Department of Pharmacology and Clinical Pharmacy

School of Pharmacy

College of Health Sciences

Addis Ababa University

Wound Healing and Antibacterial Activities of Solvent Fractions of 80% Methanol Leaf Extract of *Brucea antidysenterica J .F. Mill* (Simaroubaceae)

By: Befekadu Wolde (B.Pharm)

December, 2020

Addis Ababa, Ethiopia

Wound Healing and Antibacterial Activities of Solvent Fractions of 80% Methanol Leaf Extract of *Brucea antidysenterica J .F. Mill* (Simaroubaceae)

By: Befekadu Wolde (B.Pharm)

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

Advisor: Prof. Eyasu Makonnen (PhD), Department of Pharmacology and Clinical pharmacy, School of Pharmacy, Addis Ababa University

Co-advisors:

- Solomon Mequanente (PhD), Department of Pharmacology and Clinical pharmacy, School of Pharmacy, Addis Ababa University
- Belete Legesse (PhD), CDT Africa, Addis Ababa University

Addis Ababa, Ethiopia

December, 2020

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by **Befekadu Wolde** entitled " **Wound Healing and Antibacterial Activities of Solvent Fractions of 80% Methanol Leaf Extract of *Brucea antidysenterica J .F. Mill* (Simaroubaceae)"** 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.

Approved by the Examining Board

Examiner (External) _____ Signature _____Date ____

Examiner (Internal) _____ Signature _____Date ____

Advisor: Prof. Eyasu Makonnen (PhD) Signature _____ Date _____

Co-Advisor: Solomon Mequanente (PhD) Signature _____ Date _____

Co-Advisor: Belete Legesse (PhD) Signature _____ Date _____

_____

Head of Department

DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

Name: Befekadu Wolde

Signature: _____

This thesis has been submitted for examination with my approval as a University advisor.

Name: Prof. Eyasu Makonnen (PhD)

Signature: _____

Place and date of submission: Addis Ababa, Ethiopia, December, 2020

ABSTRACT

Wound Healing and Antibacterial Activities of Solvent Fractions of 80% Methanol Leaf Extract of *Brucea antidysenterica J .F. Mill* (Simaroubaceae)

Befekadu Wolde

Addis Ababa University, 2020

Brucea antidysenterica is a medicinal plant used for different aliments including dysentery and non-healing wounds. Wound healing, antibacterial, antiinflammatory and antioxidant activities of the crude leaf extracts were reported to be promising. The aim of this study was to examine wound healing and antibacterial activity of solvent fractions of 80% methanol leaf extract *of B. antidysenterica*. The crude leaf extract was fractionated with water, n-butanol and chloroform. Solvent fractions were formulated as 2% and 4% ointment bases. Excision and incision models were used to determine wound healing activity. Wound contraction rate, period of epithelialization and breaking strength determined. *In vitro* antibacterial activities of solvent fractions were tested. 2% and 4% aqueous fractions (AF) showed wound healing activity though n-butanol (BF) and chloroform fractions (CF) appeared to have delayed wound healing or exacerbating effect. At the end of treatment period, 2% and 4% AF significantly increased wound contraction ($p<0.001$) compared with the negative control. 4% AF significantly shortened ($p<0.05$) Period of epithelialization. 2% and 4% AF significantly increased tensile strength of wounded skin compared with untreated ($P< 0.001$). BF had the highest antibacterial activity as shown by inhibiting growth of four bacterial strains. *S. aurues* was the most susceptible species for BF with MIC of 75mg/ml. The AF of 80% methanol leaf extract *of B. antidysenterica* possesses wound healing activity. AF, BF and CF evidenced antibacterial activity against selected strains.

Key words: *Brucea antidysenterica*, wound healing activity, Agar well diffusion.

Acknowledgments

Let the Glory be to the almighty God that gave me courage and patience to complete this MSc work.

I am greatly thankful to my principal advisor Prof. Eyasu Makonnen (PhD) and co-advisors Dr. Solomon Mequanente (PhD) and Dr. Belete Legesse for suggesting me the project work and for their unreserved timely expertise guidance, start to the finish. My heartfelt gratitude also goes to Mr. Dereje Nigussie (MSc, PhD candidate) for his dedicated consultations and support during this MSc work.

My acknowledgment also goes to my classmates, pharmacology laboratory and animal house staffs, Mr. Shemsu and Mr. Aminu from the Department of Microbiology and EPHI traditional and modern medicine research center staffs for their support and encouragement during my laboratory works.

My sincere regards also goes to Addis Ababa Health bureau for sponsoring my MSc Program, Addis Ababa University for financial assistance and CDT Africa for supporting this project.

Lastly, I express my honest love to my wife Hiwot, my families and friends for their priceless support during my project work.

Table of Content

List of Tables

List of Figures

Abbreviations/Acronyms

ANOVA	Analysis of variance
ATCC	American type culture collection
AF	Aqueous fraction
BF	n-butanol fraction
BP	British pharmacopeia
CF	Chloroform fraction
CFU	Colony forming units
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPHI	Ethiopian public health institute
FGF	Fibroblast growth factor
NHS	National health service, united kingdom
IL -1	Interleukin-1
MMP	Matrix metalloproteinase
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MEBO	Moist exposed burn ointment
NHS	National health service
OECD	Organization for economic co-operation and development
PMNs	Polymorphonuclear neutrophils
ROS	Reactive oxygen species
SPSS	Statistical package for social science
TM	Traditional medicine

TIMPs	Tissue inhibitors of metalloproteinases’
TNF-α	Tumour necrosis factor α
TGF-β	Transforming growth factor β
WHO	World health organization

1. Introduction

1.1 Wounds and their health care burden

Wound is defined as damage or disruption to the normal anatomical structure and function of a living tissue (Hemamalini et al., 2011). This damage ranges from a simple break in the epithelial integrity of the skin to deeper subcutaneous tissue involvements and damage to other structures, like muscle and bone (Velnar et al.,2009).

Wounds can arise from physical, chemical, thermal, microbial or immunological damage to a tissue or can be the result of a disease process such as; pressure vascular compromise, immunodeficiency, malignancy, metabolic diseases; including diabetes, nutritional deficiencies, and adverse effects of medications (Eming et al., 2016; Frykberg & Banks, 2015; Shedoeva et al., 2019). Despite the different underline causes, wounds show a common physiological response to the noxious factor resulting in bleeding, vessel contraction, coagulation, activation of complement and inflammatory response (Eming et al., 2016).

Wounds are clinically classified based on different criteria such as time or physiology of healing (acute and chronic), aetiology (bites, burns, surgical, abrasion, laceration and other), degree of contamination (aseptic, contaminated, septic) and morphological characteristics (contusion, Incision and abration) (Puhaindran, 2008). The underline causes of wounds are also used to describe wounds like; diabetic foot ulcers, venous leg ulcers, arterial leg ulcers, and pressure ulcers (Velnar et al., 2009).

Based on Physiology or time of healing, it is broadly classified as acute wound or chronic wound. An acute wound is a wound that follows normal, timely and orderly healing process that results sustained restoration of functional and anatomical integrity. Complete healing of acute wounds is usually attained within few days or two months. Traumatic tissue damage and surgical procedures are among the causes for this type of wound (Stephan et al., 2010). Chronic wound is the one that fails to progress through the normal physiology of healing, and tissue repair is not orderly and timely. Even though there is no agreement or a clear pre-established consensus for wound chronicity, chronic wound is frequently described as ‘Wound that is not healed in 3 months’. It is also defined as ‘wound that lacks a 20–40% reduction in size after 2–4 weeks of optimal treatment or that with no complete healing after 6 weeks ‘. In chronic wounds the normal physiologic healing is disrupted at one or more phases and brings about a non-healing state. This non-healing state is linked to various underline factors including infection, tissue hypoxia, hyperglycaemia, ischemia, necrosis and excess levels of inflammatory cytokines (Kyaw et al., 2018; Velnar et al., 2009).

Wounds, especially chronic wounds or non-healing wounds have considerable humanistic and economic burdens, both at an individual and societal level. Individual Patients often suffer from pain, impaired mobility, excessive exudates, wound malodour, restricted social life, and in general impaired quality of life (Olsson et al., 2019). Chronic wounds are associated with major economic burdens to the society. Direct health care costs and indirect costs like productivity losses due to sick leave and early retirement results in substantial impacts (Shedoeva et al., 2019).

The global prevalence and health care costs of chronic wounds has been increasing alarmingly due to increases in vascular diseases, diabetes, obesity, metabolic syndrome, and aging of the population (Ellis et al., 2018; Rodrigues et al., 2019; Shedoeva et al., 2019). For example, it has been estimated that in the age group of 45-65 chronic wounds affect 120 people in every 100,000 people, but for the population of more than 75 years old the prevalence rises to 800 per 100, 000 people (Velnar et al., 2009).

Current worldwide estimate of people with chronic wound rises to 6 million each year. In developed countries 1-2% of individuals in a population acquire a chronic wound during their life time (Tessema et al.,2018). The economic burden is estimated to be nearly 2-4% of the health budgets. For instance, In USA about 8.2 million people suffer from chronic wounds each year demanding estimated more than 28 billion dollar annual health care cost (Nussbaum et al., 2018; Zielins et al., 2015). In United Kingdom, National Health Service (NHS) managing an estimated 2.2 million patients with a wounds and associated co-morbidities with annual cost of 5.3 billion pounds, which accounts 4% of NHS total expenditure in 2013. NHS also estimated that prevalence of chronic wounds will grow at the rate of 12% per annum (White et al., 2017).

In resource-poor countries, even if there are limited comprehensive epidemiological reports as developed countries; the prevalence of non-healing wounds is expected to be higher due to increased traumatic injuries and ulcers (Sasidharan, et al., 2010; Serena, 2014). According to WHO, the challenges of wound healing in the developing world are also connected to different reasons that result failure of health care delivery like , those reasons in turn are linked to poverty and social unrest. Internal displacement and the general mobility of populations add considerable unreliability to follow-up (Barchitta et al., 2019; Taye et al., 2011).

1.2 Physiological wound healing

Wound healing is a complex and dynamic process of restoring structure and function of damaged tissues. It is an intricate body's natural process which follows coordinated interactions between diverse immunological and biological systems (Davoodi-Roodbordeii et al., 2019; Hemamalini et al., 2011). The interaction involves a cascade of ordered and precisely regulated steps and events. These events include bleeding, coagulation, inflammatory response, regeneration, migration and proliferation of connective tissue and parenchyma cells,

synthesis of extracellular matrix proteins, remodeling of new parenchyma and connective tissue and collagen deposition (Stephan Schreml et al., 2010; Velnar et al., 2009). Despite the underline cause, all wound types undergo through similar wound repair events mentioned earlier, but shows a little difference from one kind of tissue to another. For example, specialized tissues such as liver and the eye follow distinct pathways and have different forms of regeneration and repair (Stephan Schreml et al., 2010).

1.2.1 Phases of wound healing

The process of wound healing is a continuous and integrated physiological process divided into different time dependent phases in order to aid understanding of biological activities that are taking place in the wound and surrounding tissue (Rodrigues et al., 2019). As shown in figure 1, these overlapping but distinct phases are i) hemostasis/coagulation, beginning immediately after injury; ii) inflammation, which begins shortly after phase one ; iii) proliferation, encompasses major healing process and starts within days of the injury and iv) remodelling, in which scar tissue formation takes place, and which may last up to a year or more (Biswas et al., 2017; D. Chen et al., 2019; Oliveira et al., 2020; Rodrigues et al., 2019).

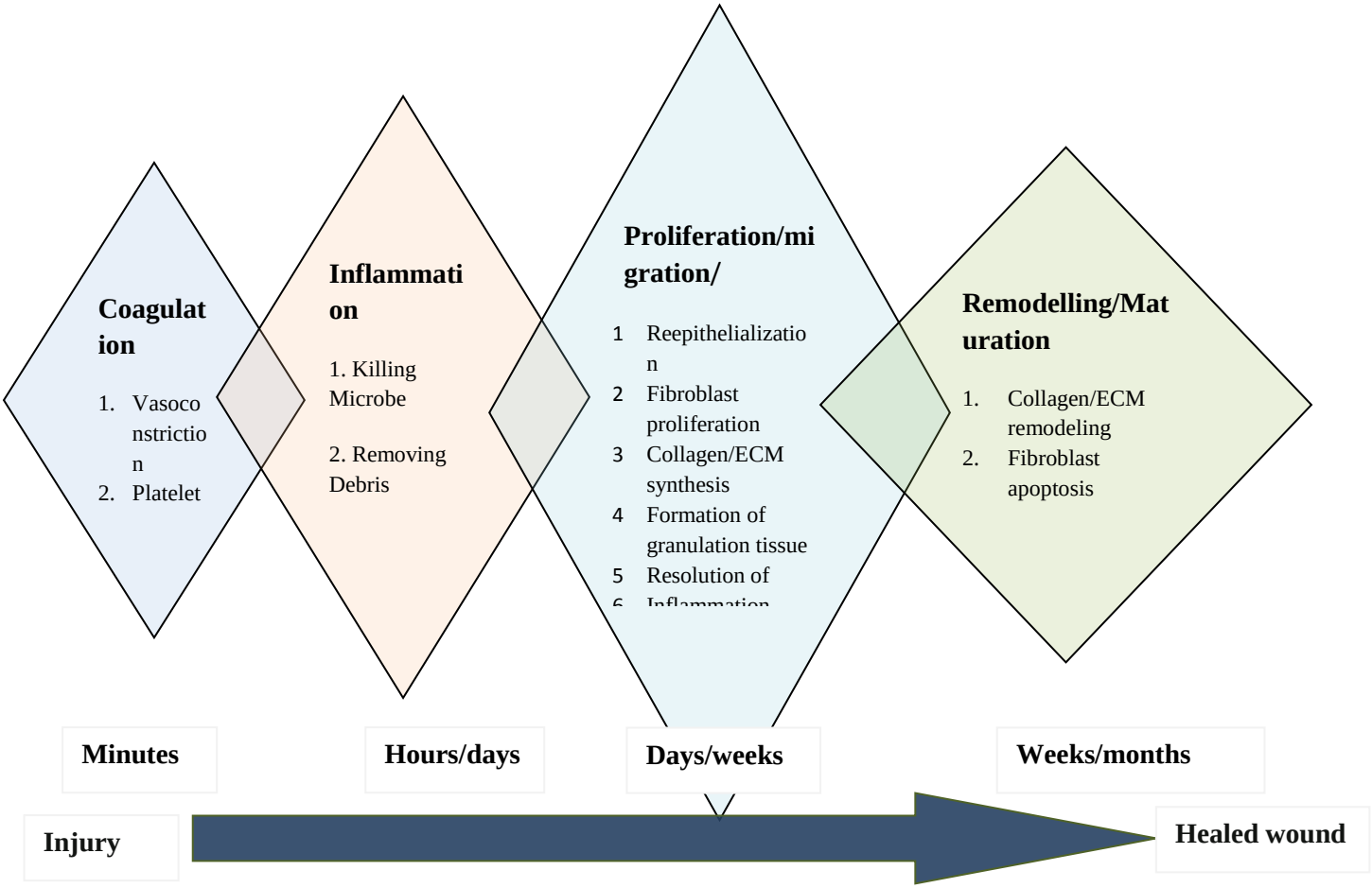

Figure 1: Wound healing cascade. Diagram adapted from Shedoeva *et al.* , 2019.

1.2.1.1 Hemostasis / Coagulation

Hemostasis is an important initial phase of the healing process that appears immediately after tissue injury. It is characterized by activation of extrinsic and intrinsic coagulation pathways and vasoconstriction (M. Chen et al., 2012; Zielins et al., 2015)

This early activity is important to protect the vascular system that is crucial for functions of vital organs and provide a matrix for invading cells (Hodges et al., 2010). Platelets are responsible cells to trigger the clotting cascade and secrete several growth factors, like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) also release inflammatory cytokines like tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) (Penn et al., 2012). These molecules promote subsequent phases of healing by activating and attracting neutrophils, macrophages, endothelial cells and fibroblasts (Hu et al., 2014).

1.2.1.2 Inflammatory phase

The next phase is humoral and cellular inflammatory phase, which is characterized by increased capillary permeability and cell migration into the wound site (Wang et al., 2018). Generally, this phase is intended to establish an immune barrier against invading micro-organisms (Stephan Schreml et al., 2010; Sorg et al., 2017). In early phase of inflammation, neutrophils are infiltrated to the wound site first, followed by monocytes and lymphocytes and start with phagocytosis in order to destroy and remove bacteria, foreign particles and damaged tissue (Rodrigues et al., 2019). Influx of inflammatory cells is mediated by chemo-attractant factors such as PDGF, TGF- β, IL-1 and other growth factors and cytokines. Neutrophil activity gradually declines within a few days and the entire neutrophils eliminated by extrusion to the wound surface and by apoptosis. At the later phase of inflammation, blood monocytes infiltrate the injury site and differentiate into macrophages. Macrophages are essential for wound healing, it secretes various enzymes and cytokines, including collagenases, which debride the wound; ILs and TNF, which stimulate fibroblasts and promote angiogenesis; and TGF, which stimulates keratinocytes (He & Marneros, 2013; Rodero & Khosrotehrani, 2010). Macrophages also phagocytize other phagocytes as well as bacteria and damaged tissue. In the absence of macrophages, wound healing and the tensile strength of the wound will be impaired (He & Marneros, 2013).

1.2.1.3 Proliferative Phase

The proliferative phase usually starts on third day after tissue injury and lasts for about two weeks (Kokane et al., 2009). Epithelialization, angiogenesis, granulation tissue formation, and collagen deposition are the principal processes of this phase (Stephan Schreml et al., 2010; Sorg et al., 2017). Three prominent cell types are responsible for those processes; fibroblasts, epithelial cells and endothelial cells (Flegg et al., 2020).

Fibroblasts and myofibroblasts migration and proliferation, which is responsible for collagen deposition, is a significant healing process of the proliferative phase. TGF-β and PDGF released by inflammatory cells and platelets are factors which attract migration of fibroblasts. Once fibroblasts are in the wound site, they proliferate profusely and produce the matrix proteins hyaluronan, fibronectin, proteoglycans and procollagen. Then, they form thick actin bundles below the plasma membrane attaching to fibronectin and collagen in the extracellular matrix. Wound contraction is achieved by retraction of these cell extensions (El Ayadi et al., 2020; Rodero & Khosrotehrani, 2010). Wound Contraction is vital for the healing process because it shortens healing time by diminishing wound size and facilitates epithelialization by shortening the distance keratinocytes should migrate (Kundu et al., 2016).

Epithelialization (re-epithelialization) is achieved by epithelial cells (keratinocytes) migration upward in the normal pattern, if basement membrane is intact. If basement membrane is destroyed epithelial cells located on the skin edge begin proliferating and projects to the center reinstate a protective barrier (Pastar et al., 2014). Keratinocytes also participate in shaping the extracellular matrix (ECM) by expressing surface markers that enhance migration across the matrix (Mazumdar et al., 2019; Shedoeva et al., 2019).

Angiogenesis, which is marked by endothelial cell migration and capillary formation, is stimulated by TNF-α released by the macrophages and tissue hypoxia. Endothelial cell growth factor, VEGF, angiopoietin-1 and PDGF also are important promoters of angiogenesis. In addition to angiogenesis, in proliferation phase development of granulation tissue is achieved. Granulation follows when the fibrin clot scaffold is replaced with new tissue rich in hyaluronan (hyaluronic acid), fibronectin, and other ECM compounds (Kondo & Ishida, 2010; Yesuf & Asres, 2013)

1.2.1.4 Remodelling Phase

The final phase of wound healing is responsible for the development of new epithelium and final mature scar tissue formation. The major feature of this phase is the deposition of collagen in an organized and mannerly network (Schultz, Chin, Moldawer, & Diegelmann, 2011). Apoptosis of macrophages and myofibroblasts as well as decline of the neovasculature are also noticeable (Oliveira et al., 2020). Remodelling leads to a normal healing through maintaining balance between degradation and synthesis of collagen (degradation of type III collagen and formation of type I collagen**)** (Oliveira et al., 2020; Pastar et al., 2014; Rodrigues et al., 2019).
Neutrophils, macrophages and fibroblasts produce matrix metalloproteinase (MMP) responsible for the degradation of collagen. MMP activity is tightly regulated and synchronized by inhibitory factors, tissue inhibitors of metalloproteinases' (TIMPs). Gradual increase in inhibitory factors and drop in MMP activity promote new matrix accumulation (Finnson, McLean, Di Guglielmo, & Philip, 2013). With progressive

collagen matrix accumulation in the process tensile strength of the wound is increased. Though, collagen fibers may regain only about 80% of their original strength of none wounding tissue (El Ayadi et al., 2020).

1.3 Pathological wound healing

Physiological wound healing processes effectively restore structural and functional states of injured tissues. However**,** the healing process may be disrupted and wounds may delay to heal, heal incompletely or become non-healing (chronic wounds) (Frykberg & Banks, 2015). Different underline factors are associated with disrupted wound healing like; systemic diseases; diabetes and vascular insufficiency; vascular hypoxia, infections, obesity, increased age, stress, nutritional status, alcoholism, smoking, and medications such as steroids and chemotherapy may disrupt healing (Blair et al., 2020; Hashemi et al., 2015; Stephan et al., 2010; Zielins et al., 2015).

These underline factors interfere with one or more cellular processes of normal physiological healing, leading to impaired healing. For instance, diabetic patients have delayed tissue repair owing to abnormal tissue inflammation, impaired fibroblast and endothelial cell proliferation and decreased collagen deposition. They are also susceptible to wound infection due to impaired neutrophil chemotaxis and phagocytosis (Okonkwo & Dipietro, 2017; Yesuf & Asres, 2013).

Nutritional status is another important factor in wound healing, malnutrition being one of the risk factors for impaired healing. Energy and protein requirements of chronic wound patients are high due to increased metabolic needs of the wound area in response to injury and loss of large amount of protein through wound exudates. Proteins are important for cell formation and activity and they are necessary for immune response. So, low protein level may delay progression from inflammatory phase to proliferative phase. Protein-energy deficiency may also decrease fibroblast activity, which in turn delays angiogenesis and reduces collagen formation (Barchitta et al., 2019).

Beyond from protein and energy, recent evidences have pointed out biochemical and molecular effects of several nutrients in the wound healing process. The amino acids, arginine and glutamine reported to have important role in wound healing. Arginine, as precursor of nitric oxide and proline, is essential for inflammatory process and collagen synthesis. Glutamine also plays several roles owing to its metabolic, enzymatic, antioxidant, and immune properties (Hung et al., 2019).

Vitamins are the most investigated micronutrients in wound healing process. It is known that Vitamin A deficiency impairs B cell and T cell function and antibody production during the inflammation (Polcz & Barbul, 2019). Deficiency of this vitamin decreases epithelialization, collagen synthesis, and granulation tissue

development in the proliferative and remodelling phases. Vitamin Bs deficiencies indirectly affect the wound healing process by impairing antibody production and white blood cell function (Barchitta et al., 2019). Likewise vitamins C and vitamin E have potent antioxidant and anti-inflammatory effects, and their deficiency may delay wound healing (Hashemi et al., 2015).

In general, chronic wounds share certain common features at the molecular level, including excessive levels of proinflammatory cytokines, proteases, reactive oxygen species (ROS), and senescent cells, and existence of persistent infection. The amplified and persistent proinflammatory cytokine cascade for a prolonged time leads to elevated levels of proteases. These elevated proteases are responsible for degradation of ECM, also attract more inflammatory cells, thus amplifying the inflammation cycle (Schultz et al., 2011).

Low concentration of ROS produced by immune cells provides defense against microorganisms. However, in chronic wounds, the predominant hypoxic and inflammatory environment increases ROS production, which damages ECM proteins and causes cell damage (Schreml et al., 2010). This leads to stimulation of proteases and inflammatory cytokines, sequentially. Improvement of wound healing resulted from application of strong antioxidants to reduce ROS has been demonstrated in animal model (Frykberg & Banks, 2015).

In contrast to delayed or non-healing wounds, Pathological scars caused by dysfunctional 'excessive healing' are another form of disrupted wound healing process. Pathological scars include hypertrophic scars and keloids, they are characterized by altered cell proliferation and increased ECM deposition (Penn et al., 2012; Schultz et al., 2011). Keloids proliferate beyond the original lesions, a clinical feature that distinguishes them from hypertrophic scars. It is also possible to differentiate them histologically based on their collagen density; thickened collagen fibrils found in keloids while fine fibers in hypertrophic scars. Pathological scars may cause debilitating contractures beyond their aesthetic implications (Eming et al., 2016).

1.4 Bacterial colonization of wounds

Wound infection is one of the most common causes for impaired wound healing. Wound infections can be caused by different groups of microorganisms like bacteria, fungi and protozoa. Though, existence of polymicrobial communities is common, bacteria are prevalent contaminants (Ayele et al., 2016). Any open wound is considered contaminated but not infected. Infection is expected when the number of microbial per gram of tissue excides 10^5.

Infections disrupt the healing process by prolonging the inflammatory phase by elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. Prolonged inflammation also leads to an increased level of MMPs, which degrade the ECM as mentioned earlier (Heil et al., 2017).

Bacterial infection is also reported to interfere with epithelialization, contraction, and collagen deposition. In addition, endotoxins from some bacteria stimulate the release of collagenase, an enzyme that contributes to collagen degradation and destruction of normal tissues surrounding the wound site. Bacterial colonization of wounds is also associated with tissue hypoxia that in turn potentially suppresses macrophage-regulated fibroblast proliferation (Guo & DiPietro, 2010).

Bacterial strains that commonly cause wound infections include *Staphylococcus aureus (s.aurus), Streptococcus pyogenes(S.pyrogenes), Escherichia coli (E.coli), Pseudomonas aeroginosa (p.aeruginosa), Streptococcus pneumonia (S.pneumonea), Klebsiella pneumoni (k.pneumone), Staphylococcus faecalis, Coliform bacilli , enterococcus , Clostridium perfringens and Clostridium tetani* (Ayele et al., 2016; Dev, Choudhury, Srivastava, & Sharma, 2019; Taye et al., 2011).

In most cases of non-healing chronic wounds *P. aeruginosa and Staphylococcus* appear to play an important role. In infected wounds these bacteria occur in the form of biofilms, which are complex communities of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix. Mature biofilms develop protected microenvironments shielding the bacteria from phagocyte activity of invading polymorphonuclear neutrophils (PMNs) and makes conventional antibiotic treatment less effective (Guo & DiPietro, 2010).

1.5 Existing therapeutics for wound management

The aim of wound care is to achieve wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient. The central modality of wound treatment is to correct the disrupted physiological process of healing, so that healing to occur (Ji et al., 2016). Managing the patient's nutritional and hydration status, addressing any co-morbidity that may contribute to non-healing state, infection control, adequate wound oxygenation and debridement are the paramount strategies to maximize wound healing potential (Eming et al., 2016).

Antimicrobial agents are among few commonly used drugs for wound management (Ayele et al., 2016). They comprise disinfectants, antiseptics, and antibiotics. Their proposed benefit is that they promote the normal healing process by protecting the wound from superficial infection (Mulisa, Asres, & Engidawork, 2015). The routine treatment strategies include application of topical antibiotics or antiseptics, or even the use of long-term systemic antibiotics. Topical antimicrobial therapy is one of the most important methods of wound care. Topical antibiotic preparations are available in the form of lotions, creams, ointments, powders, gels, foam and sprays. Reduced risk of systemic side effects, having higher concentration in the affected area and quantified drug usage are some advantages of topical agents. Though, antibiotic selection depends on activity against suspected microbes or culture and sensitivity tests there are some standard topical agents frequently used in

wound management. These topical preparations include; tetracycline, nitrofurazone, neomycin-bacitracin powder, gentamycin, polymyxin B, chloramphenicol and clindamycin.

Other wound management pharmaceuticals include herbal based healing formulations (pro-wound healing formulations) and biological products. Moist exposed burn ointment (MEBO) is one example of herbal formulation possessing β-sitosterol, baicalin, and berberine as active ingredients in a base of beeswax and sesame oil. MEBO offers the advantages of optimum moisture for wound healing. By optimizing moisture, it facilitates keratinocyte migration and interaction with growth factors. Several studies have confirmed rapid healing activity of MEBO in acute or chronic wounds (Al-Meshaan et al., 2008).

Biological formulations include topical formulation of recombinant human PDGF and other growth factors, enzymatic debridement agent like collagenase formulation and MMP inhibitors, among other (Finnson et al., 2013; Frykberg & Banks, 2015; Oliveira et al., 2020). These products are not commonly used because of unpredicted adverse effects and high production costs (Zielins et al., 2015).

1.6 Role of Medicinal Plants in Wound Management

Plant based preparations are the most commonly used traditional medicinal practices globally. WHO listed a total of 21,000 plants which are extensively used for medicinal purposes throughout the world. These plant based medicines, also called herbal medicines, are used for a number of conditions and are widely accepted across various cultures and socio-economic levels (Hassan *et al*.,2013).

One of the frequently reported uses of traditional medicines is for the treatment of wound and skin disorders. It is reported that, one-third of all traditional medicines in use are indicated for treatment of wounds and skin disorders (Hosseinkhani et al., 2017). Beyond from traditional use reports, numerous medicinal plants have been studied for their wound healing activities throughout the world. In Ethiopia, numerous ethnobotanical reports revealed that wide ranges of traditional plants are used for treatment of acute and chronic wounds (Taye et al., 2011). Many among these plants are reported to have wound healing activities in different studies. Achyranthes *aspera* (Barua et al., 2012), *Becium grandiflorum* (Beshir et al., 2016), *Rumexa byssinicus, Rhusvulgaris ficuscaricus, Acacia abysinica, Coffee Arabica* (Humaryanto & Ave, 2019), *Amorphophallus gallaensi , Allophylus abyssinicus* (Yesuf & Asres, 2013), *Bersama abyssinica* (Yemata et al., 2019) and *Brucea antidysenterica* (Mekonnen et al, 2019; Tessema et al, 2019) *are* some of these promising plants.

In addition to crude plants, several active compounds from different plants are well characterized and have been demonstrated to have properties that benefit wound healing. These include; Acemannan, a mucopolysaccharide from *Aloe Vera*, which is a potent stimulator of macrophage ; Catechins, the polyphenolic compounds from *Camellia sinensis* that stimulate the proliferation and differentiation of keratinocytes;

curcumin, an active substance found in the root of *Curcuma longa* that indicated to enhance fibroblast proliferation, granulation tissue formation, and collagen deposition; and Asiaticoside, madecassoside and triterpenes isolated from *Centella asiatica* that enhance different process of wound healing in different animal experiments (Shedoeva et al., 2019). Asiaticoside has been found to enhance collagen deposition and epithelialization in a punch wound model in the guinea pig. Triterpenes elevate collagen remodelling and glycosaminoglycan synthesis and madecassoside facilitate collagen synthesis and angiogenesis (Shedoeva et al., 2019).

1.7 Overview of Experimental Plant

B. antidysenterica is one of the most common species among more than 170 species in the family of Simaroubaceae of medium sized trees and shrubs (Alves et al., 2014). The family is widely distributed in tropical areas of America, Australia, Malaysia (Asia) and Africa (Makong et al., 2019). There have been many studies on species of simarobaceae family elucidating many potential bioactive molecules including over 200 quassinoids (Vijayan, Preeja, & Murugan, 2018). *Brucea* is one of the 32 genera of Simaroubaceae family which includes about 10 species found in Asia and Africa, two of which are found in Ethiopia; *Brucea ferruginea* and *B. antidysenterica* (Kefe et al., 2016).

B. antidysenterica had been used for dysentery in Ethiopia probably for centuries. But, it was first introduced to the scientific world after the well-known Scottish traveller James Bruce took the seed to Europe after his visit to Ethiopia between 1768 -1773. James came to know the plant after it saved his life, given by traditional herbalists, from deadly dysentery when he was in northern Ethiopia and the plant was named after his name and "*Antidysenterica*": derived from the Greek 'anti' = 'against', and 'dusenteria' = 'bad bowels' (Duke, 2019).
B. antidysenterica is a small tree or shrub (Fig.2), distributed in moist tropical African countries including; Ethiopia, Eretria, Sudan, Guinea, Nigeria, Cameroon, Burundi, Angola and Zambia. It grows 10 to 15 m height with smooth bark of grey to pale brown colour, alternate leaves and a yellow-red fruit (Dilnesa et.al, 2016; Tessema et al., 2019).

Various ethnobotanical studies reported that *B. antidysenterica,* locally called Waginos (Geeze), Yedega Abalo (Amharic), Tamija/Komeni (Affan Oromo), Meleta (Tigrigna), Hadawi (Somali) and Atanico (Sidama), is a medicinal plant of multiple uses. Reported traditional medicinal uses include treatment against scabies, wounds, leprosy, dysentery, gonorrhoea, eczema, fever, malaria, haemorrhoids, trypanosomiasis and hookworm (Amuamuta et al., 2015; Getahun, 1976; Makong et al., 2019; Mekonnen et al., 2019; Taye et al., 2011; Tessema et al., 2019).

Figure 2: Picture of *Brucea antidysenterica* (Photograph taken by principal investigator during sample collection).

Supporting the traditional use, experimental studies of different extracts and isolated compounds from this plant revealed different biological activities. Bruceantin, triterpene quassinoid, isolated from the bark of *B. antidysenterica* is one example (Gillin, Reiner, & Suffness, 1982). Bruceantin showed anti-inflammatory activity in mice model and marked amoebicidal activity *in vitro* against colony of *Entamoeba histolytica* (Alves et al., 2014). In addition, anti-inflammatory and wound healing effects of the leaf and fruit extracts were reported from investigations using animal model of wound healing and inflammation (Mekonnen et al., 2019; Tessema et al., 2019).

Bacterial growth inhibition is another biological activity of *B. antidysenterica.* Investigation of the hydro-alcoholic (80% methanol) extract of the root against wound causing bacteria reported the inhibition of bacterial growth against *S. aureus*, *S. pyogens* and *P. aeuruginosa* clinical isolates and standard strains (Ayele et al., 2016; Taye et al., 2011).

Phytochemical screening of *B. antidysenterica* showed that the plant is rich of different primary and secondary metabolites. Flavonoids, proteins, free amino acids, carbohydrates, vitamin C, alkaloids, tannins, triterpenoid, phenols, steroids and glycosides have been reported to be present in different parts of the plant (Dilnesa, Mekonon, & Abebe, 2016; Tessema et al., 2019).

1.8 Statement of the Problem

Chronic wounds are among the major challenges of the medical world. Currently, the prevalence and costs of chronic wounds is increasing globally. Increasing prevalence of diabetes and obesity and aging of the population are the major causes for higher prevalence of non-healing wounds (Olsson et al., 2019). It is estimated that by 2025 there will be more than 400 million diabetics globally, and 25% of these patients are expected to develop foot ulcers during their lifetimes (Serena, 2014). Apart from the patients suffering of pain, psychological stress and disability from non-healing wounds, its economic burden is enormous challenge for health care. Wound related complications are also related with mortality, Studies show that for every million wound patients, at least 10,000 die from microbial infections (Wong et al., 2015).

Incomplete understanding of the underlying molecular basis of tissue repair and its failure, as well as lack of preclinical animal models that properly recapitulate human conditions, has led to lack of effective therapies for treating non healing wounds or for speeding up the repair of acute wounds and reducing scar formation (Ansell et al., 2012; Eming et al., 2016; Grada et al., 2018). It is also reported that, Only1–3% of the drugs listed in Western pharmacopoeias are intended for wound management and their efficacy for fast wound healing is not satisfactory according to clinical audits (Hosseinkhani et al., 2017). In addition to this, many of the emerging drugs for wound management are expensive and linked with problems such as allergy and cancer risks (Beshir et al., 2016).

Bacterial infections reported as one of the major cause for delayed wound healing. The prevalence of the infected none-healing wounds are associated with growing resistance bacterial strains. Despite the ongoing development of new antibacterial drugs and enhancing existing ones, emergence of resistant bacteria and serious side effects of synthetic antibacterial drugs remain to be a challenge. Antibacterial resistance is among the major global problems, and the spread of multi drug resistant (MDR) bacteria in hospital and community settings remains a widely unresolved problem and a heavy burden to health services (Chaudhary, 2016; Elbossaty, 2017; Fymat, 2017; Zaman et al., 2017). More seriously, resistance to some last resort antibiotics have been reported repeatedly (Chaudhary, 2016). Those reasons demands investigations for alternative sources of antibacterial agents.

Medicinal plants show promising healing effects and constitute numerous compounds that may have different biological activities. Studying wound healing and antibacterial activities of potential medicinal plants and looking for novel wound healing and antibacterial compounds is valuable amid to address the current and emerging challenges.

2. Objectives

2.1 General Objective

The aim of this study was to examine wound healing and antibacterial activities of solvent fractions of 80% methanol leaf extract of *B. antidysenterica*.

2.2 Specific Objectives

- To investigate acute toxicity profile of solvent fractions of 80% methanol leaf extract of *B. antidysenterica*.
- To evaluate wound healing activity of solvent fractions of 80% methanol leaf extract of *B. antidysenterica*
- To assess antibacterial activity of solvent fractions of 80% methanol leaf extract of *B. antidysenterica*
- To determine minimum inhibitory concentration and minimum bactericidal concentration of solvent fractions of 80% methanol leaf extract of *B. antidysenterica.*
- To identify secondary metabolites found in solvent fractions of 80% methanol leaf extract of *B. antidysenterica*.

3. Materials and Methods

3.1 Plant material collection and identification

B. antidysenterica leaves were collected around Atawi, Wara Jarso woreda, Semen Shoa Zone, Oromia Region, Ethiopia, which is around 170km north west of Addis Ababa. Identification and authentication of the plant material was done by Getachew Addis (PhD), a taxonomist at the National Herbarium unit of Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. The collected plant materials were wrapped by a chemical free plastic bag, transported to the laboratory, cleaned by tap water and then dried at room temperature under shade for a week. When dried, the plant materials were grinded using electric mill in to course powder and stored at refrigerator until used for extraction.

3.2 Chemicals, Media and Drugs

Methanol absolute (LOBA chemie Pvt. Ltd., India), chloroform (LOBA chemie Pvt. Ltd., India), n-butanol (LOBA chemie Pvt. Ltd., India) and distilled water were used for extraction of plant material and fractionation of the crude extract. Wool fat (BDH Chemicals ltd, England), hard paraffin (BDH Chemicals ltd, England), white soft paraffin (Anonchem limited, China) , and cecostearyl alcohol (BDH Chemicals ltd, England) used to prepare simple ointment were found from the department of pharmaceutics and pharmaceutical chemistry, school of pharmacy, Addis Ababa University. Other chemicals used include; DMSO (LOBA chemie Pvt. Ltd., India), Acetic anhydride (LOBA chemie Pvt. Ltd., India), acid alcohol (LOBA chemie Pvt. Ltd., India) , ammonia (LOBA chemie Pvt. Ltd., India), ethyl acetate, ferric chloride, glacial acetic acid (LOBA chemie Pvt. Ltd., India), sulfuric acid (LOBA chemie Pvt. Ltd., India), Mueller Hinton agar (REF 24756(MM 091), SRL chemicals India,), normal saline, Nitrofurazone ointment USP 0.2% (Galentic pharm, Pvt.Ltd.Co, India), Moist Burn Ointment MEBO (Julphar pharmaceutical, Ethiopia), ketamine hydrochloride injection USP (Neon laboratories limited, India), and Ciprofloxacin 5µg standard antibiotic disc. All chemicals, and reagents used were analytical grade.

3.3 Instruments, Supplies and Apparatuses

The following apparatuses and instruments were used during the experiment; digital weighing balance (Mettler Toledo, Switzerland), mini orbital shaker (Bibby Scientific Limited, UK), rotary evaporator (Buchii model R-200, Switzerland), lyophilizer (Operan, Korea vacuum limited, Korea), deep freezer, steam sterilizer, incubator, Petri dish, Micro pipette, Cork borer, water bath, Separatory funnel, Erlenmeyer conical flask, beaker, measuring cylinder, falcon, shaver with sterile blade (Razor), ruler, mortar and pestle, ointment slab, syringe with needles, Surgical and disposable gloves, surgical threads with curved needles, forceps, Whatman filter paper (Number 1, Whatman Ltd. England), permanent marker, graph paper.

3.4 Experimental Animals

Adult, healthy Wistar albino rats (*Rattus norvegicus)* of both sex weighing 180-250g and aged 4-6 months were obtained from EPHI (animals used for excision and incision wound models). For dermal toxicity study Healthy adult female Swiss albino mice (29-40 g) found from School of Pharmacy, Addis Ababa University. The animals were housed in cages under standard conditions with 12 h light and dark cycles and had free access to standard food and water. Animals were allowed to acclimatize to the laboratory condition for a week before subjected to any experiment. All the way through the experiment, animals were handled according to international laboratory animal use and care guidelines (OECD, 2008). At the end of the experiment the animals were sacrificed by spinal dislocation.

3.5 Microbial organisms

In vitro antibacterial activity of each solvent fraction was evaluated against gram positive bacterial species; *S. aureus and S .pyogens,* and gram negative bacteria*; E. coil, P. aeuruginosa, ,* and *K. Pneumoniae.* All bacterial strains were Standard strains (American Type Culture Collections (ATCC)) and obtained from the Department of Microbiology, Parasitology and Immunology, School of medicine, Addis Ababa University, Addis Ababa.

3.6 Extraction and Fractionation of *Brucea antidysenterica* leaf

3.6.1 Extraction

The grinded leaf powder was extracted using cold maceration extraction technique, and 80% (V/V) methanol was chosen as a solvent for its better yield obtained in previous studies (Dilnesa, et al., 2016; Taye et al., 2011). Nine hundred gram of the course powder was soaked with 4,500ml of 80% methanol in Erlenmeyer conical flasks. Then, the flasks were fixed on mini orbital shakers adjusted at 145 revolutions per minute (rpm) with periodic agitation for three days. The mixture was first filtered with a nylon cloth and the mark was re-macerated to have additional yield. The filtrate was filtered for the second time with whatman's filter paper (number 1) using a pressurized suction system.

Then, total filtrate was concentrated using low pressure rotary evaporator set at a pressure of 121mbar, 45rpm rotations and temperature of 40 0c. Then to remove residual methanol, the extract was dried in water bath at 40^{o}c for a week. After the concentrate frozen in deep freezer, brownish crude extract was obtained by removing water with lyophilizer at -50 ^{o}C under vacuum pressure (200mBar).

3.6.2 Solvent Fractionation of 80% methanol crude Extract

Brownish crude extract was further fractionated by successive solvent-solvent fractionation in a separatory funnel. Water, chloroform and n-butanol were used as a solvent to differentiate active substances at different

polarity (solubility) profiles from crude extract. First the crude extract was dissolved in 1/3(W/V) of distilled water and then the same volume of chloroform was added. Then the mixture was vigorously shaken in a funnel and put on a stand awaiting a clear separation, since chloroform is heavier in density the lower part of separated mixture was collected carefully in a beaker. For maximum separation the same amount of chloroform added again to the left part (aqueous part) and fractionated.

After chloroform fraction was completely collected, n-butanol (equal volume with distilled water) was added to the funnel and the two fractions were separated in the same way previously described; the only difference was that aqueous fraction was collected first for n-butanol is lighter than water.

The fractionated mixtures were concentrated and dried to obtain the dry yield of fractions following techniques discussed previously for the crude extract. The dry Aqueous Fraction (AF), n-Butanol Fraction (BF) and Chloroform Fraction (CF) were packed in tight containers, labelled and stored in deep freezer at -20oC until used for the experiments.

3.7 Ointment formulation

Simple ointment of each solvent fraction was prepared following the formula described in British Pharmacopeia (Table 1) (British Pharmacopoeia, 1988).

Ointment preparations (50gm) for each solvent fraction with two strengths (2% w/w and 4%w/w) and simple ointment without active substance (used as a control) were formulated using a reduced formula (Table 1).

Table 1: Master and reduced formula used to formulate simple ointment.

Ingredients	Master formula	Reduced formula
Wool fat	50g	2.5 g
Hard paraffin	50g	2.5 g
Cetostearyl alcohol	50g	2.5 g
White soft Paraffin	850g	42.5g
	1000g	50g

To formulate simple ointment, the calculated amount of hard paraffin and cecostearyl alcohol were mixed and melted in a beaker. In a separate beaker the mixture of wool fat and white soft paraffin was melted while stirring to maintain its homogeneity. After removing from the water bath, the former was added to the later and then stirred until cooled.

Medicated ointment of uniform consistency and smooth texture were prepared. To prepare 2% ointments, 2g of each fraction weighed and mixed with 48 g of the ointment base. Similarly, 4g of each fraction were mixed

with 46g of ointment base. The prepared ointments were 2% Aqueous Fraction (2%AF), 4% Aqueous Fraction (4%AF), 2% n-Butanol Fraction (2%BF), 4% n-Butanol Fraction (4%BF), 2% Chloroform Fraction (2%CF) and 4% Chloroform Fraction (4%CF). And, non-medicated ointment base used as a negative control.

3.8 Grouping and dosing of experimental animals

Animals were randomly grouped in to different groups as required for the experimentation. For dermal toxicity study, six groups of female mice, three mice in each group were used (Organization for Economic Cooperation and Development (OCDE), 2004). Groups I and II were treated with 2% AF and 4% AF, respectively; Groups III, IV, V and VI were treated with 2%BF, 4%BF, 2%CF and 4%CF, respectively.

For excision model of wound healing, nine groups of rats with five rats each were used. Group I was treated with simple ointment and used as a control, Groups II, III, IV, V, VI, VII were treated with 2%AF, 4%AF, 2%BF, 4%BF, 2%CF and 4%CF, respectively. The last two groups served as positive controls: Group VIII with 0.2% nitrofurazone (NF) and Group IX with MEBO.

For Incision model of wound healing, ten groups of rats with five rats each were used. Groups I to IX were treated in the same way described for excision model and Group X was left untreated and served as negative control.

3.9 Toxicity studies

3.9.1 Acute toxicity study

Acute toxicity study was performed according to OECD guideline for the test of chemicals OECD/OCDE -425 (OECD/OCDE, 2008). Healthy female Wistar albino rats (180- 200kg) were used for the test. Before administration of test fractions, it was assessed that all animals were physically active and had been consuming food and water regularly. At the test day, rats were deprived of food but not water for 4 hours prior to dosing, and after the end of the fasting period weight was recorded and dose calculated based on weight (2000mg/kg). Then, three female rats were randomly selected and 2000mg/kg (dissolved by 1ml of DMSO) of each solvent fractions were administered via oral route. After administration of required dose, the animals were observed at least once during the first 30 minutes and periodically during the first 24 hours, with special attention given during the first 4 hours. Since death was not recorded within 24 hours, another 4 female rats for each solvent fraction were given based on their weight (2000mg/kg). After administration, the animals were observed for their behavior and physical activities for 24 hours as stated above and then daily for two weeks for any sign of delayed toxicity. Observations included for signs of toxicity like change in skin and fur, eye, mucus

membranes; also change in respiratory system, autonomic system, gross weight change, motor activity and behavioral patterns.

3.9.2 Acute dermal toxicity study

Acute dermal toxicity study was done according to OECD guideline for the test of chemicals OECD: 434 (Organization for Economic Cooperation and Development (OCDE), 2004) with slight modification. Groups of three Wistar rats with normal skin texture were used for each solvent fraction. Before the experiment, animals were acclimatized to the laboratory conditions for five days. Then 24 hours prior to application of test ointment, dorsal fur (around 10% of body surface area) was shaved and animals were housed in individual cages. On the test day, 24 hours after shaving, 2% and 4% w/w ointment preparations of each solvent fraction was applied as thin film uniformly to the shaved area. After 24 hours of exposure residual test substance was removed and animals were observed for the next 24 hours periodically for any sign of toxicity. Then animals were observed daily for development of any delayed toxicity for two weeks.

3.10 Wound healing studies

Two wound healing models commonly recommended to assess wound healing activities on rodents were used in this study; Excision and Incision models. Ointment formulations of solvent fractions, negative and positive controls were applied to group of animals arranged as described in the grouping and dosing section. Based on the experiments, wound healing activity was evaluated by rate of wound contraction and period of epithelialization for excision model and breaking strength (tensile strength) for incision model.

3.10.1 Excision wound model

Excision wound model experiment was carried out at the pharmacology laboratory of School of Pharmacy, Addis Ababa University. Appropriate experimental setting and instruments were prepared, and animals were acclimatized to the laboratory conditions. Before wounding animals were anesthetized with intra-peritoneal (IP) administration of 80mg/kg ketamine HCl and then dorsal fur was shaved and a circular area of 314mm^2 was marked using a material (a coin) of 20mm in diameter (figure 3A). Then the full thickness of marked circular area was excised out using sterile sharp surgical scissors to make a wound (figure 3B) of nearly equal area on all animals. To achieve homeostasis, wound area was blotted using a cotton swab soaked in normal saline. Wounding day was considered as day 0 and rats were housed in individual cages. After 24 hours of wounding, test and control ointments were applied and continued once daily until wound was healed. Wound closure was monitored and wound area was measured on the 2nd, 4th, 6th, 8th, 10th ... and 22nd days. Wound contraction for each day of measurement was calculated based on the initial wound size as stated in the formula below (Fikru *et al*., 2012; Wesley *et al*., 2009). In addition Period of epithelialization, the number of

days required for falling of dead tissue remnants without any residual raw wound, was evaluated (Liu et al., 2013).

$$\%\ \boldsymbol{Wound\ contraction}\ (\boldsymbol{wound\ closure}) = \frac{\boldsymbol{Initial\ wound\ area - wound\ area\ at\ day\ n}}{\boldsymbol{Intial\ wound\ area}}\boldsymbol{x}100$$

Where 'n' = number of measurement days (2^{nd}, 4^{th}, 8^{th}, 12^{th}…..22^{nd}

\

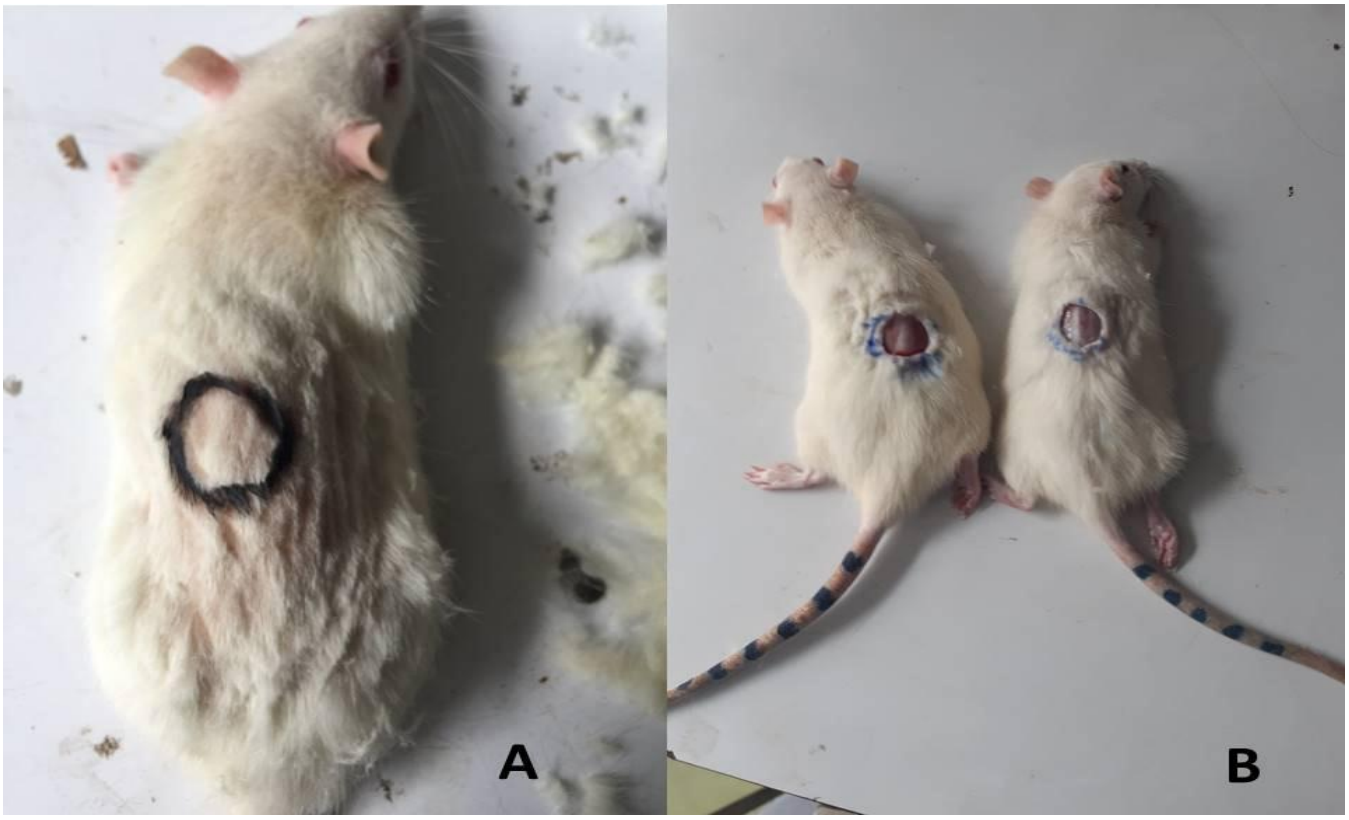

Figure 3: Excision wounding of animals. 3A- Circularly marked area to be excised, 3B - Excised wound area

3.10.2 Incision wound model

On wounding day, animals were anesthetized using the same technique as described previously for excision wound model. Then, the dorsal fur was shaved and a three cm long longitudinal paravertebral incision was made through the skin and subcutaneous tissue. Using surgical thread (silk, no. 000) and a curved needle (no. 9) the skin parted was sutured one centimeter apart. Thread on both wound edges was tightened for good closure of the wounds (Fig 4A). Starting from 24 h of post wounding (day 1), animals were treated with respective ointments as described under grouping and dosing section, except the last group of animals which was left untreated and used as references. On day eight post wounding, sutures were removed and treatment continued. Tensile strength was measured on the 10th post wounding day using continuous water flow technique (Wang et al., 2018) (Fig.4B & 4C). Based on the tensile strength measured, percent of strength was calculated using the following formulas (Kundu et al., 2016).

$$\%\ \boldsymbol{Tensile\ sterngth\ of\ Extracts} = \frac{\boldsymbol{TSextract\text{-}TSso}}{\boldsymbol{TSso}}\boldsymbol{x}100$$

$$\%\ \boldsymbol{Tensile\ sterngth\ of\ Reference} = \frac{\boldsymbol{TSreference\text{-}TSso}}{\boldsymbol{TSso}}\boldsymbol{x}100$$

$$\%\ \boldsymbol{Tensile\ sterngth\ of\ SO} = \frac{\boldsymbol{TSSo\text{-}TSLU}}{\boldsymbol{TSLU}}\boldsymbol{x}100$$

Where; 'SO' is an ointment without active substance (vehicle), and 'LU' is left untreated (negative control) (Barua et al., 2012)

Figure 4: Incision wound experiment. Showing a 3cm longitudinal wound created on dorsal area of animals, sutured 1cm apart and the continuous water flow experiment to investigate breaking strength of wound site.

3.11 Antibacterial Activity

3.11.1 Agar well diffusion

Agar well diffusion method was used to determine the antibacterial activity of solvent fractions. Muller Hinton agar (MHA) was prepared according to the manufacturer recommendation. The powder media was added to a flask containing measured volume of distilled water and placed on hot plate with stirrer until it boils. Then the media was sterilized at 121^0C for 15 minutes using an autoclave. The medium was cooled to 45-50 ºC inside water bath. For fastidious *Streptococcus* species (S. *pyogenes*) that require enriched media, 5% sterile sheep blood was added to MHA medium. After that, 20ml media was poured to pre-labelled sterile Petri-dishes (90mm) aseptically and allowed to solidify (Balouiri, Sadiki, & Ibnsouda, 2016; Mostafa et al., 2018; Yemata et al., 2019).

Broth cultures of test organism were prepared to a density of 10^8 cells ml^{-1} of 0.5 McFarland standards (Hudzicki, 2016). Then culture spread evenly (with 60^o rotation of the Petri-dishes had uniform bacterial growth) on to MHA by sterile cotton swab following aseptic condition in safety cabinet and the medium allowed to dry for 30 minutes at room temperature. After the media hardened required numbers of wells were formed using a sterile cork borer which was 6mm in diameter ensuring proper distance from the periphery and between the centres of each hole.

Test samples (solvent fractions) were prepared in concentration of 500mg/ml and 100mg/ml using DMSO as solvent. Using a micropipette 20µl of fractions samples and the negative control (DMSO) were loaded to respective wells as labelled using permanent marker on the Petri plates. Ciprofloxacin 5µg/ml disc was used as a positive control. Then, the plates were left at room temperature for two hours to allow diffusion of test samples and incubated at 37^{o}c for 24 hours. After incubation period, plates were observed for inhibition zone (zones showing no bacterial growth). Inhibition zones were measured using a scale (Balouiri et al., 2016; Mostafa et al., 2018).

3.11.2 Determination of minimum inhibitory concentration (MIC)

For fractions which showed zone of inhibition greater than and equal to 7mm for a certain organism, MIC was determined using broth macrodilution method (Balouiri et al., 2016; Ullah et al., 2016). For fractions which showed activity at the concentration of 500mg/ml, the concentration was further diluted to 1:2 and 1:4, to have 250mg/ml and 125mg/ml. Fractions that showed activity at 100mg/ml were further diluted to 75mg/ml, 50mg/ml (1:2) , 25mg (1:4), 12.5mg/ml (1;8) and 6.25mg/ml (1;16). Broth media prepared as per the manufacturer recommendation. Inoculums preparation and standardization was done aseptically as described in section 3.11.1. Each concentration of fractions was diluted in 1:2 ratio with the broth media and 1ml was added to standard test tube. The standard inoculums were diluted in broth media as (1:150), and 1ml of the diluents added to each tube, containing different concentrations of fractions. A tube without any test sample was used as control for bacterial growth. The tubes were covered and incubated at 37^{o}c for 24 hour. After incubation, bacterial growth was analyzed visually; the minimum concentration that did not show any visible bacterial growth was taken as MIC (Ullah et al., 2016).

3.11.3 Determination of the minimum bactericidal concentration (MBC)

MBC was determined by sub-culturing solvent fractions having a value of lesser or equal to MIC value. From the MIC test solutions contents were streaked using sterile cotton swabs on agar plate and incubated at 37 ^{o}C for 24 hours. The lowest concentration that yielded no single bacterial colony was taken as MBC (Ullah et al., 2016).

3.12 Preliminary Phytochemical screening

Qualitative Screening for the presence of secondary metabolites in aqueous, n-butanol and chloroform fractions of 80% methanol extract of *B. antidysenterica* leaf was performed using standard tests described previously. Presence of alkaloids, saponins, flavonoids, terpenoids, phenols, glycosides and tannins was tested, following the procedures discussed below:

A) Test for Saponins (foam test)

From each of the solvent fractions 0.25g of sample was taken and dissolved in 5ml of distil water. After vigorous shaking the formation of persistent foam observed for 30 minute was taken as an indication for the presence of saponins (Tessema et al., 2018; Yemata et al., 2019).

B) Test for Alkaloids

Each fraction (0.5g) was stirred with 1 % HCl (10 mL) on a water bath for 5 min and filtered. The filtrate was divided into three equal parts. To one portion of filtrate, 1 ml Mayer's reagent (Potassium mercuric iodide solution) was added. Formation of orange color precipitate gives an indication of the presence of alkaloids (Vijayan et al., 2018).

C) Test for Tannins

Each fraction (0.25g) was boiled in 10 mL of water in a test tube and filtered. To the filtrate, few drops of 0.1 % FeCl3 (ferric chloride) was added to give a brownish green or a blue-black color which confirms the presence of tannins (Bhosale et al., 2012; Fentahun M et al., 2017).

D) **Test for Terpenoids**

Each solvent fraction (0.25g) was mixed with 2 ml of chloroform, and 3 ml concentrated H2SO4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids (Tessema et al., 2019).

E) Test for flavonoids

Ten ml of ethyl acetate was added to 0.2gm of each solvent fraction and heated for 3 min on a water bath. The mixture was filtered, and the filtrate was mixed with 1mL of dilute ammonia solution. Formation of intense yellow colour ratifies the presence of flavonoids (Oumer et al., 2014).

F) Test for steroids

Each of the solvent fractions (0.25g) was dissolved in 0.25mL dichloromethane to produce a dilute solution. To this solution 0.25 ml of acetic anhydride was added, followed by three drops of concentrated sulphuric acid. Formation of a blue-green coloration indicated the presence of steroids (Mekonon, et al., 2016; Krishnaveni & Thaakur, 2008).

G) Test for glycoside

Each solvent fraction (0.25g) was diluted with 5ml of water and 2ml of glacial acetic acid containing one drop of ferric chloride solution was added. The 1ml of concentrated sulphuric acid was added to the solution. The formation of a brown ring at the interface indicates the presence of glycosides (Vijayan et al., 2018).

H) **Test for phenols**

Ten mg sample from each solvent fraction was dissolved in 1ml of water. Half ml of 5% ferric chloride solution was added to the solution and development of deep blue or black colour was taken as an indicator for the presence of phenols (Tessema et al., 2019).

3.13 Statistical analysis

Raw data 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 -tests and data were considered significant at $p < 0.05$. Statistical analysis was done using SPSS version 23.

4. Results

4.1 Yields of extraction and fractionation

From the total of 900 g course leaf powder of *B. antidysenterica* extracted using 80% methanol, 120gm of dark brown paste was obtained; the yield was calculated to be 13%. The 120gm crude extract was fractionated using distilled water, n-butanol and chloroform. The yields of aqueous, n-butanol and chloroform fractions were found to be 73gm (61%), 25gm (21%) and 16gm (13%) respectively.

4.2 Toxicity study

4.2.1 Acute toxicity

The results from an acute toxicity study showed that aqueous, n-butanol and chloroform fractions of 80% methanol extract of *B. antidysenterica* leaf appeared to be safe up to the dose of 2000 mg/kg which was confirmed by the death of none of the animals and absence of any sign of gross and behavioural toxicity up to two weeks. Based on this, it can be said that the LD_{50} of the three solvent fractions are greater than 2000 mg/kg.

4.2.2 Acute dermal toxicity

The study revealed that neither 2% nor, 4% ointment preparations of all solvent fractions showed any sign of inflammation, redness, rush or irritation when observed 24 hours after applications. No overt signs of toxicity were observed during monitoring for further 14 days.

Although acute dermal toxicity showed safety of sample ointments, when the 5% w/w ointment preparations of fractions were applied at excision wound area during a pilot study the n-butanol and chloroform fractions showed inflammation and irritation of wound area revealed after three days of application of sample ointments. Continuous application of 5% CF ointment caused one and two deaths out of five animals on days six and eight. In excision and incision models, application of 2% and 4% ointments of both BF and CF showed inflammation and irritation of wound area in the majority of the animals with repetitive doses starting from day 3, and was exacerbated with continued application of treatment. No death was, however, recorded with continuous treatment applications up to day 10. Comparing BF and CF based on simple observation of wound areas, CF showed higher degree of inflammation and irritation of wound area in the majority of the animals. However, all 5% AF, 4% AF and 2% AF ointment preparations showed no sign of irritation, redness and inflammation.

4.3 Wound healing effects

4.3.1 Effect on excision wound model

The solvent fractions of 80% methanol leaf extract of *B. antidysenterica* revealed varied activities on excision wounds. Only aqueous fraction showed promising effect on wound contraction and period of epithelialisation as shown in tables 2 and 3, respectively; while n-butanol and chloroform fractions appeared to affect the healing negatively.

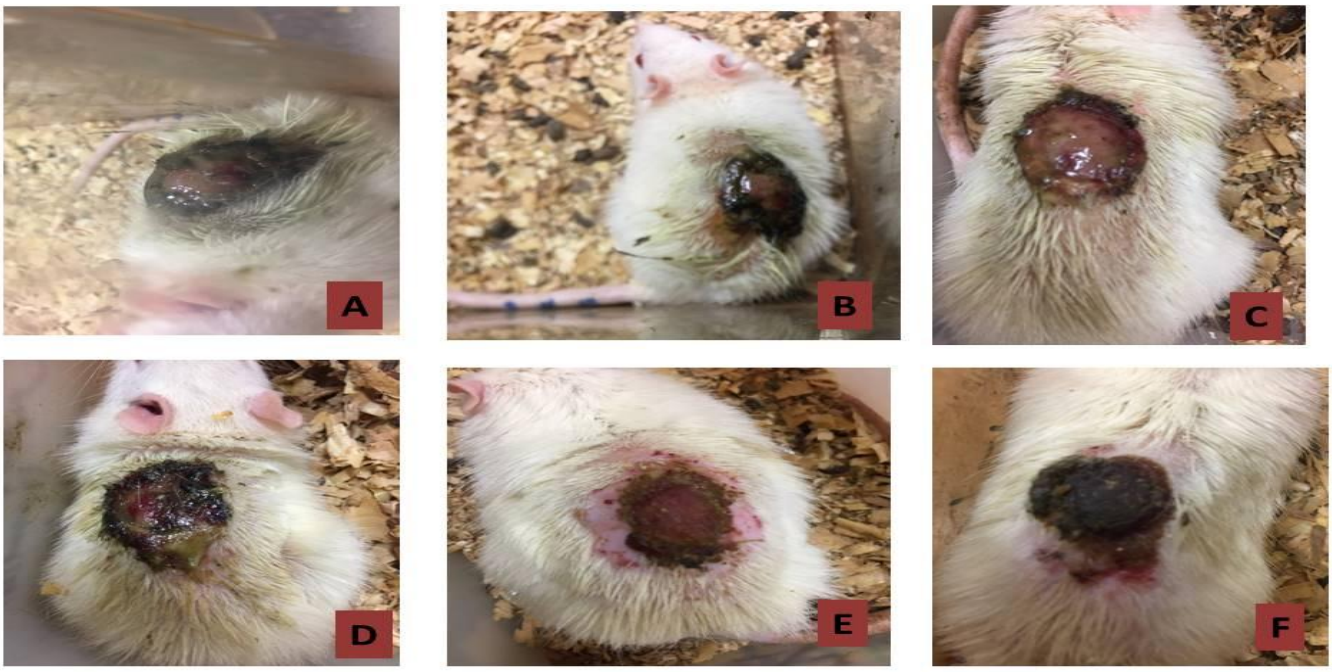

Figure 5: Pictograph of wound site after application of n-butanol and chloroform fractions on Excision wounds. 5A = Day 4 2% n-butanol fraction treated animal, 5B = Day 4 4% Chloroform fraction treated group, 5C=Day 8 4% n-butanol treated animal, 5D= Day 8 4% chloroform treated animal, 5E= Day 10 2% n-Butanol treated animal, 5F= day 10 2% Chloroform treated animal.

Majority of 2%BF, 4%BF, 2%CF and 4%CF treated animals showed slow progressive wound closure for the first few days of treatment, but from day six onward with continued application of ointments of the two fractions, wounds and the surrounding tissue became erythematic, inflamed, irritated and increased pain sensitivity to touch had been noticed (figure 5). After day 11, application of CF and BF ointments was discontinued considering loss of healing progress and increased wound area observed in majority of animals treated. Animals treated with CF showed more wound site exacerbation than BF treated groups (Table 2). In

both BF and CF treated animals, majority of 4% ointment treated animals showed higher degree of wound exacerbation than 2% ointment treated animals.

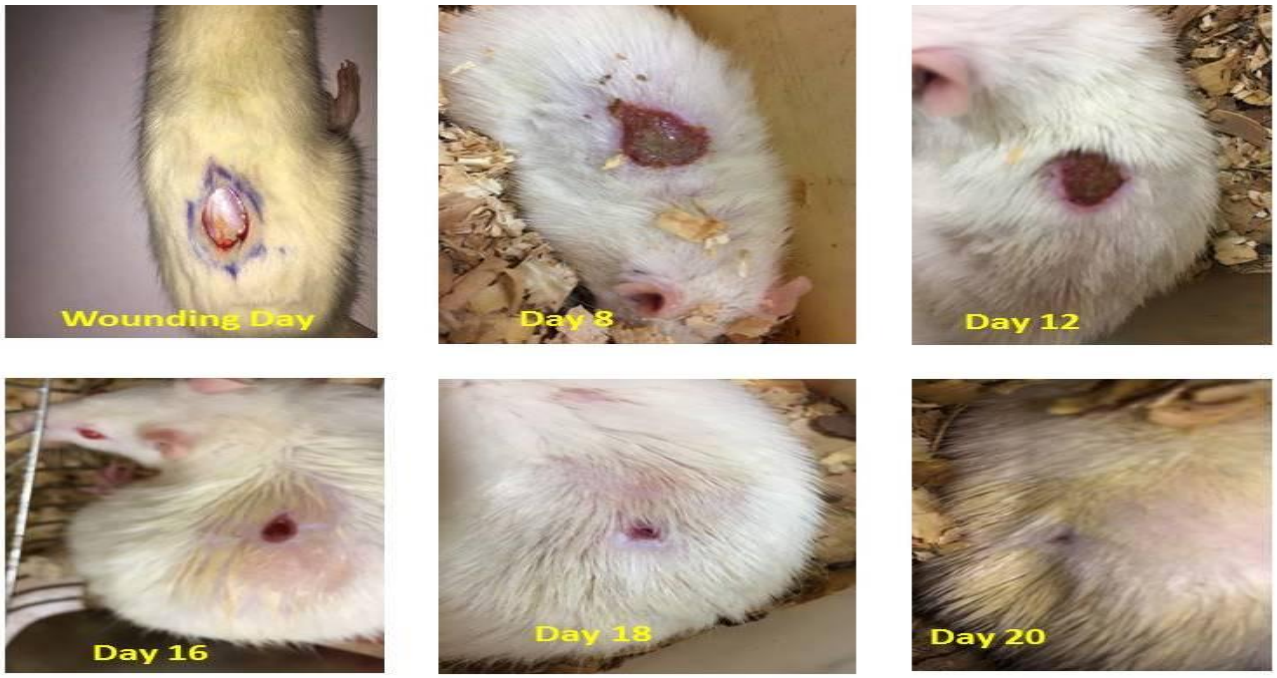

Figure 6: Pictograph of wound contraction of animal treated with aqueous fraction of 80% methanol extract of B. antidysenterica leaf on different days of measurement.

On the contrary to n-butanol and chloroform fractions, 2%AF, 4%AF (figure 6) and standard treatments have shown significant effect on wound contraction compared with the control. Standard drugs (NF and MEBO) showed significant ($p<0.001$) wound contraction starting day 6 post wounding; while 2%AF and 4%AF showed significant wound contraction on day 10 ($p<0.001$) and day 8 ($p<0.05$), respectively. The aqueous fraction and standard drugs showed significant wound contraction compared with negative control up to final day of measurement. On final day, 2%AF had 97.5% of wound contraction and 4%AF 98.39%.

Table 2: Effect of solvent fractions of 80% methanol extract of *B. antidysenterica* leaf on percentage wound contraction of excision wound in rat.

Group	Wound area (mm2)±SEM (% contraction)									
	Day 2	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**	**Day 14**	**Day 16**	**Day 18**	**Day 20**
SO	298.66 ±6.85 (4.89)	266.02±8.35 (15.27)	257.54±9.35 (17.98)	230.4±17.75 (26.63)	194.28±11.86(38.1)	137.2±7.7 (56.3)	104.06±14.14 (66.85)	69.38±9.55 (77.9)	42.84±6.40 (86.35)	26.02±3.70 (91.71)
2%AF	295.7 ±8.83 (5.82)	257.38±7.02 (18.03)	237.96±7.93 (24.22)	184.22±8.83 (41.33)	124.76±4.8 * b (60.24)	79.04±7.03 *c (74.83)	50.8±5.18 *a (83.82)	24.38±4.07 *c (92.23)	16.82±3.84 *c (94.64)	7.86±3.36* c (97.5)
4%AF	284.02±13.33 (9.54)	251.88±9.37 (19.78)	222.04±9.95 (29.29)	173.4±16.14 *a (44.77)	103.12±10.7*c (67.15)	72.58±11.48 *c (76.89)	42.72±9.66 *c (86.4)	21.68±7.75 *c (93.1)	9.46±3.9 *c (96.99)	5.04±2.78 *c (98.39)
2%BF	307.86 ±6.14 (1.95)	268.82±6.46 (14.38)	261.4±9.91 (16.75)	266.6±14.83 (15.09)	248.9±5.5 (20.73)	238.24±10.73 (24.13)	-	-	-	-
4%BF	310.9 ±3.1 (0.99)	286.34±3.04 (8.78)	302.11±12.56 (3.78)	308.2±11.76 (1.83)	307.86±6.14 (1.95)	-	-	-	-	-
2%CF	314 ±0 (0)	308.2±11.76 (1.83)	326.96±7.94 (-4.13)	320.48±6.48 (-2.06)	307.86±6.14 (1.95)	-	-	-	-	-
4%CF	307.86 ±6.14 (1.95)	314.3±9.95 (-0.1)	333.63±13.15 (-6.25)	333.32±7.89 (-6.15)	339.76±6.44 (-8.2)	-	-	-	-	-
NF	280.74 ±9.89 (10.58)	243.4±6.74 (22.48)	201.3±7.96 *b (35.89)	160.94±8.25 *a (48.74)	103.8±15.96 *c (66.94)	58.78±6.71 *c (81.28)	30.7±3.87 *c (90.22)	7.44±1.68 *c (97.63)	1.4±0.71 *c (99.56)	0 *c (100)
MEBO	283.6 ±8.27 (9.67)	246.6±12.08 (21.46)	211.42±7.46 *b (32.67)	163.6±11.66 *a (47.89)	105.82±9.63 *c (66.29)	65.24±4.61 *c (79.22)	35.56±3.14 *c (88.68)	10.74±2.56 *c (96.58)	2.33±1.28* c (99.26)	0.04±0.04 *c (99.99)

n=5 Wistar rats in each group; analyzed by one way ANOVA followed by Tuckey post hoc test ; Day 2-20 are wound contraction measurement days; SO-simple ointment base; AF-Aqueous fraction, BF- n-Butanol fraction, CF- Chloroform fraction, NF=nitrofurazone ointment, MEBO- Moist Exposed Burn Ointment *: compared to the control; $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$. Missing data shows that n-butanol and chloroform fraction treatments were discontinued after day 10.

Although both aqueous fraction and standard drug showed significant effect compared to the control, wound contraction by standard drugs on each day of measurement and the final percentage of contraction; 100% and 99.99% for NF and MEBO, respectively, showed no significant difference against both 2% AF and 4% AF. Additionally, there was no significant difference among 2%AF and 4%AF in wound contraction and epithelialisation period at each day of measurements.

Table 3: Effect of solvent fractions of 80% methanol extract of *B. antidysenterica* leaf on epithelialization period of excision wound in rats

Group	**Epithelialization Period (Mean ± SEM), Days**
SO	19.6 ± 0.5
2%AF	18.2 ± 0.4
4% AF	17.2 ± 0.6*a
NF	16.4 ± 0.4*b
MEBO	16.4 ± 0.5*b

n=5 Wistar rats in each group; analyzed by one way ANOVA followed by Tuckey post hoc test ; SO-simple ointment base; AF-Aqueous fraction, NF= nitrofurazone ointment, MEBO- Moist Exposed Burn Ointment *: compared to the SO; $^{a}p<0.05$; $^{b}p<0.01$;

Epithelialization period is the other important parameter determined from excision wound model. Time for complete epithelialization was short in AF ointment and standard drugs treated groups compared to control (Table 3). Although 2%AF ointment failed to produce a statistically significant difference in period of epithelialization, those treated with 4%AF had a significantly shorter ($p< 0.001$) epithelialization period compared to the control group. Apart from this, there was no significant difference between groups treated with standards and aqueous fraction as well as among the two ointment strengths of aqueous fractions.

4.3.2 Effect on incision wound model

Aqueous fraction of *B. antidysenterica* 80% methanol extract was found to increase the breaking strength of incision wounds, i.e., tensile strength compared with negative control group. As shown in Table 4, 2%AF ($p<0.05$) and 4%AF ($p< 0.001$) significantly increase the tensile strength compared against simple ointment treated group. But, it was revealed that BF and CF did not increase the tensile strength. The standard drugs,

nitrofurazone and MEBO showed significant increment in breaking strength compared to both BF and CF. Ointment of 4%AF also significantly increase breaking strength compared to that of 2% BF ($p< 0.05$). Nevertheless, no significant differences were observed among the different concentrations of aqueous fractions and standards drugs.

Table 4: Effect of solvent fractions of 80% methanol extract of *B. Antidysenterica* leaves on breaking strength of incision wound site in rats.

Group	Breaking Strength (g)	Tensile Strength (%)
LU	460.2 ± 43.41	
SO	513.4 ± 19.37	10.36
2%AF	642.2 ± 27.11*c $^{\#}$a	25.09
4% AF	684 ± 24.31*c$^{\#}$c **a	33.23
2% BF	546.6 ± 12.83	6.47
4%BF	575.6 ± 34.29	12.12
2% CF	565.4 ± 22.12	10.13
4% CF	564.2 ± 17.18	9.89
NF	711 ± 21.47*c $^{\#}$ c **b $^{\#\#}$a***a $^{\#\#\#}$a	38.49
MEBO	699.6 ± 16.34*c $^{\#}$ c **b***a $^{\#\#}$a $^{\#\#\#}$a	36.27

Values are expressed as mean ± SEM (n=5 Wistar rats in each group) and analyzed by one way ANOVA followed by Tuckey post hoc test; LU=left untreated control; SO=simple ointment base; AF-Aqueous fraction, BF- Butanol fraction, CF- Chloroform fraction; NFO=nitrofurazone ointment, *: compared against LU, $^{\#}$: compared against SO;** compared against 2%BF, ***compared against 4%BF, $^{\#\#}$ compared against 2%CF, $^{\#\#\#}$ compare against 4%CF ; a-$p<0.05$; b-$p<0.01$; c-$p<0.001$

4.4 Antibacterial activity

4.4.1 Bacterial growth inhibition

Aqueous, n-butanol and chloroform fractions of *B. antidysenterica* 80% leaf extract showed moderate bacterial growth inhibition of different standard organisms tested. Bacterial growth inhibitory activity,(measured as zone of inhibition) of the fractions ranged from 7mm (chloroform fraction against *K. pneumoneae*) to 16mm (butanol fraction against *S. aureus*). Butanol fraction showed the highest growth inhibition against the gram positive bacteria *S. aureus and S.pyogens,* and gram negative bacteria *E. coil and P. aeuruginosa* (Table 5).

Table 5: Mean bacterial growth inhibition zone of solvent fractions of 80% methanol extract of *B. antidysenterica* leaf in agar well diffusion method (20µl sample volume).

		Bacteria				
Fractions & Standard	Concentration	*S. aureus*	*E. coli*	*K. pneumoneae*	*P. aeuruginosa*	*S. pyogens*
Aqueous Fraction	100mg/ml	-	-	-	-	-
	500mg/ml	10	-	-	-	8
n- Butanol Fraction	100mg/ml	12	-	-	-	-
	500mg/ml	16	8		11	10
Chloroform Fraction	100mg/ml	8	-	-	-	-
	500mg/ml	10	8	7	-	-
Ciprofloxacin	5µg/ml	23	24	-	34	24

Where; MIC= minimum inhibitory concentration, MBC =minimum bactericidal concentration

4.4.2 Minimum inhibitory concentration and minimum bactericidal concentration

The MIC and MBC values were determined for fractions that showed growth inhibitions in agar well diffusion method. The lowest MIC (75mg/ml) observed was that of n-butanol fraction against *S. Aureus* (Table 6). The lowest MBC observed (125mg/ml) was that of n-butanol fraction (against *S. pyogens* and *S. aureus)* and chloroform fraction (against *S. Aureus*

Table 6: MIC and MBC of solvent fractions of 80% methanol extract of *B. antidysenterica* leaf using tube dilution method.

	Bacteria							
Fractions	***S. aureus***		***E. coli***		***P. aeuruginosa***		***S. pyogenes***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Aqueous Fraction	250mg/ml	250mg/ml	-	-	-	-	250mg/ml	-
n- Butanol Fraction	75mg/ml	125mg/ml	500mg/ml	-	250mg/ml	-	125mg/ml	125mg/ml
Chloroform Fraction	125mg/ml	125mg/ml	500mg/ml	-	-	-	-	-

Where; MIC= minimum inhibitory concentration, MBC =minimum bactericidal concentration

4.5 Phytochemical screening

Phytochemical screening of aqueous, n-butanol and chloroform fractions of 80% methanol extract of *B. antidysenterica leaf* showed the presence of different secondary metabolites. Majority of secondary metabolites tested were shown to be present in all fractions tested in the experiment (Table 7).

Table 7: Results of phytochemical screening of solvent fractions of 80% methanol extracts of *B. antidysenterica* leaf.

Secondary Metabolites	**Solvent fractions**		
	Aqueous fraction	**n-butanol fraction**	**Chloroform fraction**
Alkaloids	+	+	+
Tannins	-	-	-
Saponins	+	+	-
Flavonoids	+	+	+
Terpenoid	+	+	+
Phenols	+	+	+
Steroids	+	-	+
Glycosides	+	+	-

Note: (+) indicates presence and (-) indicates absence of particular metabolite

5. Discussion

Wound healing and antibacterial activities of *B. antidysenterica* reported in a range of ethnobotanical studies. In the previous study, wound healing activity of 80% methanol leaf extract of *B. antidysenterica* showed significant wound contraction (99.9%) compared with simple ointment, and it shortened the epithelialization period and increased the tensile strength of wounds (Tessema *et al.*, 2018). In another study, aqueous crude extract of the leaf on excision and incision wound healing models showed promising results (Mekonnen et al., 2019). In addition to wound healing effects, extracts of root, seed and leaf of *B. antidysenterica*, were reported to show antibacterial activity against different wound causing bacterial strains (Fantahun *et al.*, 2017; Taye *et al*., 2011).

In the present acute toxicity study, aqueous, n-butanol and chloroform fractions of the 80% crude leaf extract, revealed that all the three fractions had oral median lethal dose (LD_{50}) of more than 2000mg/kg indicating the safety nature of the fractions. Similarly, acute dermal toxicity showed that 2% and 4% ointment preparations were non-irritant and cause no rash, redness or other unexpected reactions. This was in line with the recommendation that products used for topical wound treatments should be non-toxic, biocompatible and have intended clinical activity without adversely affecting physiological healing process.

On the contrary to acute dermal toxicity results, application of 5% chloroform ointment at wound site during the pilot study showed wound exacerbation and 2 out of 5 animals treated within six days of continuous treatment died. Same concentration of n-butanol fraction also adversely affected wound healing though no death was recorded up to the 10^{th} day of treatment. Additionally, 2% and 4% of n-butanol and chloroform fractions revealed irritation, inflammation and adversely affected wound healing. The present findings are supported by that of the previous study which reported that 10% ointment of 80% methanol extract had a lethal effect when applied to excision wounds, the study reported that 50% of animals tested with 10% methanol leaf extract and 4% of methanol fruit extract killed 50% of treated animals (Tessema et al., 2019). The toxic effect might be attributed to the non-polar compound/s (Tessema et al., 2019) which are not present in the aqueous fraction for no adverse effect was observed in mice treated with aqueous fraction in the current experiment. The negative effect of n-butanol and chloroform fractions may be attributed to the increased or prolonged inflammation, considering that the effect revealed after two or three days of treatment characterized by redness, swelling and pain around the wound site, and defected scarring that may suggest pathological fibrosis. Fibrosis is a natural process of physiological wound healing to repair tissue function. Defected fibrosis, however, leads to suboptimal healing, dysfunctional tissue and detrimental scarring. Prolonged inflammation, mainly due to persistent activation of TGF- β signaling, is the major cause for pathological fibrosis (El Ayadi et al., 2020).

Wound repair involves different process including contraction, formation of epithelialization and fibrosis (Malviya & Jain, 2009). As there is no single representative model or reference standard for studying wound healing effect, two reputed *in vivo* models were used in this study; excision and incision wound healing models. Excision wound model is popularly used to examine effective wound closure; contraction of wound area and epithelialization (restoring the epidermis) being the reference parameters. In the present study, 2% and 4% ointments of the aqueous fraction of 80% methanol extract of *B. antidysenterica* leaf enhanced wound contraction rate compared with simple ointment treated animals. The effect however was less than that of the standards. On day 20 of wounding, the 2% AF and 4% AF treated animals attained 97.5% and 98.4%, respectively of wound closure while standard drugs treated animals had complete wound closure, These results can be considered a promising wound healing activity compared with the 91.7% wound closure recorded with simple ointment treated animals.

Wound contraction facilitates healing in short time as it decreases the size of the wound and reduces the amount of extracellular matrix needed to repair defected tissues. Contraction also facilitates healing by promoting epithelialization. Epithelialization (re-epithelialization) is achieved by epithelial cells (keratinocytes) migration from basement membrane upward or from wound edge, contraction shortens this distance keratinocytes must travel (Beshir et al., 2016). The wound contraction observed by aqueous fraction of *B. antidysenterica* 80% methanol leaf extract may be associated with *B. antidysenterica* anti-inflammatory activity (Tessema et al., 2019) and potent antioxidant property (Dilnesa, Mekonon, et al., 2016). Prolonged inflammation may lead to generation of ROS which in turn damages cells and tissues in the wound site. In addition, increase in free radical production augmented by impaired antioxidant activity may worsen the condition resulting in impaired healing (Patel, Srivastava, Singh, & Singh, 2019). In this regard, the potent antioxidant activity of aqueous fraction and rich content of antioxidant phytochemicals like vitamin C in *B. antidysenterica* leaf (Mekonnen et al., 2019) may be mentioned as one contributing property for its wound healing activities shown in this study.

As mentioned earlier, epithelialization period is another parameter examined by excision wound model. Epithelialization is considered to be a defining parameter of successful wound closure, and impaired re-epithelialization is associated with chronic non healing wounds (Pastar et al., 2014). In addition, loss of skin barrier function might cause dehydration, infection, or even death. In the present study, 4% AF shortens the epithelialization period to 17.2 days compared with 19.6 days of simple ointment treated group. Also, the effect of aqueous fraction on re-epithelialization was comparable with standard drugs (nitrofurazone and MEBO) with no significant difference observed.

Closure of wound site and regeneration of the epithelium is not enough for better wound healing. Strength or durability of the wounded area is important and is achieved by increased formation of collagen and concentration and stabilization of fibres (Heil et al., 2017). In incision wound model measuring, the tensile strength of the wound is important parameter that implies durable healing. The aqueous fraction of *B. antidysenterica* 80% methanol leaf extract increased tensile strength of incision wounds at the strength of both 2% and 4% ointment preparations. Breaking strength or tensile strength was achieved by aqueous fraction treated animals was significantly higher than those left untreated and animals treated by simple ointment.

Enhanced strength of the wound matrix implies that cell to cell and cell to matrix interactions are regained and shows repair of framework for angiogenesis which in turn facilitates blood circulation that provide oxygen and nutrient for the healing tissue. So increasing strength in the process of wound healing, such as observed by aqueous fraction of *B. antidysenterica* leaf extract treatment in this study, may suggests functional recovery of injured tissue.

This study examined that treatment with aqueous fraction of 80% methanol leaf extract of *B. antidysenterica* leaf facilitated wound contraction, shortened period of epithelialization, and enhanced breaking strength. These effects may be related with induction or stimulation of cellular proliferation, enhancement of collagen synthesis and cross-linking of proteins, increased anti-inflammatory activity and potent antioxidant property of the fraction.

Wound infections are among the major causes for chronic non-healing wounds. Most infections are caused by bacteria that may reside in the wound area, migrate from other body part or colonize the wound from the environment. Because of this, effective antimicrobial therapy has been one of the most important wound care mechanism(Mostafa et al., 2018).

Plants with antibacterial activities have been reported and gave hope to be alternative sources for anti-infective medications and fighting emerging resistance microbial strains (Valle, Andrade, Puzon, Cabrera, & Rivera, 2015). *B. antidysenterica* is one of the plants which showed antibacterial activity against different bacterial strains, including common bacteria infecting wounds (Dilnesa, et al., 2016; Fentahun M, 2017). In the present study aqueous, n-butanol and chloroform fractions of 80% methanol leaf extract of *B. antidysenterica* showed activity against different gram positive and gram negative standard bacterial strains. *S. aureus* was observed to be the most sensitive bacteria among the tested organisms to the all three fractions. N-butanol and chloroform fractions showed bactericidal activity against this organism at the concentration of 125mg/ml. The most active fraction that had activity against most bacteria strains tested was n-butanol

fraction and the least one was aqueous fraction which had minimal activity against *S. aureus* and *S. pyogenes.* From the present study no association was observed between antibacterial and wound healing activities indicating that wound healing process seen by the aqueous fraction treatment may not be mediated by antibacterial activity.

Medicinal plants have been recognized to have much broad biological activities. In the process of wound healing they may stimulate production of critical cytokines such as PDGF, FGF, VEGF, TGF-β and IL-1β to accelerate epithelialization, angiogenesis, granulation tissue formation, proliferation of fibroblasts and collagen deposition in the wounds. These comprehensive healing activities and other broad biological properties of medicinal plants are attributed to their phytochemical contents (Aslam et al., 2018; Walton, 2014).

Qualitative phytochemical tests of aqueous, n-butanol and chloroform fractions of 80% methanol leaf extract of *B. antidysenterica* showed that all the three fractions are rich is different phytochemicals such as alkaloids, flavonoids, terpinoids and phenols, which is in agreement with the findings of the previous studies (Dilnesa, Mekonon, *et al*., 2016; Fantahun M *et al*., 2017; Tessema *et al*., 2018b). The biological activities of *B. antidysenterica* leaf shown in the present study may be attributed to these phytochemicals. Phenols, for example, have a potent antioxidant and free radical scavenging activities. Aqueous fraction of 80% methanol extract of *B. Antidysenterica* leaf was shown have phenols in this study, and higher amount of phenols were reported to be present in the leaf extract (Dilnesa, Mekonon, et al., 2016). This high phenolic content may have contributed to the better wound healing property of this fraction through suppressing ROS and other oxidants and promoting cell proliferation. The other phenolic molecules, flavonoids are also potential natural antioxidants shown to be present in the fractions of *B. antidysenterica* leaf methanol extract and with higher amount in aqueous fraction (Alemayehu, et al., 2016; Dilnesa et al., 2016).

Phenols including flavonoids; and terpinoids were reported to have antibacterial, anti-inflammatory and astringent properties (Bahramsoltani, Farzaei, & Rahimi, 2014), all of which may have contributed to the wound contraction, rate of epithelialization and antibacterial activities shown in this study.

Alkaloids were other pharmacologically important phytochemicals shown to be present in all fractions of the plant in the present study. Antibacterial, antifungal, vasoconstriction, analgesic and cytotoxic properties are some of the pharmacological activities of alkaloids (Bahramsoltani et al., 2014; Inbaneson et. al., 2012; Juneja et al., 2020). Wound healing, antibacterial and even toxic effects observed in the present study may be associated with the alkaloid contents of the fractions.

Although phytochemicals presented in the fractions may be associated with the activities reported in this study, a single phytochemical alone may not be responsible for the effects observed. There is a possibility for a combination of two or more phytochemicals to be responsible for the effects as well.

6. Conclusion

The present study showed that the aqueous fraction of 80% methanol *B. antidysenterica* leaf extract has wound healing activity supporting the traditional claim. The n-butanol and chloroform fractions, on the other hand, showed delayed wound healing effect. The study also showed that all the three fractions have antibacterial activity; n-butanol fraction revealed better bacterial inhibition and aqueous fraction was the least active against the selected bacterial strains. The fractions did not demonstrate dermal toxic effects though the n-butanol and chloroform fractions showed dermal adverse reactions upon prolonged administration.

7. Recommendations

Based on the findings of the present study, the following further studies are suggested:

- Investigation of wound healing activity of compounds isolated from the aqueous fraction.
- Study on antibacterial activity of compounds isolated from the fraction
- Study on the possible mechanism/s of action of the aqueous fraction for its wound healing activity
- Carry out toxicity study of the isolated compounds

8. References

Al-Meshaan, M., Abdul Hamid, M., Quider, T., Al-Sairafi, A., & Dham, R. (2008). Role of mebo (moist exposed burn ointment) in the treatment of fournier's gangrene. *Annals of Burns and Fire Disasters*, *21*(1), 20–202.

Alves, I. A. B. S., Miranda, H. M., Soares, L. A. L., & Randau, K. P. (2014). Simaroubaceae family: Botany, chemical composition and biological activities. *Brazilian Journal of Pharmacognosy*, *24*(4), 481–501.

Amuamuta, A., Mekonnen, Z., & Gebeyehu, E. (2015). Traditional therapeutic uses and phytochemical screening of some selected indigenous medicinal plants from Northwest Ethiopia. *African Journal of Pharmacology and Therapeutics*, *4*(3), 80–85.

Ansell, D. M., Holden, K. A., & Hardman, M. J. (2012). Animal models of wound repair : Are they cutting it ? *Journal of Experimental Dermatology*, *21*, 581–585.

Aslam, M. S., Ahmad, M. S., Riaz, H., Raza, S. A., Hussain, S., Qureshi, O. S., … Javed, O. (2018). Role of Flavonoids as Wound Healing Agent. *Intechopen*, 0–7.

Ayele, T. T., Regasa, M. B., & Delesa, D. A. (2016). Evaluation of Antimicrobial Activity of Some Traditional Medicinal Plants and Herbs from Nekemte District against Wound Causing Bacterial Pathogens. *Science, Technology and Arts Research Journal*, *4*(2), 199.

Bahramsoltani, R., Farzaei, M. H., & Rahimi, R. (2014). Medicinal plants and their natural components as future drugs for the treatment of burn wounds: An integrative review. *Archives of Dermatological Research*, *306*(7), 601–617.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79.

Barchitta, M., Maugeri, A., Favara, G., San Lio, R. M., Evola, G., Agodi, A., & Basile, G. (2019). Nutrition and wound healing: An overview focusing on the beneficial effects of curcumin. *International Journal of Molecular Sciences*, *20*(5).

Barua, C. C., Talukdar, A., Begum, S. A., Pathak, D. C., Sarma, D. K., Borah, R. S., … Chandra, D. (2012). In vivo wound-healing efficacy and antioxidant activity of Achyranthes aspera in experimental burns. *Pharmaceutical Biology*, *50*(7), 892–899.

Beshir, K., Shibeshi, W., Ejigu, A., & Engdawork, E. (2016). In vivo wound healing Activity of 70% Ethanolic Leaf Extract of Becium grandiflorum Lam. (Lamaceae) in Mice. *Ethiopian Pharmaceutical Jornal*, *32*(2), 117–130.

Bhosale, U., Pophale, P., Somani, R., & Yegnanarayan, R. (2012). Effect of aqueous extracts of Achyranthes aspera Linn. on experimental animal model for inflammation. *Ancient Science of Life*, *31*(4), 202.

Biswas, T. K., Pandit, S., Chakrabarti, S., Banerjee, S., Poyra, N., & Seal, T. (2017). Evaluation of Cynodon

dactylon for wound healing activity. *Journal of Ethnopharmacology*, *197*, 128–137.

Blair, M. J., Jones, J. D., Woessner, A. E., & Quinn, K. P. (2020). Skin Structure-Function Relationships and the Wound Healing Response to Intrinsic Aging. *Advances in Wound Care*, *9*(3), 127–143.

British Pharmacopoeia. (1988). *British Pharmacopoeia*. 713.

Chaudhary, A. S. (2016). A review of global initiatives to fight antibiotic resistance and recent antibiotics' discovery. *Acta Pharmaceutica Sinica B*, *6*(6), 552–556.

Chen, D., Hou, Q., Zhong, L., Zhao, Y., Li, M., & Fu, X. (2019). Bioactive Molecules for Skin Repair and Regeneration: Progress and Perspectives. *Stem Cells International*, *2019*.

Chen, M., Chen, R., Wang, S., Tan, W., Hu, Y., Peng, X., & Wang, Y. (2012). Chemical components, pharmacological properties, and nanoparticulate delivery systems of Brucea javanica. *International Journal of Nanomedicine*, *8*, 85–92.

Davoodi-Roodbordeii, F., Afshar, M., Haji Abas Tabrizi, F., Choopani, S., Torkaman, G., Moayer, F., & Salimi, M. (2019). Topical hydrogel containing Fumaria vaillantii Loisel. extract enhances wound healing in rats. *BMC Complementary and Alternative Medicine*, *19*(1), 1–9.

Dev, S. K., Choudhury, P. K., Srivastava, R., & Sharma, M. (2019). Antimicrobial, anti-inflammatory and wound healing activity of polyherbal formulation. *Biomedicine and Pharmacotherapy*, *111*(December 2018), 555–567.

Dilnesa, A., Alemayehu, M., & Atakilt, A. (2016). Green Synthesis of Silver Nanoparticles and Their Antibacterial Activities of the Crude Extracts of Brucea Antidysenterica Leaves. *International Journal of Mathematics and Physical Sciences Research*, *4*(1), 90–95.

Dilnesa, A., Mekonon, A., & Abebe, A. (2016). Phytochemical screening and antioxidant activity investigations on the crude extracts of brucea antidysenterica leaves. *International Journal of Research and Development*, *1*(10), 1–15.

Duke, T. N. (2019). Isolation and Structural Elucidation of an Alkaloid Constituent from the Berries of Brucea Antidysenterica Tariku. *Chemical and Process Engineering Research*, *59*(2), 1–12.

El Ayadi, A., Jay, J. W., & Prasai, A. (2020). Current approaches targeting the wound healing phases to attenuate fibrosis and scarring. *International Journal of Molecular Sciences*, *21*(3).

Elbossaty, W. F. (2017). Public Health & Safety Antibiotic Drugs and Multidrug Resistance Bacteria. *International Journal of Public Health and Safety*, *2*(3), 2–4.

Ellis, S., Lin, E. J., & Tartar, D. (2018). Immunology of Wound Healing. *Current Dermatology Reports*, *7*(4), 350–358.

Eming, S. A., Martin, P., Tomic-canic, M., Park, H., & Medicine, R. (2016). Wound repair and regeneration: Mechanisms, signaling, and translation. *Science Translational Medicine*, *6*(265), 1–36.

Fentahun M, Ayele Y, Amsalu N, Alemayehu A, A. G. (2017). Antibacterial Evaluation and Phytochemical Analysis of Selected Medicinal Plants against Some Pathogenic Enteric Bacteria in Gozamin District, Ethiopia. *Journal of Pharmacovigilance*, *5*(5).

Fikru, A., Makonnen, E., Eguale, T., Debella, A., & Abie, G. (2012). Evaluation of in vivo wound healing activity of methanol extract of Achyranthes aspera L . *Journal of Ethnopharmacology*, *143*(2), 469–474.

Finnson, K. W., McLean, S., Di Guglielmo, G. M., & Philip, A. (2013). Dynamics of Transforming Growth Factor Beta Signaling in Wound Healing and Scarring. *Advances in Wound Care*, *2*(5), 195–214.

Flegg, J. A., Menon, S. N., Byrne, H. M., & McElwain, D. L. S. (2020). A Current Perspective on Wound Healing and Tumour-Induced Angiogenesis. *Bulletin of Mathematical Biology*, *82*(2).

Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care*, *4*(9), 560–582.

Fymat, A. L. (2017). Antibiotics and Antibiotic Resistance. *Biomedical Journal of Science & Technical Research*, *1*(1).

Getahun, A. (1976). *Some Common Medicinal and Poisonous Plants Used in Ethiopian Folkmedicine*. PDF available on the web.

Gillin, F. D., Reiner, D. S., & Suffness, M. (1982). Bruceantin, a potent amoebicide from a plant, Brucea antidysenterica. *Antimicrobial Agents and Chemotherapy*, *22*(2), 342–345.

Grada, A., Mervis, J., & Falanga, V. (2018). Research Techniques Made Simple: Animal Models of Wound Healing. *Journal of Investigative Dermatology*, *138*(10), 2095–2105.e1.

Guo, S., & DiPietro, L. A. (2010). Critical review in oral biology & medicine: Factors affecting wound healing. *Journal of Dental Research*, *89*(3), 219–229.

Hashemi, S. A., Madani, S. A., & Abediankenari, S. (2015). The review on properties of aloe vera in healing of cutaneous wounds. *BioMed Research International*, *2015*.

Hassan, M. M., Khan, S. A., Shaikat, A. H., Hossain, M. E., Hoque, M. A., Ullah, M. H., & Islam, S. (2013). Analgesic and anti-inflammatory effects of ethanol extracted leaves of selected medicinal plants in animal model. *Veterinary World*, *6*(2), 68–71.

He, L., & Marneros, A. G. (2013). Macrophages are essential for the early wound healing response and the formation of a fibrovascular scar. *American Journal of Pathology*, *182*(6), 2407–2417.

Heil, N., Bravo, K., Montoya, A., Robledo, S., & Osorio, E. (2017). Wound healing activity of Ullucus tuberosus, an Andean tuber crop. *Asian Pacific Journal of Tropical Biomedicine*, *7*(6), 538–543.

Hemamalini, K., Ramu, A., Mallu, G., Venkata Srividya, V., Sravani, V., Deepak, P., & Reddy, U. V. (2011). Evaluation of wound healing activity of different crude extracts of anogeissus acuminata and gymnosporia emerginata. *Rasayan Journal of Chemistry*, *4*(2), 466–471.

Hodges, L. R., Rose, L. J., O'Connell, H., & Arduino, M. J. (2010). National validation study of a swab protocol for the recovery of Bacillus anthracis spores from surfaces. *Journal of Microbiological Methods*, *81*(2), 141–146.

Hosseinkhani, A., Falahatzadeh, M., Raoofi, E., & Zarshenas, M. M. (2017). *An Evidence-Based Review on Wound Healing Herbal Remedies From Reports of Traditional Persian Medicine*. *22*(2), 334–343.

Hu, M. S., Maan, Z. N., Wu, J. C., Rennert, R. C., Hong, W. X., Lai, T. S., … Lorenz, H. P. (2014). Tissue engineering and regenerative repair in wound healing. *Annals of Biomedical Engineering*, *42*(7), 1494–1507.

Hudzicki, J. (2016). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology*, (December 2009), 1–13. Retrieved from https://www.asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro

Humaryanto, & Ave, O. R. (2019). Exploring the Potential of Green Coffee Extract for Wound Healing Treatment. *IOP Conference Series: Earth and Environmental Science*, *391*(1).

Hung, S. Y., Tsai, J. S., Yeh, J. T., Chen, K. H., Lin, C. N., Yang, H. M., … Huang, Y. Y. (2019). Amino acids and wound healing in people with limb-threatening diabetic foot ulcers. *Journal of Diabetes and Its Complications*, *33*(10), 107403.

Inbaneson, S. J., Ravikumar, S., & Suganthi, P. (2012). In vitro antiplasmodial effect of ethanolic extracts of coastal medicinal plants along Palk Strait against Plasmodium falciparum. *Asian Pacific Journal of Tropical Biomedicine*, *2*(5), 364–367.

Ji, Y., Zhang, A., Chen, X., Che, X., Zhou, K., & Wang, Z. (2016). Sodium humate accelerates cutaneous wound healing by activating TGF-β/Smads signaling pathway in rats. *Acta Pharmaceutica Sinica B*, *6*(2), 132–140.

Juneja, K., Mishra, R., Chauhan, S., Gupta, S., Roy, P., & Sircar, D. (2020). Metabolite profiling and wound-healing activity of Boerhavia diffusa leaf extracts using in vitro and in vivo models. *Journal of Traditional and Complementary Medicine*, *10*(1), 52–59.

Kefe, A., Giday, M., Mamo, H., & Erko, B. (2016). Antimalarial properties of crude extracts of seeds of Brucea antidysenterica and leaves of Ocimum lamiifolium. *BMC Complementary and Alternative Medicine*, 1–8.

Kokane, D. D., More, R. Y., Kale, M. B., Nehete, M. N., Mehendale, P. C., & Gadgoli, C. H. (2009). Evaluation of wound healing activity of root of Mimosa pudica. *Journal of Ethnopharmacology*, *124*(2), 311–315.

Kondo, T., Ishida, Y. (2010). Molecular pathology of wound healing. *Forensic Science International*, *203*(1–3), 93–98.

Krishnaveni, A.,Thaakur, S. R. (2008). Pharmacognostical and preliminary phytochemical studies of Passiflora foetida. *Ancient Science of Life*, *27*(3), 19–23.

Kundu, A., Ghosh, A., Singh, N. K., Singh, G. K., Seth, A., Maurya, Laloo, D. (2016). Wound healing activity of the ethanol root extract and polyphenolic rich fraction from Potentilla fulgens. *Pharmaceutical Biology*, *54*(11), 2383–2393.

Kyaw, B. M., Järbrink, K., Martinengo, L., Car, J., Harding, K., & Schmidtchen, A. (2018). Need for improved definition of "chronic wounds" in clinical studies. *Acta Dermato-Venereologica*, *98*(1), 157–158.

Liu, H., Lin, S., Xiao, D., Zheng, X., Gu, Y., Guo, S. (2013). Evaluation of the Wound Healing Potential of Resina Draconis (Dracaena cochinchinensis) in Animal Models. *Evidence Based Complementry and Alternative Medicine*, *2013*, 1–10.

Makong, Y. S., Happi, G. M., Djouaka Bavoua, J. L., Wansi, J. D., Nahar, L., Waffo, A. F. K., … Sarker, S. D. (2019). Cytotoxic Stilbenes and Canthinone Alkaloids from Brucea antidysenterica (Simaroubaceae). *Molecules*, *24*(23), 1–10.

Malviya, N., & Jain, S. (2009). Wound healing activity of aqueous extract of Radix paeoniae root. *Acta Poloniae Pharmaceutica - Drug Research*, *66*(5), 543–547.

Mazumdar, S., Ghosh, A. K., Dinda, M., Das, A. K., Das, S., Jana, K., & Karmakar, P. (2019). Evaluation of wound healing activity of ethanol extract of Annona reticulata L. leaf both in vitro and in diabetic mice model. *Journal of Traditional and Complementary Medicine*, (xxxx), 1–11.

Mekonnen, Z., Amuamuta, A., & Abere, Y. (2019). Wound healing effect of aqueous extracts of Brucea antidysenterica and Croton marcostachyus from Northwest Ethiopia in albino mice. *African Journal of Pharmacology and Therapeutics Vol.*, *8*(1), 14–19.

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N., & Bakri, M. M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences*, *25*(2), 361–366.

Mulisa, E., Asres, K., & Engidawork, E. (2015). Evaluation of wound healing and anti-inflammatory activity of the rhizomes of Rumex abyssinicus J. (Polygonaceae) in mice. *BMC Complementary and Alternative Medicine*, *15*(1).

Nussbaum, S. R., Carter, M. J., Fife, C. E., DaVanzo, J., Haught, R., Nusgart, M., & Cartwright, D. (2018). An Economic Evaluation of the Impact, Cost, and Medicare Policy Implications of Chronic Nonhealing Wounds. *Value in Health*, *21*(1), 27–32.

Okonkwo, U. A., & Dipietro, L. A. (2017). Diabetes and wound angiogenesis. *International Journal of Molecular Sciences*, *18*(7), 1–15.

Oliveira, A., Simões, S., Ascenso, A., & Reis, C. P. (2020). Therapeutic advances in wound healing. *Journal of Dermatological Treatment*, *0*(0), 000. https://doi.org/10.1080/09546634.2020.1730296

Olsson, M., Järbrink, K., Divakar, U., Bajpai, R., Upton, Z., Schmidtchen, A., & Car, J. (2019). The humanistic and economic burden of chronic wounds: A systematic review. *Wound Repair and Regeneration*, *27*(1), 114–125.

Organization for Economic Cooperation and Development (OCDE). (2004). *Organization for Economic Cooperation and Development guidelines for testing of chemicals 434 : Acute Dermal Toxicity – Fixed Dose Procedure*.

Organization for Economic Cooperation and Development (OCDE). (2008). *Oecd guidelines for the testing of chemicals*.

Oumer, A., Bisrat, D., Mazumder, A., & Asres, K. (2014). A new antimicrobial anthrone from the leaf latex of Aloe trichosantha. *Natural Product Communications*, *9*(7), 949–952.

Pastar, I., Stojadinovic, O., Yin, N. C., Ramirez, H., Nusbaum, A. G., Sawaya, A., … Tomic-Canic, M. (2014). Epithelialization in Wound Healing: A Comprehensive Review. *Advances in Wound Care*, *3*(7), 445–464.

Patel, S., Srivastava, S., Singh, M. R., & Singh, D. (2019). Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomedicine and Pharmacotherapy*, *112*(October 2018), 108615.

Penn, J. W., Grobbelaar, A. O., & Rolfe, K. J. (2012). The role of the TGF-β family in wound healing, burns and scarring: a review. *International Journal of Burns and Trauma*, *2*(1), 18–28.

Polcz, M. E., & Barbul, A. (2019). The Role of Vitamin A in Wound Healing. *Nutrition in Clinical Practice*, *34*(5), 695–700.

Puhaindran, M. E. (2008). Principles of wound healing. *Diabetic Foot Problems*, *9*(2), 395–402.

Rodero, M. P., & Khosrotehrani, K. (2010). Skin wound healing modulation by macrophages. *International Journal of Clinical and Experimental Pathology*, *3*(7), 643–653.

Rodrigues, M., Kosaric, N., Bonham, C. A., & Gurtner, G. C. (2019). Wound healing: A cellular perspective. *Physiological Reviews*, *99*(1), 665–706.

Sasidharan, S., Nilawatyi, R., Xavier, R., Latha, L. Y., & Amala, R. (2010). Wound healing potential of Elaeis guineensis Jacq leaves in an infected albino rat model. *Molecules*, *15*(5), 3186–3199.

Schreml, S., Szeimies, R. M., Prantl, L., Karrer, S., Landthaler, M., & Babilas, P. (2010). Oxygen in acute and chronic wound healing. *British Journal of Dermatology*, *163*(2), 257–268.

Schreml, S., Szeimies, R. M., Prantl, L., Landthaler, M., & Babilas, P. (2010). Wound healing in the 21st century. *Journal of the American Academy of Dermatology*, *63*(5), 866–881.

Schultz, G. S., Chin, G. A., Moldawer, L., & Diegelmann, R. F. (2011). Principles of wound healing. *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists*, (December 2018), 423–450. https://doi.org/10.1017/UPO9781922064004.024

Serena, T. E. (2014). A Global Perspective on Wound Care. *Advances in Wound Care*, *3*(8), 548–552.

Shedoeva, A., Leavesley, D., Upton, Z., & Fan, C. (2019). Wound healing and the use of medicinal plants. *Evidence-Based Complementary and Alternative Medicine*, *2019*. https://doi.org/10.1155/2019/2684108

Sorg, H., Tilkorn, D. J., Hager, S., Hauser, J., & Mirastschijski, U. (2017). Skin Wound Healing: An Update on the Current Knowledge and Concepts. *European Surgical Research*, *58*(1–2), 81–94.

Taye, B., Giday, M., Animut, A., & Seid, J. (2011). Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia. *Asian Pacific Journal of Tropical Biomedicine*, *1*(5), 370–375.

Tessema, Z., Makonnen, E., Debella, A., & Molla, Y. (2018). Evaluation of in vivo wound healing and anti-inflammatory activity of crude extract of the fruits of Brucea antidysentrica in mice. *Wound Medicine*, *21*(May), 16–21.

Tessema, Z., Makonnen, E., Debella, A., & Molla, Y. (2019). Evaluation of in vivo wound healing and anti-inflammatory activity of 80% methanolic extract of the leaves of B. antidysentrica J. F. Mill (Simaroubaceae) in mice. *Asian Journal of Complementary and Alternative Medicine*, *7*(1), 1–8.

Ullah, N., Parveen, A., Bano, R., Zulfiqar, I., Maryam, M., Jabeen, S., … Ahmad, S. (2016). In vitro and in vivo protocols of antimicrobial bioassay of medicinal herbal extracts: A review. *Asian Pacific Journal of Tropical Disease*, *6*(8), 660–667.

Valle, D. L., Andrade, J. I., Puzon, J. J. M., Cabrera, E. C., & Rivera, W. L. (2015). Antibacterial activities of ethanol extracts of Philippine medicinal plants against multidrug-resistant bacteria. *Asian Pacific Journal of Tropical Biomedicine*, *5*(7), 532–540.

Velnar, T., Bailey, T., & Smrkolj, V. (2009). The wound healing process: An overview of the cellular and molecular mechanisms. *Journal of International Medical Research*, *37*(5), 1528–1542.

Vijayan, C., Preeja, P., & Murugan, K. (2018). Analysis of phytochemical profile and genotoxicity of crude methanolic extracts of Quassia indica (Gaertn .) with anti- inflammatory and antioxidant properties . *IOSR Journal Of Pharmacy*, *8*(2), 10–17.

Walton, E. W. (2014). Topical phytochemicals: Applications for wound healing. *Advances in Skin and Wound Care*, *27*(7), 328–332.

Wang, P. H., Huang, B. S., Horng, H. C., Yeh, C. C., & Chen, Y. J. (2018). Wound healing. *Journal of the Chinese Medical Association*, *81*(2), 94–101.

Wesley, J. J., Christina, A. J. M., Chidambaranathan, N., & Ravikumar, K. (2009). Wound healing activity of

the leaves of Tribulus terrestris (linn) aqueous extract in rats. *Journal of Pharmacy Researh*, *2*(5), 841–843.

White, R., Smerdon, G., Bishop, A., Witts, S., Reeves, J., & Ashford, C. (2017). 'The health economic burden that acute and chronic wounds impose on an average clinical commissionin group/ health board in the UK. *Journal of Wound Care*, *26*(6), 353–355.

Wong, S. Y., Manikam, R., & Muniandy, S. (2015). Prevalence and antibiotic susceptibility of bacteria from acute and chronic wounds in Malaysian subjects. *Journal of Infection in Developing Countries*, *9*(9), 936–944.

Yemata, G., Desta, B., & Fetene, M. (2019). In vitro antibacterial activity of traditionally used medicinal plants against Xanthomonas campestris pv. musacearum in Ethiopia. *Biodiversitas*, *20*(2), 555–561.

Yesuf, A., & Asres, K. (2013). Wound healing Activity Guided Isolation of Active Constituents From the Leaves of Allophylus abyssinicus (Hochst.) Radlk. *Phytopharmacology*, *4*(2), 442–453.

Zaman, S. Bin, Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., & Hossain, N. (2017). A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*, *9*(6).

Zielins, E. R., Brett, E. A., Luan, A., Hu, M. S., Walmsley, G. G., Paik, K., … Longaker, M. T. (2015). Emerging drugs for the treatment of wound healing. *Expert Opinion on Emerging Drugs*, *20*(2), 235–246.