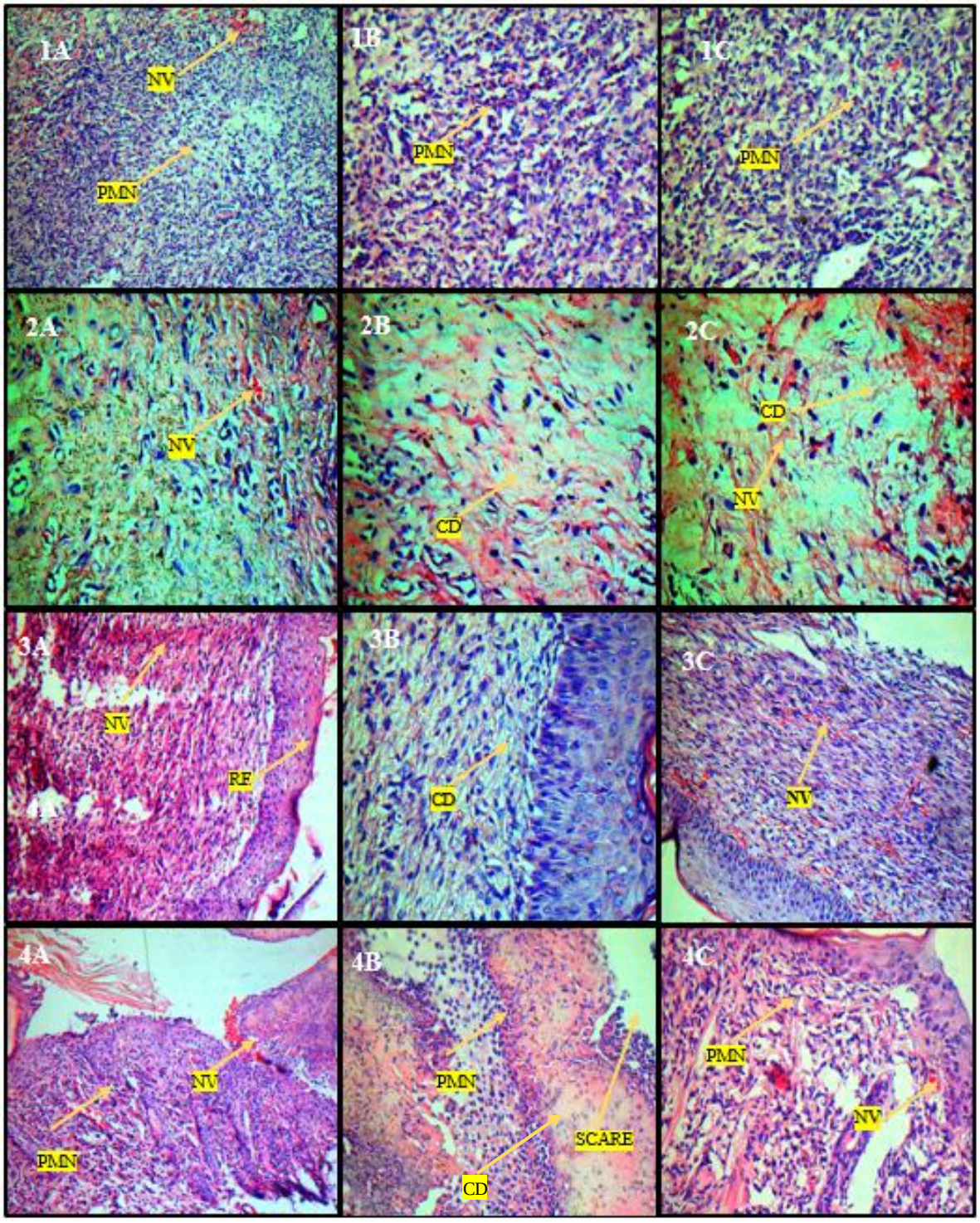


Figure 7: Photomicrograph of hematoxylin and eosin staining of tissues from wound model in mice. 1: simple ointment 2: Nitrofurazone 3: 5% extract 4: 10% extract. RE: re-epithelization, CD: collagen depositions, PMN: polymorphonuclear cells, NV: neovascularization. A, B and C represents pictures from three different positions of the wound specimen of respective formulations.

As Figure 7 depicts, histology of granulation tissue of healed wounds treated with simple ointment contain less collagen fibers, fibroblasts and blood capillaries, and more inflammatory cells (Figure 7. 1A, 1B, 1C). Whereas, granulation tissue of healed wound in extract-treated group contained few inflammatory cells, and more collagen fiber, fibroblasts and proliferating blood capillaries (angiogenesis) (Figure 7, 3A, 3B, 3C and 4A, 4B, 4C). Standard drug treated wound exhibited higher collagen fiber, but lesser blood capillaries as compared to the 10% extract. There was full thickness re-epithelization (Fig 7, 3A), high content of neovascularization (Fig 7, 3C) and better content of collagen deposition in 5% ointment treated wound as compared to 10% ointment treated wound, which observed to contain fewer neovascularization, less collagen deposition and greater content of PMN (Figure 7, 4A) as an evidence for delayed healing.

ii. Incision wounds

Tensile strength of incision wound model

Both 5 and 10% ointment preparations of the crude extract showed significant increase ($p < 0.001$) in wound tensile strength (Table 5) as compared to controls. Similar to the case of excision wounds, better activity was exhibited in lower dose (5% extract) than the higher dose (10% extract). However, there was no significant tensile strength difference between the reference drug and the two extract doses. Similarly, no significant difference in tensile strength was noted when the two strengths of the extract were compared with each other.

Table 5: Effect of topical application of ointments of 70% ethanol leaf extract of *B. grandiflorum* on tensile strength in mice incision wound

Group	Tensile strength in gram	% Tensile strength
Untreated control	204.1167 ± 14.39	-
Simple ointment	246.2300 ± 12.76	20.60%
0.2% w/v Nitrofurazone	384.1333 ± 11.24 [ab]**	56.00%
5% w/w extract	393.7733 ± 12.87 [ab]**	59.12%
10% w/w extract	371.6450 ± 10.39 [ab]**	50.90%

n = 6 Swiss albino mice per group, values represents mean ± SEM, [a]as compared to simple ointment, [b]as compared to untreated control. **P< 0.001.

4.4. Antibacterial activity

i. Zone of inhibition

According to the agar well diffusion test, the growth of all test bacterial strains were inhibited by the tested concentrations of the crude extract of the plant in a concentration dependent manner. Table 6 shows zone of inhibition of the test samples on different bacterial strains.

Among the test bacteria, gram positive bacterial species (*S. pneumoniae* and *S. pyogens*) were more susceptible than that of the gram negative bacterial strains at the corresponding tested concentrations of the crude extract, especially at 100 µg/µl and 200 µg/µl. As depicted in Table 6, the most susceptible bacteria at 200 µg/µl were standard strains of *S. pyogens* and *S. pneumoniae*. However, the strains of *S. aureus* were relatively less susceptible compared to the other bacterial species at comparable concentration of the crude extract.

Among the gram negative bacteria, standard strains of *P. mirabilis* were most susceptible followed by that of clinical strains of *Enterococcus* at 200 µg/µl of the crude extract. However, the strains of *K. pneumoniae* were relatively less susceptible compared to the other bacterial species at comparable concentration of the crude extract.

Zone of inhibition of crude extract at 50 µg/µl was significantly different ($p<0.05$) compared to zone of inhibition at 100 µg/µl against the growth of each test bacterium with the exception of standard strain *S. pnuemonia*. Moreover, zone of inhibition of 200 µg/µl was significantly different ($p<0.05$) compared to that of zone of inhibition at 50 µg/µl and 100 µg/µl against the growth of each test bacterium. The zone of inhibition of 200 µg/µl was significantly ($p<0.05$) greater than that of the standard drugs against *S. pyogens*, *S. pnuemonia* and *Enterococcus*. The three extract doses also showed significantly ($p< 0.05$) higher zone of inhibition compared to standard drugs against *S. pnuemonia*.

Table 6: Mean zones of inhibition of 70% ethanol leaf extract of *B. grandiflorum* against gram positive and gram negative bacteria

Bacterial strain	Zone of inhibition in mm						
	A	B	C	Cip	Amo	Cef	Amp
S. aureus (Clinical isolate)	$6.52^{1*2*6*7*}$	$8.39^{1*2*5*7*}$	$13.08^{1*2*5*6*}$	0	0	-	-
S. aureus (ATCC 25923)	$11.21^{1*2*6\Phi7*}$	$12.21^{1*2*5\Phi7*}$	$16.32^{1*2*5*6*}$	26.32	24.13	-	-
S. pyogens (ATCC 19615)	15.8^{1*6*7*}	$21.14^{1*2*5*7}$	$24.17^{1*2*5*6*}$	18.15	16.12	-	-
S. pnuemonea (ATCC 49619)	$17.17^{4\Phi7*}$	$18.00^{4*7\Phi}$	$20.00^{4*5*6\Phi}$	-	-	-	15.50
P. mirabilis (ATCC 35659)	16.44^{1*6*7*}	$19.90^{1*2*5*7*}$	$22.39^{1*2*5*6*}$	25.18	15.80	-	-
K. pneumonia (ATCC 700609)	$14.10^{1*2*3*6*7*}$	$17.02^{1*2*3*5*7\#}$	$18.00^{1*2*3\#5*6\#}$	22.03	20.00	19.02	-
E. coli (ATCC 25922)	16.00^{3*6*7*}	19.01^{3*5*7*}	22.02^{3*5*6*}	25.02	-	-	-
P. aeruginosa (ATCC 28753)	7.30^{3*6*7*}	$10.16^{3*5\#7\#}$	$11.10^{5*6\#}$	11.21	0	-	-
Enterococcus (Clinical isolate)	$9.20^{1*2*6*7*}$	$10.47^{1*2*5*7*}$	$14.49^{1*2*5*6*}$	0	13.02	-	-

Analysis was performed with One-Way ANOVA followed by Tukey test; [1] compared to ciprofloxacin, [2] to Amoxacillin, [3] to ceftriaxone, [4] to Ampicillin, [5] to 50 µg/µl, [6] to 100 µg/µl, [7] to 200 µg/µl, *P< 0.001, $^{\Phi}$P< 0.01, $^{\#}$P< 0.05,. A=50 µg/µl, B=100 µg/µl, C=200 µg/µl of *B. grandiflorum* extract, Amo= Amoxicillin 25µg, Cip= Ciprofloxacin 5µg, Cef= Ceftriazone 30µg, Amp= Ampicilline10µg, - = not done, 0= no activity.

ii. Minimum inhibitory concentration and Minimum bactericidal concentration

The more susceptible the bacterium is, the lower is the concentration of the extract required for growth inhibition in most of the test bacteria. The minimum MIC (highest dilution) was 12.50 µg/µl against *Streptococcus pyogen*. Based on the MBC determination method (Table 7), the crude extract was bactericidal except for *K. pneumonia* in which it was bacteriostatic. Among the gram positive bacteria, *Streptococcus pyogen* required minimum concentration 18.75 µg/µl and from the gram negative bacteria *E. coli* 37.5 µg/µl.

Table 7: Minimum inhibitory concentrations and minimum bactericidal concentration of 70% ethanol leaf extract of *B. grandiflorum*

Name of Bacterial strains	**MIC (µg/µl)**	**MBC (µg/µl)**
S. aureus (ATCC 25923)	28.13	112.50
S. pnuemonea (ATCC 49619)	15.62	31.25
S. pyogens (ATCC 19615)	12.50	18.75
K. pneumonia (ATCC 700609)	37.50	-
E. coli (ATCC 25922)	14.65	37.50
P. aeruginosa (ATCC 28753)	37.50	112.50

MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration.

4.5. Antiinflammatory activity

Table 8: Antiinflammatory activity of the 70% ethanol leaf extract of *B. grandiflorum* using carrageenan induced paw edema

Group	Mean increase in the paw volume (ml)				
	Basal	1 h	2h	3h	4h
Control	0.74±0.002	0.45±0.009	0.51±0.012	0.43±0.011	0.43±0.029
Diclofenac (10mg/kg)	0.82±0.003	0.21±0.012$^{abc\Phi}$ (53.33)	0.11±0.014$^{abc\Phi}$ (78.43)	0.07±0.008$^{abc\Phi}$ (83.72)	0.06±0.010$^{abc\Phi}$ (86.05)
Indomethacin (10 mg/kg)	0.63±0.004	0.25±0.016$^{abc\Phi}$ (44.44)	0.16±0.008$^{abc\Phi}$ (68.63)	0.13±0.009$^{abc\Phi}$ (69.77)	0.12±0.008$^{abc\Phi}$ (72.10)
B. grandiflorum (100 mg/kg)	0.66±0.005	0.43±0.002 (04.44)	0.41±0.009$^{a\Phi}$ (19.61)	0.39±0.008 (09.30)	0.33±0.018^{a}* (23.25)
B. grandiflorum (200 mg/kg)	0.60±0.017	0.36±0.005$^{ab\Phi}$ (20.93)	0.34±0.016$^{ab\Phi}$ (33.33)	0.33±0.014$^{a\Phi b*}$ (23.25)	0.27±0.011$^{a\Phi}$ (37.21)
B. grandiflorum (400mg/kg)	0.73±0.010	0.29±0.017$^{ab\Phi c}$* (35.56)	0.26±0.004$^{abc\Phi}$ (49.02)	0.19±0.012$^{abc\Phi}$ (55.81)	0.15±0.008$^{abc\Phi}$ (65.11)

n=6 Swiss albino mice per group, data represent mean± S.E.M, *P< 0.05, $^{\Phi}$P< 0.001, ^{a}as compared to negative control, ^{b}as compared to 100 mg/kg, ^{c}as compared to 200 mg/kg, Values in parenthesis represent percentage inhibition of paw Volume.

After administration of carrageenan, all the extracts and standard drugs treated animals showed significant reduction of edema as compared to the negative control (Table 8), except with100 mg/kg extract, which failed to be significant at first and third hour. The middle dose 200 mg/kg, higher dose 400 mg/kg and standard drugs showed significant ($p < 0.001$) suppression of edema at all-time points as compared to controls. There were significant differences between 200 and 100 mg/kg doses at 1 h and 2h ($p < 0.001$), and 3h ($p < 0.05$), whereas at all-time points ($p < 0.001$) with 400 mg/kg. It was also observed that 400 mg/kg

showed significant difference from the 200 mg/kg dose at 1h ($p < 0.05$), 2h, 3h and 4h ($p < 0.001$).

4.6. Phytochemical screening

Preliminary phytochemical screening of the leaves of *B. grandiflorum* indicated the presence of six classes of secondary metabolites as shown in Table 9. However, alkaloids, cumarins and anthraquinones were not detected.

Table 91: Preliminary phytochemical screening of 70% ethanol leaf extract of *B. grandiflorum*

Secondary Metabolite	Result
Alkaloids	-
Saponins	+
Tannins	+
Coumarins	-
Anthraquinones	-
Terpenoids	+
Flavonoids	+
Phenols	+
Steroids	+

(+ = Present, - = absent)

5. DISCUSSION

Seventy percent (70%) ethanol were used to extract leaf of *B. grandiflorum*. It is a universal solvent that can possibly extract polar and less polar compounds. It is also preferable for extracting secondary metabolites like flavonoids. Pure ethanol and ethanol 70% are safe solvents with lower toxicity than methanol. Also, good yield and high concentration of secondary metabolites like bioactive flavonoid compounds can be isolated with these safe solvents from a plant matrix (Bimakra *et al*., 2011).

To carry out the wound healing study, formulation of semi-solid preparations with the extracts were preferred to apply topically than the crude extracts (Esimone *et al*., 2009). Simple ointment base possesses multiple roles on wound healing process; prevents escape of moisture from the skin and causes hydration of the stratum corneum, provides a medium for dissolution of the drug and keratinocyte migration. Wool fat and cetostearyl alcohol are thickeners and are used for stabilization of ointment (Ansel, 1985; Schultz *et al*., 2003).

In the present study, two different models were used to assess the effects of 70% ethanol leaf extract ointment of *B. grandiflorum* on the various wound phases which run concurrently but independent of each other. The use of a single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing. Hence, two or more models are used in wound healing studies. Even though a large effort has been made to study in vitro wound healing activity, in vivo studies still remain indispensable for wound healing activity investigation as wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state (Abdulla *et al*., 2010).

Wound contraction is a process that occurs throughout the healing process (Tang *et al.*, 2007). It mainly depends on the repairing ability of the tissue, type and extent of damage and general state of health of the tissue. Contraction decreases healing time because it decreases the size of the wound and reduces the amount of ECM needed to repair the defect. Contraction also facilitates re-epithelization by shortening the distance migrating keratinocytes must travel (Strodtbeck, 2001). The greater wound contraction effect of the test samples might be due to their higher antiinflammatory effect (Getie *et al.*, 2003). The wound contractions observed indicate that *B. grandiflorum* possess a definite pro-healing action, since 88% of the healing of wound occur due to contraction, and the other 12% occur due to scar formation (Ejaz *et al.*, 2009). In this study mice treated with 5% and 10% ointment extract exhibited about three folds increase in wound closure as compared to negative control treated mice. Besides 5% extract ointment exhibited about 96% wound closure on day twelve whereas the closure of negative control was only 70% (Table 2).

Wound re-epithelization is a hallmark of successful wound care (Stadelmann *et al.*, 1998). Moreover, better healing activity is distinguished with short epithelization period (Süntar *et al.*, 2010). The epithelization time was significantly reduced from 16 days (control) to 13 days for 5% extract (Table 3). The occurrence of enhanced epithelization and wound contraction could be due to the ability of *B. grandiflorum* extracts to enhance collagen synthesis as evidenced by the histopathological analysis (Table 4 and Figure 7), as wound contraction begins almost concurrently with collagen synthesis (Esimone *et al.*, 2008). Therefore, the shorter epithelization time in the 5% extract might be due to facilitated proliferation and migration of epithelial cells and/or increased viability of epithelial cells (Sanwal and Chaudhary, 2011).

Enhanced healing activity has been attributed to increased collagen formation, angiogenesis, stimulation of epithelial cell proliferation and increase in tensile strength of the wounds (Buntrock *et al.*, 1982; Suguna *et al.*, 1996; Shukla *et al.*, 1999). Collagen played a central role in the healing of wounds and it is a principal component of connective tissue and provides a structural framework for the regenerating tissue (Cohen *et al.*, 1992). Angiogenesis in granulation tissues improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process (Szabo *et al.*, 1995). In the present study, granulation tissue of healed wound in reference drug and 5% w/w extract treated groups showed less concentration of inflammatory cells, and more collagen fiber, fibroblasts and proliferating blood capillaries (angiogenesis) as compared with control treated group, which contain less collagen fibers, fibroblasts and blood capillaries, and more inflammatory cells, thus showing delayed wound healing processes (Table 4 and Figure 7). Reasons for the delay in wound healing are concordant with other studies described in Habibipour *et al*. (2003).

Further efficacy of the crude extract in wound healing was evidenced by the tensile strength in incision wounds. The tensile strength of both 5% and 10% ointments of the crude extract treated groups was highly significant ($p<0.001$) as compared to simple ointment treated and untreated groups and specially 5% ointment showed slightly greater strength as compared to standard treated group (Table 5). Tensile strength indicates how much the repaired tissue resists tensile under tension and may indicate the quality of repaired tissue (Shivhare *et al.*, 2010). Tensile strength of a wound mainly depends upon the increase in collagen concentration and stabilization of the fibers. Hence, based on the histopathological analysis detected (Figure 7, 4B), it could be assumed that the extract enhances the strength of the

incision wound by increasing the collagen levels, which could stick the wound edges together at the repaired site (Stadelmann *et al*., 1998). Since incisional wounds treated with the test samples showed greater tensile strength as compared to the negative control, it might be plausible to say that the test samples not only increased collagen synthesis per cell, but also aided in cross-linking of the proteins (Prasad and Dorle, 2006; Agarwal *et al*., 2009; Wang *et al.*, 2011).

Five percent of the crude extract of the plant produces better effect than 10% of the ointment. According to this result, it can be said that the irritant property of the extract which seems to increase with dose may be responsible for the slight delaying of wound healing in the larger dose, reasons for the delay in wound healing are concordant with other studies described in Mekonnen *et al*. (2013). As the extract directly contact the wounded skin, irritant compounds in the extract may cause recurrent inflammation on the wounded site and thus interfere with the healing processes (Robert *et al*., 2004).

Wounds are known to be complicated by infection (Deshmukh *et al.*, 2009) that can seriously delay the healing process by disrupting the normal clotting mechanisms, promoting disordered leukocyte function and ultimately delaying angiogenesis (Annan and Houghton, 2008). Microbes can also cause poor quality granulation tissue formation, reduced tensile strength of connective tissue, impaired epithelization and odor (Maryanne *et al.*, 2003). Microbes and endotoxins can lead to a prolonged elevation of pro-inflammatory cytokines such as IL-1 and TNF-α. These can cause a chronic inflammatory state that promotes development of MMPs, thus inhibiting wound healing. Earlier studies have shown that the antimicrobial activity of various plants support wound healing (Deshmukh *et al.*,

2009), and therefore, the antibacterial and antiinflammatory activity of the test samples can significantly contribute to their wound healing activities.

The extract showed dose dependent bacterial inhibition activity against both Gram-positive and the Gram-negative bacteria; *S. aureus, S. pnuemonea, S. pyogenss, E. coli, P. mirabilis, Enterococcus* and *P. aeruginosa* (Table 6) which appear to play an important role in infection of wounds (Esimone *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 (Menke *et al.*, 2007). Excessive inflammation, however, impedes wound healing. This arises from the fact that prolonged inflammation leads to an increased level of MMPs that can degrade the ECM. Previous reports indicate that a number of plants with antiinflammatory activity do also possess wound healing effect (Menke *et al.*, 2007).

Carrageenan-induced hind paw edema model has been widely used for the discovery and evaluation of antiinflammatory drugs (Padilha *et al.*, 2010). Carrageenan is a sulphated polysaccharide obtained from sea weed and is commonly used to induce acute inflammation. Inflammation induced by carrageenan develops immediately following injections, and produces three distinct phases. The first phase (0-1.5 h) is mediated by histamine and serotonin. The second phase (1.5-2.5 h) is mediated by bradykinin, while prostaglandins are implied in the third phase (2.5-5 h). The third phase of edema is sensitive to most clinically effective antiinflammatory drugs, which have been frequently used to assess the anti-edematous effect of natural products (Di Roso *et al.*, 1971; Marrassini *et al.*, 2010).

Inhibition of edema formation of the extract was observed at all points of time after carrageenan injection (Table 8). Hence, it is conceivable to summarize that antiinflammatory activity of the extract could involve inhibition of effect mediated by histamine, bradykinin and prostaglandin. Despite the fact that many antiinflammatory agents are used for wound treatment, they may also inhibit wound repair via their global antiinflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis (Guo and Dipietro, 2010).

Preliminary phytochemical screening of the leaves of *B. grandiflorum* gave positive results for six classes of secondary metabolites as shown in Table 9, even though their quantity cannot be determined by the test procedures used. A plant of the same genus *Becium obovatum*, shows similar result during a phytochemical screening done in Uriri District – Kenya (Okach, 2013) which support the findings of the present study.

The wound healing activity of the *B. grandiflorum* extract may be attributable to the phytochemicals present in the plant, which is composed of flavonoids, tannins, saponins, steroids, terpenoids and phenolic compounds. But, in this study, it is difficult to say which component/s of the plant extract is/are responsible for these activities since fractionation of the constituents in the extract was not carried out. Terpenoids and flavonoids are known to promote the wound healing process, mainly due to their astringent and antimicrobial properties, which could be responsible for wound contraction and increased rate of epithelization (Tsuchiya et. al., 1996; Sasidharan *et al.*, 2010; Kumar, 2013; Muralidhar *et al.*, 2013).

B. grandiflorum is a Lamiaceae family, which is known for its flavonoids content (Sytar *et al.*, 2015). Medicinal efficacy of many flavonoids as wound healing, antibacterial, antiinflammatory, anticancer and antiviral agents is well established (Kumar, 2013; Muralidhar *et al.*, 2013). Experimental assessment of wound healing activity of flavonoid fraction showed increased rate of wound contraction, epithelialization and increased granuloma tissue formation. In addition, they increase the skin tensile strength and hydroxyproline content which was a reflection of increased collagen levels by increased cross linking of collagen fibers (Muralidhar *et al.*, 2013). Moreover, flavonoids are known to reduce LPO not only by preventing or slowing the onset of necrosis but also by improving vascularity. Hence, any drug that inhibits LPO is believed to increase viability of collagen fibrils by increasing the strength of collagen fibers, increasing the circulation, preventing cell damage and by promoting the DNA synthesis (Getie *et al.*, 2002). Secondary metabolites like tannins, steroids and saponins are known to have antimicrobial and antiiflammatory effect that can be responsible for the treatment of gastrointestinal infection, swellings and wounds (Okach, 2013).

An ideal drug should fulfill the criteria such as rapid contraction, reduced epithelization time and increased tensile strength. Biochemically, tissue DNA, RNA, total protein and hydroxyproline are markers of good healing property of a drug (Kanti *et al.*, 2001). A plant based remedy should affect at least two different processes of wound healing before it can be said to have wound healing activity (Houghton *et al.*, 2005). Even though, tissue DNA, RNA, quantification of collagen content and other markers were not performed due to resource constraints, the present study provided evidence for the wound healing potential of *B. grandiflorum*.

CONCLUSIONS

In the present study, the different phases of wound repair; wound contraction, epithelization and tensile strength were improved by ointments prepared from 70% ethanol leaf extract of *B. grandiflorum* as compared to the control group. In addition, the extract was proved to exhibit antibacterial and antiinflammatory activities. Hence, the present research results support the traditional claims of the plant for the treatment of wounds. The wound healing activity of the plant could be due to its antibacterial, antiinflammatory, or improved collagen synthesis or a combination of these, probably due to the presence of flavonoids, tannins, saponins, steroids, terpenoids and phenolic compounds.

RECOMMENDATIONS

- Further toxicological studies such as sub-acute, sub-chronic and chronic toxicities should be done in order to assess the long term effect of the extract and fractions.
- The results of the present study should be corroborated with hydroxyproline assay.
- The plant should be further fractionated to identify which fractions (s) of the plant extract responsible for wound healing activity, antiinflammatory and antibacterial activities.
- Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for wound healing activity, antiinflammatory and antibacterial activities.
- As chronic wounds such as diabetic wounds are major global burden, it is worthwhile to study the activity of the plant on chronic wounds

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