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Evaluation of *invivo* wound healing and anti-inflammatory activity of 80% methanol crude extracts of the leaves and fruits of *Brucea antidysentrica J .F. Mill* (Simaroubaceae) in mice

By:

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Addis Ababa, Ethiopia

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A Thesis submitted to the Department of Pharmacology, School of Medicine, College of Health Sciences in partial fulfilment of the requirements for the Degree of Master of Science in Pharmacology.

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School of Graduate Studies

This is to certify that the thesis prepared by Zenaw Tessema, entitled: Evaluation of *in vivo* wound healing and anti-inflammatory activity of 80% methanol crude extracts of the leaves and fruits of *Brucea antidysentrica J.F.Mill (*Simaroubaceae) in mice and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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

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ABSTRACT

Evaluation of *invivo* wound healing and anti-inflammatory activity of 80% methanol crude extracts of the leaves and fruits of *Brucea antidysentrica J .F. Mill* (Simaroubaceae) in mice.

Zenaw Tessema

Addis Ababa University, 2016

Introduction: *Brucea antidysentrica* locally known as "Abalo" is traditionally used to treat conditions like scabies and external parasites, dysentery, gonorrhea, eczema, cancer, malaria, and trypanosomosis among others. The fruits and leaves of *B. antidysentrica* are also claimed to promote wound healing and anti-inflammatory activities. However, there is no scientific confirmation that substantiates the traditional claims.

Objective: to evaluate the wound healing and anti-inflammatory activities of both fruits and leaves extracts of *B. antidysentrica* in mice model.

Materials and methods: Mice were used for wound healing and anti-inflammatory studies, while rats were used for skin irritation test. For studying healing activity, 80% methanolic extracts of the leaves and fruits were formulated in strength of 2% and 4% and 1% and 2% as ointment base respectively for topical applications of excision and incision wound models. The negative controls were treated with simple ointment while positive controls with nitrofurazone (0.2%) skin ointment. Extract solutions of the leaves and fruits in 2% Tween 80 at a dose of 100 mg/kg, 200 mg/kg and 400 mg/Kg body weight were used for anti-inflammatory activity tests orally against the inflammation produced by carrageenan injection. Negative controls for anti-inflammatory test were treated with 2% Tween80 and the positive controls with Indomethacin 10mg/kg. Parameters, including rate of wound contraction, period of complete epithelialization, skin breaking strength and edema inhibition were evaluated.

Results: On the last day of treatment, 80% methanol fruits and leaves extracts showed a significant wound healing activity in strengths of 2% compared with negative control as evidenced by an increase in % wound contraction ($p < 0.01$) and a decrease in epithelization period ($p<0.05$). The 4% MLE also showed the highest % wound contraction ($P<0.001$) and the

shortest epithelialization period than the rest of the extracts (P<0.01). One percent MFE was found to increase the % wound contraction significantly on the last day of treatment (P<0.01) and its effect on the epithelialization period was insignificant. In the incision wound model, both 2% and 4% extract ointments of the leaves and only the 2% MFE resulted in a significant increase in tensile strength ($p < 0.01$) compared with negative control. The same extracts also revealed a significant anti-inflammatory effect compared with negative control particularly 3 to 4 h after extract administration as shown by a decrease in edema expressed as % reduction of edema. All doses of the leaves extract exhibited a higher effect on the 3^{rd} (P<0.05) and the 4^{th}h (P<0.001) compared to the negative control. Similar effect was also found for the 200mg/kg and 400mg/kg doses of the fruits extract, while its 100mg/kg dose reduced the edema significantly on the 4^{th} h (P<0.001).

Conclusion: The 80% methanol extracts of the fruits and leaves of *B. antidysentrica* supports the traditional claims for healing of wounds as evidenced by an increase in wound contraction rate and tensile strength, decrease in epithelization period and anti-inflammatory activity.

Key words: wound healing, anti-inflammatory, excision, incision, carrageenan induced paw edema, *Brucea antidysentrica*.

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LIST OF ABBREVATIONS AND ACRONYMS

AAU	Addis Ababa University
AFRO	Africa Regional Office
ANOVA	Analysis of Variance
BP	British Pharmacopeia
CHS	College of Health Science
COX	Cyclooxygenase
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EP	Epithelialization Period
EPHI	Ethiopian Public Health Institute
FGF	Fibroblast Growth Factor
HIV/AIDS	Human Immune Virus/Acquired Immune Deficiency virus
IL-1	Interleukin –One.
ILAR	Institute for Laboratory Animal Research
IP	Intrapertonial
IV	Intravenous
LD50	Medial Lethal Dose
MF	Master Formula
MFE	Methanol Fruits Extract

MLE	Methanol Leaves Extract
OECD	Organization for Economic Cooperation and Development
PDGF	Platelet-Derived Growth Factor
PGE	prostaglandin E
PII	Primary Irritation Index
PMN	PolyMorphonuclear Neutrophils
RF	Reduced Formula
RNA	Ribonucleic Acid
RPM	Revolution Per Minute
SEC	Scientific Ethical Committee
SEM	Standard Error of the Mean
SoM	School of Medicine
SPI	Scoring of Primary Irritation
SPSS	Statistical Package for the Social Sciences'
TMMRD	Traditional and Modern Medicine Research Directorate
TGF-b	Transfer Growth Factor b
TNF-a	Tumor Necrosis Factor a
TS	Tensile Strength
UK	United Kingdom
USD	United States Dollar
USP	United States Pharmacopeia
WHO	World Health Organization
WHS	Wound Healing Society

1. INTRODUCTION

1.1. Wound and basic principle of its formation

The skin is the largest organ of the body that acts as a barrier against external agents. The loss of skin tissue integrity can cause lesions or illnesses that bring disability or even death (Panda *et al*., 2011; Asghar *et al*., 2015).

Wound which is inescapable event of life (Majumdar, 2005) is a clinical problem as old as mankind and may be defined in different ways. But the most acceptable one is "a loss or breaking of cellular and anatomical or functional continuity of living tissues" (Raina *et al*.,2008; Kumar *et al*., 2013; Mulisa *et al*., 2015). According to the Wound Healing Society (WHS), wounds are physical injuries that result in an opening, breaking or interrupting of tissue integrity that cause disturbance in the normal skin anatomy and function (Murthy *et al*., 2013; Hussain *et al*., 2014; Subalakshmi *et al*., 2014; Ositadimma *et al*., 2015) which in turn have a significant impact on public health and expenditure of health care resources (Fikru *et al*., 2012).

Physical, chemical, thermal, microbial, or immunological insults to the tissue are among the factors mentioned in wound production (Majumdar, 2005; Thakur *et al.,* 2011; Hussain *et al*., 2014).

Based on different classification criteria such as etiology, location, type of injury or presenting symptoms, wound depth and tissue loss or clinical appearance (Udaya *et al* .,2010; Sabale *et al*.,2012); there are different types of wounds, including injuries, cuts and bites, diabetic, gastric and duodenal ulcers. These wounds can be broadly classified as acute or chronic depending on physiology or the time it takes to heal. Without complications, most wounds are acute wounds and tend to heal within few weeks. Chronic wounds in contrast, require prolonged time to heal, do not heal, or recur frequently. These wounds tend to occur when the normal wound healing process has been compromised due to microbial infection, metabolic disturbances, or an underlying disease (Agyepong *et al*., 2015).

Based on the underlying cause of wound creation; it can also be categorized as open and closed wounds (Alam *et al*., 2011). In open wounds, the blood escapes the body and bleeding is clearly visible. It can be further classified as incised wound, laceration or tear wound, abrasions or superficial wounds, puncture wounds, penetration wounds and gunshot wounds. On the other hand in the case of closed wounds, blood escapes the circulatory system but remains in the body and includes contusion or bruises, heamatomas or blood tumor, crush injury etc (Alam *et al*., 2011; Shrimanker *et al*., 2013).

Acute wounds are tissue injuries that normally proceed through an orderly and timely reparative process that result in sustained restoration of anatomic and functional integrity. They are usually caused by cuts or surgical incisions and complete the wound healing process within the expected time frame (Diegelmann and Evans, 2004).

Chronic wounds, rarely seen in healthy individuals and usually associated with diseases like diabetes and obesity, are defined as wounds, which have failed to progress through an orderly and timely reparative process of healing and therefore enter a state of pathologic inflammation. As a result, the healing process is delayed, incomplete, and does not proceed in a coordinated manner, subsequently resulting in poor anatomic and functional integrity over a period of 3 months (Menke *et al*., 2007;Trostrup *et al*., 2013).To mention some from this category; foot ulcers and pressure ulcers are complications of diabetes and spinal cord injuries, respectively. All wound types have the potential to become chronic and, as such, chronic wounds are traditionally divided etiologically. Identifying and treating the underlying aetiology of a chronic wound such as venous insufficiency, arterial perfusion, diabetes, or unrelieved pressure as well as systemic factors such as nutritional status, immunosuppression, and infection that may contribute to poor wound healing are key to successful wound treatment (Werdin *et al*.,2009). Chronic wounds result in significant functional impairment, reduction in quality of life, and large financial costs for patients and the health care system. Yet the epidemiological profile of chronic wounds hasn't been well established (Graves and Zheng, 2014). Current estimates indicate about 6 million people suffer from chronic wounds worldwide (Agyepong *et al*., 2015) which is responsible for loss of USD 25 billion for its clinical management representing an incredible burden in public health expenditure. Also in developed countries, the population

experiencing chronic wound during their lifetime is estimated to be 1-2% posing a public health problem. Loss of 2-4% of the total health care expenses for the clinical management of chronic wounds in Scandinavian countries is a proof of the reality (Sen *et al*., 2009).

1.2. Wound healing processes

The wound healing process, particularly in skin, has been well characterized histologically in studies extending back more than 100 years (Shawi and Martin, 2009). The term "wound healing" embraces all types of wounds, burns, and ulcerations. Complete wound healing includes restoration of function hardly ever achieved in those disfigured by wounds, especially when one includes the appearance of the skin or absence of an appendage (WHO, 2010).Wound healing is a complex and dynamic interplay between various cell types, the extracellular matrix (ECM), cytokines, and growth factors (Pakyari *et al*.,2012). It is a normal biological process that is initiated by trauma and often terminated by scar formation which reveals that healing is essentially a survival mechanism and represents an attempt to maintain normal anatomical structure and function (Fikru *et al*., 2012; Kumar *et al*., 2013; Mohsenikia *et al*., 2015). It comprises a series of coordinated and overlapping processes that have been characterized over many years (Ansell *et al*., 2012). The processes involved include hemostasis, inflammation, fibroblast activation and migration, re-epithelization, proliferation of endothelial cells, and remodeling (Fikru *et al*., 2012; Hussain *et al*., 2014; Mohsenikia *et al*., 2015). Wound healing remains a challenging clinical problem, and requires appropriate and efficient management. Much has been focused on wound care with an emphasis on new therapeutic approaches and the development of technologies for acute and chronic wound management (Velnar *et al*., 2009).

The process of wound repair differs a little from one kind of tissue to another and is independent of the form of injury. Even though the different steps in the wound healing process occur in a continuous, integrated manner (Majumdar, 2005), it is convenient to classify the physiological process involving through four temporarily and spatially overlapping phases: hemostasis, inflammation, proliferation, and remodeling phases (Ud-Din and Bayat, 2014; Frykberg and Banks., 2015) and for proper healing to occur these phases need to be well controlled (Gould *et al*, 2008

1.2.1. **Hemostasis phases**

As soon as injury occurred, an important step of initiation and continuation of the healing process called hemostasis appears. It is characterized by vasoconstriction, platelet degranulation and aggregation, and fibrin deposition leading to formation of a clot and bleeding cessation (Pakyari *et al*., 2012). Platelets being the primary subset of cells that enter to the injured site release various types of growth factors such as platelet-derived growth factor (PDGF), ,epidermal growth factor (EGF), and fibroblast growth factor (FGF) and inflammatory cytokines like tumor necrosis factor alpha (TNF-a), transforming growth factor beta (TGF-b), all together encourage the inflammatory phase and some of them function as chemo-attractants (Frykberg and Banks., 2015).Immediately after the production of these initiation factors, epithelial cells travel under the newly formed granulation tissue being activated by several cytokines and growth factors; specifically, interleukin (IL)-1a appears to be expressed within the epidermis and released upon the dermal injury, which in turn stimulates various genes including adhesion molecules, chemokines, cytokines, proteolytic enzymes, and matrix proteins in different types of skin cells (Pakyari *et al*.,2012,Bodnar, 2014).

1.2.2. Inflammatory Phase

The inflammatory response following tissue injury and lasts from day 0 to 5 plays crucial roles both in normal and pathological healing (Koh and DiPietro, 2013). During this phase of wound healing macrophages, epithelial cells, and lymphocytes secret to much amount of proangiogenic molecules (growth factors and cytokines) (Bodnar, 2014).The response from inflammatory phase is initiated at the moment of injury. Shape and architecture of tissues are disrupted owing to surgical or traumatic wounds and cause hemorrhage. In the beginning, blood fills the wound and exposure of this blood to collagen in the wound leads to platelet deregulation and activation of a plasma protein (coagulation factor XII also known as Hageman factor). As a result a number of biological amplification systems including the complement kinin and clotting cascades and plasmin generation are followed. This condition serves to amplify the original injury signal and lead not only to clot formation, which unites the wound edges, but also to the accumulation of a number of mitogens and chemo-attractants at the site of wound. Production of both kinins and prostaglandins leads to vasodilatation and increased

small vessel permeability in the region of the wound leading to edema in the area of the injury. Within 6 hours, circulating immune cells start to appear in the wound. Polymorphonuclear neutrophils (PMN) are the first blood leukocytes to enter the wound sites. Their main functions appear to be phagocytes of the bacteria, which have been introduced into the wound during injury. In the absence of infection, Polymorphonuclear neutrophils (PMNs) have a relatively short life span in the wound and their numbers decrease rapidly after the third day. The next cellular, immune component enter to the wound is macrophages. These macrophages have a much longer life span than the Polymorphonuclear neutrophils (PMN) and persist in the wound until healing is complete (Kumar *et al*., 2013).

Like neutrophils, macrophages phagocyte and digest organisms responsible for pathological process and secrete collagenase and elastases which break down the affected tissues and release cytokinins. Macrophages release different types of biologically active substances; in addition growth factors and other substances are also released which are essential for the initiation and progression of granulation formation (Majumdar, 2005).

1.2.3. Proliferation/granulation/ contraction Phase

This phase lasts approximately from days 3-14 and in the absence of significant infection or contamination, the inflammatory phase is short, and after the wound has been successfully cleared of devitalized and unwanted material, it gives away to the proliferative phase of healing. Granulation tissue consists of a combination of cellular elements, including fibroblasts and inflammatory cells. Fibroblasts which are the primary synthetic element in the repair process and are responsible for production of the majority of structural proteins first appear in significant numbers in the wound on the third day post-injury and achieve peak numbers on the seventh day. This rapid expansion in the fibroblast population at the wound site occurs via a combination of proliferation and migration (Majumdar, 2005) and the migration of fibroblasts to the wound site is assisted by contraction of extra cellular matrix (ECM) and the formation of granular tissue (Ayuk,2012). Then the fibroblasts produce large quantities of collagen which forms the main constituent of the extracellular wound matrix, and are ultimately responsible for imparting tensile strength to the scar which finally leads to restoration of an epithelial integrity at the wound surface. This phase comprised of events such as angiogenesis, fibroblasia and

granulation tissue formation, collagen deposition, epithelialization and contraction that overlap each other (Guo and Dipietro, 2010;Ayuk, 2012).

1.2.4. Remodeling /Maturation Phase

The final stage of wound healing process which starts from day 7 and involves remodeling, realignment and well organization of the collagen tissue to produce greater tensile strength, cell and capillary density reduction and a balance between synthesis and degradation can take up to 2 years and results in the development of normal epithelium and maturation of the scar tissue. Eventually they will regain a structure similar to that seen in unwounded tissue. The main cells involved in this process are the fibroblasts (Orsted *et al*., 2004; Sinno and Prakash, 2013). The four phases of wound healing process are shown in Table1.

Table1. The four phases of wound healing process

Phase of healing	Time post injury	Cells involved in the phases	Function or activity
Homeostasis	immediately	Platelets	Clotting
inflammation	Day 0-5	Neutrophils or macrophages	Phagocytosis
Proliferation(granulation or contraction)	Day 3-14	Macrophages	Fill defect
		Lymphocytes	Re –establish
		Neurocytes	Skin function closures
		Fibroblasts	
		Keratinocytes	
Remodeling (maturation)	Day7-2 yrs	Fibrocytes	Develop tensile strength

1.3. Factors that can interfere with healing

"The germ is nothing. It is the terrain in which it is found that is everything." Stated by Louis Pasteur. This is similar with wounds! Factors that affect wound healing must be addressed in a holistic fashion as stated above, at the terrain in which the wound is found. The individual with a wound has a wide terrain, from the local wound environment to the environment in which he or she lives, and that terrain may determine the healing ability. In other words, wounds do not exist in isolation from the patient as a whole (Orsted *et al*., 2004). Multiple factors can lead to impaired wound healing and these factors can be categorized into local and systemic. Local factors are those that directly influence the characteristics of the wound itself, while systemic factors are the overall health or disease state of the individual that affect his or her ability to heal (Guo and DiPietro, 2010). Local factors affect the features of the wound and they are mainly oxygenation, infection, presence of a foreign body and venous insufficiency while the systemic factors are provoked by the physiological state of an individual which may impair wound healing. Some of these factors include age and gender, temperature, chemicals, sex hormones, stress, moisture, nutritional status, diabetes, HIV/AIDS, cancer heredity healing disorders and obesity. Alcoholism, smoking, and certain medications such as steroids and chemotherapy also affect the wound healing processes (Thomas, 2011; Ayuk, 2012).

1.4. Management of wounds

The correct approach of treating wounds should effectively assist the healing process, and can have an important impact on the final clinical outcome. Physiological, endocrine and nutritional support at a clinical level significantly influence repair and, without which, wound healing often fails completely. Assessment of the wound and the patient starting with a diagnosis of the wound's aetiology and continues with optimizing the patient's medical condition, particularly blood flow to the wound area is considered to be the first stage in wound management. The wound needs to be debrided and dressed correctly. The next important stage in wound management is the lavage of micro organisms, dead tissues and foreign bodies which decrease tissue bacterial count using bacitracin or normal saline solution. Currently novel techniques such as topical growth factor application and incisional priming with PDGF or IL-1 can optimize both the cellular and molecular environment, thus decreasing healing time by

modifying inflammation and accelerating the proliferative phase. Electrical field stimulation may also optimize the remodeling phase by promoting more efficient fibroblast recruitment and collagen deposition (Velnar *et al*., 2009).

1.5. Plant medicines traditionally used in would healing

According to World health organization(WHO) traditional medicine is defined as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises applied to treat, diagnose and prevent illnesses or maintain wellbeing(Lulekal *et al*.,2008).

Traditional people around the world possess unique knowledge of plant resources on which they depend for food and medicine (Bekele and Reddy, 2015). Trends in the use of traditional and complementary medicine are on the increase in many developed and developing countries (Limenih *et al*., 2015).

As estimated by the World Health organization, 80% of the populations of Asia, Africa and Latin America use traditional medicine to meet their primary health care needs (WHO-AFRO, 2010).

In Ethiopia, it has been estimated that traditional remedies are the most important and sometimes the only source of therapeutics for nearly 80% of the population of which 95% of traditional medicinal preparations are of plant origin (Getaneh and Girma, 2014) due to the cultural acceptability, relatively low cost and limited access to modern health facilities (Kassaye *et al*., 2006).

There are many plants which are traditionally used for wound healing in Ethiopia, These include *Achyranthes aspera (*Fikru *et al*., 2012*),Rumexa byssinicus (*Mulisa *et al*., 2015*), Brucea antidysentrica, Datura stramonium, Croton macrostachyus, Acokanthera schimperi (*Taye *et al*,2011*), Rhusvulgaris ficuscaricus, Acacia abysinica, Vernonia amygdalina Del (*Gebeyehu *et al*.,2014*), Commelin abengalensis L, Solanum incanum, Ximenia americana (*Teklehaymanot,2009*), Acalypha volkensii Pax, Amorphophallus gallaensi (*Gidey *et al*.,2009*),Clematis hirsute Guill.&Perr (*Gidey *et al*.,2007*), Bersama abyssinica, Cynodon*

*dacytylon, (*Abera,*2003), Cordial africana, Coffee arabica (*Regassa,2013*)* and many others are being used in the treatment of wounds and other diseases in the traditional health care system of the country.

The study done on the *in vivo* wound healing activity of methanol extract from *Achyranthes aspera L.* leaves showed significant wound healing activity compared to group of rats treated with simple ointment (Fikru *et al*., 2012). In addition, it was also reported that Wound treated with 5 % and 10 % (w/w) hydroalcoholic extract ointment from rhizomes of *Rumexa byssinicus J*. exhibited significant wound healing activity in both excision and incision models (Mulisa *et al*., 2015). Since the *in vivo* wound healing activity of *Brucea antidysentrica* is not reported, this study will focus on evaluation of its wound healing and anti-inflammatory activity using incision & excision wound models and carrageenan induced hind paw edema model.

1.6. Overview of the experimental plant

The Simaroubaceae family includes 32 genera and more than 170 species of trees and brushes of pantropical distribution. It is characterized by its content of bitter substances, mostly responsible for its pharmaceutical properties. The family is characterized by the presence of quassinoids, secondary metabolites responsible for a wide spectrum of biological activities such as antitumor, antimalarial, antiviral, feeding deterrent, amebicide, antiparasitic and herbicidal (Alves *et al., 2014*) and antimicrobial and antioxidant (Viswanad,2011) .

Brucea (a family of Simaroubaceae) is widely distributed genus occurring in tropical Africa and tropical Asia. It is very bitter monoecius or dioecius shrub or small tree which is grouped into ten species. But the most common acceptable species are the following: *B.javanica, B.mollis, B.antidysentrica*, and *B.quineensis (*Roberts, 1994*).*

The study on the Methanolic-chloroform and methanolic-aqueous root extracts of *Brucea mollis* showed significant *in vitro* antiplasmodial activity which was also supported by their promising *in vivo* activity, respectively (Sharma *et al*., 2013).

The fruit of *Brucea javanica* is currently recorded in the Pharmacopoeia of the People's Republic of China (2010 edition) for treatment of fever, malaria and amebic dysentery (Liang *et al.*, 2015).

Brucea antidysenterica J. F. Mill belongs to a genus *Brucea* and a family of Simaroubaceae is commonly known as Waginos (Geeze) (Limenih *et al*., 2015), Abalo (Amharic), Meleta (Tigrigna), Hadawi (Somaligna), Atanico (Sidamigna) (Getahun, 1976), and it is among the commonly used traditional medicinal plants. The plant *Brucea'*: is named after James Bruce (1730-1794), a Scottish man who travelled to Ethiopia in the years 1768-1773 and took seeds of this plant to Europe. '*Antidysenterica*': derived from the Greek 'anti' = 'against', and 'dusenteria' = 'bad bowels'; so, active against e.g. dysentery (Jansen, 1981).

It is an ever green shrub or tree up to seven meters high (fig.1). The plant grows at moderate elevations, usually to 2,500 metres and exceptionally to 3,700 metres in the moisture tropics of Africa (Getahun, 1976). The plant is mainly distributed in Ethiopia, Sudan, Tanzania, Cameroon, Nigeria, Angola, Malawi and Zambia (Jansen, 1981).

Brucea antidysenterica J. F. Mill has a number of therapeutic applications like treating scabies and external parasites (Bekele and Reddy, 2015), as an antidysentric agent (Limenih *et al*., 2015; Teklehaymanot, 2009), wound healing effect (Getahun, 1976;Taye *et al*,2011; Regassa,2013;Getaneh and Girma, 2014; d'Avigdor *et al*.,2014;), treatment of Gonorrhoea (Lulekal *et al*.,2008), eczema and hookworm (Gebeyehu *et al*.,2014), as anticancer and anti-malaria (Abera,*2003*) and treatment of Trypanosomosis (Tamiru *et al*., 2013) among others.

Fig, 1. Leaves (A) and Fruits (B) of *B.antidysentrica* J. F. Mill

(Source: Photograph taken from floral site (Debre Markos) during collection in January, 2016)

The methanolic extract *in vitro* wound healing activity from *Brucea antidysenterica* showed 35% growth inhibition on wound causing bacteria like *S. aureus, S. pyogens, E. coli and P.aeuruginosa* (Taye *et al*., 2011). Since its wound healing and anti-inflammatory activity is not investigated; the traditional claim enforces to evaluate its *in vivo* activity.

1.7. Statement of the problem

Wound is one of the most common diseases often having severe complications in relation to health and posing high costs for therapy. In order to establish integrity of the damaged tissue; series of events must be progressed orderly in well controlled manner that unless otherwise cause physical disability even lead to death (Paulan, 2013; Taye *et al*, 2011; Gautama *et al*., 2011).

Wounds are also significant causes of morbidity and mortality worldwide. Studies show that for every million wound patients, at least 10,000 die from microbial infections (Wong *et al*., 2015). Currently available methods of wound management including debridement, irrigation, antibiotics, tissue grafts and proteolytic enzymes are found to be associated with major drawbacks such as invasiveness and being expensive (Werdin *et al.,* 2009). Emergence of resistant strains along with lack, high cost and retarded rate of newly generated antibiotics increase wound related mortality and morbidity (Akinsulire *et al*., 2007).

Hence it is paramount important to urgently intensify research to emerge new, cheap and effective wound healing agents. Now a day, scientists and researchers turn their attention to the medicinal plants as a noble source in the development of wound healing agents. In line with this, there is a need for conducting investigation towards medicinal plant claimed to be effective in the management of wound and inflammation.

2. OBJECTIVES

2.1. General objective

- ❖ To evaluate the wound healing and anti-inflammatory effect of 80% methanol extract of *B.antidysentrica* fruits and leaves in mice.

2.2. Specific objectives

- ✓ To determine wound healing activity of 80% methanol extract of *B.antidysentrica* leaves on excision and incision wound models.
- ✓ To evaluate the wound healing activity of 80% methanol extract of *B. antidysentrica* fruits on excision and incision wound models.
- ✓ To evaluate the anti-inflammatory effect of the 80% methanol extract of *B. antidysentrica* leaves on carrageenan induced hind paw edema model.
- ✓ To evaluate the anti-inflammatory effect of the 80% methanol extract of *B. antidysentrica* fruits on carrageenan induced hind paw edema model.
- ✓ To identify the secondary metabolites found in *B. antidysentrica*.
- ✓ To investigate the acute toxicity of leaves and fruits extracts in mice.

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3. MATERIALS AND METHODS

3.1. Drugs and chemicals

Wool fat, hard paraffin, white soft paraffin, cecostearyl alcohol, ethanol absolute , carrageenan (type I, lot 102k0871), Tween 80 (BDH Laboratory Supplies Poole, BH151TD lot ZA2088516, England),distilled water, ferric chloride (Hopkin and Williams Ltd, England), potassium iodide BP (Evans medical Ltd, England), sodium hydroxide, hydrochloric acid (Lot 80k3493), sulfuric acid (Park scientific Ltd, Lot 8114/10, UK), Wagner's reagent, chloroform, Lead acetate, Acetic anhydride, Sodium chloride were all obtained from Ethiopian public health institute, methanol absolute (Blulux, India ,Purchased from ZAF pharmaceuticals Pvt.Ltd.Co.), Nitrofurazone ointment USP 0.2% (Galentic pharm, Pvt.Ltd.Co, India), ketamine hydrochloride injection USP (Neon laboratories limited, India), normal saline (IV infusion BP Medsol pharmaceuticals) were all obtained from Black Lion Hospital).All the drugs, chemicals, and reagents used were of the required standard and analytical grade.

3.2. Instruments and Apparatus

The apparatus and instruments used in this research work were Plethysmometer (Ugo Basil 7140, Italy), sensitive digital weighing balance (Mettler Toledo, Switzerland), mini orbital shaker (Bibby Scientific Limited Stone Staffo Reshire, UK), Whatman filter paper (Number 1) (Maidstone, UK) , electrical hair clipper series 3000, syringe with needles, gloves, mortar and pestle, Erlenmeyer conical flask, rotary evaporator (Buchii model R-200, Switzerland), lyophilizer (Operan, Korea vacuum limited, Korea), deep freezer, sharp sterilized scissors, surgical threads with curved needles, forceps, permanent marker, graph paper, cotton swab, water bath, beaker, ointment slab, gauze and elastic bandage.

3.3. **Collection of plant materials**

Fresh leaves and fruits of *B. antidysentrica* were collected from Debre Markos, East Gojjam Zone, Amhara Region, and 300 km away from Addis Ababa in January, 2016. The plant materials were then wrapped with plastic sheets and transported to the Laboratory of Pharmacology Department, School of Medicine, College of Health sciences, AAU. Identification and authentication of the plant material was done by a taxonomist at the National Herbarium unit of Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia and deposited with a voucher specimen (number ZT-001) for future reference.

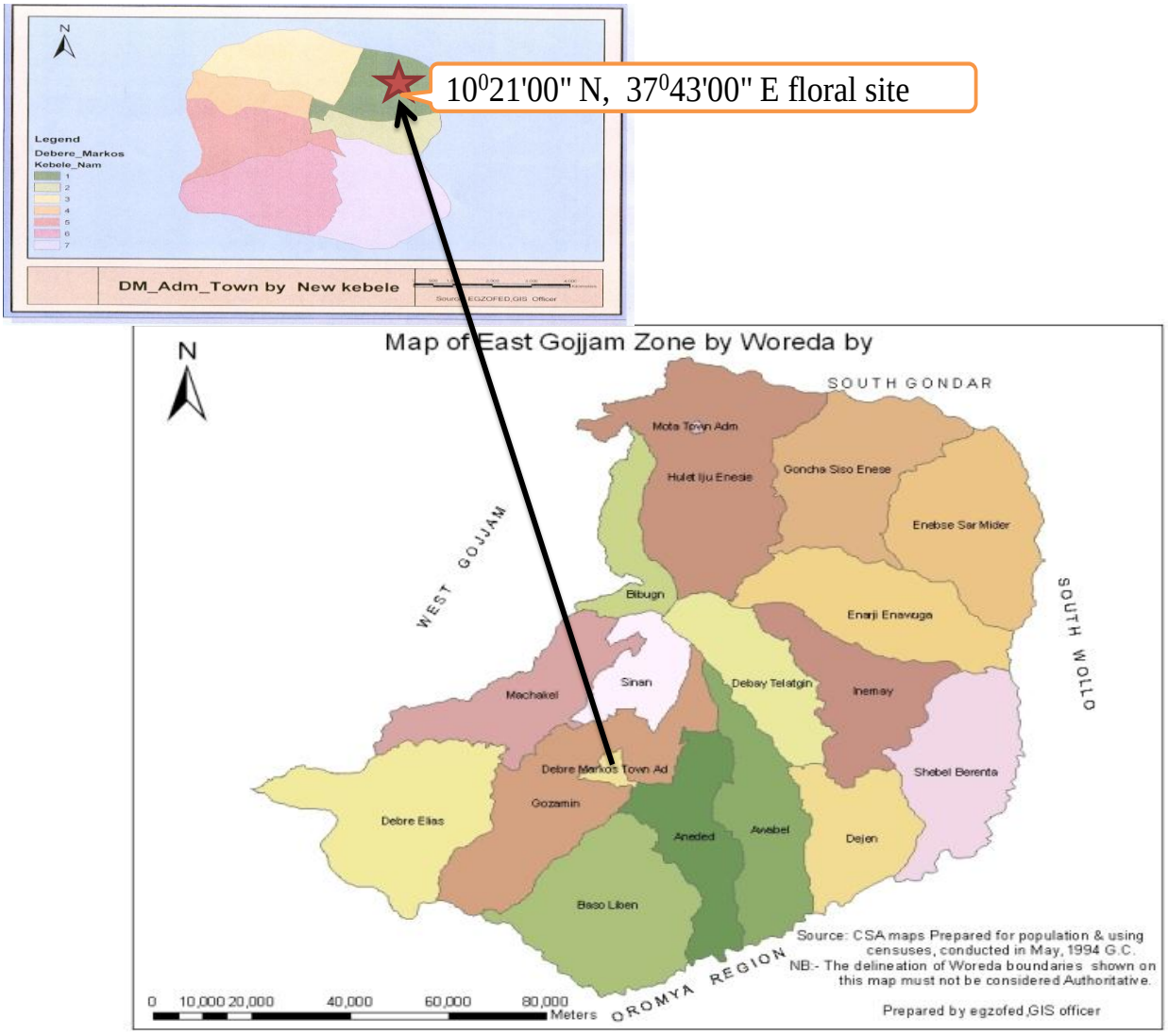

Fig 2. Map of *B.antidysentrica* plant collection area

The collected plant materials were cleaned to eliminate any dead matter or other unwanted particles and then dried at room temperature under shade area without exposing to sunlight in the laboratory of Pharmacology Department. The dried materials were grounded using mortar and pestle and then prepared for extraction.

3.4. Experimental animals

Healthy, adult Swiss albino mice of both sex (29-40 g, and 6–8 weeks of age) and adult healthy Wistar rats (*Rattus norvegicus)* of both sex weighing150-200g and aged 3‑4 months were obtained from the animal house of EPHI, Addis Ababa, Ethiopia. The animals were housed in cages under standard conditions (22 ± 3 °C, 40-70 % relative humidity, and 12 h light and dark cycles) and had free access to food (standard pellet diet) and water *ad libitum.* The animals were allowed to acclimatize to the laboratory condition for a week before the starting of the experiment. Animals were handled according to international laboratory animal use and care guidelines throughout the experiment. At the end of the experiment the animals were sacrificed under high dose of anesthesia (ILAR, 1996).

3.5. Ethical approval

The study protocol was approved by the Scientific and Ethics Committee (SEC) of the Department of Pharmacology, School of medicine, College of Health Sciences, AAU.

3.6. Preparation of the crude extracts of *B. antidysentrica* leaves and fruits.

Cold maceration extraction technique was used to extract the experimental plant materials as outlined by (Mulisa *et al*., 2015). Three hundred gm of the course powdered leaves and fruits from *Brucea antidysentrica* was soaked with 1500 ml of 80 % (V/V) methanol for 72 h in an Erlenmeyer conical flask separately with frequent agitation using mini orbital shaker adjusting at 170 revolution per minute (rpm) and at 90 minutes for 3 times a day at room temperature until the soluble matter gets dissolved. Each mixture was then strained; the marc (the damp solid material) was pressed and first filtered using folded gauze and nylon cloth. The residues were re-macerated twice in order to maximize the yield. The combined filtrate from each of the

plant materials was pooled together and was sieved through Whitman's filter paper (No-1) using pressurized suction filtration system. The methanol from the combined filtrate of the 80 % methanol extract was removed under a reduced pressure by a rotary evaporator at 45 rpm and 40^{o}C to obtain the crude extract. The extract was further concentrated to dryness in dry oven for 10 days to completely remove the methanol from the filtrates. Then the filtrates were frozen overnight using deep freezer followed by drying with a lyophilizer at -50^{o}C and vacuum pressure (200 mBar) to remove water. The dried products from both the extracts were stored in tight containers in deep freezer at -20^{o}C until used for formulation of ointments.

3.7. **Ointment formulation**

Simple ointments of the 80 % methanol extracts were prepared following the formula (Table 2) described with some modification (British pharmacopeia, 1988).

Table 2. Formula used for preparation of simple and medicated ointment

Ingredients	MF	RF
Wool fat	50 g	5 g
Hard paraffin	50 g	5 g
Cecostearyl alcohol	50 g	5 g
White soft paraffin	850 g	85 g
Total	1000 g	100 g

MF, Master formula; RF, reduced formula

Five ointment preparations (each 100 g), with (1 % w/w and 2%w/w 80% methanol fruits and 2% w/w and 4% w/w 80% methanol leaves) and without (simple ointment only and served as a control) the extracts were formulated using the reduced formula from the master formula (Table 2). To prepare the simple ointment, the calculated amount of hard paraffin and cecostearyl alcohol were mixed and melted in a separate beaker. In another beaker the mixture of wool fat and white soft paraffin was melted by stirring to maintain its homogeneity. After removing from the water bath the former was added to the later and then stirred until cooled. To prepare the medicated ointment, 1g of 80 % methanol fruits extract, 2g of 80 % methanol

leaves and fruits extracts and 4 g of 80 % methanol leaves extract were mixed with 99 g, 98g and 96g of the ointment bases, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture (Ansel, 1985). To prepare the control ointment, 100 g of the entire base ingredients were taken and treated in the same way to formulate ointment exclusive of an active ingredient.

3.8. **Grouping and dosing of experimental animals**

For excision model, six groups of mice, each containing six mice were used. The first group was treated with simple ointment, and served as a negative control. Groups II and III were treated with 1% and 2% of 80% methanol fruits extract ointments, respectively and groups IV to V were treated with 2% and 4% of 80% methanol leaves extract ointments, respectively. Group VI was treated with nitrofurazone (0.2 %) and served as a positive control.

For incision model, seven groups of mice, containing six mice per group were used. The animals of Group I-VI were treated in a similar fashion with excision wound model, but animals in Group VII were left untreated and served as untreated negative control.

For the determination of anti-inflammatory activity, eight groups of mice each containing six animals were used. Group I was given the vehicle (2% Tween 80) and served as a negative control. Groups II, III and IV received 100mg/kg, 200mg/kg and 400mg/kg crude extracts of the leaves whereas groups V, VI and VII were given the same dose of crude extracts of the fruits. The eighth group was treated with indomethacin (10mg/kg) and served as positive control. All administrations were performed orally using gavage with a maximum volume of 1ml/100g. Extracts as well as standards were dissolved using 2% Tween 80.

3.9. **Wound healing studies**

Based on the arrangements of experimental animals described in the grouping and dosing part, the effect of 80% methanol extracts of both the leaves and fruits were evaluated on excision and incision wound models in mice. After treatment of the mice with the extracts; the wound-healing activity of both leaves and fruits extracts was assessed by the period of

epithelialization and rate of wound contraction (Malviya and Jain, 2009) and by the extent of breaking strength (Sharma *et al*.,2011).

3.9.1. Excision wound model

The wound site was prepared following the excision wound model. The mice were anesthesized prior to and during infliction of the experimental wounds. The surgical process was carried out using ketamine (80mg/kg) plus diazepam (5mg/kg) through intrapertonial (IP) route of administration. After the mice were anesthetized, the fur from their dorsothoracic area was removed using electrical hair clipper. Then after a full thickness of circular area of approximately 314 mm^2 was marked with thin permanent marker and excised carefully using forceps and small sharp sterilized scissors, on the shaved region to create nearly the same size of wounds in all mice. Hemostasis was achieved by blotting the wound with cotton swab drenched in normal saline. Then the animals were left until recovery from anesthesia. After recovery, they were returned to their individual cage with the wound undressed and the day was considered to be day 0. Starting from day one the wound was treated with topical application of the ointments prepared daily after classifying them into their respective groups. The animals were observed for wound closure and measurement was taken at 2, 4,6,8,10,12 and14^{th} post wounding days using transparency sheet and permanent marker. Period of epithelialization, the number of days required for falling scar without any residue raw wound were also observed (Kokane *et al*., 2009). The wound healing effect of the extracts was calculated taking the initial size of wound, *i.e*., 314mm^2 as 100 % as follows (Sharma *et al*.,2011, Mekonnen *et al*.,2012).

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

Where n=the days where measurement was taken; 2^{nd}, 4^{th}, 6^{th}……..14^{th} day

3.9.2. Incision wound model

On wounding day, experimental animals were anesthetized in the same manner described for excision wound model. The dorsal fur of each mouse was then shaved and a three cm long

longitudinal paravertebral incision was made through the skin and subcutaneous tissue. The parted skin was then sutured one cm apart using a surgical thread with curved needle. The continuous thread on both wound edges was tightened for good closure of the wounds (Fig 2A).

After 24 h of wound creation (on day 1), animals were treated as described under grouping and dosing section, with topical formulation of vehicle, extract or standard daily for nine days, leaving out the last group without applying any of the interventions. The sutures were removed on day 8 post-incision and tensile strength was measured on the 10^{th} post-wounding day using continuous water flow technique (Wang *et al*.,2011) (Fig.2B &C).The percent strength was also calculated using the following formulas (Suntar *et al.,* 2011).

$$\text{Tensile strength (TS) of extract (\%)} = \frac{\text{TSextract} - \text{TSvehicle}}{\text{Tsvehicle}} \times 100$$

$$\text{Tensile strength (TS) of reference (\%)} = \frac{\text{TSreference} - \text{TSvehicle}}{\text{Tsvehicle}} \times 100$$

$$\text{Tensile strength (TS) of vehicle (\%)} = \frac{\text{TSvehicle} - \text{TS l.u}}{\text{TS l.u}} \times 100$$

Where l.u= left untreated

A B C

Fig. 3 Incised mice (A) and continuous water flow method for determination of tensile strength (B &C).

3.9.3. Anti-inflammatory activities

The acute anti-inflammatory activity of the test substances against the carrageenan-induced hind paw edema model in mice was determined according to the methods described earlier with some modifications. Following an overnight fasting of food with free access of water, mice were divided into eight groups of six mice each. And the basal volume of each of the mouse was determined before administration of any of the substances using Plethysmometer (Alamgeer *et al*., 2015). Group I served as control and was treated with 2% Tween 80 (on the bases of 1 mL/100g) orally by oral gavage. Groups from II-IV were given methanol leaf extracts of 100,200 and 400 mg/kg p.o. respectively and groups from V-VII were treated with the same dose of fruits extract, while the last group was treated with standard drug (indomethacin 10mg/kg) p.o 1 h before carrageenan injection. The standard and the test substances were suspended with 2% Tween 80. Then the inflammation in the hind paw was induced by injecting 0.05 mL of freshly prepared carrageenan suspension (1%) in normal saline into the sub planer-surface of the right hind paw. The linear circumference of the injected paw was measured at 1^{st}, 2^{nd}, $3^{rd}$and 4^{th}h of the administration of carrageenan, with the help of Plethysmometer (Gebrehiwot *et al*., 2015). Paw circumference increase at 1, 2, 3 and 4 h after carrageenan injection was taken as the parameter for measurement of inflammation. Extracts capacity to suppress the paw inflammation was expressed in terms of edema percent inhibition (Kumar *et al*., 2012) and was calculated as follows:

$$\text{Percentage of inhibition (\%)} = (1 - x/y) * 100$$

Where x = mean increase in paw circumstance of treated mice and y = mean increase in paw circumstance of control mice.

3.10. Phytochemical screening

The 80% methanol extracts of *Brucea antidysentrica* leaves and fruits were screened for the presence of secondary metabolites to relate the wound healing and anti-inflammatory activity of the plant with the presence or absence of these constituents. Thus the test for alkaloids, saponins, flavonoids, terpenoids, phenols, glycosides and tannins was performed according to standard tests described with little modification (Yadav and Agarwala, 2011, Hossain *et al*.,

2013, Ugochukwu *et al*., 2013, Wadood *et al.*, 2013, Geetha *et al, 2014*, Ndam *et al*., 2014, Yadav *et al*., 2014,).

A) Test for Alkaloids (Wagner's test)

Ten mg of each of the crude extracts were dissolved in 1ml of distil water. With this solution three drops of Wagner's reagent(solution prepared by dissolving 2 gm of Iodine in a solution of 6 gm of potassium iodide in 100ml distill water) was added. The presence of alkaloids was confirmed by the formation of reddish brown colored solution.

B) Tannins test (Lead acetate and Ferric chloride test)

For the lead acetate test, 0.1 gm of each of the extracts was dissolved in 2ml of distil water. Then 1ml of each of the solution was taken and 0.5 ml of 1% lead acetate was added to it. Formation of yellowish precipitate was observed for the presence of tannins. Another crosschecking test done for identification of tannins was ferric chloride test. In this test 0.5 ml of 5% ferric chloride solution was added to the same solution used for lead acetate test and development of dark bluish or black color was observed for the presence of tannins.

C) **Test for Triterpenoid**

The dry crude plant extracts of each of the leaves and fruits (10 mg) were dissolved in 2ml chloroform and then 1 mL acetic anhydride was added to each of the solution. Then 1mL concentrated sulphuric acid was added to the solution. Formation of reddish violet colour shows the presence of Triterpenoid.

D) Test for flavonoids (Alkaline reagent or NaOH test)

The crude extracts (0.3g) of leaves and fruits were dissolved in 2ml of distil water. To these, three drops of 20% sodium hydroxide solution was added. An intense yellow color was formed which turned to colorless on addition of three drops of 20 % hydrochloric acid which indicated the presence of flavonoids in each of the extracts. In addition, lead acetate test was performed. To the same solution used above 3 drops of 10% lead acetate was added and formation of yellow precipitate was observed for the presence of flavonoids.

E) Test for saponins (foam test)

About 0.3g of each of the crude extracts was taken and dissolved in 20 ml 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.

F) **Test for phenols (Ferric chloride test)**

Ten mg of each of the extracts was dissolved in 1ml of water. Half ml of 5% ferric chloride solution was added to it and development of deep blue or black color was taken as an indicator for the presence of phenols.

G) Test for steroids (Liebermann-Burchard test).

About one half gram (0.5 g) of each of the crude extracts was dissolved in 0.5mL dichloromethane to produce a dilute solution. To this solution 0.5 mL of acetic anhydride was added, followed by three drops of concentrated sulphuric acid. Formation of a blue-green colouration indicated the presence of steroids.

H) Test for glycoside (Glycoside tests)

Small amount of the extracts (0.1g) of leaves and fruits were dissolved in 1 ml of distil water and then three drops of 20% sodium hydroxide solution was added and formation of yellow color confirms the presence of glycosides.

3.11. Acute toxicity studies

3.11.1. Acute oral toxicity study

The experiment was conducted on healthy Swiss albino female mice. The oral acute toxicity study was performed by preparing aqueous hydroalcoholic solutions from each of the extracts according to the OECD 425:2008 guideline for the crude extracts (OECD, 2008).The mice were withheld food but not water for 4 hours prior to dosing and at the end of the fasting period weight was recorded and the dose was calculated based on the measured weight. A single female mouse was given 2000mg/kg of the leaves extract prepared. After administration, food

was withheld for further 2 h period and observed at least once during the first 30 minutes after dosing, periodically during the first 4h. Since death was not recorded within the 24 h ; another 4 female mice were given the same dose and gross physical and behavioral observation was done daily for the occurrence of any clinical sign of toxicity like lacrimation, hair erection and loss of motor/or feeding activities and mortality as well as reduction of weight. The same procedure was followed for the extract from fruits.

3.11.2. Skin irritation test

Skin irritation test was done according to occluded dermal irritation test with slight modification (Nair *et al*, 2012**,** More *et al*, 2013). Both sex of 20 rats (10 male and 10 female) showing normal skin texture were grouped into four groups (Group I and II received 1% and 2% fruits extract and group III & IV received 2% & 4% leaves extract) each group with five rats and the animals were housed in a cage and acclimatized to the laboratory condition prior to the test. At the end of acclimation a day before test procedure, the fur was shaved from the dorsal area of the trunk and two demarcated areas were marked in such a way that one area serves for the test substance and the other was treated with simple ointment for comparison. Topical preparations of leaves, fruits and simple ointments were applied to the respective groups for 24 h. Without delay, the area was covered by dressing gauze over which the occlusive material, plastic sheet was placed. Then the wrapper was loosely attached with the skin by non-irritant adhesive tapes and in order to ensure its contact with the skin till the end of the exposure period, it was tied across the diameter of the experimental animals using elastic bandage. At the end of the exposure period, the entire materials used to adhere the formulation were removed with care not to damage the skin followed by washing of the test site with distil water. After 1 h the animals were observed for skin irritation and thereafter the observation was made at 24, 48 and 72 h of interval. The skin reactions, in terms of edema and erythema, were investigated based on the skin reactions scoring system (Table 3).

Table 3: Classification of erythema and oedema scores used to determine the primary irritation index

Erythema	Value
No erythema	0
Very slight erythema	1
Well- defined erythema	2
Moderate to severe erythema	3
Sever erythema (beef redness) to eschar formation	4
Edema formation	Value
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1mm)	3
Sever edema(raised more than 1mm extending beyond the area of exposure)	4
Total possible score for primary irritation	**8**

The Scoring of primary irritation (SPI) for each rat was calculated as:

$$\frac{\sum \text{(Erythema and edema graded at 24, 48 and 72 hrs)}}{\text{Number of observations}}$$

The difference between the summation of the SPIs of all animals from the treated site and the control site were calculated and used for determination of Primary Irritation Index which was calculated as:

$$PII = \frac{\sum SPI\ (Test) - \sum SPI\ (Control)}{\text{Number of animals}}$$

Finally the degree of irritation was categorized as negligible, slight, moderate and sever based on the value of PII (Table4).

Table 4: Categories of irritation response in rats:

Category	Primary Irritation Index(PII)
Negligible	0-0.4
Slight irritation	0.5-1.9
Moderate irritation	2.0-4.9
Sever irritation	5-8

3.12. **Statistical analysis**

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

4. RESULTS

4.1. Yields of extraction

From a total of each of the leaves and fruits of 300g *B. antidysentrica* macerated with 80% methanol; residues of 45.8 and 39.9 g were obtained making the yield 15.3 and 13.3%, respectively that were kept in the refrigerator until used.

4.2. Wound healing Effect of the extracts

4.2.1. Excision wound model

The 80% methanol extracts from the fruits and leaves of *B. antidysentrica* formulated in simple ointment was found to be active on excision wounds (Fig.3) which was revealed by the % of wound closure and epithelialization period that shows the rate at which wound healing progresses. As depicted in Table5, only the standard drug showed significant wound contraction against the control ($P<0.05$) on day two. But on day 4, the 2% MFE contracted the wound significantly ($P<0.05$) compared to the control. Two and four percent leaves extracts, 2%MFE and the standard drug were found to be comparably effective ($P<0.05$) against the simple ointment on days six & eight, and the effect was maintained till the 14^{th} day with increased degree of significance.

On the other hand, significant wound contraction effect was observed with the 1%MFE on the 12^{th} and 14^{th} day ($P<0.05$ and $P<0.01$, respectively). Among the extracts, 4% MLE showed significant ($P<0.001$) and maximum percentage (99.9 %) of wound contraction which was followed by 2% MLE (99.45%; $P<0.01$) and 2% MFE (99.00%; $P<0.01$) on the last day of treatment compared to the control. Although this effect was observed, none of the extracts was found to be significantly different from at least one of the extracts even to the 1%MFE which failed to achieve significant wound contraction effect till the 10^{th} day of treatment. The reality of failing achievement of significant effect against all doses of extracts was also true for the standard nitrofurazone.

Another wound healing parameter that can be derived from excision wound is determination of epithelization period. The data from Table5 confirmed that significantly shorter healing time was achieved by 2% MFE & MLE ($P<0.05$), 4% MLE ($P<0.01$) and the standard drug ($P<0.001$) compared to the control. However, 1% MFE was unable to reduce the healing time

significantly. Similarly the difference in the healing time between extract formulations and standard nitrofurazone drug was found to be insignificant

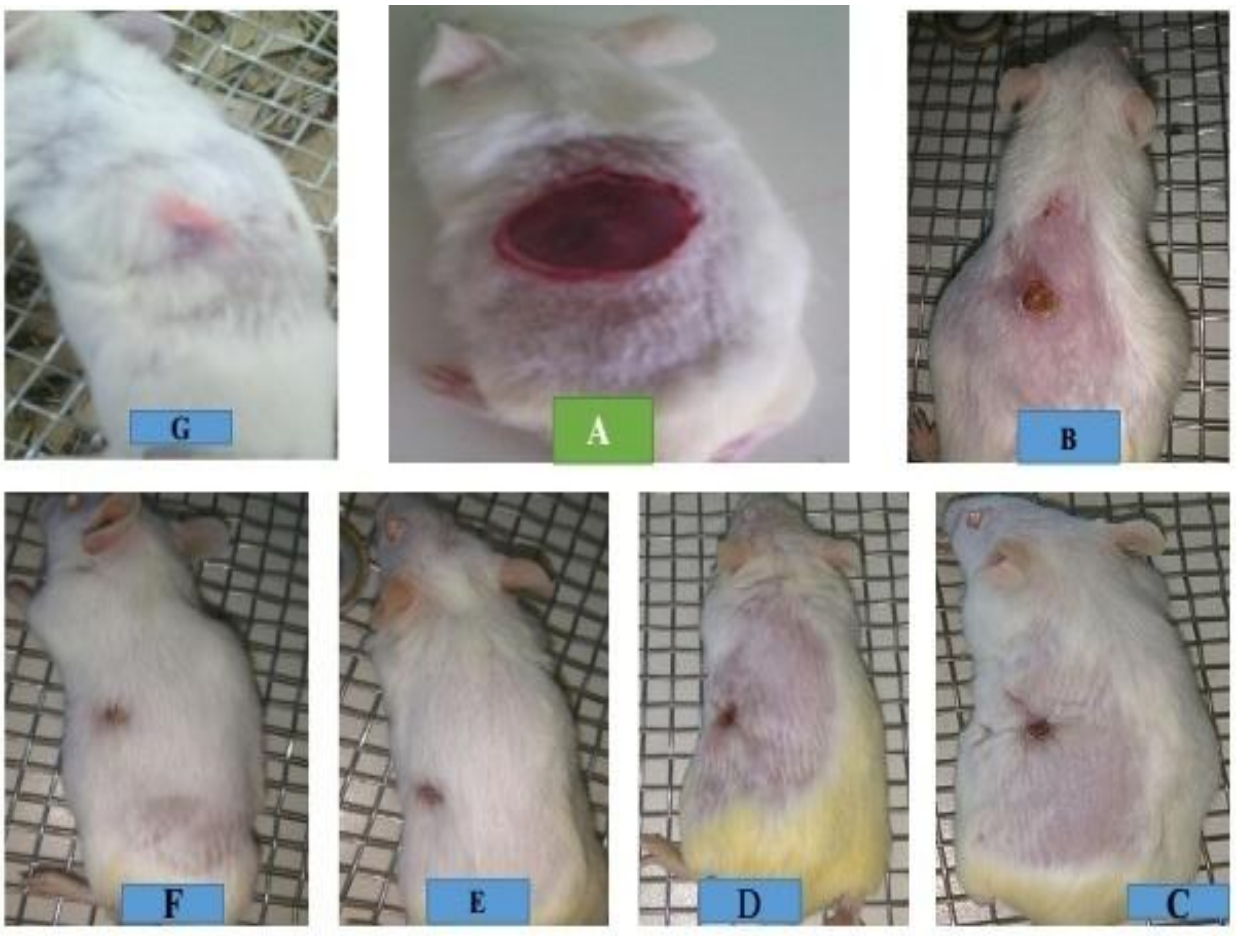

Fig.4. Excision wound immediately after wounding (A) and a healing progresses on excision wound after application of the 80% methanol fruits and leaves extract of *B. antidysentrica* (all doses) and the standard for 10 days (Letters from B –G represent groups treated with SO, 1%MFE, 2%MFE, 2%MLE, 4%MLE and NFO respectively) where MFE=methanol fruit extract, MLE=methanol leaves extract and NFO= nitrofurazone.

Table 5: Effect of topical application of methanol extracts of *B.antidysentrica* leaves and fruits on percentage wound contraction and epithelization time of an excision wound in mice

Groups	Wound area (mm2)±SEM (% contraction)							EP (days)
	2	4	6	8	10	12	14	
SO	217.13±13.18 (30.90)	175.72±16.24 (44.00)	146.05±21.96 (53.50)	100.36±18.96 (68.00)	60.99±13.05 (80.60)	39.25±9.83 (87.50)	28.26±8.92 (91.00)	17.60±1.08
1%MFE	148.88±15.39 (52.59)	113.55±15.68 (63.84)	85.76±14.72 (72.69)	59.19±8.17 (81.15)	36.74±4.07 (88.30)	18.21±1.41***1** (94.20)	5.65±0.38***2** (98.20)	15.20±0.20
2%MFE	137.57±18.07 (56.19)	97.10±13.96***1** (69.08)	68.92±9.29***1** (78.05)	42.90±6.75***1** (86.34)	26.61±7.28***1** (91.53)	13.50±4.90***2** (95.70)	3.14±1.43***2** (99.00)	14.40±0.68***1**
2%MLE	145.62±26.70 (53.63)	116.38±24.53 (62.94)	73.83±14.75***1** (76.49)	48.87±10.20***1** (84.44)	21.00±6.36***2** (93.31)	7.07±2.51***2** (97.75)	1.73±1.35***2** (99.45)	14.00±0.63***1**
4%MLE	140.91±24.06 (55.13)	111.82±22.41 (64.39)	70.69±9.99***1** (77.49)	41.45±5.88***1** (86.80)	16.01±3.65***2** (94.9)	3.45±1.58***3** (98.90)	0.31±0.19***3** (99.90)	13.60±0.68***2**
0.2% NFO	127.68±11.57***1** (59.34)	91.26±11.30***1** (70.94)	68.14±10.53***1** (78.30)	43.69±8.44***1** (86.09)	10.99±4.76***3** (96.50)	3.14±2.43***3** (99.00)	0.00±0.00***3** (100.00)	12.00±0.71***3**

Values are expressed as mean ± SEM (n=6 animals in each group) and analyzed by one way ANOVA followed by tuckey post hoc test; numbers from 2-14 indicate the day on which contraction rate measurement was taken; EP = epithelization period; SO=simple ointment base; MFE= methanol fruits extract; MLE=methanol leaves extract NFO=nitrofurazone ointment, *: compared against the control. [1]$p<0.05$; [2]$p<0.01$; [3]$p<0.001$

4.2.2. Incision wound model

The effect of 80% methanol extracts of leaves and fruits of *B. antidysentrica* on incision wound model with the topical application of the extracts formulated with simple ointment were made known to be effective in increasing the breaking strength of the healing wound as shown in Table6. MFE (2%), 2%MLE and 4%MLE significantly increased the tensile strength ($P<0.01$) by 79.90%, 84.04%, and 89.62%, respectively and the standard ($P<0.001$) by 96.75% compared to simple ointment. Comparing with the animals left untreated, the 1%MFE had a comparable higher increasing effect on the tensile strength ($P<0.05$). Similarly both 2% and 4% leaves extracts, the standard ($P<0.001$) and the 2%MFE ($P<0.01$) increased the tensile strength. No significant differences were observed among the extracts and the standard.

Table 6: Effect of topical application of 80 % methanol extracts of *B. antidysentrica* leaves and fruits on breaking strength of an incision wound on day 10 of wound creation

Dose	Breaking strength (g)	% tensile strength
LU	141.0±10.6	-
SO	160.4±13.9	13.75
1% MFE	254.5±34.7 $+_1$	58.68
2% MFE	288.5±19.8$^{*}{}_2{}^{+}{}_2$	79.90
2% MLE	295.1±29.1$^{*}{}_2{}^{+}{}_3$	84.04
4% MLE	304.1±27 $^{*}{}_2{}^{+}{}_3$	89.62
0.2%NFO	315.5±7 $^{*}{}_3{}^{+}{}_3$	96.75

Values are expressed as mean ± SEM (n=6 animals in each group) and analyzed by one way ANOVA followed by tuckey post hoc test; tensile strength was measured on the 10th post-wounding day using continuous water flow technique; SO=simple ointment base; LU=left untreated control; MFE= methanol fruits extract; MLE=methanol leaves extract NFO=nitrofurazone ointment, *: compared against the control, + :compared against left untreated $^{1}p<0.05$; $^{2}p<0.01$; $^{3}p<0.001$.

4.3. **Anti-inflammatory effect of the extracts**

The study on the anti-inflammatory activity of the 80% methanol extracts of *B. antidysentrica* leaves and fruits in carrageenan-induced hind paw edema model demonstrated that the extracts were effective in inhibiting edema in mice (Table 7).

After 1 h of carrageenan injection, none of the extracts exhibited significant reduction on carrageenan induced edema except the standard drug (indomethacin) ($P<0.05$). On the second hour, only the maximum dose (400mg/kg) of the leaves extract reduced the edema comparably ($P<0.05$). On the last consecutive two hours, the standard indomethacin showed to reduce the edema ($P<0.001$) compared to the negative control and ($P<0.05$) to the smallest dose of MFE (100mg/kg). The maximum dose of the leaves extract also reduced the edema volume significantly at the third ($P<0.01$) and the 4^{th} h ($P<0.001$) compared to the negative control. Similarly on the third hour, the rest of the extracts except MFE (100mg/kg) decreased the edema volume significantly ($P<0.05$) showing more significant effect on the last hour ($P<0.001$) including the lowest dose of MFE (100mg/kg). Though the effects seem to be dose dependent; comparable reduction of edema was not noticed among extracts.

Table 7: Anti-inflammatory effect of 80 % methanol extract of leaves and fruits of *B. antidysentrica* on carrageenan-induced paw edema following oral administration in mice

Groups	Mean change in the hind paw volume(ml)				
	BV	1h	2h	3h	4h
Control	0.6±0.01	1.15±0.07	1.2±0.07	1.20±0.07	1.17±0.07
MFE (100mg/kg)	0.60±0.04	0.93±0.05 (19.62%)	0.98±0.19 (18.94%)	1.09±0.17 (10.97%)	0.81±0.06(30.43%)♥3
MFE(200mg/kg)	0.63±0.0	0.90±0.03 (22.05%)	0.90±0.06 (25.08%)	0.84±0.06 (31.42%)♥1	0.70±0.06 (39.83%)♥3
MFE(400mg/kg)	0.59±0.03	0.88±0.04 (23.96%)	0.88±0.10 (26.58%)	0.81±0.04 (33.72%)♥1	0.63±0.02(46.38%)♥3
MLE(100mg/kg)	0.65±0.06	1.04±0.05 (9.55%)	0.87±0.04 (7.41%)	0.84±0.08 (31.10%)♥1	0.76±0.05 (34.87%)♥3
MLE(200mg/kg)	0.64±0.08	1.02±0.15 (11.81%)	0.86±0.03 (27.74%)	0.85±0.06 (30.44%)♥1	0.68±0.04 (42.22%)♥3
MLE(400mg/kg)	0.63±0.03	0.94±0.04 (18.40%)	0.77±0.03 (36.05%)♥1	0.73±0.02 (39.93%)♥2	0.59±0.03 (49.23%)♥3
Indomethacin(10mg/kg)	0.59±0.50	0.77±0.04(32.81%)♥1	0.76±0.02 (37.21%)♥1	0.68±0.03 (44.19%)♥3♣1	0.57±0.03 (51.28%)♥3♣1

Values are expressed as mean ± SEM (n=6 animals in each group) and analyzed by one way ANOVA followed by tuckey post hoc test; BV=basal volume; MFE= methanol fruits extract; MLE=methanol leaves extract, ♥: compared against the control, ♣: Compared against MFE (100mg/kg), [1]$p<0.05$; [2]$p<0.01$; [3]$p<0.001$.

4.4. Phytochemical screening

The results of phytochemical screening of leaves and fruits of *B. antidysentrica* showed the presence of different secondary metabolites as shown below (Table 8).

Table8. Results of phytochemical screening of 80% methanol extracts of leaves and fruits of *B. antidysentrica*

		Test results from 80% methanol extracts	
Phytochemicals	Test used	Leaves	Fruits
Alkaloids	Wagner's test	+	+
Tannins	Lead acetate and ferric chloride	+	+
Saponins	Frothing test	+	-
Flavonoids	NaOH test	+	+
Triterpenoid	--------	+	+
Phenols	Ferric chloride test	+	+
Steroids	Liebermann-Burchard	+	+
Glycosides	Glycoside test	+	+

Note: (+) indicates the presence and (-) indicates the absence of particular metabolites.

4.5. Acute toxicity studies

4.5.1. Acute oral toxicity study

The results from an acute oral toxicity study showed that the plant extracts of both leaves and fruits were appeared to be safe up to the dose 2000 mg/kg which was confirmed by the death of none of the mice and absence of any sign of toxicity till the end of the 14^{th} day. Therefore, the LD_{50} of both leaves and fruits is greater than 2000 mg/kg.

4.5.2. Skin irritation study

After 24 h of treatment with 1% and 2% fruits extract and 2% and 4% leaves extract along with their bases, the score of skin irritation in terms of edema and erythema was found to be 0 and in between 0 and 2 in all mice respectively (Table9 and fig.4). This indicates that none of the animals showed formation of edema after application of any of the ointments (medicated or none medicated). On sites applied with ointments containing leaves and fruits with their respective simple ointments, erythema with scores of 3 and more than 3(moderate to severe irritation) was not found in any of the animals. At the end of 72 h none of the animals showed either formation of edema and erythema. The Scoring of Primary Irritation (SPI) for the medicated ointments was found in between 0.067 to 0.33 which were falls under the category of negligible irritants. Apart from this, what attention seeking finding was that the higher percent (10% MLE and even 4%MFE) ointment formulations from the study plant caused death of more than 50% of the experimental animals when applied on the excised wound area during the pilot study.

Table 9: Score of irritation and edema after application of ointments containing extracts of *B. antidysentrica* with their respective bases

Mice No	Reaction	Score of skin irritation														
		Ointments of fruits extract						Ointments of leaves extract						Ointments of bases		
		1%			2%			2%			4%					
		24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
1	Erythema	0	0	0	2	0	0	1	0	0	1	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Erythema	1	0	0	2	0	0	0	0	0	1	0	0	1	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Erythema	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Erythema	0	0	0	2	2	0	0	0	0	1	1	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Erythema	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PII		**3/30=0.1**			**10/30=0.33**			**2/30=0.067**			**4/30=0.13**			**2/30=0.067**		

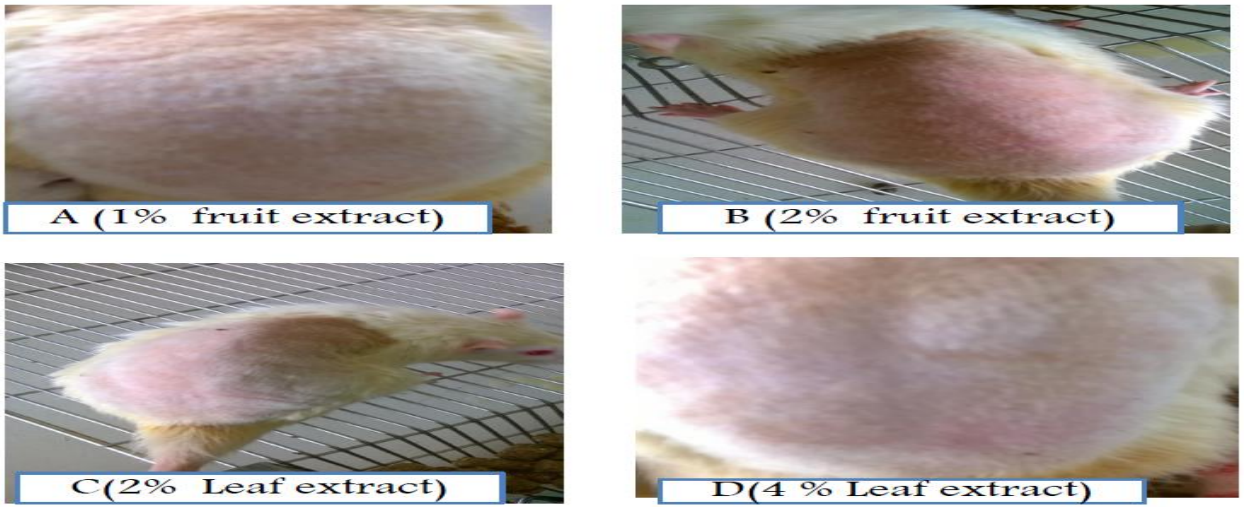

Fig.5.Animals tested for the skin irritation with the respective indicated medicated formulations.

5. DISCUSSION

The use of herbal medicine worldwide has given an excellent opportunity to look for therapeutic compounds from ancient systems of medicine which can be used for the development of new therapeutically active compounds (Samanta *et al.,* 2016). People used a number of plants and/or their derivatives as a wound healing agents assuming that they are effective and safe without scientific validation of their pharmacological parameters (Sabale *et al*., 2012). The objective of this study was to evaluate the wound healing and anti-inflammatory activities of both the fruits and leaves of *B.antidysentrica*.

Since a single model may not be sufficient to collectively present all the components of wound healing processes, two different animal models (excision and incision) were used to evaluate the wound healing activities of 80% methanol extracts of both leaves and fruits of *B. antidysentrica* in the present study.

In excision wound model, the topical application of ointments formulated from 80% methanol extracts of both fruits and leaves of *B.antidysentrica* resulted in an improved wound contraction rate compared with animals treated with simple ointments which may be attributed to the enhanced wound healing progression and the display of noticeable wound margin hydration resulted from tissue regeneration.

On the last day of treatment, complete healing was observed with the standard drug while the percentage closure of all extracts was fallen between 98.2%-99.9%. On the same day, simple ointment base treated group showed 91.00% healing. Wound contraction, which contributes to wound closure, is expressed as a reduction in percentage of the original wound size (Thakur *et al*., 2011).

During healing, contraction plays crucial role as it decreases the dimension of the wound and hence shortens the healing time. Moreover contraction reduces the amount of extracellular matrix needed to repair the defect and assists re-epithelization by shortening the distance migrating keratinocytes travel (Mulisa *et al*., 2015).

Additionally, the efficacy of the medication is dependent on the rate of wound closure. The wound contraction effect of the extracts of both leaves and fruits of *B. antidysentrica* may be associated with inhibition of microbial growth particularly in the inflammatory phase and their mitogenic activity which enhance fibroblast motility and its cellular proliferation as well as sub sequential transformation to myofibroblast during wound healing mainly being dermal (Thakur *et al*., 2011). Stimulation of fibroblasts is believed to be one of the mechanisms of plant extracts to facilitate wound healing as result of their migration from the wound edge to the wound site, proliferation and consequently production of collagen which is considered to be the main constituent of extracellular matrix (Abood *et al*., 2015).

In this study, the animal groups treated with the preparations containing the crude extracts except those treated with 1% MFE significantly shortened the period of epithelization. Epithelialization which is an essential component of wound healing is used as a defining parameter of successful wound closure ability (Pastar *et al*., 2013). As epithelialization proceeds, contractile property of myofibroblasts is enhanced, epithelial cells are proliferated and crawl across the wound bed to cover it where these proliferation and migration processes along with contractile property of myofibroblasts are attributed to the significant effect of the extracts on the epithelialization period (Samanta *et al*., 2016). The significant reduction in the period of epithelization shown by the 80% methanol extracts of the leaves and 2%MFE shows their potential healing effect.

In the incision wound model the extracts increased the tensile strength showing their wound healing effect. The increased tensile strength may be due to collagen synthesis which is a component of growing cells, its maturation, angiogenesis and stabilization of fibers. The cumulative effect of all these phenomena improves circulation, thus providing oxygen and nutrients, essential for the healing process of the wound site (Murti and Kumar, 2012; Samanta *et al.*, 2016).

Although inflammation is a biological response as a defense mechanism to avoid harmful stimuli followed by healing process, uncontrolled inflammation could lead to serious illnesses which cause great impact on public health and economy (Devasvaran and Yong, 2016).

This calls up on to investigate for possible anti-inflammatory activity which the plant might possess in order to facilitate wound healing process.

In this study, the two lower doses (100mg/kg,200mg/kg) of 80% methanol extract of the leaves and the two higher doses (200mg/kg and 400mg/kg) of the fruits extract from *B. antidysentrica* showed significant anti-inflammatory effect on carrageenan induced mouse paw edema on the 3^{rd} and 4^{th} h. The 400mg/kg of MLE, however, reduced the paw edema significantly starting from the 2^{nd} h while the smallest dose of the fruits (100mg/kg) had a significant pronounced effect only at the last hour. Carrageenan-induced edema has been widely considered to be an important experimental animal model to determine acute inflammation and is supposed to be biphasic. An initial phase lasting up to 2 h is attributed to the release of serotonin, histamine, bradykinin and substance-P. In contrast, the late phase lasting from 3–5h is mainly due to the neutrophils infiltration into the inflammatory site and the production of large amounts of pro-inflammatory mediators such as PGE2 and various cytokines (proinflammatory cytokines) such as IL-1β, IL-6,and TNF-α (Ma *et al*., 2013, Kuedo *et al*.,2016).Carrageenan induced paw edema is sensitive to cyclooxygenase inhibitors and are used to evaluate the effect of non steroidal anti-inflammatory agents, which primarily inhibit the cyclooxygenase involved in prostaglandin synthesis (Sarkhel,2016). The expression of COX-2 is maximal at the late phase of carrageenan induced paw edema, which could subsequently increase prostaglandin levels in inflammatory reactions (Chang *et al*., 2012).

The results from this study showed that the extracts affect the second phase of inflammation induced by carrageenan, i.e., the extracts inhibit the release of inflammatory mediators like prostaglandins and leukotrienes through inhibition of cyclooxygenase pathway (Amdekar *et al.,* 2012). Inhibiting carrageenan induced inflammation is mediated more through inhibition of cyclooxygenase pathway than lypoxygenase pathway. Prostaglandins and leukotrienes attract PolyMorphonuclear leucocytes towards the inflammation site and lead to tissue damage probably by releasing free radicals (Kumar *et al.,* 2012).

The other possible mechanism which may be attributed to the effect of the plant extract is the effect on the regulation of proinflammatory or anti-inflammatory cytokines.

When proinflammatory cytokines (IL-1β and IL-6) are up regulated and anti-inflammatory ones (IL-10, IL-4, and IL-13) are dawn regulated the production of prostaglandins is positive affected and the reverse has a negative effect on prostaglandins production. Therefore, the plant extract may up regulate the anti-inflammatory cytokines and reduce production of proinflammatory mediators like prostaglandins (Amdekar *et al*., 20112).

Phytochemical screening of the 80% methanol extracts of both leaves and fruits of *B. antidysentrica* showed the presence of various secondary metabolites including alkaloids, tannins, flavonoids, phenols, steroids, glycosides, and triterpenoids. The phytochemical test showed that only the leaves extract contained saponins. Previous study on phytochemical screening of *B. antidysentrica* leaves revealed the presence of vitamin C, free amino acids and flavonoids as well (Amuamuta *et al*., 2015).

These biologically active ingredients are directly responsible for antioxidant, antimicrobial, antifungal and anticancer activities through different mechanisms (Hossain *et al*., 2013). Tannins promote the wound healing through several cellular mechanisms, chelating of the free radicals and reactive species of oxygen and shrinking of proteins due to their astringent effect, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts (Pawar and Toppo, 2012, Ashok and Upadhyaya, 2012).

Another report on tannins indicated that they reduce bacterial proliferation by blocking key enzymes during microbial metabolism and hence could act as an efficient antimicrobial agent. In addition, phenolic compounds are strongly believed to have biological and pharmacological properties like antimicrobial, antioxidant and anti-inflammatory (Aberoumand, 2012).

Since infected wounds attract high levels of phagocytic cells which release reactive oxygen species in an attempt to fight infection and damage the host cells leading to delay the healing process, agents suppressing these reactive species are found to be of paramount importance in wound healing (Adetutu *et al*.,2011).

Flavonoids are responsible to reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and improving vascularity that results in increasing the viability of collagen fibrils

by increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. Flavonoids and Triterpenoids are also known to promote the wound-healing process mainly due to their anti-microbial and antioxidant ability, which seems to be responsible for wound contraction and increased rate of epithelialization (Mittal *et al*., 2016). Another study also reported that certain flavonoids have astringent effects playing an important role on wound contraction and rate of epithelialization (Lesschaeve and Noble, 2005, Ambiga *et al.*, 2007). In addition they have the ability to inhibit enzymes like phospholipaseA_2 that plays a pivotal role in the production of prostaglandins (Safari *et al*., 2016). The association of antibacterial activity to steroids, anti-inflammatory activity to saponins and anti bacterial and anti analgesic activities to alkaloids is also reported by Kumar Bargah (2015).

Although the potential wound healing and anti-inflammatory effect of both leaves and fruits was demonstrated in this study, precisely correlation of the effect to specific metabolites was difficult as the observed effect may be attributed to a single active ingredient or to the combined activity of various active ingredients.

According to the results from this experiment of acute oral toxicity test, the median lethal dose (LD_{50}) was found to be >2000 mg/kg for both leaves and fruits extract. Generally, the (OECD, 2001) guideline recommends the test chemical to be categorized under experimentally safe substance for use if the LD_{50} value of the test chemical is more than 3 times the minimum effective dose. Since the 80% methanol extract of both parts had LD_{50} value of more than the recommended dose (100 mg/kg), it was taken as a good candidate for further studies. Based on the LD_{50} value, (LD50 >2000 mg/kg) both extracts can be designated as 'unlikely to be hazardous' (WHO, 1992).

The SPI value for the medicated ointments being between 0.067 – 0.33 in the skin irritation test indicated that the extracts are under the category of negligible irritants (More *et al*., 2013). The allergy to a substance is a state of hypersensitivity of the skin, immune response to antigen that appears so excessive or inappropriate, and is also manifested as erythema and edema (Omale and Sunday, 2014). The absence of the reaction described in terms of erythema and edema in this study indicated that the extracts are non-irritant which might have contributed to the quick healing process and safety of the formulation. Higher strength of the formulation (10% of MLE and 4% MFE), however, killed the experimental animals when applied on the excised wound area in the pilot study.

6. CONCLUSION

The present study indicated that 80% methanol extracts of the leaves and fruits of *B. antidysentrica* possess wound healing activity proving the traditional claim. The wound healing effect is strengthened by the observed anti-inflammatory effect of the extract. Both the wound healing and anti-inflammatory activities may be attributed to the presence of biologically active secondary metabolites including flavonoids, tannins, steroids, triterpenoids, saponins, phenols and alkaloids that act either individually or collectively to bring about the overall effect. These findings provide a scientific support for folkloric repute of *B.antidysentrica* leaves and fruits as wound healing and anti-inflammatory agent.

7. RECOMMENDATIONS

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

- ✓ Performing wound healing and anti-inflammatory activity tests with various solvent fractions.
- ✓ Carrying out *invitro* tests for the wound healing and anti-inflammatory activity of the crude extract and its fractions.
- ✓ Carrying out quantitative phytochemical study to clearly quantify the active components against wound and inflammation from the plant.
- ✓ Carrying out chronic toxicity studies of the extract in animal models.

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9. APPENDIXES

9.1. Photos of plant material collection from Debre Markos, E/Gojjam Zone, Amhara Region

9.2. Photos showing the drying of plant materials at Pharmacology Department laboratory, SoM, CHS, AAU

9.3. Some of the instruments used during the experiment

Plethysmometer

Rota vapour

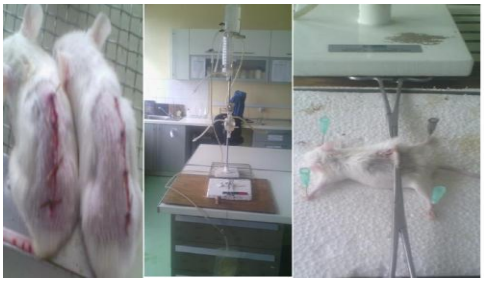
lyophilizer

9.4. Photos showing some of the procedure during experiment

Anesthesizing of mice

Hair removal from their dorsal area

Marking the circular area



Incision and determination of tensile strength

Excision