Evaluation of Wound Healing Activity of 80% Methanol Leaf Extract of *Discopodium penninervium* Hochst (Solanaceae) in Mice

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ABSTRACT

Evaluation of Wound Healing Activity of 80% Methanol Leaf Extract of *Discopodium penninervum* Hochst (Solanaceae) in Mice

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Discopodium penninervum is among the Ethiopian medicinal plants claimed for wound healing activity. However, to date, there is no scientific report on evaluation of its wound healing activity. Thus, this work was initiated to investigate wound healing activity of 80 % methanol leaf extract of *D.penninervum* in mice.

Acute oral and dermal toxicity test was studied by observing sign of toxicity for oral and dermal toxicity. Wound healing activity was investigated using excision and incision wound models by topical application of 5% and 10% (w/w) ointments of 80% methanol leaf extract of the plant.Parameters such as wound contraction and period of epithelialization were determined from excision wound model, whiletensile strengthwas evaluated fromincision wound model.In addition,anti-inflammatory, antibacterial and antioxidant activity studies of the extract were also performed.Carrageenan-induced hind paw edema model was usedfor anti-inflammatory study at a concentration of 100, 200 and 400 mg/kg. Antibacterial activity of the extract was examined using agar well diffusion technique against several common bacterial strains for wound infection. For antioxidant activity test, DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was used. In parallel, total flavonoid content of the extract was determined by using aluminum-chloride colorimetric method.

The extract and formulated ointments were found to be safe in oral and dermal toxicity studies, respectively. Both 5% and 10% (w/w) ointments exhibited significant ($p< 0.001$) increase in wound contraction and shorter epithelialization periodin excision wound model and, increased tensile strength in incision wound model. Plant extract also showed significant ($p<0.001$) anti-inflammatory, antibacterial and antioxidant activities, which could indicate the possible mechanism(s) of wound healing.The crude extract showed antibacterial activity against both gram positive and gram negative bacteria with MIC $_{index}$ of 2. Radical scavenging potential against DPPH free radicals was found to be effective with IC_{50} value of 2.77mg/ml. In addition,

quantification of total flavonoid content revealed the presence of 43.03mg quercetin equivalent flavonoids in the crude extract.

The findingscollectively indicate that traditional use of the plant for wound treatment has a scientific basis. Flavonoids alone or in concert with other phytoconstituents through anti-inflammatory, antibacterial and antioxidant action could be responsible for wound healing.

Key words: *Discopodium penninervum*,Wound healing, Excision model, Incision model, Anti-inflammatory, Antibacterial, Antioxidant, Total flavonoid content.

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LIST OF ACRONYMS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BP	British Pharmacopoeia
CFU	Colony Forming Units
DPPH	1,1-Diphenyl -2-Picryl Hydrazyl
EGF	Epidermal Growth Factor
ECM	Extracellular Matrix
IC	Inhibition Concentration
IL	Interleukin
LPO	Lipid Peroxidation
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistance
MHB	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Multi Drug Resistant *Staphylococcus aureus*
MMP	Matrix Metalloproteinase
OECD	Organization of Economic Corporation and Development
PGE2	Prostaglandin E2
QE	Quercetin Equivalent
TFC	Total Flavonoid Content

TGF- β	Transforming Growth Factor- beta
TNF- α	Tumor Necrosis Factor alpha
SEM	Standard Error of the Mean
SSTI	Surgical Site Infections
SPSS	Statistical Package for Social Sciences

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1. INTRODUCTION

1.1 .Overview of Wound

Wounds are disruption of functional continuity and anatomical structure of cells and tissues at the sites of injury (Velnar*et al*.,2009).Wounds can be caused by physical, chemical, microbiological or immunological processes. It has been estimated that 14 million people suffer from wounds and burns annually with over 80 percent of these living in low and middle income countries.However, this may be under reported since wounds are a daily occurrence that can happen to anyone at any time and people do not always seek medical attention for simple wounds and burns(Namunana *et al*., 2018).

Wounds can be categorized as acute or chronic(Namunana et al., 2018). Acute wounds heal in a very orderly and efficient manner,whereas chronic wounds fail to maintain this phase and it doesn't healwithin the expected period of time (Diegelmannand Evans,2004). Wound healing capabilities can be impaired by factors such as age, dessication, necrosis, pressure at wound site, body type (e.g. obesity), chronic diseases (e.g. cancer, peripheral vascular disease, diabetes mellitus, etc.), immunosuppression, nutritional status, vascular insufficiency, and infection with microbes such as bacteria(Hess, 2011). There is, therefore, a need for intervention in form of treatment.

1.2 .Physiology of WoundHealing

Wound healing is a biological process, which is initiated by trauma and often terminated by scar formation.Thus, healing is essentially a survival mechanism and represents an attempt to maintain normal anatomical structure and function (Broughton *et al*., 2006).All tissues in the body are capable of healing either by regeneration or repairmechanisms. Regeneration is the replacement of damaged tissues by identical cells and is more limited than repair.In humans, complete regeneration occurs in a limited number of cells for example, epithelial, liver and nerve cells. Repair is the main healing mechanism, where damaged tissue is replaced by connective tissue which then forms a scar.Healing process is awell-organized cascade of overlapping events with homeostasis, inflammation, proliferation and remodeling phases(Diegelmann and Evans, 2004).

Hemostasis Phase

Hemostasis starts immediately after injury through clot formation,which is followed by a number of significant eventsand vasoconstriction. Fibrinolysis and degranulation of platelets within the fibrin clot release growth factors and cytokines that attract many mediators and cells, including leukocytes and fibroblasts, to the wound bed. Vasoconstrictionand the resulting hypoxia in the wound environment act as signals to recruit endothelial responder cells and stimulate angiogenesis, which occurs during the proliferative phase. Together with the chemical signals, the clotting system initiates the plasma protein systems, the complement system, and the kinin system and results in direct pathogen destruction, as well as activation and regulation of the inflammatory response. These events are central to initiating the entire wound-healing cascade by providing the substances and communication that transition the wound to the next phase of healing(Velnar *et al*., 2009).

Inflammatory Phase

The inflammatory phase is the immune system's reaction to injury and insult. Inflammation begins shortly after hemostasis and aims to remove invading pathogens, as well as necrotic and damaged tissue, to provide a clean wound base in preparation for the proliferative phase of healing. Thus, the use of anti-inflammatory medications during this phase should be limited(Van Hengel et al., 2013).Ten to fifteen minutes after injury, vasoconstriction subsides and vasodilatation is induced,which results in the classic inflammatory presentation signaled by increased redness, warmth, induration, and pain around the wound, as well as white blood cell migration.Neutrophils and macrophages, which are chemo-tactically attracted to the site of injuryClean-up the wound environment through phagocytosis. They begin to arrive at the wound site within the first hour after injury and are the dominant cell for the first 2–3 days. Growth factors released by the neutrophils attract additional leukocytes to the area. Later on neutrophil starts to be replaced by the macrophage as the primary white blood cell in the wound on about the third day after injury(Broughton *et al a*., 2006).

Proliferative Phase

The proliferative phase of healing is initiated during the inflammatory stage. Cells that regulate the inflammatory stage are also involved in the initiation of the proliferative phase once most of

the necrotic and damaged tissue is removed. The goal is to fill the wound defect with new tissue and to restore the integrity of the skin(Broughton *et al a*., 2006).

The key processes of the proliferative phase include synthesis of extracellular matrix, wound contraction, epithelialization and formation of granulation tissue that occurs concurrently.

Wound Contraction

Wound contraction occurs in the later stages of proliferation. Differentiated fibroblasts called myofibroblasts contain bundles of parallel actin and myosin fibers similar to those found in smooth muscle. They contract their fibers while anchoring themselves to the wound bed and neighboring cells(Atiyeh *et al*., 2002).

Epithelialization

It is the resurfacing of the wound. The primary cell is the keratinocyte, derived predominantly from epidermal stem cells located in the bulge area of the hair follicle and the epidermis at the edges of the wound. Keratinocytes respond to signals from the macrophages, neutrophils, and other factors within hours after injury. Later, they respond to growth factors and oxygen delivered by the newly established vascular network, by advancing in a sheet to reestablish the epidermis. In full-thickness wounds, however, the sheet will be slightly thinner, as it is covering scar and lacks the anchoring structure of undamaged tissue, thus making it more prone to friction injury. The new skin and tissue have a tensile strength of approximately 15% of normal and must be protected from re-injury (Mutsaers et al., 1997).

Granulation

Granulation is the term used to describe the new wound matrix made up of collagen and an extracellular material called ground substance. These provide the scaffolding into which new capillaries will grow to form connective tissue. The growth of new blood vessels is termed angiogenesis. This is stimulated by macrophage activity and tissue hypoxia resulting from the disruption of blood flow at the time of injury. The role of oxygen in wound healing is complex and not yet fully understood. It may be significantly different in epidermal and connective tissue repair. Macrophages produce a variety of substances that stimulate angiogenesis. These include transforming growth factor (TGF), which promotes formation of new tissue and blood vessels,

and tumour necrosis factor (TNF), which facilitates the breakdown of necrotic tissue, stimulating proliferation. Healthy granulation tissue does not bleed easily and is a pinky red color. The condition of granulation tissue is often a good indicator as to how the wound is healing. Granulation tissue which is dark in color may signal that the wound is ischemic or infected(Percival, 2002).

Remodeling Phase

The remodeling phase is marked by the activities of growth factors, matrix metalloproteinases(MMPs), fibroblasts, macrophages, and epidermal cells to rebuild scar tissue under the reformed epidermis. The result is increased tensile strength, but decreased vascularity. This process of extra cellular matrix (ECM) degradation and deposition begins in the proliferative phase and extends for one to two years after closure. As a result, the tensile strength increases from the initial 15% of thenormal, for the newly formed scar in proliferative phase, to 80% of the normal for matured scar tissues in remodeling phase (Gantwerker and Hom, 2012).

1.3. Pathophysiology of Wound Healing

A delicate physiological balance must be maintained during the healing process to ensure timely repair or regeneration of damaged tissue. Wounds may fail to heal or have a greatly increased healing time when unfavorable conditions are allowed to persist. An optimal environment must be provided to support the essential biochemical and cellular activities required for efficient wound healing and to remove or protect the wound from factors that impede the healing process(Guo and DiPietro, 2010).The wound healing cascade may be arrested in any of the phases, leading to formation of a chronic non-healing wound. Many mediators including inflammatory cells, growth factors, proteases such as MMPs and cellular and extracellular elements play important roles in the process of wound healing. Alterations in one or more of these components may lead to impaired healing(Thiruvoth *et al*., 2015) .

Most of the symptoms associated with acute inflammatory response last for approximately two weeks. If inflammation persists for months or years, it is called chronic inflammation. Chronic inflammation associated with wounds often occurs when a wound is sealed by necrotic tissue, is contaminated with pathogens, or contains foreign material that cannot be phagocytized or

solubilized during the acute inflammatory phase.Whereas acute wounds go through the linear and overlapping events of the four wound healing phases, healing-impaired chronic wounds do not progress through the orderly process. Some areas of the wound are found in different phases, having lost the ideal synchrony of events that leads to normal (rapid) healing. Eventhough MMPs play an important role in wound remodeling, unbalanced expression of MMPs and tissue inhibitors of MMPs may also contribute to delayed healing or excessive fibrosis,as a result abnormal production of MMPs impairs function of cytokines to digest bacteria and necrotic tissue and result in arresting of wound healing in the inflammation stage(Li *et al*., 2007).

1.4. Factors Affecting Wound Healing

Factors affecting wound healing can be extrinsic or intrinsic depending on their source. Extrinsic factors impair healing from the external environment, whereas intrinsic factors directly affect the performance of bodily functions through the patient's own physiology or condition(Guo and DiPietro, 2010).

Extrinsic factors affecting wound healing from the external enviromentincludemechanical stress like pressure, shear, and friction; debris such as necrotic tissue or foreign material;temperature,desiccation and maceration,infection,chemical stress;medications like anti-inflammatory drugs such as steroids and non-steroidal anti-inflammatory drugs; chemotherapeutic agents,immunosuppressive drugs. Otherextrinsicfactors that affect wound healing include; alcohol abuse, smoking, and radiation therapy. Some of intrinsic factors include; chronic diseases such as diabetes mellitus, circulatory conditions, anemia and autoimmune diseases;age , and nutritional status(Guo and DiPietro, 2010).

Desiccation removes physiological fluids that support wound healing activity. Dry wounds are more painful, itchy, and produce scab material in an attempt to reduce fluid loss. Cell proliferation, leukocyte activity, wound contraction, and revascularization are all reduced in a dry environment. Epithelialization is drastically delayed in the presence of scab tissue that forces epithelial cells to burrow rather than freely migrate over granulation tissue. Maceration resulting from prolonged exposure to moisture may occur from incontinence, sweat accumulation, or excess exudates and it can lead to enlargement of the wound, increased susceptibility to mechanical forces, and infection(Guo and DiPietro, 2010).

An infection mostly from *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*and *Bacillus species* delay natural healing process by protracting the inflammatory phase or disrupting the normal clotting mechanism. Hence, finallyit interferes and delays epithelialization, contraction, collagen deposition and angiogenesis(Arun et al., 2016).The endotoxinsthemselves also stimulate phagocytosis and release of collagenase, which contribute to collagen degradation and destruction of surrounding of previously normal tissue(Broughton *etal*., 2006). During the prolonged inflammatory phase, neutrophils produce free radicals, which will result in oxidative stress leading to lipid peroxidation (LPO), DNA breakage and enzyme inactivation (MacKay and Miller, 2003).

1.5. Management of Wound

The first stage of wound management should be an assessment of the wound and the patient. It begins with a diagnosis of the wound's etiology and continues with optimizing the patient's medical condition, particularly blood flow to the wound area(Velnar *et al*., 2009).

If wound healing process is affected negatively, it can result in chronic wounds. Therefore, management of wound is essential for non-delayed wound healing and prevention from development of chronic wounds. Management of infection and inflammation are the keys to a successful wound healing(Hajimehdipoor *et al*., 2017).Medical treatment of wound includes administration of drugs either locally (topical) or systemically (oral or parenteral) in an attempt to aid wound repair .The topical agents used include antibiotics and antiseptics,desloughing agents (chemical debridement, e.g. hydrogen peroxide, eusol and collagenase ointment),wound healing promoters (e.g. honey, benzoyl peroxide, dexpanthenol, tetrachlordecaxide solution (Raina *et al*., 2008).

Topical antimicrobial therapy is one of the most important methods of wound care, which aims to control microbial colonization and subsequent proliferation, thus promotinghealing of the wounds(Odimegwu *et al*., 2008). They are selected based on their ability to destroy or inhibit the growth of pathogenic organisms, while the tissue is left undamaged.Some examples of preparations include amikacin (in gel or cream), bacitracin, chloramphenicol, clindamycin (cream, lotion, and foam), gentamicin (in ointment or cream), nitrofurazone (solution, or soluble dressing) and polymyxin B(Agarwal *et al*., 2010).Adequate calories, protein, fluids,

vitamins, and minerals are required to maintain tissue integrity, to prevent breakdown, and to support the body's natural healing processes as a result nutrition deficiencies may contribute to delayed wound healing(Atiyeh *et al*., 2002).

Negative pressure wound therapy is another wound management , it uses sub-atmospheric pressure to promote healing by: (i) maintaining a moist wound healing environment while removing stagnant wound fluid that may contain pro-inflammatory mediators; (ii) optimizing wound perfusion by decreasing peri-wound edema; (iii) mechanical stretching of the cells, resulting in increased vessel formation (neoangiogenesis) and granulation tissue formation; and (iv) managing the bacterial colonization in the wound(Atiyeh et al., 2002).In general, the main objective of wound management is to facilitate healing in the shortest time possible, with minimal pain, discomfort, and scarring (Mulisa *et al*., 2015).

1.6. Medicinal Plants Used in the Management of Wound

Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns (Kokane et al., 2009).

Over 80% of the world's population depends on traditional medicines for numerous skin disorders(Dickson *et al.*, 2010).About 70% of wound healing drugs are plant origin, 20% of mineral origin, and the remaining 10% consisting of animal products(Chopda and Mahajan, 2009). These drugs are stated to be effective in different conditions such as wounds, ulcers, sinuses, abscess, syphilitic ulcers, and maggots in wounds, septic wounds, and inflammatory changes of wounds, cellulitis, purulative ulcer, diabetic carbuncle, and fistula-in-ano(Chopda and Mahajan, 2009).However, the availability of modern drugs used for the treatment of wound accounts only for 1- 3% of the total drugs. Many of these drugs are not only expensive but also pose problems such as allergy and drug resistance (Prasad and Dorle, 2006).

Plants have an extensive potential for the management and treatment of wounds and burns with their antioxidant, anti-inflammatory and antimicrobial activities. Plants like *Adhato davasica*

Linn. Aloevera, Hippophaer hamnoidesL, Hibiscus rosasinesis, Tephrosiapurpurea Linn, Tribulusterrestris Linn, Gymnemasylvestre R.B, Calophyllum inophyllum, Morindacitrifolia, etc. are scientifically proved to exhibit wound healing activity(Rawat *et al*., 2012).

In Ethiopia, different medicinal plant crude extract and various fractions have been tested for their wound healing activities, such as *Allophylus abyssinicus*(Yesuf and Asres, 2013),*Kalancho epetitiana*(Mekonnen *et al*., 2013),*Rumex abyssinicus*(Mulisa *et al*., 2015)and*Becium grandiflorum(Beshir et al., 2017)*were found to be effective on different animal wound models.

1.7. Overview of the Experimental Plant

Discopodium penninervium Hochst (Solanaceae) (Figure1) is locally known as alumi (Agawgna), ameraro (Amaharic), mararo (Oromiffa) and alhem (Tigirgna). It is a shrub or small tree up to 5 m high,stems slightly fleshy, branchlets brown and hairy. It usually grows in dry,moist, and wet,dega and wurchagroclimatic zones of Tigray, Wolo, Gojam, Shoa, Harereghe, Arsi, Sidamo and Kefa regions(Getachew and Biruk, 2014).

In various regions of Ethiopia, different parts of *Discopodium penninervum*are thought to be an effective medicinal remedy. In Southern Ethiopia, the leaves are used for treatment of liver disease. In North Shewa zone,Amhara region, it is used for treatment of different health problems :-fresh roots for snake bite(boil and drink the decoction when cool),leaves for treatment of wound and eczema(the leaves dried, grinded and the powder is applied to the affected area) (Hamill *et al*., 1993)**.**In western Ethiopia also,it is used for wound treatment (Hamill *et al*., 1993). In Wonago region, it is used for persons, who are unable to walk properly(rubbing affected part with fresh/dry crushed leaf) (Wubetu *et al*., 2017).However, although *Discopodium penninervum* is traditionally claimed for having wound healing activity, it is not yet proven scientifically.

A B

Figure 1:Photograph of *D.penninervum* at time of flowering(A) and fruiting(B)

1.8. Rational for the Study

Each year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring (Hajimehdipoor *et al*., 2017).Open wounds are particularly prone to infection, especially by bacteria, and also provide an entry point for systemic infections.Wound infections are responsible for significant human mortality and morbidity worldwide. It results in sepsis, limb loss, long hospital stays andhigher costs(Azene and Beyene, 2011).There is anecdotal, yet well-observed, evidence that in many hospitals in Africa wound infection rates are high and in some hospitals almost every wound will become infected (Loefler *et al*., 2002). *S .aureus* was the most frequently isolated pathogen in wound which accounted for 38.2% of isolates followed *by P.aeroginesa* (22.3%) (Agra *et al*., 2013).

Skin disorders, primarily wounds, are the third most common causes of people seeking medical care in developing countries. Medical costs and lost productivity from the workforce suggest that chronic wounds cost several billions of dollars annually. Contributing to these staggering costs is

treatment regimen that is expensive and/or ineffective and associated with recurrence rate (Dickson *et al*., 2010).

Chronic wounds represent a huge challenge that inflicts a considerable encumbrance on patients, society and health care providers and thus poses a major threat to public health and the global economies(Rondas, 2016). In developed countries, it has been estimated that 1 to 2% of the population will experience a chronic wound during their lifetime (Guest *et al*., 2015, Heyer *et al*., 2016)similar to the prevalence rate for heart failure (Roger, 2013). The National Health Service in the United Kingdom has estimated the costs of chronic wounds to be €38–59 per inhabitant (€2.34–3.51 billion in total) per year (Rondas, 2016).

The prevalence of wound infection from 2006 - 2010 ranged from 61.5% -76.2%,and the overall prevalence of wound infection was 70.1% in a 5 year surveillance study of wound infection done in Nigeria (Oladeinde *et al*., 2013). In Ethiopia, a study conducted at Tikur Anbessa Specialized Hospitalindicated that the overall bacterial prevalence in postoperative wound was 75.6% and predominant bacterial isolates were *S.aureus* (33.3%) and *E.coli* (14.3%). Double infections accounted for 11.4% ,of which 23.5% were due to *S.aureus* and *Pseudomonas spp.*Co-infection (Asres *et al*., 2017).

Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced with concomitant killing of regenerating cells. Consequently, there is a need to stimulate healing and restore the normal functions of the affected part of the body to ease the discomfort and pain associated with wounds, preventing infection, and activating tissue repair processes (Agra *et al*., 2013).

Nowadays, a broad range of antibiotics are being used for management of wound infections, but these have been proved to have undesirable effects on the human body and the pathogens have been successful in developing resistance against various commonly used antibiotics.The rapid emergence of antimicrobial resistance among bacteria is a public health crisis globally. The overall multi-drug resistance (MDR) (resistant to three or more antibiotics) rate of gram positive and gram negative bacteria was 73.6% and 67.6% , respectively (Sani *et al*., 2012)**.** Wound infections with antimicrobial-resistant bacteria increase patient morbidity and mortality and greatly increase the cost of medical care (Zaoutis, 2009)**,** as a result, the demand

of herbal drugs is increasing day by day in developed as well as developing countries because they are safer and well tolerated as compared to those allopathic drugs (Arun *et al*., 2016). Moreover, in developing countries, the use of herbs is not only an alternative to the mainstay, which is western medicine, but also the most easily accessible and affordabletreatment (Hinz, 2007).

More than 80% of the world's population still depends upon traditional medicines for various skin diseases (Abirami *et al*., 2011). Herbal treatment for wounds provides fibro-genetic and concentration of collagen resulting in faster wound healing (Bhat *et al*., 2007) . The growing popularity of natural and herbal medication, easy availability of raw materials, cost effectiveness and paucity of reported adverse reactions prompted me to formulate anherbal topical preparation and assess its wound healing ability on experimentally induced cutaneous wounds in mice models. *D.penninervum* is one of the traditionally claimed plants for wound healingwithout anyscientific evidence yet. Thus, this work wasundertaken to explore the wound healing activity of *D.penninervum*plant extract.

2. OBJECTIVES

2.1.General Objective

The general objective of the study was to evaluate wound healing activity of 80% methanol leaf extract of *Discopodium penninerуum* in mice.

2.2.Specific Objectives

The specific objectives of the study were:

- To evaluate acute oral toxicity in mice
- To evaluate acute dermal toxicity in mice
- To evaluate effectiveness of the formulated ointments in excision and incision wound models
- To evaluate anti-inflammatory activityusing carrageenan induced paw edema model
- To determine antibacterial activity using agar well diffusion method
- To determine antioxidant activity using DPPH assay
- To determine quantity of antioxidant principles in the extract

3. MATERIALS AND METHODS

3.1.Materials

3.1.1. Chemicals, Drugs and Reagents

Methanol (Carlo Erba Reagents, Italy), distilled Water (Addis Abeba Univeristy Pharmaceutics laboratory, Addis Ababa, Ethiopia), Ketamine hydrochloride (Neon laboratories, India,), Diazepam (Gland Pharma limited, India), Mueller Hinton Agar (Himedia, Laboratories Pvt.Ltd India), Muller Hinton Broth (Himedia, Laboratories Pvt.Ltd India), blood agar, ampicillin (Himedia Laboratories Pvt. Ltd, India), Cefoxitin (HimediaLaboratories Pvt. Ltd, India), Ciprofloxacin (Himedia Laboratories Pvt. Ltd, India),Amoxicillin (Himedia Laboratories Pvt. Ltd, India), Nitrofurazone USP 0.2% ointment (Shanghani General Pharmaceutical co, Ltd,China),Wool fat, Hard paraffin, White soft paraffin (queens Hygore industriePlc), Cetostearyl alcohol (Banbury, Oxon..UK), Indomethacin (LebanLaboratories Pvt.Ltd India), Carrageenan (Sigma-Aldrich Steinheim, Germany), DPPH (Sigma Aldrich,Germany), quercetin(Sigma Aldrich, Germany), Aluminum chloride and Ascorbic acid were used. Chemicals and reagents used were of analytical grade. Additionally, the following materials were used: sterile syringe filters, syringes, needles, scissors, oral gavage, electronic balance, gloves, etc.

3.1.2.Plant Material

The leaves of *D.penninervum*were collected from one of its natural habitat around Debre Markos town (297Kms from the capital), Amhara region, North West Ethiopia. Plant identification and authentication was done by a taxonomist Ato Melaku Wonidafrashat the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a plant sample was deposited with specimen numberM.A-001for future reference.

3.1.3.Experimental Animals

Healthy, adult white albino mice of both sex (25–35g, and 6–8 weeks of age) were obtained from animal house of School of Pharmacy, Addis Ababa University. They were housed in clean polypropylene cages with laced steel roofs under standard conditions (25 ± 2°C, 55 ± 5 % relative humidity, and 12 h light and dark cycles). The animals were fed with standard laboratory diet and clean drinking water *ad libtum* ,and they were acclimatized to laboratory

condition for one week prior to the experiment. Each group was kept in a separate cage. Their cages were cleaned daily, food and water changed daily.

3.2. Methods

3.2.1. Plant Extraction

Leaves of*D. penninerуum* were collected, washed under running tap water, and dried for two weeks under shade. The dried leaves werethen crushed to course powder using grinder. Then 500g of the coursepowder was macerated once only with 5L of 80% methanol for 72 h in conical flask with occasional stirring and shaking. The extract was thenfiltered by Whatman qualitative filter paper (What man No.1). The filtrate was combined and concentratedusing a Rota vapor with a temperature not exceeding 40^0C to remove methanol.Concentrated extract was then frozen in a deep freezer and dried in a lyophilizer to remove its aqueous content(Pirbalouti *et al*., 2010).

Out of 500 g powder, the resulting dry extract was weighed and provided a percentage yield of 19.2%. At last,the dried extract was packed in a closed vesseland stored in a refrigerator for the preparation of topical formulation (ointment).

3.2.2.Ointment Formulation

Simple ointment of plant extract was prepared following the formula (Table 1) described in the Britishpharmacopoeia (BP, 1988a.).Three ointment preparations (each 200 g),were formulated using the reduced formula, i.e. 200g simple ointment base (non-medicated), 5%(w/w)medicated ointment (10g of extract was incorporated in 200g of simple ointment base) and 10%(w/w) medicated ointment (20g of extract was incorporated in 200g ofsimple ointment base). Fusion method was employed in the preparation of non-medicated ointment base (Namunana et al., 2018).To prepare medicated ointments, levigation method was used to make ointment of uniform consistency and smooth texture (Sawant and Tajane, 2016). Simple ointment base as a negative control and Nitrofurazone ointment (0.2%w/w) as standard drug were used for comparing the wound healing potential of plant extract in the models used.

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

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

3.2.3.Acute Oral Toxicity Test

Acute oral toxicity test was performed according to Organization of Economic Corporation and Development OECD 420:2001 guideline (OECD, 2001). Female mice of 6-8 weeks were used for the test. The mice were fasted (3-4 h) before administering the test substance and for a further 1-2 hoursafter administering the test substance. First, a sighting study was performed to determine the starting dose. For this, a single female mouse was given 2000 mg/kg of the extract (1 ml/100 g) as a single dose by oral gavage. After observation for mortality or any sign of toxicitylike, changes inbehavioral, neurological, irritability, spontaneous activity, convulsions, urinary, salvation and pilo-excitation within 24 h, another 4 female mice were given the same dose and observed for onset, duration, and severity of toxic signs ; continuously for the first 30 min then intermittently for 4 h over a period of 24 h and, daily thereafter, for a total of 14 days.

3.2.4.Acute Dermal Toxicity Study

Acute dermal toxicity were tested as described elsewhere (Kokane *et al*., 2009, Mulisa *et al*., 2015). For dermal toxicity, 5 female albino mice wereused. Animals showing normal skin texture were housed individually in a cage and acclimatized to the laboratory condition for five days prior to the test. Around 10 % of the body surface area fur was then shaved from the dorsal part 24 h before the study. A limit test dose of 2000 mg/kg of the 10 % formulation of the extract was applied on the shaved area. At the end of the exposure period (24 h), the residual test substance was removed and the animals were observed for 24 h

and for the next 14 days for development of any adverse skin reactions, like inflammation , irritation or redness.

3.2.5.Grouping and Dosing of Animals

For excision model the animals were randomly divided into four groups (6 per group) as follows: Group I micewere treated with the reference standard (0.2% w/w Nitrofurazon). Group II and III mice were treated with 5% w/w & 10% w/w of extract ointment, respectively, andGroup IV mice were treated with simple ointment base (negative control). For incision model the animals were divided into five groups (6 per group) to start ointment application. Three groups were control groups (negative, positive and untreated controls-group I, II and III, respectively) and the other two were test groups (with 5 & 10% solvent extracts ointment i.e. group IV and V). For assessment of anti-inflammatory activity, mice were grouped in to five groups. Group I,II and III(treatment groups) weretreated with three different doses of the extract (100 mg/kg, 200 mg/kg and 400 mg/kg). Group IV (positive control group) were treated with Indomethacin 10mg/kg and group 5 (negative control group) were treated withvehicle (distilled water). Both, extract and indomethacin powder weredissolvedindistilled water andmaximum volume administered was 10 ml/kg (Wolde‑Mariam et al., 2013). Dose levels were chosen based on acute oral toxicity result described in OECD (2001). A middle dose, which is one-tenth of the maximum dose obtained during acute toxicity study; a low dose, which is half of the middle dose, and a high dose which is twice of the middle dose.

3.2.6. Wound Healing Activity Test

Wound healing activity was evaluated by excision and incision models. This was achieved by observing the rate of wound contraction, complete epithelialization time and wound tensile strength.

I. Excision wound model

Animals were anesthetized using subcutaneous injection of ketamine (1 ml/kg) and diazepam (1 ml /kg). The back hair of the animals was shaved. After this, about 300 mm^2 circular area was marked (Figure4A). The full thickness of the marked area was then excised by using sharp sterilized scissors (Figure 4B). After 24 h of wound creation, the ointments wereapplied once daily, for each group accordingto the respective grouping as described under grouping and dosing section, to cover the wound area until complete healing was achieved. Wound closure rate was assessed by tracing the wound area every three daysuntil complete wound healing. The wound was left undressed to the open environment (Charde *et al*., 2010).

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

$$\text{Wound contraction} = \frac{\text{Area on day zero} - \text{Area on day of measurement}}{\text{Area on day zero}} \times 100\%$$

Complete Epithelializationtime

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

II Incision wound model

First the animals were anaesthetized by using subcutaneous injection of ketamine (1 ml/kg) and diazepam (1 ml /kg). Para vertebral straight incision of 3 cm length was then made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel (Figure 2A). After complete hemostasis, the wound was closed by means of interrupted sutures placed at equidistant points about 1 cm by asurgical thread (silk no. 00 round) (Figure2B). Then, animals were treated once a day according to the respective grouping as described under grouping and dosing section,from 0 day to 9 post-wounding day (Beshir *et al*., 2017). The sutures were removed on day 8 post- incision and wound breaking strength was estimated on 10^{th} post wounding day using continuous water flow technique (Wang *et al*., 2011)**.** Finally, the breaking strength was expressed as minimum weight of water necessary to bring about gaping of wound area.

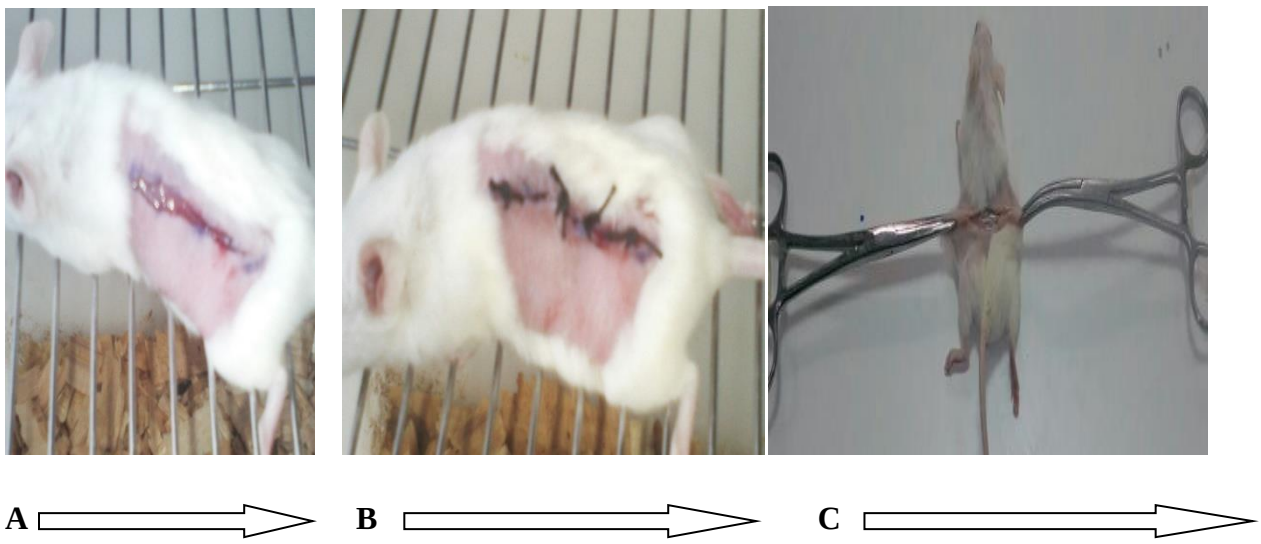

Figure 2: Photograph of incision wound model:-Para vertebral straight incision (A), interrupted suturing (B),and measurement of tensile strength (C).

Measurement of tensile strength

Mice were anesthetized and secured to the operating table (Figure2C). Two forceps were firmly applied 3mm away from the edge of wound facing each other on opposite side of the incision wound. One of the forceps was fixed on stands, while the other was connected to a freely suspended lightweight plastic of volume 1000 ml through a string

run over to a pulley. Water was allowed to flow continuously from the reservoir slowly and steadily into the container. The moment at which the wound opened, the water flow arrested and the volume of water collected in the container (approximately equal to its weight) was noted as tensile strength (Ilango and Chitra, 2010). Percent tensile strength was calculated by using the formula shown below (Akkol *et al*., 2011).

$$\text{Percent tensile strength (TS) of extract} = \frac{\text{TS extract} - \text{TS simple ointment}}{\text{TS simple ointment}} * 100$$

$$\text{Percent tensile strength (TS) of reference} = \frac{\text{TS reference} - \text{TS simple ointment}}{\text{TS simple ointment}} * 100$$

$$\text{Percent tensile strength(TS) of simple ointment} = \frac{\text{TS simple ointment} - \text{TS left untreated}}{\text{TS left untreated}} * 100$$

3.2.7.Anti-Inflammatory ActivityTest

Anti-inflammatory activity ofthe extract was determined using mouse paw edema model. Before administration of any drug, the mice were fasted overnight with free access to water and the basal volume of the right hind paw of each mouse was determined using plethysmometer (Ilango and Chitra, 2010). After determination of basal volume, the animals were assigned into five groups (each containing 6 mouse) such that the mean volumes of thosegroups were not significantly different. The mice were then treated as described under grouping and dosing section.One hour later, the animals were injected with 0.05 ml of a solution of 1% carrageenan in 0.9% saline (w/v) in the sub-plantar region of the right hind paw to induce inflammation. The inflammation were then quantified by measuring the paw volume by dislocation of the water column in a Plethysmometer (UGO Basile 7140, Italy) at 0, 1, 2, 3 and 4 hourafter Carrageenan injection .

The percentage inhibition of edema for each group was calculated using the following formula (Mahomed and Ojewole, 2004).

$$\text{Percentage inhibition of edema} = \frac{C_o - C_t}{C_o} \times 100\%$$

Where C_o is the average inflammation (hind paw edema) of the control group at a given time; and C_t is the average inflammation of plant extract or indomethacin treated mice at the same time.

3.2.8.*In Vitro*Antibacterial Activity Test

I.Inoculum preparation and standardization

Standard bacterial strains including *E. coli (ATCC 2592), P. aeruginosa (ATCC 2785), S. aureus (ATCC 25923), and S. pyogens (ATCC 1961)*were obtained from Akililu Lemma Institute of Pathobiology and *K. pneumonia (ATCC 1705)*, from Ethiopian Public Health Institution (EPHI). The bacteria were selected by considering the likely bacterial strains that can cause skin and soft tissue infections or wound infections. The test organisms were grown in a Muller Hilton agar (MHA) medium and three to five well-isolated colonies of same morphological type were selected from an agar plate culture. Each isolate was grown in 4 ml Muller Hilton broth (MHB) in Erlenmeyer flask. Then, turbidity of each bacterium was standardized to a density of1.5×10^8 CFU/ml of 0.5 McFarland by following the guideline of Clinical and Laboratory Standard Institute (CLSI, 2014).

II. Agar well diffusion

The antibacterial activity of crude extract was determined using agar well diffusion method as described elsewhere (Taye *et al*., 2011, Andualem *et al*., 2014)**.** The standardized bacterial suspensions prepared were streaked evenly on sterile MHA plates or MHA with 5% blood agar (for *Streptococcus pyogens*) with cotton swab. Fifty minutes later, fourequidistant wells were made on each plate with a 6 mm diameter sterilized cork borer. The labeled wells were filled with 100 µlof 25, 50, and 100mg/ml of crude extract, making the final concentration of 2.5, 5and 10 mg/well, respectively, for *S. aureus and S. pyogen*and, for *E. coli*, *P. aeruginosa* and *K. pneumonia*the labeled wells were filled with 100µl of 300, 400, and 500 mg/ml of the crude extract making the final concentration of 30, 40 and 50 mg/well, respectively. Dose selection was based on pilot study doneusing concentration ranging from 25-500mg/ml for those bacterial species.In addition, commercial antibiotic discs of ampicillin 0.01 mg/disc and amoxicillin0.01mg/disc(for *Streptococcus pyogens*), cefoxitin 0.03 mg/disc (for *E. coli* and *S. aureus* species) and ciprofloxacin 0.005 mg/disc (for *P. aeruginosa*and *K. pneumoniae*) as a

positive control and sterilized distilled water as a negative control were used (Kumar *et al*., 2010a, Govindappa and Poojashri, 2011). The plates were then incubated at 37 °C for 24 h and zone of inhibition was determined. The experiment was performed in three independent tests for each bacterial strain. Hence, the antibacterial activity was determined by measuring of inhibition zone diameters (mm) and evaluatedaccording to the parameters suggested elsewhere (Wafa *et al*., 2016).

- ✓ <9 mm, inactive ;
- ✓ 9–12 mm, less active ;
- ✓ 13–18 mm, active;
- ✓ >18 mm, very active

III .Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum Inhibitory Concentration **(**MIC) values of the plant extract was determined using micro-well dilution methodaccording to the protocol of Clinical and Laboratory Standard Institute (CLSI, 2014) for the bacterial strains, which were found to be sensitive in the agar well diffusion assay.The first row of microtiter plate was filled with 100 µl (25 µg/µl for gram positive bacteria and 700µg/µl for gram negative bacteria) of the test materials in distilled water. All the wells of microtitre plates were then filled with 100 µl MHB.Serial two-fold dilutions were prepared in 96-well plates with MHB at a concentration varying between 25 to 0.05µg/µl for gram positive bacteria and between700 to 21.88µg/µl for gram negative bacteria by transferring 100µl test material from the first column to the subsequent wells in the next column of the same raw. Finally, a volume of 10 µl bacterial suspension was added to each well to achieve a final concentration ofapproximately5×10^6 CFU/ml. The last well containing 100 µl of MHB only served as negative control (sterility control) and 100µl MHB and 10µl of inocula without extract was used as positive control (growth control). The plates were then incubated at 37°C for 24 h by covering with parafilm to prevent dehydration. After incubation, 25µl of 0.2%(w/v) resazurin dye solution, as indicatorwas added and MIC was recorded.The color change in the well was then observed visually. Any color change observed from purple to pink or colorless was taken as positive. The MIC is the lowest concentration of the compound at which the microorganism tested does not demonstrate visible growth. Minimum bactericidal concentration (MBC) was determined by sub-culturing the test dilutions on to a fresh solid

medium and incubated further for 18-24 h. The lowest concentration that revealed no visible bacterial growth after sub-culturing was taken as MBC.

The mechanism of antibiosis (static or cidal) of the extracts was calculated using the ratio of MBC/MIC or MIC_{index} as described elsewhere (Olajuyigbe and Afolayan, 2012). When the ratio of MBC/MIC was ≤2.0, the extract was considered bactericidal or otherwise bacteriostatic. If the ratio is ≥16.0, the extract was considered ineffective.

3.2.9.Antioxidant Activity Test

I. Free radical scavenging activity

The ability of plant extracts to have free radical scavenging activity was determined using 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay as described elsewhere (Blois, 1958),with minor modifications**.** A test sample stock solution (5 mg/ml in methanol) was prepared. Then, serial dilutions (2.5, 1.25, 0.625, and 0.315) were made by diluting the stock solution with methanol. Ascorbic acid as a standard stock solution(0.5mg/ml in methanol) was prepared andthen serial dilutions (0.25, 0.125 0.0625 and 0.0315mg/ml) were prepared.

Five milliliters of 0.004% DPPH solution (4mg/100ml in methanol) was added to each 50ul of various concentrations of test solution, ascorbic acidsolution and methanol in test tubesand allowed to reactat 25^{o}C in dark for 30 min. The absorbance was then measured at 517 nm in a UV spectroscopy (Nahak and Sahu, 2011).All experiments were performed in triplicate and the results were averaged. DPPH scavenging activity is usually presented by IC_{50} value,whichwas calculated from concentration vs %inhibition graph .IC_{50} values represent the concentration of sample, which is required to scavenge 50% of the DPPH free radicals (Ekuadzi *et al.*, 2012).A lower IC_{50} value corresponds to a higher antioxidant activity (Wafa *et al*., 2016).

The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation-:

$$\% \text{ of Inhibition} = \frac{\text{Absorbance of control - Absorbance of Test}}{\text{Absorbance of control}} \times 100$$

The DPPH solution plus methanol as a negative control and ascorbic acid as a the positive control were used(Barku *et al*., 2016)**.**

Finally,to determine the IC_{50} value of extract and ascorbic acid, regression equations of calibration curves of concentration versus % inhibition were calculated using MS Excel software (Mohammadi-Motamed *et al*., 2014).

II.Total flavonoid Content Determination

Total flavonoid content (TFC) was determined as described elsewhere(Ayoola *et al*., 2008) with minor modification. The analysis was based on the formation of yellow color of flavonoid-aluminum complex. Aluminum chloride(2ml,2%) was mixed with the same volume of the leaf extract (1mg/ml). Blank was prepared consisting of 2ml of sample solution without aluminum chloride. The absorbance readings at 415nm were taken after 1h of incubation at room temperature against ablank sample. The TFC was determined using a standard curve of quercetin at 2.5-40µg/µl ($y=0.035x+0.519, R^2=0.997$) (Figure7),and value was calculated as milligram quercetin equivalents/gram of dried extract (mg QE/g)

3.4Statistical Analysis

The experimental results was expressed as mean ± standard error of the mean (SEM). Analysis wasdone using statistical package for social sciences(SPSS) version 20. Test of statistical significance was carried out by employing one way analysis of variance (ANOVA) followed by Tukey‟s post Hoc test. The analysis was performed with 95% confidence interval and the significance was set at $p<0.05$.

4. RESULTS

4.1. Acute Oral Toxicity Test

Acute oral toxicity of the plant was done at 2000 mg/kg dose and the result showed no sign of toxicity (changes in behavioral, neurological, irritability, spontaneous activity, convulsions, urinary, salvation and pilo-excitationor mortality) in the first 24 h as well as in the following 14 days.Therefore, the dose causing 50% death of the animals (LD_{50}) of the plant is greater than 2000 mg/kg.

4.2. Acute Dermal Toxicity

After 24h of application of alimit test dose of 2000 mg/kg of the 10% formulation of the extract, there was no any dermal toxicity (inflammation, irritation or redness) observed. There was also no sign and symptom as well as mortality observed when the animals were monitored for 48 h and for 14 consecutive days of cage side observation.Hence, the extracts were considered to be safe (Figure 3).

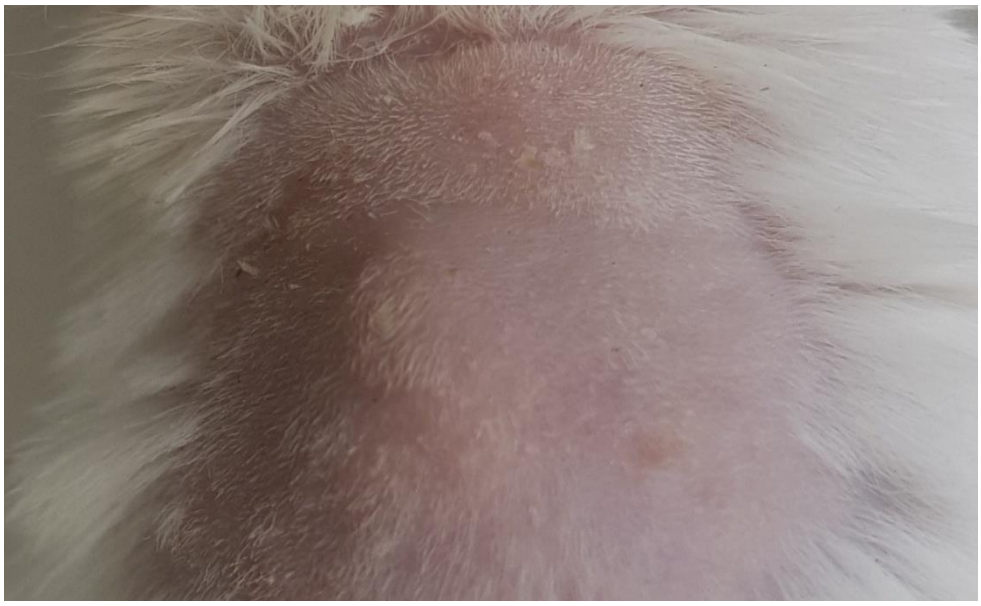

Figure 3: Photograph of acute dermal toxicity test result.

4.3. Wound Healing Activity

4.3.1.Excision Model.

Wound Contraction and Complete Epithelilization time

As presented in Table 2, 80% methanol leaf extract of *D.penninervum* showed significant increase in percentage closure of wound and shorter epihelilization time when compared to control. The percentage wound contraction was significantly higher ($p< 0.01$) for extract and nitrofurazon as compared with the negative control in all days of contraction measurement. However, there was no significant difference observed in activity and rate of wound closure between the extract and standard drug. In addition, there was no significant difference in wound closure between 5% and 10% ointment preparations. Complete epithelilization time was significantly shorter ($p<0.01$) in 5%, 10%extract and nitrofurazon treated groups as compared to control(Table2).There was no significant difference between extract and standard as well as among the different ointment preparations of the extract in complete epithelilization time.

Table 2.Effect of topical application of ointments of *Discopodium penninervum* in excision wound model

Group	Wound area in mm^2 (Values in parenthesis represent percentage of wound closure)					Complete Epithelilization time(days)
	Day 3	Day 6	Day 9	Day 12	Day 15	
Simple ointment	278.50±6.90 (7.17%)	251.33±7.47 (16.22%)	212.17±8.59 (29.28%)	121.00±9.63 (59.67%)	33.50±5.56 (88.83%)	21.50±.992
0.2%w/v Nitrofurazon	215.83±5.58** (28.06%)	172.33±10.56** (42.56%)	107.50±11.955** (64.17%)	63.67±11.66** (78.78%)	2.83±0.98** (99.06%)	16.17±.401**
5% w/w extract	235.17±9.59** (21.6%)	183.33±14.43** (3889%)	130.17±13.642** (56.61%)	72.83±7.56** (75.72%)	9.00±2.42** (97%)	17.00±.516**
10% w/w extract	209.83±9.29** (30.06%)	167.83±18.88** (44.06%)	128.33±13.76** (57.22%)	64.33±10.01** (78.56%)	5.83±0.79** (98.06%)	16.50±.224**

n = 6, Values are expressed as mean ± SEM; Analysis was performed by one way ANOVA followed bytuckey post-hoc test; *p < 0.05, **p < 0.001, compared to simple ointment.

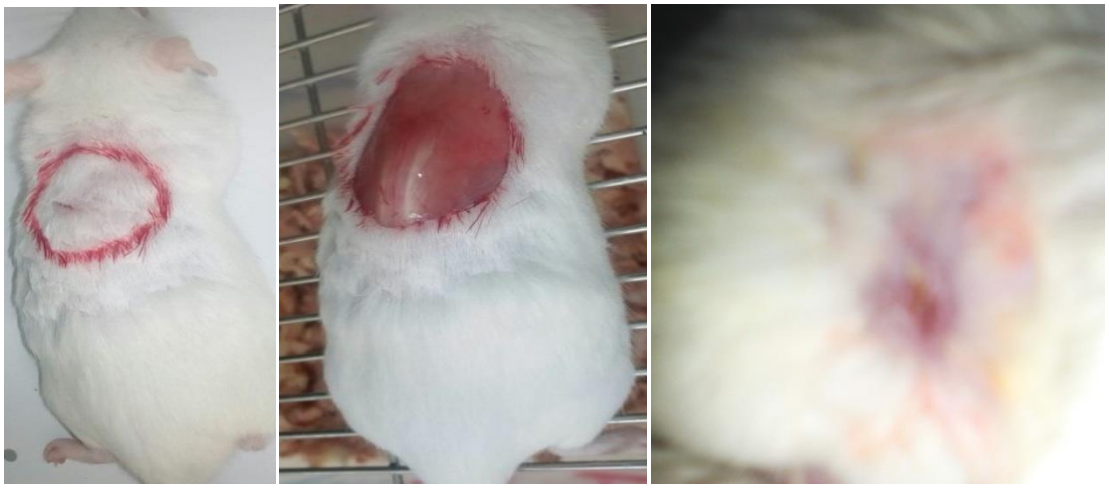

Figure 4:Photograph of excision wound model;marked skin(A),excised skin(B),healed skin(C)

4.3.2.Incision Model.

Both 5 and 10% ointment preparations of crude extract showed significant increase ($p<0.001$) in wound tensile strength (Table 3) as compared to control. However, there was no significant tensile strength difference between the reference drug and the two extract doses. Similarly, no significant difference in tensile strength was noted when the two strengths of the extract were compared with each other.

Table 3: Effect of topical application of ointments of 80% Methanol leaf extract *of D. penninervum* in incision wound model

Treatment groups	Tensilestrength(gram)	**%**tensile strength
Non_ treated	232.50±14.361	
Simple ointment	263.75±15.462	13.44
0.2% w/v Nitrofurazon	402.50±11.637**	52.61
5% w/w extract	398.75±11.614**	51.18
10% w/w extract	405.00±12.583**	53.55

n = 6, Values are expressed as mean ± SEM; Analysis was performed by one way ANOVA followed bytuckey post-hoc test; **p < 0.001, compared to simple ointment.

4.4 Anti-Inflammatory Activity

The anti-inflammatory activity of 80% methanol leaf extract of *D. penninervum* was evaluated by carrageenan-induced paw edema model in mice. After one hour of administration of carrageenan, there was no significant difference between treated and control animals.At 2h, only the extracts showed significant ($p<0.01$) reduction of paw edema, while the standard drug failed to be significant as compared to control.However,standard drug,100mg/kg,200mg/kg and 400mg/kg extracts treated animals showed significant ($p<0.01$) reduction of edemaat 3h and 4h as compared to the negative control with maximum inhibition of65.45% 56.27%,57.36% and 58.18% ,respectively (Table 4).There was no significant difference among the different doses of the extractas well as between the extract and standard drug at all -time points when compared with each other**.**

Table 4: Anti-inflammatory activity of 80% methanol leaf extract of *D. penninervum* using carrageenan induced paw edema model in mice.

Groups	Mean increase in paw volume				
	0h	1h	2h	3h	4h
Control	0.2160± 0.020	0.2380±0.093	0.2240±0..024	0.2280 ±0.183	0.2200±0.019
Indomethacin 10mg/kg	0.2100± 0.014	0.1940±0.008 (27.73%)	0.1640±0.0163 (26.79%)	0.0940±0.004** (58.77%)	0.880±0.002** (65.45%)
100mg/kg extract	0.200± 0.010	0.1900±0.003 (14.23%)	0.1200±0.089** (46.42%)	0.1100±0.004** (51.76%)	0.0940±0.004** (56.27%)
200mg/kg extract	0.2020± 0.016	0.1900±0.025 (20.17%)	0.118±0.012** (47.32%)	0.1100±0.013** (51.75%)	0.0960±0.013** (57.36%)
400mg/kg extract	0.2100±0.022	0.1960±0.178 (19.33%)	0.1020±0.124** (54.46%)	0.1040±0.150** (54.39%)	0.0920±0.005** (58.18%)

n = 6, Values are expressed as mean ± SEM; Analysis was performed by one way ANOVA followed bytuckey post-hoc test; *p < 0.05, **p < 0.001 compared to distilled water;Values in parenthesis represent percentage inhibition of paw Volume.

4.5.Antimicrobial Activity

4.5.1. Zone of Inhibition

All test bacterial strains were inhibited by the tested concentrations of the crude extract of the plant in a concentration dependent manner. Among the tested bacteria, Gram-positive bacteria were more sensitive than Gram-negative ones.As depicted in Table 5, the extract was active on gram positive bacteria at 50 and 100µg/µl,with a maximum inhibition observed with *S.aures*(19.3±1.076) at 100µg/µl. In case of gram-negative bacteria,the crude extractwas active at the higher concentration (500µg/µl) in all studied organisms (Table 6).

Table 5: Mean zone of inhibition of 80% methanol leaf extract of *D. penninervum* against gram positive bacteria

	S.aureus (ATCC 25923)	*S.pyogen* (ATCC 1961)
	Zone of inhibition ±Sd(mm)	
25µg/µl (crude extract)	10.9±1.140	10.3±0.476
50µg/µl (crude extract)	15.3±0.635	13.8±1.184
100µg/µl (crude extract)	19.3±1.076	15.1±0.405
Amoxicillin (0.01mg/disc)	NT	15.6±1.402
Ampicillin (0.01mg/disc)	NT	-
cefoxitin (0.03mg/disc)	15.3±1.26835	NT
DW (negative control)	-	-

DW= Distilled water, NT= Not tested, - = no inhibition

Table 6: Mean zone of inhibition of 80% methanol leaf extract of *D. penninervum* against gram negative bacteria.

	E.coli (ATCC 2592)	*P. aeruginosa* (ATCC 2785)	*K.pneumonia* (ATCC 1705)
	Zone of inhibition ±SD (mm)		
300µg/µl (crude extract)	8.3±0.497	-	-
400µg/µl (crude extract)	9.3±0.541	9.0±0.141	9.2±0.355
500µg/µl (crude extract)	13.1±0.900	13.0±0.970	13.1±0.938
Cefoxitin(0.03mg/disc)	14.3±0.499	NT	NT
Ciprofloxaxillin(0.005mg/disc)	NT	30.3±0.499	20.3±0.499
DW(negative control)	-	-	-

DW= Distilled water, NT=Not tested,- =no inhibition

4.5.2.Minimum inhibitory concentration and Minimum bactericidal concentration

The MIC values of the crude extract against different isolates was found to be in the range of 2.6-350 µg/µl and MBC values was in the range of 5.2-700µg/µl(Table 7).The lowest MIC value was observed for*S. aureus*, (2.6µg/µl) and highest (350µg/µl) for all gram negative bacteria included in the study. A similar trend was also observed with MBC. The MIC_{index}wasfound to be 2.

Table 7:Minimum Inhibitory Concentration, Minimum Bactericidal Concentrationand Minimum Inhibitory Concentration indexof 80%methanol leaf extract of *D.penninervum*

Tested bacteria					
	S. aureus	*S. pyogen*	E. coli	*P. aeruginosa*	*K. pneumonia*
MIC (µg/µl)	2.6	5.2	350	350	350
MBC(µg/µl)	5.2	10.4	700	700	700
MIC_{index}	2	2	2	2	2

4.6. Antioxidant Activity

4.6.1. Free Radical Scavenging Activity

Regression equations were found to be y = 17.11x + 2.65, R^2 = 0.992 for the plant extract and y = 94.9x -0.923, R^2 = 0.999 for ascorbic acid(Figure 5). From these equations the IC_{50} value for the extract and ascorbic acid were calculated as 2.77mg/ml and 0.54 mg/ml, respectively(Figure6).

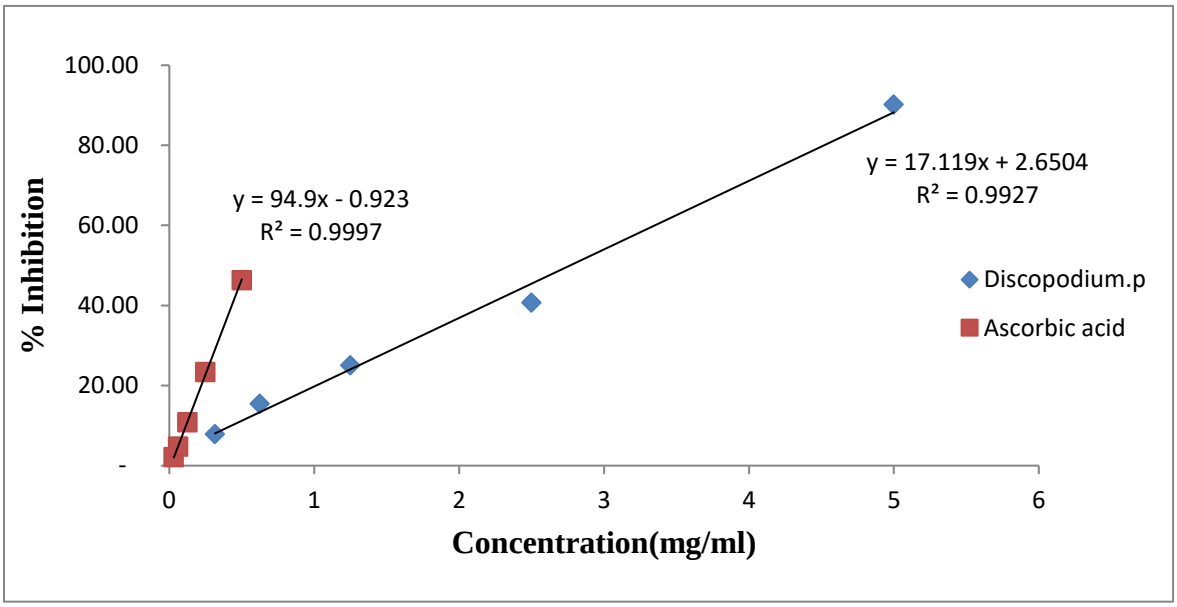

Figure5: Free radical scavenging activity (%) of 80% methanol leaf extract of*D. penninervium*

Values are average of triplicate measurements (mean ± SD).

DPPH scavenging IC_{50} (mg/ml)

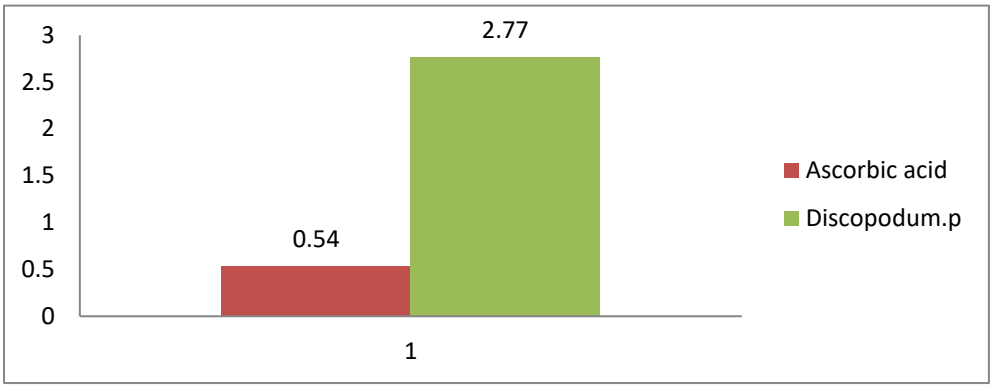

Figure 6: IC_{50}values of80% methanol leaf extract of *D.penninervium* .

4.6.2.Total Flavonoid Content Determination

Total flavonoid content of 80% methanol leaf extract of *D.penninervum* was found to be 43.03 mg/g, which is expressed as mg quercetin equivalent per gram of dry extract.

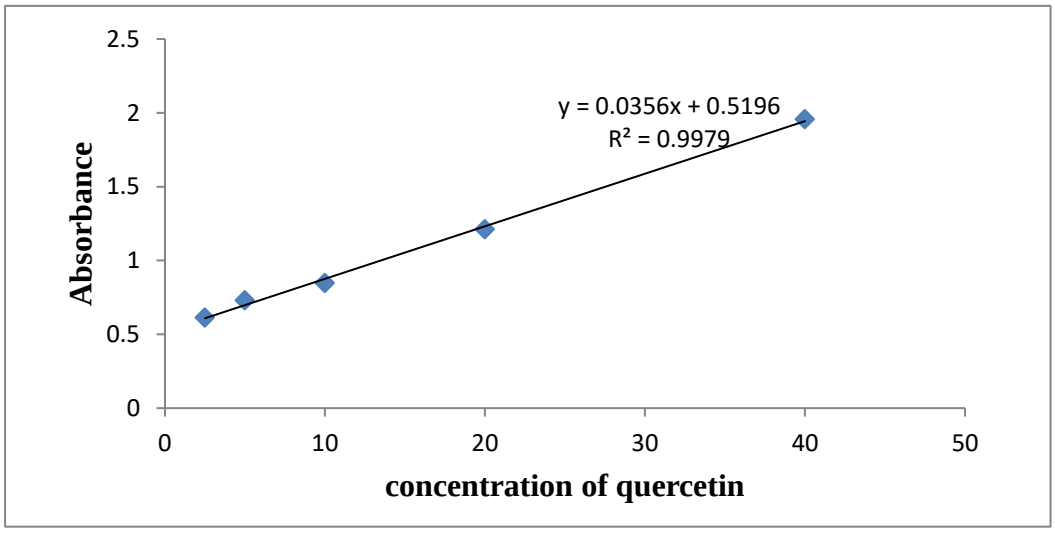

Figure7: Total flavonoide content of 80% methanol leaf extract of *D.penninervum*

5. DISCUSSION

The leaves of *D.penninervum*was extracted with 80% methanol using maceration technique, in line with the conditions similar to those used by traditional medical practitioners for their preparation. Methanol is also abest solvent to get the highest phenolic and flavonoid contents, followed by 80% ethanol, 80% acetone and distilled water from different parts of the plants(Butsat and Siriamornpun, 2016).

In order to evaluate wound healing activity, no single model is adequate to collectively represent the various components of the wound healing process as a whole. Hence, in the present study, two different wound models were used to establish the healing potential of methanol extract of *D.penninervum* on various phases (Arun *et al*., 2016). Although a large effort has been made to study *in vitro* wound healing activity, *in vivo* studies still remain indispensable for wound healing activity investigation as wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state(Abdulla and Fard, 2010).

Wound contraction, the centripetal movement of the wound boundary and adjacent uninjured skin toward the wound center,is a hallmark ofhealing full-thickness cutaneous wounds(Tranquillo and Murray, 1993). In the granulation tissue, fibroblasts are activated and acquire α-SmoothMuscle actin expression and become myofibroblasts. These myofibroblastic cells synthesize and deposit the ECM components that eventually replace the provisional matrix. These cells exhibit contractile properties, due to the expression of α-Smooth Muscle actin in microfilament bundles or stress fibers, playing a major role in contraction and maturation of granulation tissue(Darby *et al*., 2014).Contraction commences approximately a week after wounding, when fibroblasts have differentiated into myofibroblasts. The greater wound contraction effect ofcrude extract as compared to negative control might be due to its effect on fibroblast synthesis and differentiation. In fullthickness wounds, contraction peaks at 5 to 15 days post wounding. Contraction can last for several weeks and continues even after the wound is completely reepithelialized.A large wound can become 40 to 80% smaller after contraction(Janis and Attinger, 2006).A successful contraction results in a smaller wound to be repaired by scar formationand it reduces healing time because less granulation tissue needs to be produced to replace tissue loss (Porter, 2007). Hence, a small scar wound healingwithin a short

period of time observed in extract treated mice could be due to the effect of extract on wound contraction.Wound contraction depends on the shape of the wound, tissue type and the general state of the hosts health(Verma *et al*., 2013).

The process of epithelilization involves cellular detachment, proliferation,and differentiation. The marginal basal cells migrate toward the center of the wound as a monolayer and exhibit contact guidance. The epithelilal cells fill in the area of the wound that is left after wound contraction, provided the area to be covered is not too large. These cells migrate until they reach cells migrating from the opposite direction, and the migration stops by contact inhibition(Thiruvoth *et al*., 2015).It is stimulated by the presence of EGF(epidermal growth factor) and TGF-α (transforming growth factor alpha) that are produced by activated wound macrophages, platelets and keratinocytes(Diegelmann and Evans, 2004).The total duration of epithelialization can range from days to weeks, depending on wound size and the condition of the granulation tissue(Thiruvoth *et al*., 2015).The epithelization time was significantly reduced from 21 days (control) to 17 and 16 days for 5% and 10% extract, respectively. Consequently, the shorter epithelialization time of the extract may be due tofacilitated wound contraction and proliferation and migration of epithelial cells.

Collagen is a predominant extracellular protein in the granulation tissue of ahealing wound and there is arapid increase in the synthesis of this protein in the wound area soon after an injury, which provides strength and integrity to tissue matrix(Arun *et al*., 2016). The extract showed faster healing and increased tensile strength of the healed incision wounds, as a result an increase in tensile strength of treated wounds was observed and this may be due to the increase in collagen concentration and stabilization of the fibers(Agarwal *et al*., 2009).

In normal wound healing,inflammation is a protective process conducted by the organism with the purpose of removing harmful stimuli and initiating the operation of healing. However, the excessive and unbalanced inflammation could delay the healing period and enhance scarringTherefore, anti-inflammatory compounds are considered as effective agents in wound healing(Fahimi *et al*., 2015)**.**

Carrageenan induced hind paw oedema is the most sensitive and reliable model for evaluating acute phase and orally active anti-inflammatory agents(Kumar *et al.*, 2014). Carrageenan is a

sulphated polysaccharide obtained from sea weed (Rhodophyceae) and is commonly used to induce acute inflammation and is believed to be bi-phasic(Hafeez *et al*., 2013).The initial phase (0-1 h) is attributed to the release of serotonin, histamine, bradykinin and substance P. The late phase (after 1h) is mainly due to neutrophil infiltration into the inflammatory site and the production of large amounts of pro-inflammatory mediators such as prostaglandin E2(PGE2)and various cytokines such as IL-1β, IL-6, IL-10 and TNF-α(Ismail *et al*., 2017) . The extract showed significant inhibition of edema in the late phase of caraggennen induced inflammation(Table 4). Hence, the anti-inflammatoryactivity of the extract could be due to inhibition of the effect mediated by pro-inflammatory mediators such as PGE2 and various cytokines.The anti-inflammatory capacity of plant extracts is evaluated by calculating percentage reduction of edema. The maximum percentage reduction of edema shown by different methanol plant extracts was found to vary andranged from 21.66%(Palmeiro*et al*.,2002) to 86%(Selvan*et al* 2014). The maximum reduction of methanol leaf extract of *D.penninervium* at 100mg/kg,200mg/kg,400mg/kg doses was 56.27%,57.36% and 58.18%,respectively, which is within the above range. The difference in percentage of reduction of edema by methanol extract of different plants may be due to methodological issues, including, among others, animal species, dose of caraggenan,dose of extract used and time of measurement of edema.

Bacterial infections of different types of wounds present a challenge in the treatment of skin injuries and wound healing(Vittorazzi *et al*., 2016).Hence , an infection can delay the process by several mechanisms such as decreasing blood supply, promoting disordered leukocyte function and debridement phases ,and producing proteolytic enzymes(Fahimi *et al*., 2015). Therefore,infected wounds heal less rapidly and often result in the formation of unpleasant exudates and toxins, which would lead to the killing of regenerating cells in the healing process, increased pain and discomfort and other complications(Vittorazzi *et al*., 2016). In addition, wound infections can lead to prolonged elevation of pro-inflammatory cytokines such as IL-1 and TNF-α and production of free radicals at or around the wound site, which finally inhibits wound healing. So, infection is the major complication of wounds and antibacterial agents play an important role in the wound healing process(Fahimi *et al*., 2015)**.**The crude extract showed antibacterial activity against both gram positive and gram negative bacteria with MIC $_{index}$of 2. This suggests that the plant has a bactericidal effect against the tested bacteria. It is to be noted that crude extractinhibited*S.pyogenes* and *S.aureus*,which are the most common gram

positivebacteria in wound infection, at lower concentration than gram negative bacteria.This may be due to the presence of high amount of hydrophilic flavonoids in the crude extract rather than uncharacterized nonflavonoid lipophilic antimicrobial compounds such as residual terpenes, sterols and psoralens(Mandalari *et al*., 2007). Because 80% methanol could only extract polar and semi- polar secondary metabolites,including flavonoids than the non -polar one, which is supported by astudy done on determination of antioxidant activity in methanol and chloroform extracts of *Momordicacharantia*, it was found that the methanol extract(polar solvent) contained significantly higher flavonoids (approximately 19 fold) than chloroform extract(nonpolar solvent)(Rezaeizadeh *et al*., 2011).Hence,the low susceptibility of Gram negative bacteria to the crude extract could be due to the higher content of lipids in their cell membrane, which prevents penetration of flavonoids to the membrane, if the antibacterial activity observed was due to the presence of quantified flavonoids.

The imbalance between oxidants and antioxidants contribute to oxidative stress and is involved in many pathological processes such as inflammation, atherosclerosis, cancer, aging etc(Ekuadzi *et al*., 2012).Oxygen radicals are generated principally in ischemic or inflamed tissues(Shetty, 2013).Now a-days, most of the antioxidants are manufactured synthetically. Such synthetic antioxidants are known to have potential side effects and possess some degree of carcinogenicity when taken *in vivo*. Hence, their use is being restricted. However, antioxidant substances from plant materials are safeand protect the organisms from being damaged by the excess of free radicals thereby stimulating wound healing process(Reddy and Grace, 2016, Mzindle, 2017).

The antioxidant activity of the extract was evaluated using DPPH free radical scavenging activity.The stable DPPH radical method is a widely used, relatively quick, most accepted and precise method for the evaluation of free radical scavenging activity of a plant extract(Reddy and Grace, 2016).DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 517 nm induced by antioxidants(Reddy and Grace, 2016).The extract showed strong free radical scavenging activity with IC_{50} value of 2.77mg/ml. The antioxidant capacity of plants is varied. Methanol extract of *D.penninervum* showed higher antioxidant activity than methanol extract of other fruits including mangosteen, orange, pomelo, grapes, and papaya, which have a moderate antioxidant activity (IC_{50} from 11.18-32.80

mg/ml)(Anagnostopoulou *et al*., 2006).However, it is lower than methanol extracts of *A.pyrethrum*(Selles *et al*., 2012) and *RhodiolaimbricataEdgew*(Kumar *et al*., 2010b),with IC_{50} value of 0.056mg/ml and 0.33mg/ml, respectively. The antioxidant activity of the extract could bebecause of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation.

Flavonoids are widely present in the plant kingdom exhibiting abroad range of biological activities,including antibacterial, antifungal,antiviral, anti-allergic,anti-inflammatoryand antioxidant activities(Mohammed *et al*., 2014).Their general occurrence, broad spectrum, multiplicity and natural origin make them suitable chemical scaffolds for novel drugs (Umesh *etal.*, 2018). Flavonoids have been found to possess antioxidants, antimicrobial and anti - inflammatory properties in various studies(Govindappa *et al*., 2011). Hence, quantitative determination of flavonoids is one of the important parameters to assess wound healing activity of a plant. Consequently,quantitative colorimetric assay in this study showed the presence of43.03 mgQE/g of extractof flavonoid contentin 80%methanol extract of *D.penninervum*. The amount of flavonoids found differs from plant to plant with the same extraction method. The amount of flavonoid found in methanol extract of *D.penninervum* was lower than found in methanol extracts of *Amaranthusspinosus* (63.16mgQE/g extract ±11)(Barku *et al*., 2013) and *Anacycluspyrethrus*(92.5mgQE/g extract ±4.2)(Selles *et al*., 2012) but higher than found in methanol extracts of *Momordicacharantia*(7.63mgQE/g extract ±1.013)(Rezaeizadeh *et al*., 2011) and bothstem(12.8mgQE/g extract ±0.58) and leaf(3.2mgQE/g extract ±0.78)methanol extract of*Cosciniumfenestratum*(Goveas and Abraham, 2013).Hence, the wound healing activity of the extract may be either due to the individual effect of the presence of moderately high concentration of flavonoids or additive effect with other phytoconstituents present in it.Flavonoids as antioxidant, stabilize the reactive oxygen species by reacting with the reactive compound of the radical, because of the high reactivity of the hydroxyl group of flavonoids(Nijveldt *et al*., 2001).Hence, flavonoids play a major role in the wound healing by preventing and protecting oxidative damage from free radicals(Agyare *et al*., 2013).Additionally, Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing onset of cell necrosis, but also improving vascularity, hence increasing the strength of collagen fibers by increasing circulation or by preventing cell damage through promoting DNA synthesis(Ukwueze *et al*., 2013).Quercetin, which is amember of flavonoids in particular, inhibits both

cyclooxygenase and lipoxygenase activities and thus diminishing the formation of these inflammatory metabolites.Another anti-inflammatory property of flavonoids is their suggested ability to inhibit neutrophil degranulation, which is a direct way to diminish the release of arachidonic acid by neutrophils and other immune cells(Nijveldt *et al*., 2001).Hence, the anti-inflammatory activity of the extract is either due to the presence of flavonoids or may be other metabolites such assaponins(Kumar *et al*., 2014),tannins(Muthu and Durairaj, 2015) and glycosides(Dickson *et al*., 2010).The proposed antibacterial activity of flavonoids includes inhibition of nucleic acid synthesis, inhibition of energy metabolism, and inhibition of cytoplasmic membrane function, which causes bacterial cell death (Cushnie and Lamb, 2005).

CONCLUSION

In conclusion, while plant-based traditional medicine has been used throughout generations, the efficacy of such treatments requires experimental backup and scientific verification.Thus, the wound healing activities of *D.penninervum* was evaluated using excision and incision wound models. In addition, antioxidant, antibacterial and anti-inflammatory activity of the plant was evaluated, in parallel with quantitative determination of flavonoids. Hence,the results obtained in the present study clearly suggest that 80% Methanol leaf extract of *D.penninervum* is endowed with significant wound healing activitythat could be attributed toantibacterial,anti-inflammatory and antioxidant activities of the plant. Particularly,the effectiveness of the crude extract on wound healing may be due to the presence of moderately high amount offlavonoids that either act alone or in concertwith other secondary metabolites. Hence,findings collectively uphold the traditional use of the plant for wound treatment.

RECOMMENDATIONS

- Sub-acute, sub-chronic and chronic oral and dermal toxicities should be done to evaluate the long term effect of the plant.
- The plant should be fractionated to identify the fraction (s) of the plant extract which is responsible for wound healing activity including, anti-inflammatory, antibacterial and antioxidant activities.
- Isolation of the various compounds responsible for these activities should be done.
- Study has to be done to evaluate the activity of the plant on chronic wounds,like diabetic wound.

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