WOUND HEALING AND ANTI-INFLAMMATORY ACTIVITIES OF SOLVENT FRACTIONS OF 80 % METHANOL LEAF EXTRACT OF *ACHYRANTHES ASPERA* L. (AMARANTHACEAE) IN RATS

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This is to certify that the thesis prepared by Teklie Mengie, entitled "Wound healing and anti-inflammatory activities of solvent fractions of 80 % methanol leaf extract of *Achyranthes aspera* L.(Amaranthacea)" in rats and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abstract

Wound healing and anti-inflammatory activities of solvent fractions of 80 % methanol leaf extract of *Achyranthes aspera* L. (Amarenthaceae) in rats

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The leaf of *Achyranthes aspera* L.(Telenj), has been used traditionally for the treatment of wound in various parts of Ethiopia. The leaf had many confirmed *in-vitro and in-vivo* activities that can promote wound healing and anti-inflammatory effects. However, the various fractions of *A. aspera* have not been explored scientifically for its wound healing and anti-inflammatory activities in animal model. The objective of this study was to evaluate the wound healing and anti-inflammatory activities of solvent fractions of 80% methanol leaf extract of *A. aspera* in rats. Air dried leaf of *A. aspera* were grounded and macerated for 72 hours successively by 80 % methanol three times. Then the dried extract was fractionated with chloroform, n-butanol and distilled water. Acute oral and dermal toxicity was determined in rats. Wound healing activity of solvent fractions was evaluated using excision and incision wound models. Anti-inflammatory activity was evaluated using carrageenan induced hind paw edema and cotton-pellet induced granuloma models in rats. 2000 mg/kg oral and 10 % w/w solvent fraction ointment test doses were safe in rats. The 10 % w/w chloroform fraction ointment revealed high percentage of wound contraction ($p<0.01$) and reduced period of epithelialization ($p<0.01$). In the carrageenan induced paw edema and the cotton pellet induced granuloma models chloroform, n-butanol and aqueous fractions showed a statistically significant effect with different onset and magnitude. Chloroform fraction was found to be the most active fraction, and the 400 mg/kg dose demonstrated the maximum percent of inhibition of edema (52.50 %; $p < 0.01$) at 4 hr of post induction which is comparable to indomethacin at the dose employed. Chloroform fraction was also found to be the most active fraction in inhibiting the exudative and proliferative component of chronic inflammation in the cotton pellet induced granuloma model, where the maximum percentage of exudate inhibition (37.52 %, $p < 0.01$) and granuloma formation inhibition (52.81 %, $p < 0.01$) was exhibited at the dose of 400 mg/kg. Data obtained from the present study collectively indicated that the chloroform fraction of 80 methanol leaf extract of *A. aspera* possessed significant wound healing and anti-inflammatory activities, upholding the folkloric use of the plant.

Key words: *Achyranthes aspera*, wound healing activity, anti-inflammatory, carrageenan induced paw edema, cotton pellet granuloma, rat.

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List of Acronyms/Abbreviations

AF	Aqueous Fraction
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
BFGF	Basic Fibroblast Growth Factor
BF	Butanol Fraction
BP	British Pharmacopoeia
C5a	Complement 5a
CF	Chloroform Fraction
COX	Cyclooxygenase
CDT-Africa	Center For Innovative Drug Development & Therapeutic Trails For Africa
DAMPs	Damage-Associated Molecular Patterns
EC	Endothelial Cells
ECM	Extracellular Matrix
EPHI	Ethiopian Public Health Institute
HA	Hyaluronic Acid
IRFs	Interferon Regulatory Factors
JAK	Janun Kinase
LD_{50}	Lethal Dose 50%
LOX	Lipooxygenase
LTs	Leukotrines
MAPKs	Mitogen-Associated Protein Kinases
MMP	Matrix Metalloproteinase
NLRs	Nucleotide Binding Oligomerization Domain-Like Receptors
NSADs	Non steroidal Anti-inflammatory Drugs
OECD	Organization for Economic Corporation and Development
PAMP	Pathogen-Associated Molecular Pattern
PDGF	Platelet-derived Growth Factor
PGs	Prostaglandins
PMNLs	Polymorph Nuclear Leucocytes
PRRs	Pattern Recognition Receptors
SEM	Standard Error of the Mean

TGF-β	Transforming Growth Factor-Beta
TIMPs	Tissue Inhibitors of Metalloproteinases
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor Alpha
TXs	Thromboxanes
WHO	World Health Organization
WHS	Wound Healing Society

1. Introduction

1.1 Overview of wound and management

In normal skin, the epidermis and dermis form a protective barrier against the external environment. Once this barrier is broken, a wound will be inflicted (Nagori & Solaki, 2011). Wound is a rupture in the epithelial integrity of the skin which arises due to physical or chemical injuries or microbial infections (Farahpour & Habibi, 2012). On the basis of physiology of wound healing, wounds can be classified as acute or chronic. Acute wounds are tissue injuries that heal through an orderly sequence of physiological events which results in sustained restoration of anatomic and functional integrity, and have generally less than 8 weeks. In this kind of wound *Staphylococcus aureus* is the most important pathogen (Bowler &Davies, 1999). On the other hand, chronic wounds are wounds that have failed to proceed through an orderly and timely process to produce anatomic and functional integrity even after 3 months (Bowler, 2002; Lazarus *et al.*, 1994). Wound Healing Society (WHS) classifies chronic wounds into four categories based on their etiologies: pressure ulcer, diabetic ulcer, venous ulcer and arterial insufficiency ulcer (Järbrink *et al*., 2016). The latent microorganisms of both aerobic and with higher prevalence of anaerobic bacteria (e.g. *Peptostreptococcus species, Bacteroides species, Porphyromonas species and Prevotellas*) are common in chronic wounds (Bowler & Davies, 1999).

In the majority of chronic wounds, the healing process is thought to be 'stuck' in the inflammatory or proliferative phases (Enoch & Price, 2004). In addition, oxidative damage by free radicals or condition-specific factors such as neuropathy in diabetes or ischemia in peripheral vascular disease may lead to the non-healing nature of chronic wounds (Fadeev & Nemtseva, 2009).

Annually, millions of people experience burns, to be ill with chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring (Meier & Nanney, 2006). The prevalence and costs of chronic wounds is increasing globally. Venous leg ulcers alone in most cases consume 1–3% of healthcare budgets. The number of diabetic foot ulcers is expected to reach 380 million by 2025, representing 7.1% of the adult population worldwide (Lantis & Price, 2011). In developing countries, such as sub-Saharan African and South Asian countries, about 1% to 2% of the population experiences a chronic wound during their lifetime. The prevalence of chronic wounds was reported as 4.5 per 1,000 populations, whereas that of acute wounds was nearly double at 10.5 per 1,000 populations (Sasidharan *et al.*, 2010; Siddiqui & Bernstein, 2010). In Africa the rate of surgical site infection varied from 2.5% to 30.9% following various types of surgical procedure (Bagheri *et al*., 2011). In Ethiopia, *S. aureus, Klebsiella, E. coli, and P. aeruginosa* were the most important bacteria responsible for post-

operative wound infection in the hospital and high rates of drug resistance to some commonly used antibiotics were observed (Guta *et al.*, 2014). Another study from Ethiopian armed force referral and teaching hospital, reported that multi-drug resistant (MDR) gram positive and gram negative bacteria isolates which were 73.6% and 67.6%, respectively and 79.2% of *S. aureus* were resistant to three or more antibiotics (Ayalew, 2014).

1.2 Wound healing cascade

Wound healing is the course of repairing the injured part of skin and other soft tissues (Nayak *et al.*, 2007). Healing inaugurates the instant the wound is created (Molnar *et al.*, 2014). All tissues in the body are capable of healing by one of two mechanisms: regeneration or repair. Regeneration is the replacement of damaged tissues by the same cells and is more limited than repair. In humans, it occurs in a limited number of cells for example, epithelial, liver and nerve cells. The main wound healing mechanism is repair where damaged tissue is replaced by connective tissue which then forms a scar (Diegelmann & Evans, 2004). Wound healing occurs as a result of a sequential cascade of four overlapping but well-defined phases (Clark, 1988; Deore *et al.*, 2014); hemostasis, inflammation, proliferation and remodeling (Datta *et al*., 2011).

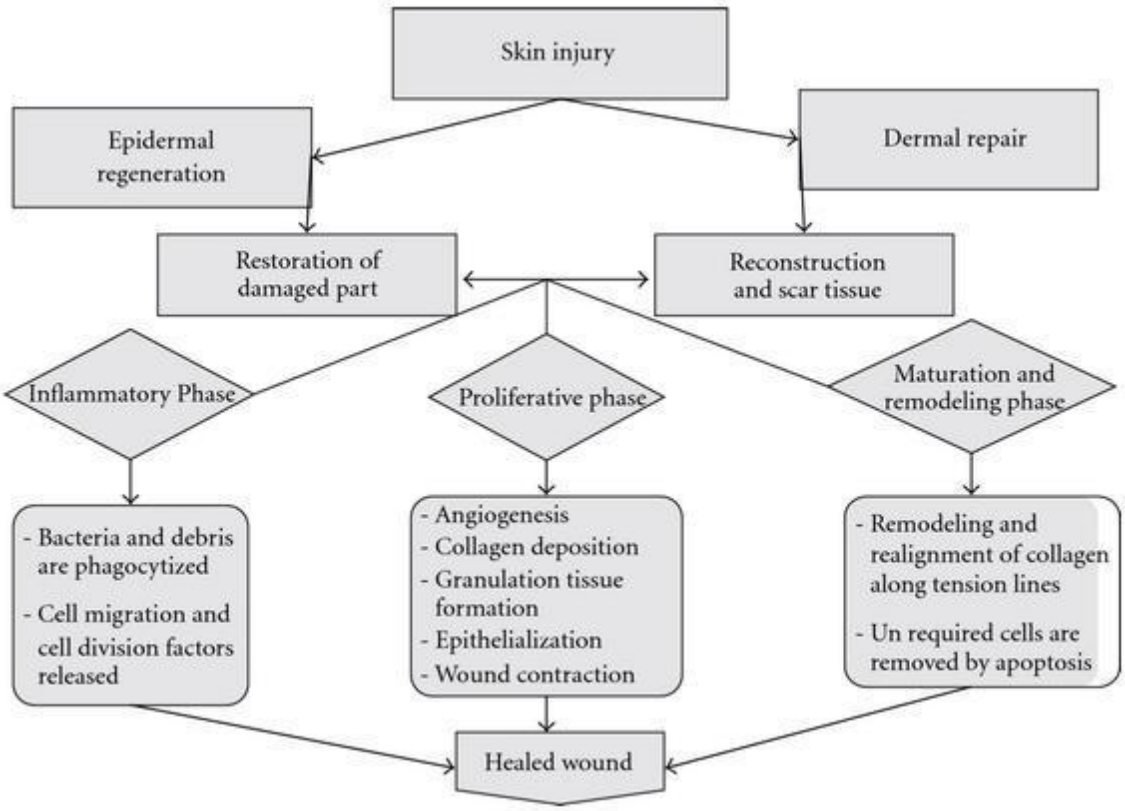

Figure 1: Mechanism of Wound Healing (Datta *et al.*, 2011).

1.2.1 Hemostasis

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

1.2.2 Inflammation

The inflammatory phase starts as early as two hours after injury. Polymorphonuclear leukocytes (PMNLs), crucial inflammatory cell, mostly neutrophils from granulocytes and activated monocytes or macrophages from agranulocytes. These cells are responsible for bound wound bed preparation of the healing wound (Menke & Diegelmann, 2006). Early stage of inflammation begins with the activation of complement and infiltration of the wound with granulocytes or PMNLs. Then these cells are attracted to the wound site by complement 5a (C5a), platelets, formyl-methionyl peptide products from bacteria and Transforming Growth Factor-beta (TGF-β) (Enoch & price, 2004). Proteolytic enzymes, such as elastase and matrix metalloproteinase (MMP)-8 as a result of Neutrophils release, to assist in their movement through the tissue and needed for the removal of damaged extracellular matrix (ECM). Once in the wound environment, they begin the debridement of devitalized tissue and phagocytize bacteria and foreign particles (Jie *et al*., 2007). On the other way, other type of PMNL, mast cell granules are filled with enzymes such as chymase, tryptase, histamine and other active amines which cause acute signs of inflammation: rubor (redness), calor (heat), tumor (swelling), and dolor (pain) when released (Diegelmann & Evans, 2004).

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

1.2.3 Proliferative phase

The proliferative phase starts at about day three and lasts for two weeks after wounding. The proliferative phase includes; neoangiogenesis, formation of granulation tissue and ECM, and re-epithelialization (Schreml *et al.*, 2010). Key cells for these processes are the fibroblasts and keratinocytes (Eaglstein, 2001).

i. Fibroblast migration

Fibroblasts are dermal cells that produce hyaluronan (glycosaminoglycans), fibronectin, proteoglycans, collagen and numerous other substances that comprise the ECM (Ghosh *et al*., 2006; Lee *et al*., 2004). Wound fibroblasts tend to be involved in the process of wound contraction by the help of actin after differentiation into myofibroblasts (Junker *et al*., 2008). Contraction decreases healing time because it decreases the size of the wound and reduces the amount of ECM needed to repair the defect and shorten re-epithelization by the distance migrating keratinocytes must travel.

ii. Collagen synthesis

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

iii. Angiogenesis

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

iv. Granulation tissue formation

Granulation occurs as the fibrin clot scaffold is replaced with new tissue rich in hyaluronan, fibronectin and other ECM components. The predominant cell type found in granulation tissue is the fibroblast (Strodtbeck, 2001). ECM is composed of substances that promote adhesion and migration (fibronectin); glycoaminoglycans that promote tissue hydration (hyaluronan); proteoglycans involved in the regulation, migration, storage and expression of growth factors, enzymes, and coagulation proteins (chon-

droitin sulfate); and glycoproteins that provide tissue strength and resiliency (collagens, elastin) (Gurtner *et al.,* 2008). The structure and composition of granulation tissue undergoes constant change in collagen composition as it matures (Palpandi *et al*., 2010).

v. Re-epithelization

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

1.2.4 Remodeling phase (maturation stage)

Matrix synthesis and the remodeling phase are initiated concurrently with the expansion of granulation tissue and continue over extended periods of time up to two years (Clark *et al*., 1995). The wound develops its final power during this stage of wound healing. However, a maximum of 80 % unwounded skin strength can be achieved (Levenson *et al*., 1965). The key cells for remodeling are macrophages and fibroblasts. ECM reshaping by cross-linking collagens, cell maturation, and program cell death are the mechanisms used in wound remodeling (Strodtbeck, 2001). There is ongoing collagen synthesis and breakdown as the ECM is continually remodeled, equilibrating to a steady state about 21 days after wounding. This breakdown continues for 6 months to 1 year after injury. The initial type III collagen is replaced by type I collagen until a type I: type II ratio of 4:1 is reached, which is equal to normal skin (Ferguson & Kane, 2004). Collagen degradation is achieved by specific MMPs that are produced by fibroblasts, granulocytes and macrophages. As remodeling of the wound continue MMP activity decreases and tissue inhibitors of metalloproteinases (TIMPs) activity increases. TGF-β plays an important role in mediating this, as it promotes matrix accumulation (Enoch & prices, 2004). With time, the density of cells is reduced by apoptosis triggered by unknown sources (Desmoulire *et al.*, 1995). Keratinocytes are the first cells to undergo programmed cell death; myofibroblasts are the second. It has been suggested that apoptosis may be signaled by the withdrawal of cytokines as the wound heals, other theories exist

myofibroblast differentiation itself signaling apoptosis (Jiirgensmeier *et al.*, 1994). Remodeling is thus a balance between the synthesis of new collagen and the degradation of old tissue in the wound healing process is switched off, the new connective tissue matures and changes from pinkish-red to a white color. (Robson *et al*., 2001).

1.3 Factors affecting wound healing

A number of factors can lead to impaired wound healing. These factors can be generally categorized into local and systemic. Local factors are those that directly influence the characteristics of the wound itself, while systemic factors are the overall health or disease state of the individual that affect his or her ability to heal (Guo & Dipietro, 2010). Oxygenation, infection, foreign body, venous insufficiencies are some of the local factors affecting wound healing. Some of the systemic factors include age, gender, sex hormones, stress, ischemia; diseases like diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia and obesity; medications like glucocorticoids, non-steroidal anti-inflammatory drugs, chemotherapy; alcoholism and smoking; immune-compromised conditions like cancer, radiation therapy, acquired immune deficiency syndrome (AIDS); and nutrition (Nagori & Solaki, 2011; Guo & Dipietro, 2010; Stadelmann *et al.*, 1998).

Infection may extend the inflammatory phase of the wound and thus leads to the failure of wound healing (Abo *et al.*, 2004). The hypothetical mechanism by which wound infection occurs is believed to be liberation of bacterial enzymes and MMPs that may degrade fibrin as well as wound growth factors (Stadelmann *et al*., 1998). During the extended inflammatory phase, neutrophils produce free radicals, which will result in oxidative stress leading to lipid peroxidation, DNA breakage and enzyme inactivation (Kumar *et al.*, 2007). Nutritional deficiencies can slow down wound healing. Vitamin A is necessary for epithelial and bone formation, cellular differentiation and immune function. Vitamin C is crucial for collagen formation, proper immune function and as a tissue antioxidant. Vitamin E is the most important lipid-soluble antioxidant in the skin. Glucosamine appears to be the rate-limiting substrate for hyaluronic acid (HA) production in the wound. Tissue levels of the amino acids arginine and glutamine may influence wound repair (Desmoulire *et al.*, 1995).

1.4 Wound management

The foremost objective of holistic wound management is to facilitate healing in the shortest time possible, with minimal pain, discomfort and scarring to the patient and must occur in a physiologic environment conducive to repair and regeneration (Mohanty *et al*., 2010). The overall treatment of wound depends on the type, cause and depth of the wound. Treatment of recent lacerations involves examining, cleaning and closing the wound. However, since chronic wounds harbor microbes, antibiotics are chosen

based on their ability to inhibit the growth of pathogenic organisms (Lipsky & Hoey, 2009). Some examples of the preparations include amikacin (in gel or cream), bacitracin, chloramphenicol, clindamycin (cream, lotion, and foam), gentamicin (in ointment or cream), nitrofurazone (solution, or soluble dressing) and polymyxin B (Lipsky & Hoey, 2009; Sasidharan *et al.*, 2010). Further, negative pressure wound device and advanced silver-containing dressing products help the body achieve the ideal moist and warm state. In addition, protected wound healing environment, skin substitutes for burn wounds, growth factors and biologic wound products and hyperbaric oxygen are among the modern treatments (Esimone *et al*., 2005).

1.5 Inflammation and its management

1.5.1 Overview of inflammation

Inflammation is an invasive form of defense that is broadly defined as a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat pathogenic intruders *(*Ashley, *et al., 2012).* Based on apparently visual observation, it is characterized by the cardinal signs of: sensation of heat, redness, swelling, pain and loss of function. Inflammation is a major feature of many diseases, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease and atherosclerosis (Shu *et al,* 2013).

1.5.2 Types of inflammatory responses

Inflammatory response is a regular defense mechanism that is designed to limit invasions and damage after injury, a process which has been essential for the survival of Homo sapiens in the absence of medication such as antibiotics (Boer, 2012). It promotes pathogen killing as well as tissue repair processes and helps to restore homeostasis at infected or damaged sites (Calder, 2014). In spite of the fact that such self-limiting acute inflammation is a beneficial response, deregulated and chronic inflammation is a phenomenon that may go in front to a host of devastating chronic pathologies (Munari & Cervera, 2015).

Acute inflammation is such a nonspecific and short lived response which occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed, broken down, or walled off by scarring (fibrosis) (Vetriselvan *et al.,* 2013). The initiation of acute inflammation involves expression of two families of pathogen-associated molecular pattern (PAMP) receptors of endothelial cells (ECs) including membrane bound Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These Pattern Recognition Receptors (PRRs) are able to sense components of exogenous microbes, toxins, cytoplasmic pathogen associated molecular patterns (PAMPs),

as well as dangerous endogenous DAMPs (Cantu *et al.*, 2013; Mai *et al*., 2013). The activation of TLRs and NLRs by their respective ligands then activates downstream signaling pathways which finally converge on activation of pro-inflammatory transcription factors, such as nuclear factor-*kappa*B (NF B), activator protein-1 (AP-1) or interferon regulatory factors (IRFs) *(*Oviedo-boyso *et al.,* 2014). Then it leads to expressions of many genes which involved in acute inflammation (Mogensen, 2009). The vascular response of acute inflammation is initiated by the local blood vessels as a result of tissue injury (Vetriselvan *et al.,* 2013). At its basic level ; vasodilatation of precapillaries arterioles leads to blood flow to injured tissue while post capillaries venules vasoconstriction lead to formation of hydrostatic pressure further potentiating exudation of plasma proteins and leukocytes in edema formation (Rubin & Strayer, 2007). These responses are mediated by the release of specific vasoactive mediators such as nitric oxide, histamine and eicosanoids (Larsen & Holt, 2000).

The hallmark of acute inflammation is mainly mediated by eicosanoids which are released at the site of tissue injury and prominently contributed to the development of cardinal signs of acute inflammation due to their effect on the microvasculature to cause vasodilation/constriction (Massoumi & Sjölande*r,* 2007). Generally speaking, products of the two enzymes, COX and 5-LOX, are important mediators of inflammation. High levels of LTs and up-regulated expressions of COX-2 and its main products are characteristic hallmarks of inflammation (Massoumi & Sjölander, 2007). Inflammatory reactions could be as the result of cellular response in ECs at the inner side of all vessel types. Because of their location, Endothelial cell are one of the first cell types to detect foreign pathogens and endogenous metabolite-related danger signals in the blood stream and hence function as danger signal sensors that orchestrate the trafficking of leucocytes from intravascular space to extravascular sites of inflammation (Mai *et al.,* 2013).

Resolution of acute inflammation occurs through a highly regulated cell death mechanism that prevents the release of histotoxic cellular contents as the result of neutrophils apoptosis (Barnig& Levy, 2015). Although it is often defined simply in terms of time course, with lesions of over 6 weeks' duration traditionally being regarded as chronic and it is most appropriately defined in terms of the process, in which continuing active inflammation and attempted tissue healing by repair occur concurrently, rather than consecutively. This is in fact chronic inflammation may develop from unresolved symptomatic acute inflammation or may advance insidiously over a period of months without apparent acute onset of clinical manifestations (Tandon *et al.,* 2014).

Even though limited inflammation is beneficial, extreme or continual inflammation which runs unchecked can lead to a host of chronic inflammatory pathologies, such as remodeling of the artery wall in atherosclerosis *(*Razavian *et al.,* 2011). More over chronic inflammation is not characterized by the typical cardinal signs of acute inflammation. In contrasting to acute inflammation in which neutrophils predominate, chronic inflammation is well characterized histopathologically by predominant infiltration of mononuclear immune cells (monocytes, macrophages and lymphocytes), with angiogenesis and fibrosis *(*Vetriselvan *et al.,* 2013).

1.5.3 Management of inflammation

Non-steroidal anti-inflammatory drugs and Corticosteroids are the foremost classes of drugs extensively used for the management of inflammatory conditions. These modern anti-inflammatory drugs have been developed for their capacity to inhibit or antagonize the production or action of pro-inflammatory mediators (Ayroldi *et al.*, 2012; Sousa *et al.,* 2013). In the recent years an intension have been made to develop biological agents targeting inflammatory cytokines such as anti-TNF- and anti- IL-1 therapy *(*Murdaca *et al.*, 2009).

NSAIDs have been used over years as the therapeutic agents of first choice for the management of inflammatory diseases conditions through inhibiting the enzyme COX, and hence block the production of pro-inflammatory prostanoids (PGs and TXs) (Croff, 2013). In terms of composition, most NSAIDs are organic acids with low pK values that tend themselves to their accumulation at sites of inflammation, areas that often exhibit lower pH than uninvolved sites and besides they act through single- or dual-action drugs (Shah & Piyush, 2015). Most conventional NSAIDs put forth their anti-inflammatory effects principally via inhibition of COX-2, but prone to adverse effects; GI mucosal damage and nephrotoxicity due to inhibition of COX-1. Now a day, a variety of new and specific COX-2 inhibitors, such as celecoxib and rofecoxib, were developed with the anticipation of reducing possible GI side effects. However, emerging awareness of the cardiovascular adverse effects of selective COX-2 inhibitors has raised the question of safety, especially for patients with established cardiovascular (Al-Saeed, 2011; Stanos, 2013).

Corticosteroids are another classes of drugs used for management of different inflammatory diseases (Mukhopadhyay, 2018). They represent the first successful development of an endogenous anti-inflammatory mediator, cortisol (Sousa *et al*., 2013). Even though there are several mechanisms by which glucocorticosteroids halt inflammation, the predominant one is to switch off multiple inflammatory genes encoding for cytokines, chemokines, adhesion molecules such as intracellular adhesion molecule-1, inflammatory enzymes such as COX-2, receptors and proteins (Barnes, 2006; Desmet *et al.*,

2017). Furthermore, glucocorticoids interfering pro-inflammatory transcription factors through blocking mitogen-activated protein kinases (MAPKs**)** (Ayroldi *et al*., 2012)**.**

1.6 Perspective of drug development for wound healing and inflammation

Recent advancement in biotechnology has permitted the launch of commercial development of biological agents. They are used worldwide for the treatment of inflammatory conditions like rheumatoid arthritis, psoriatic arthritis, Crohn's colitis and ankylosing spondylitis (Jangid *et al.,* 2015; Tousoulis *et al.,* 2016). These biological products antagonize the effect of pro-inflammatory cytokines such as TNF-α and IL-1(Tousoulis *et al.,* 2016). The advance to TNF-α inhibition has followed two different mechanistic paths: i) the selections of monoclonal antibody which binds with high affinity to TNF-α and inhibits the effective binding of TNF-α with its receptors, and ii) the synthesis of a soluble TNF-receptor (TNF-R) fusion protein which directly binds to and blocks TNF-α receptors (Fu *et al.*, 2013; Jangid *et al.*, 2015). Infliximab, adalimumab, golimumab, and certolizumab, are examples of monoclonal antibodies that act by the first mechanism, whereas Etanercept is a direct receptor block (Murdaca *et al*., 2009). Small molecule drugs also remain a journey of drug discovery which inhibit novel inflammatory targets such as Janus kinase (JAK). Drugs like tofacitinib, ozanimod and other siphingosine -1- phosphate receptor modulators inhibitors; laquinimod, mongersen are approved by FDA for treatment of chronic inflammatory disease (Olivera *et al.,* 2017). Furthermore, targets such as micro RNA-124, a critical mediator for inflammation, were identified in rodents and could be a potential therapeutic target for the treatment of inflammatory diseases (Sun *et al*., 2013).

Development of primary health care herbal ointments has been proposed for the invention of ointment, which covers the antimicrobial, wounds healing and anti-inflammatory activities (Pradesh, *2008*). Many standardized herbal preparations showed antioxidant and anti-inflammatory activities due to the isolation of many phytoconstituents (Rahimi *et al., 2010*). Moreover, it was known that phytochemicals are known to have potential anti-inflammatory activity through inhibiting the release of pro-inflammatory cytokines (Salim *et al.,* 2014; Shah *et al*., 2010)**.**

It has been reported that many plant-derived compounds present significant anti-inflammatory activities through modulation of various pro-inflammatory cytokines (Calixto *et al.,* 2004). For instance curcumin significantly inhibit the release of pro-inflammatory cytokines via suppressing NF-κB activation via the translocation of p65 into the nucleus (Jin e*t al.,* 2007). Compounds isolated from Boswellia serrata resin (12-ursene, 2-diketone) blocked expression of pro-inflammatory cytokines by inhibiting the activation of kinases (MAPK and JAKs), TNFα, IL-1β and iNO in chronic inflammation (Gayathri *et al.*, 2007). Naringenin (flavonoids) isolated from citrus fruits and grapefruits attenuates the production of IL-1β and TNF-α through suppressing the expression of mRNAs and NF-κB (Park *et al.,* 2012). In addition to these, zedoarondiol (sesquiterpene lactone), isolated from rhizoma of *Curcuma heyneana* inhibit the expression of pro-inflammatory cytokine by suppressing the phosphorylation of several kinases and subsequent inactivation of NF-κB pathway (Cho *et al.,* 2009).

1.7 Herbal Medicines in the management of wound and inflammation in Ethiopia

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). The widespread use of traditional medicine among both rural and urban population could be due to their cultural acceptability, physical accessibility and economic affordability as compared to modern medicine (Prasad *et al.*, 2012). Herbal preparations and their products are considered an essential and major source of modern medicine around the globe (Garg & Sardana, 2016). As a result of this, an enormous number of drugs are produced from such preparations. Studies estimate that at least 25% of all modern medicines are derived from medicinal plants and approximately one-third of all traditional medicines in use are applied for the treatment of wounds and skin disorders. 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 & Dorle, 2006). Several studies from different parts of the world show that various medicinal plants have wound healing properties. Plants like *Alternanthera sessilis, Carica papaya, Catharanthus roseus, Cecropia peltata, Clerodendrum serratum, Euphorbia hirta, Euphorbia nerrifolia, Ginkgo biloba, Morindacitrifolia,*

Ocimum sanctum, Pterocarpus santalinus, Sesame indicum, Trigonellafoenum-graecum are scientifically shown to exhibit wound healing activity through various processes of the wound healing, such as coagulation, inflammation, epithelization, collagenation and wound contraction (Asif *et al.,* 2007;Nagori & Solaki, 2011).

The phyto-medicines for wound healing are not only economical and inexpensive but are also evidently safe as hypersensitivity reactions are rarely encountered with the use of these agents compared with synthetic agents (De Moraes *et al,* 2006). Many plants have been proved to possess significant healing properties (Mittal & Dixit, 2013). Between 70% and 95% of citizens in the majority of developing countries and 70% to 90% of populations in some industrialized nations use traditional medicine as primary health care to address their health-care needs and concerns. Populations using traditional medicine for primary care in African countries accounts for 90% in Ethiopia, 75% in Mali, 70% in Rwanda, 60% in Tanzania, and 60% in Uganda (Robinson & Zhang, 2011). In Ethiopia, the practice of traditional medicine in general and herbal medicines in particular has been the main stay of management for majority of the rural population since long time ago and about 800 species of plants that are used in the traditional health care system to treat nearly 300 mental and physical ailments .Many of these are skin disorders including wounds and they are very common in the country (Teklehaymanot *et al*., 2006). Herbal medicines are known to make the wound area moist to facilitate healing. So that discoveries are shifted to wound healing promotions to reduce hospitalization costs and complications.

Medicinal plants are tested for their wound healing activities. In this regard, many plants are being used for treatment of different wound and inflammatory conditions and symptoms in traditional medical practice of Ethiopia. Some of these plants include crude extracts and various fractions of *Rumex abyssinicus* (Mulisa *et al.*,2015), *Allophylus abyssinicus* (Yesuf & Asres, 2013), *Eucalyptus globules Labill* ('mich')(Mesfin *et al.*, 2009), *Kalanchoe petitiana* (Mekonnen *et al.*, 2013) and *calpurnia aurea(ait.)* (Ayal *et al.*, 2019) were proved for their wound healing, anti-inflammatory and antibacterial activities. Thus, plants are the best and cost-effective substitutes of wound healing (Mulisa *et al.*, 2015).

1.8 *Achyranthes Aspera* L.

Achyraaspera L.belongs to family Amaranthaceae, is found throughout tropical Asia, Africa, Australia and America and it grows as wasteland herb everywhere. It is a shrub that grows up to one meter height; leaf simple, opposite, obovate or elliptic; flowers bracteate and bracteolate, greenish, in spikes; fruits oblong urticle; seeds single, inverse (Figure 2). It is used as folk medicine. It holds a reputed position as medicinal herb in different systems of medicine in India. According to Ayurveda, it is bitter,

pungent, heating, laxative, stomachic, carminative and useful in treatment of vomiting, bronchitis, heart disease (Lakshmi *et al.*,2018).

Different researchers have investigated *A. aspera* both *in-vitro* and *in-vivo* for different biological activities; for instance, it is used in the indigenous system of medicine as emenagogue, antiarthritic, antifertility, laxative, ecbolic, abortifacient, anti-helminthic, aphrodisiac, antiviral, antihypertensive, anticoagulant, diuretic, immunostimulant, antihyperlipidemic, antioxidant, anti-tumor (Dey, 2011; Hasan, 2014), antiplasmodial (Inbaneson *et al.,* 2012) and leishmanicidal (Sharma *et al.,* 2016) activity. Additionally, crude and solvent fractions *A. Aspera* was investigated for antimicrobial activity in *in-vitro* using a variety of solvent systems for both clinically isolated and laboratory screened microorganisms (Begum *et al*, 2002; Jebashree *et al*., 2011; Kaur *et al*. 2005; Ndhlala *et al*., 2015; Rao *et al.,* 2018). Besides these, a different part of the plant was evaluated against the spectrum of pathogens (Elumalai *et al*., 2009; Kalaivanan *et al*., 2013).

Various solvent extracts of *A. Aspera* parts were investigated (Afridi *et al.,* 2016; Yadav *et al.,* 2016), and showed significant activity comparable with conventional anti-microbiological agents (Suresh *et al.,* 2003). Other reports also showed that *A. Aspera* have promising antibacterial and anthelmintic activity (MIC<1mg/ml) (Ndhlala *et al.,* 2015). Different solvent extracts exhibited different activities. For instance, studies reported that chloroform, and methanol root and shoot extracts of *A. aspera* showed good antibacterial activity against *Klebsiella* species while petroleum ether root extract showed the activity against *B Substiles*. Antifungal activity of roots was found in extracts with petroleum ether, chloroform and methanol against *fusarium* species. Only methanol and aqueous shoot extracts showed weak activity against Penicillium (Kaur *et al*., 2005). These observed antimicrobial activities could be due to the presence of some antimicrobial substances in the extracts (Dey, 2011).

Several studies also paid attention non-infectious disease. For instance, *A. aspera* showed a significant anticonvulsant (Gawande *et al.,* 2017; Viswanatha & Venkataranganna, 2017), anxiolytic and antifertility (Barua *et al*., 2012; Mookerji & Basak, 1975; Shibeshi *et al*., 2006), immuno-stimulant (Rao & Chakrabarti, 2004; Rao *et al*, 2006), abortifiecnt (Awe *et al.*, 2007), anti-inflammatory (Bhosal *et al*., 2012;Vetrichelvan & Jegadeesan, 2003; Reddy,2018), antioxidant (Raut, 2013; Talukder *et al.,* 2012), cytotoxic (Arora *et al*, 2014; Subbarayan *et al*., 2013), hepatoprotective (Prasad *et al.*, 2012), antiarthritis (Kothavade *et al.*, 2015), anti-asthmatic (Goyal *et al*., 2008) activities and are scientifically proved. Rajpal *et al.* (2012) investigated that the aqueous extract of *A. aspera* showed no eye irritation potential in both *in-vitro* and *in-vivo* methods. Furthermore, several assays were conducted to asses

acute and sub acute toxicity profile of *A.aspera* and assured the safe dose in animal studies. For instance, Emmanuel *et al*.(2015) showed that intra-peritoneal LD_{50} of the extract was found to be 1224.7mg/kg and the topical skin irritation was negative.

Figure 2: *A. aspera* from site of collection (Captured by Teklie on 20/04/2019)

1.9 Rationale of the study

Various nonsteroidal anti-inflammatory drugs have been shown to trim down pain and inflammation by blocking the metabolism of arachidonic acid by isoform of cyclooxygenase enzyme, thereby dropping the production of prostaglandin. However, there are many side effects associated with the administration of nonsteroidal anti-inflammatory drugs. On top of this, the majority of existing drugs suffer from various adverse events, especially at higher doses and longer duration of therapy (Spies *et al.,* 2011). For instance, NSAIDs, are confer with a development of gastric or duodenal ulceration, nephrotoxicity, bronchospasm and exacerbation of symptoms of asthma, an increase in blood pressure, and increased incidence of myocardial infarction and stroke side effects (Al-Saeed, 2011; Lirk *et al*., 2007). This remains concern regarding adverse cardiovascular effects have emerged and associated with considerable morbidity and mortality (Al-Saeed, 2011).

Virtually all acute and chronic diseases are either driven or modulated by inflammation (Vodovotz *et al*., 2010). Despite this fact, the complex interplay between beneficial and harmful arms of the inflammatory response underlies the lack of safe and fully effective therapies for many of the pathologies (Vodovotz *et al.*,2009). Thus, research and development movement in the pharmaceutical sector is focused on development of new drugs, innovative/indigenous processes for known drugs and development of plant-based drugs through investigation of leads from the traditional systems of medicine (Lirk *et al*., 2007, 2010).

Corticosteroids are associated with manifold side-effects such as diabetes mellitus/glucose intolerance, hypertension, obesity, osteoporosis, immune suppression, glaucoma, and growth retardation in children (Rhen & Cidlowski, 2005; Spies *et al.,* 2011). There are medicinal plants with anti-inflammatory therapeutic effects with low or no side effects (Oguntibeju, 2018). In view of this, WHO advocates the inclusion of herbal medicines of proven safety and efficacy in the healthcare programs because of the great potential they hold in combating various diseases (WHO, 2005).

Achyranthes aspera is one of these plants in which crude extracts of various solvent system showed promising wound-healing activities (Barua *et al.,* 2012; Edwin *et al.,* 2008; Fikru *et al.,* 2012; Ghosh *et al.,* 2011; Mondal *et al.,* 2016; Staden, 2015) and ani-inflammatory activity (Lee *et al*., 2017; Lee *et al*., 2012; Vetrichelvan & Jegadeesan, 2003b; Vijaya Kumar *et al.,* 2009; Young *et al.,* 2012) in both acute and chronic animal models. In spite of many claims and *in-vitro* studies with supportive results in wound healing and anti-inflammatory activity, no scientific study has been conducted on the wound healing and anti-inflammatory activity of solvent fractions of *A. aspera* leaf in animal model. Moreover Fikru *et al.* (2012) recommended further investigation on solvent fractions of *A. aspera* leaf for their

wound healing and anti-inflammatory activity. Hence it was essential to evaluate wound healing and anti-inflammatory activities of solvent fractions of 80 % methanol leaf extract of *A. aspera* as the leaf may be a possible source of effective, safe and affordable wound healing agents and attempted to ascertain in which fraction(s) of *A. aspera* leaf responsible for wound healing and anti-inflammatory activities were concentrated so as to provide a clue about the nature of the phytochemical constituents responsible for its actions.

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2. Objectives

2.1 General Objective

- To evaluate wound healing and anti-inflammatory activities of solvent fractions of 80 % methanol leaf extract of *A. aspera* in rats.

2.2 Specific Objectives

- To asses acute oral and dermal toxicity of solvent fractions of 80 % methanol leaf extract of *A. aspera* in rats
- To evaluate wound healing activity of chloroform, n-butanol and aqueous fractions of 80 % methanol leaf extract of *A. aspera* using excision and incision wound models in rats
- To evaluate anti-inflammatory activity of chloroform, n-butanol and aqueous fractions of 80 % methanol leaf extract of *A. aspera* using carrageenan induced paw edema and cotton pellet induced granuloma models in rats.
- To determine preliminary phytochemical constituents of chloroform, n-butanol and aqueous fractions of 80 % methanol leaf extract of *A. aspera*.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals, Drugs and Reagents

Chemicals, drugs and reagents used in the study include: distilled water (Social Pharmacy and Pharmaceutics Laboratory, Addis Ababa University), chloroform, ethyl acetate and mercuric chloride (Bulex Laboratory, India), nitrofurazone ointment 0.2 %, Ketamine (NEON Laboratories, India), Sulphuric acid, n-butanol , methanol and hexane (Loba Chemie, India),wool fat, hard paraffin, white soft paraffin, cetostearyl alcohol, indomethacin (Cadila, Ethiopia), Tween 80 (Uni-Chem, India), diethyl ether (BDH Laboratory Supplies, England) and carrageenan (Sigma Aldrich, Germany), acetic anhydride (Lot A13/45/67/A), ammonia and ferric chloride anhydrous (Sisco Research Laboratories, India), Potassium iodide (Calibre Engineering, India), normal saline (Addis Pharmaceutical Factory, Ethiopia). Chemicals and reagents to be used were of analytical grade.

3.1.2 Instruments, apparatus and supplies

Instruments, apparatus and supplies used in the study include: rotary evaporator (Heidolph, Germany), lyophilizer (OPR-FDU-5012, Korea), digital plethysmometer (Ugo Basile-Cat no 7140, Italy), electronic balance (KERN-ALJ 220-4, Germany), mini orbital shaker (SSM1-STUART), Tissue Drying Oven (Laboratory Oven.Lab-Tech, India), separatory funnel, flasks, Cotton pellets, syringes with needles, Guavage, Whatman filter paper (Number 1), blunt forceps, scissors, suturing set.

3.2 Collection of Plant Material

The fresh leafs of *Achyranthes aspera* which is called "ተለንጅ" in Amharic and "Maxxannee" in Afaan Oromo were collected from natural habitat around Debre Tabor, South Gondar, North West Ethiopia. The plant was identified and authenticated by Dr Getachew Addis, herbal taxonomist, and a voucher specimen GDBT/002/19 was deposited at the Ethiopian Public Health Institute Herbarium.

3.3 Experimental animals

Healthy, adult Wistar albino rats of both sex (6–8 weeks of age) weighing 200 -250 g obtained from the animal house of the Ethiopian Public Health Institute, Addis Ababa were used. They were kept 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) with free access to standard laboratory pellet and clean drinking water *ad libitum* . Rats were acclimatized to laboratory condition for one week prior to commencement of the experiments. All procedures and techniques used in this experiment were carried in accord-

ance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Council, 2011).

3.4 Preparation of crude extract and solvent fractions of *A. aspera* leaf

Air dried and powdered leaf of *A. aspera* was first defatted by macerating with n-hexane for 72 hours at room temperature with occasional shaking followed by filtration. The remnants of solvent (n-hexane) were removed from the residue through exposing to open air. Cold maceration extraction technique was used to extract the plant material. A 1.07 kg of powder was macerated in a flask containing 80% methanol (1:6 w/v) for 72 hrs. Then, the extract was filtered using Whatman filter paper (No 1) and the marc was re-macerated for a second and third time by adding another fresh solvent. The filtrates from the three batches of the 80 % MeOH (methanol) were combined and concentrated in a rotary evaporator at temperature of 40^0C. The remaining solvent (water) was removed using a lyophilizer. After solvent removal, a black sticky residue weighing 123.51 gm was obtained, giving rise to a percentage yield of 11.54%.

The 80% methanol extract was subjected to successive fractionation using methods previously mentioned (Kalaivanan *et al.*, 2013) with solvents of different polarities (chloroform, n-butanol and water) (Sarker *et al*., 2005). One hundred twenty- three gram of the crude extract was suspended in a separatory funnel in 180 ml of distilled water. The same volume of chloroform was added and mixed well. The mixture was then allowed to form a distinct layer and the chloroform fraction was separated by eluting the lower layer. This was repeated three times. Then the aqueous residue was similarly mixed with equal volume of n-butanol, and separated. The chloroform and n-butanol fractions were concentrated by rotary evaporator and dried by dry oven at 40^oC. The aqueous fraction was lyophilized. The % yields of the dried fractions were 48.3 g (39.11%), 35.21 g (28.5%) & 38.43 g (31.1%) of aqueous, n-butanol and chloroform respectively. Then fractions were stored in tight container at −4°C until initiation of the actual experiment (Fentahun *et al*., 2017). All fractions were reconstituted in 2% tween 80 at appropriate concentration for the various experiments to be conducted.

3.5 Formulation of ointments

Simple ointment and medicated ointments of aqueous, n-butanol and chloroform fractions were prepared as per British Pharmacopea.2009. To prepare 50 g of simple ointment; 2.5 g of hard paraffin and 2.5 g of Cetostearyl alcohol were melted in a beaker. A 2.5 g wool fat and 42.5 g white soft paraffin were melted in another beaker. Finally, the contents of the two beakers were mixed and stirred until cooled. A 5 % w/w and 10 % w/w ointments of each fraction were prepared by incorporating 2.5 g and 5 g of the aque-

ous, n-butanol and chloroform fraction each into a 47.5 g and 45 g of simple ointment base respectively to get a 50 g medicated ointment of each fraction.

3.6 Acute oral and dermal toxicity tests

Acute oral toxicity test for solvent fractions of the leaf of *A. aspera* was performed according to the Organization for Economic Cooperation and Development guideline (OECD) 425; "Limit Test at 2000 mg/kg" (OECD, 2008). Four Wistar albino female rats of 6-8 weeks were selected randomly and used for the test. The rats were fasted overnight before administering the fractions and for a further 1-2 hrs after administering the fractions. First, a sighting study was performed to determine the starting dose, in which, a single female rat for each fraction was given 2000 mg/kg of the respective fraction as a single dose by oral gavage. Since no death was observed within 24 hrs, additional four rats were used for each of the fractions, and administered the same dose of fractions. The rats were observed continuously for 4 hrs with 30 min interval and then for 14 consecutive days with an interval of 24 hrs for the general signs and symptoms of toxicities such as changes in skin and fur, eyes and mucous membranes, somatomotor activity, and behavioral pattern, salivation and diarrhea, weight loss, tremor and convulsions, lethargy and paralysis, food and water intake and mortality.

Acute dermal toxicity was also carried out as per OECD (2002.Adopted:2017). Three female rats showing normal skin texture for each fraction were randomly selected and housed individually in a cage and acclimatized to the laboratory condition for a week prior to the test. Then, the rats were anesthetized by ketamine 1m1/kg i.p injection and around 10 % of the body surface area fur was shaved from the dorsal area of the trunk 24 h before the study. A limit test dose of 2000mg/kg of the 10% w/w of the solvent fraction formulations were applied uniformly over the shaved area for 24 hours. During the exposure period, rats were caged individually. At the end of the exposure period, residual test substance was removed and the rats were observed daily for the development of any adverse skin reactions for 14 days. The reactions, defined as erythema and edema, were evaluated and graded according to the OECD404 grade (2002).

3.7 Grouping and dosing of animals

For excision wound model, animals were randomly divided into eight groups of six rats per group as follows: the rats in the first group were treated with simple ointment. The second group was treated with nitrofurazone (NF 0.2%) ointment. The third and fourth groups were treated with 5 & 10 % w/w ointments of aqueous fraction respectively. The fifths & sixth groups were treated with 5 & 10 % w/w ointments of n-butanol fraction, respectively while seventh & eighth groups were treated with 5 & 10 %

w/w ointments of chloroform fractions of 80 % methanol leaf extract of *A. aspera*, respectively. For incision wound model the animals were randomly assigned into nine groups of 6 rats per group with the same grouping and dosing as that of excision wound model with except inclusion of untreated group. For anti-inflammatory activity evaluation in two models, the rats were randomly assigned into eleven groups of six rats in each group. The first two groups served as negative (2% tween 80 at a dose of 10 ml/kg) and positive (indomethacin 10 mg/kg) controls for both models. The first three test groups (3-5) received three different doses (100, 200 and 400 mg/kg) of the aqueous fraction. The other three test groups (6-8) received n-butanol fraction at the same three dose level, while the rest three test groups (9-11) received the chloroform fraction at the same three dose levels. The fractions and indomethacin powder were dissolved in 2% tween 80 to prepare suspension (Doyo, 2016). The doses (100, 200 and 400 mg/kg) were selected based on results of oral limit test.

3.8 Wound Healing Models

3.8.1 Excision wound model

Rats were anesthetized with intraperitoneal (i.p) 50 mg/kg ketamine (Thakur *et al.*, 2011). The fur was removed from dorso-thoracic area. A circular mark of 314 mm^2; as described by Nagar *et al.*, (2016) was prepared using permanent marker and a full-thickness was excised using sharp sterilized scissors, and considered day 0. Starting from day one; i.e after 24 hours of wound area created; the rats were treated and ointments were applied as described above. All ointments were applied once daily to the wound area until the wound got completely healed. The rats were observed for wound closure, and measurement was taken every two days post wounding using a transparency paper and a permanent marker (Kokane *et al.*, 2009; Wang *et al.*, 2011). Then, the traced area for each rat was calculated by measuring the diameter using a millimeter scaled ruler. The percentage of wound contraction was evaluated as described by the previous study (Shivhare *et al*., 2010).

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

Where n= number of days i.e. 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th}, 12^{th}, 14^{th}, 16^{th}, 18^{th} and 20^{th} (the day the wound in the fraction treated groups including the standard completely healed).

The number of days required for the dead tissue remnants to fall off from the wound surface exclusive of leaving a raw wound behind was taken as the endpoint of complete epithelialization and the days required for this was considered as period of epithelialization (Liu *et al.*, 2014; Pawar *et al.*, 2013).

3.8.2 Incision wound model

Rats were anesthetized and their fur was removed as described for the excision wound model. Three centimeters long, linear- paravertebral incision was made through the full thickness of the skin on either side of the vertebral column 1 cm from the midline. The removed skin was kept together and stitched using chromic catgut (2/0 metric-1/2 Circle) with curve needle at the intervals of 1 cm (Thakur *et al.*, 2011) which was considered day 0. Starting from day one, the ointments were applied as described in section 3.7. The ointments were applied topically once per day for 9 days. The sutures were removed on the 8th day of post wounding (Wang *et al.*, 2011). Then the tensile strength was measured (Ilango & Chitra, 2010) on the 10th day to measure the extent of healing. It was measured through continuous constant water flow technique by considering the weight of water required to break the skin (Fikru *et al.*, 2012; Wang *et al.*, 2011).

The breaking strength of the groups treated with fraction ointments were compared with those of the standard, simple ointment and untreated control. The percentage of tensile strength was calculated as follows;

$$\text{Percent tensile strength of fractions} = \frac{\text{Tensile Strenth (fractions)} - \text{Tensile Strength (so)}}{\text{Tensile Strength (so)}} \times 100$$

$$\text{Percent tensile strength of reference} = \frac{\text{Tensile Strength (reference)} - \text{Tensile Strength (so)}}{\text{Tensile Strength (so)}} \times 100$$

$$\text{Percent tensile strength of simple ointment} = \frac{\text{Tensile strengh (so)} - \text{Tensile Strength (lu)}}{\text{Tensile Strenth (lu)}} \times 100$$

Where so=simple ointment and lu=left untreated (Mulisa *et al.*, 2015)

3.9 Determination of anti-inflammatory activity

3.9.1 Carrageenan-induced paw edema

The method described by Ayal *et al.*,(2019) was followed with minor modification to study the effect of solvent fractions of 80 % methanol leaf extract of *A. aspera* on acute inflammation. Rats fasted overnight with free access to water until the experiment commenced. The basal volume, i.e. displacement of water by the left hind paw of each rat was determined using calibrated plethysmometer and randomly assigned in their respective groups before administration of the substance as described in section 3.7. Then rats were treated with test substance by making use of oral gavage. After 1hr of test administration, inflammation was induced in the left hind paw by injecting 0.05 ml of freshly prepared 1% carrageenan suspension in normal saline into the sub-plantar-surface of the left hind paw. The change in volume of the injected paw was measured after 1, 2, 3 and 4 hrs of post induction with carrageenan using

plethysmometer (*Mulisa et al., 201*5). A change in paw volume at1, 2, 3 and 4 hrs after carrageenan injection was considered the parameter for measurement of inflammation. The average foot swelling in fraction treated rats as well as standard were compared with that of the negative control and the percent inhibition (anti-inflammatory activity) of edema was determined using the following formula (Muhammad *et al.*, 2012):

$$\text{Percentage inhibition of edema} = \frac{\text{PEC- PET}}{\text{PEC}} \times 100$$

PEC= Paw edema of negative control and PET = paw edema of test groups including the standard.

3.9.2 Cotton pellet induced granuloma

The method previously used by Afsar *et al*., (2013) was used to evaluate the transudative and proliferative (granulomatous) components of chronic inflammation. Male albino Wistar rats (200-250 g) fasted overnight with free access to water untill the commencement of the experiment. The control, standard and test groups of rats received 2% tween 80, indomethacin and fractions, respectively as mentioned in section 3.7.

Cotton pellets weighing 10 ±1 mg were sterilized in an autoclave for 30 min at 120 ^{0}C under 15Ib pressure. Twenty minutes after treatment with the standard drug and fractions the rats were anesthetized with ketamine hydrochloride (50 mg/kg, i.p) and subcutaneous tunnel was made aseptically using blunted forceps in both sides of previously shaved groin region of each rat. Two sterilized cotton pellets weighing 10 ±1 mg each were then implanted bilaterally in the subcutaneous tunnel and stitched with chromic catgut (2/0 metric-1/2 Circle). Treatment with 2% tween 80, indomethacin and fractions continued for a total of seven consecutive days (p.o., once a day). On day 8, the rats were sacrificed with ether anesthesia, thereafter; the pellets surrounded by granuloma tissue were dissected out carefully and freed from extraneous tissue. The wet weight of the cotton was taken immediately after removal and then dried up to constant weight at 60 ^{0}C for 24 hrs and the net dry weight, that is, after subtracting the weight of the cotton pellets, was determined.

Measure of exudate formation = Immediate wet weight of pellet - Constant dry weight of pellet

Measure of granuloma tissue formation = Constant dry weight - Initial weight of cotton pellet

The exudate amount (mg), granulation tissue formation (mg), percent inhibition of exudate and granuloma tissue formation were calculated according to the formula described before (Aziz *et al.*, 2014).

$$Exudate\ inhibition\ (\%) = (1 - \frac{exudate\ in\ treated\ group}{exudate\ in\ controls} \times 100)$$

$$Granuloma\ inhibition\ (\%) = (1 - \frac{granuloma\ in\ treated\ group}{granuloma\ in\ controls} \times 100)$$

3.10 Phytochemical screening of solvent fractions

Preliminary phytochemical screening of secondary metabolites of aqueous, n-butanol and chloroform fractions of 80 % methanol leaf extract of *A. aspera* were carried out using standard tests (Ayoola *et al.*, 2008; Sasidharan *et al.*, 2011);

Test for saponins

To 0.25 g of each fraction (AF, BF and CF), 5 ml of distilled water was added. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of a stable froth that persists for about half an hour indicated the presence of saponins.

Test for terpenoids

To 0.25 g of each fraction, 2 ml of chloroform was added. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for tannins

About 0.25 g of each fraction was boiled in 10 ml of water in a test tube and then filtered with filter paper (Whatman No. 1). A few drops of 0.1% ferric chloride were added to the filtrate. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2 g of each fraction, and heated on a water bath for 3 min and cooled. About 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonia layer indicated the presence of flavonoids.

Test for cardiac glycosides

To 0.25 g of each fraction was diluted with 5 ml of water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.

Test for steroids

Two ml of acetic anhydride was added to 0.25 g of each fraction with 2 ml sulfuric acid. The color change from violet to blue or green in some samples indicated the presence of steroids.

Test for alkaloids

0.5 g each fraction was taken and a few drops of freshly prepared Mayer's reagent were added. The formation of cream was taken as positive for the presence of alkaloids.

3.11 Data Analysis

Data were analyzed using SPSS version 20.0 for Windows. Experimental results were expressed as mean ± standard error of the mean (S.E.M) and statistical significance test was carried out by using one way analysis of variance (ANOVA) followed by Tukey post Hoc test for multiple comparisons to compare results among groups, p values < 0.05 were considered statistically significant. The analyzed data were then presented using tables and figures.

4. Results

4.1 Acute oral and dermal toxicity test

The results of acute oral and dermal toxicity tests of fractions (AF, BF and CF) of 80 % methanol leaf extract of *A. aspera* indicates that the solvent fractions did not show gross behavioral changes, dermal toxic effects or mortality within 24 hrs as well as for the next 14 days. According to the "Limit Test at 2000 mg/kg" of OECD guideline 425 (2008), the oral LD_{50} of solvent fractions was greater than 2000 mg/kg in rats.

4.2 Evaluation of wound healing

4.2.1 Excision wound model

Rats treated with chloroform and n-butanol fraction ointments revealed wound healing. The percentage of wound contraction of the rats treated with the 10 % w/w ointment of chloroform fraction was significant ($p<0.01$) in most post wounding days compared with the negative control (Table-1 and Figure-3) and reduced period of epithelialization (Table-2). The wound contraction of the group treated with 10% w/w chloroform fraction ointment was comparable with that of the positive control (NF 0.2%) in most post wounding days. These were, 44.96 and 47.59% on day 8; 97.85 and 98.17% on day 16; 99.37 and 99.56% on day 18; 99.92 and 99.95% on day 20; for 10 % w/w CF and NF 0.2% ointments respectively. On the other hand, rats treated with 5 % w/w and 10 % w/w ointments of aqueous and n-butanol fractions showed statistically insignificant wound healing activity compared with the negative control until the fourth day. In addition, all 5%w/w fraction ointments showed statistically insignificant wound contraction until the sixth day. There was significant difference in the wound contractions between the rats treated with the three fraction ointments. However, 5 % w/w CF ointment started significant wound healing on eighth day and revealed significant difference in wound contractions compared with 5 % w/w AF and simple ointments ($p<0.01$) on the sixteenth, eighteenth and twenty days. The wound contraction of the group treated with 10 % w/w CF ointment revealed significant wound healing activity compared with 5 % w/w and 10 % w/w AF and BF on fourteenth day. Thus, CF is the most active fraction evidenced by higher percentage of wound contraction and reduced period of epithelialization (Figure-3, Table-1 and Table-3).

Table 1: Effect of solvent fractions of 80 % methanol leaf extract of *A. aspera* on percentage wound contraction in rats

Treatment group	Days of wound area measurement in mm^2 (mean±SEM) and % contraction									
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
SO	307.97±2.5 [1.92]	297.85±2.91 [5.14]	282.13±1.14[(11.30]	247.82±2.56 [21.08]	221.44±3.59 [29.48]	183.5±2.78 [41.56]	95.50±6.31 [69.59]	59.80±3.05 [80.95]	38.99±4.02 [87.58]	35.33±3.61 [88.75]
NF 0.2%	300.22±0.89 [4.3])	284.10 ±1.33 [9.24] ^{*a}	240.60±7.52 [31.15] ^{*a}	164.37±6.55 [47.59] ^{*aef}	131.39±6.63 [58.16] ^{*a}	75.88±6.59 [75.83] ^{*a}	30.24±2.10 [90.37] ^{*ae}	5.76±0.83 [98.17] ^{*aef}	1.39±0.41 [99.56] ^{*adef}	0.15±0.13 [99.95] ^{*af}
5% w/w AF	303.93±1.79 [3.2]	290.82±1.11 [7.38]	247.82±2.56 [26.77]	229.03±7.23[27.06]	131.75±15.89 [58.04] ^{*a}	107.64±13. 01[65.72] ^{*a}	54.95±9.84 [82.62] ^{*a}	30.23±4.21 [90.37] ^{*a}	26.36±3.52 [91.60]	15.99±2.15 [94.0]^{*a}
10%w/w AF	305.48±1.56 [2.71]	286.05±1.34 [8.9]	227.20±7.71 [39.01] ^{*a}	186.14±10.55 [40.72] ^{*af}	102.88±10.36 [67.24] ^{*a}	83.81±8.17 [73.31] ^{*a}	53.56±0.75 [82.81] ^{*a}	10.50±1.18 [96.66] ^{*af}	7.44±0.59 [97.63] ^{*af}	5.33±0.77 [98.30] ^{*af}
5%w/w BF	308.22±2.1 [1.83]	289.49±1.88 [7.81]	252.85±7.70 [24.74]	229.66±7.72 [26.86]	157.84±6.81 [49.73] ^{*a}	114.21±8.4 [63.63] ^{*a}	62.70±1.27 [80.03] ^{*a}	21.23±4.29 [92.51] ^{*a}	14.79±3.74 [95.29] ^{*a}	8.87±2.14 [97.18] ^{*a}
10%w/w BF	302.52±1.5 [3.66]	284.130±1.27[9.51]	198.53±11.9 1[36.77] ^{*a}	170.09±5.36 [45.83] ^{*af}	113.49±5.10 [63.85] ^{*ad}	73.58±7.04 [76.57] ^{*ad}	41.07±2.81 [86.92] ^{*a}	20.61±2.44 [93.44] ^{*a}	15.19±1.65 [95.31] ^{*a}	8.94±0.98 [97.19] ^{*a}
5%w/w CF	303.48±1.72 [3.35]	290.67±2.83 [7.43]	264.00±3.45 [19.04]	201.29±7.26 [35.89] ^{*a}	149.73±3.62 [52.32] ^{*a}	102.85±2.5 [67.25] ^{*a}	43.00±2.94 [86.31] ^{*a}	9.16±1.64 [97.08] ^{*af}	5.69±0.67 [98.19] ^{*af}	0.58±0.09 [99.82] ^{*af}
10%w/w CF	298.06±0.89 [5.08]	284.5±0.76	199.49±10.0 7[39.30] ^{*abcd}	172.82±8.51 [44.96] ^{*adf}	127.80±4.29 [59.30] ^{*a}	67.52±4.75 [78.50] ^{*af}	23.38±4.2 [92.55] ^{*adefg}	6.74±1.31 [97.85] ^{*af}	1.97±0.53 [99.37] ^{*aef}	0.27±0.17 [99.92] ^{*af}

Values are expressed as mean ± SEM (n=6 rats in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; ^{a} compared with simple ointment; ^{b} compared with NF 0.2%; ^{c} compared with 5% chloroform fraction; ^{d} compared with 5% n-butanol fraction; ^{e} compared with10% n-butanol fraction; ^{f} compared with 5% aqueous fraction; ^{g} compared with10% aqueous fraction; w/w = weight by weight ; SO = Simple ointment; * = $p<0.01$;NF 0.2%=Nitrofurazone 0.2% ointment; AF, BF &CF are aqueous, n-butanol and chloroform fractions of 80% methanol leaf extract of *A. aspera* (Amaranthaceae) respectively. Initial wound area was 314 $mm^{2.}$ .Values in the bracket are % contractions.

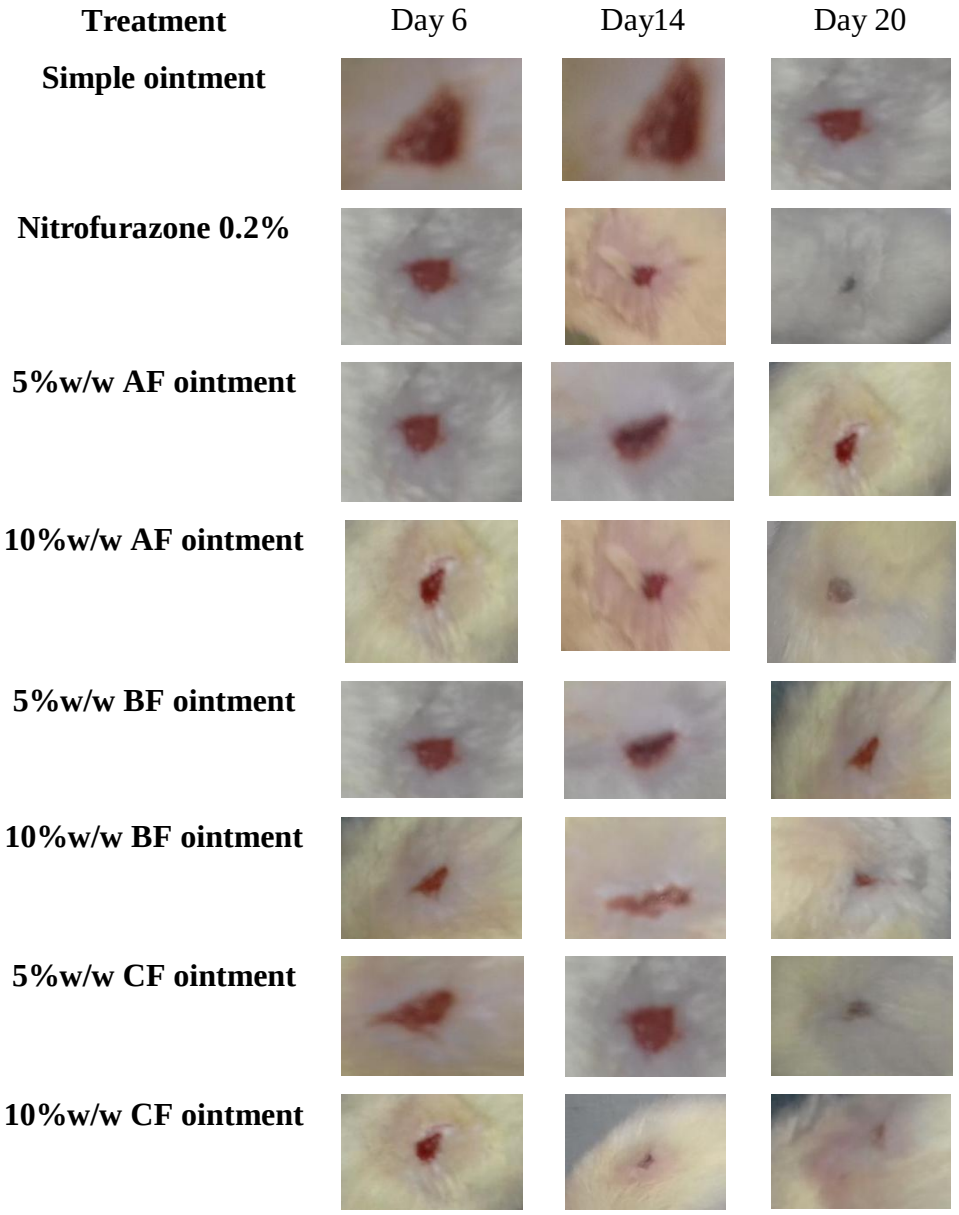

Figure 3: Progression of healing process of excision wound on rats treated with solvent fractions ointments of 80 % methanol leaf extract of *A. aspera* in rats

Table 2: Effect of topical application of solvent fractions of 80 % methanol leaf extract of *A. aspera* on period of epithelialization (no. of days) post wound creation in rats

Treatment group	Period of epithelialization in days (mean ± S.E.M)
SO	19.33±0.21
NF0.2%	14.17±0.31^{*abc}
5%w/w AF	16.67±0.21^{*a}
10%w/w AF	15.50±0.22^{*a}
5%w/w BF	16.17±0.21^{*a}
10%w/w BF	14.83±0.21^{*a}
5%w/w CF	15.33±0.33^{*a}
10%w/w CF	14.00±0.26^{*abcd}

Values are expressed as mean ± SEM (n=6 rats in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; [a] compared with simple ointment , [b] compared with 5% n-butanol fraction , [c] compared with 5% aqueous fraction, [d] compared with10% aqueous fraction; w/w = weight by weight; SO = Simple; * = $p<0.01$; NF 0.2% = Nitrofurazone 0.2% ointment ; AF, BF & CF are aqueous, n-butanol and chloroform fractions of 80 % methanol leaf extract of *A. aspera* (Amaranthaceae) respectively.

4.2.2 Incision wound model

In the incision wound model, groups treated with 5% w/w and 10% w/w ointments of the three fractions showed significant ($p<0.01$) increase in tensile strength compared with the control groups (simple ointment treated and untreated). Tensile strength was significantly increased by NF 0.2% & 10 %w/w CF ointments compared with the 5% w/w AF, BF and CF ointments ($p<0.01$). The tensile strength recorded in the group treated with 10 % n-butanol fraction was statistically significant compared with the group treated with 5% aqueous fraction (Table-3) ($p<0.01$).

Table 3: Effect of the solvent fraction ointments of 80 % methanol leaf extract of *A. aspera* on tensile strength in rats

Treatment group	Tensile strength in gram (mean ±S.E.M)	Percent of tensile strength [%]
LU	445.82 ± 42.21	-
SO	484.37 ± 73.22	8.74
NF 0.2%	876.15 ± 42.32^{*abcde}	80.89
5%w/w AF	698.10 ± 65.61^{*a}	43.09
10%w/w AF	779.14 ± 65.89^{*ae}	60.89
5%w/w BF	736.12 ± 51.66^{*ae}	51.97
10%w/w BF	835.58 ± 21.68^{*ade}	72.51
5%w/w CF	739.31 ± 51.19^{*ae}	52.63
10%w/w CF	873.15 ± 69.38^{*abcde}	80.26

Values are expressed as mean ± SEM (n=6 rats in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; [a] compared with simple ointment, [b] compared with 5% chloroform fraction , [c] compared with 5% n-butanol fraction, [d] compared with 5% aqueous fraction, [e] compared with untreated group; LU = left untreated; w/w = weight by weight ; SO = Simple ointment; * = $p<0.01$;NF 0.2% = Nitrofurazone 0.2% ointment; AF, BF & CF are aqueous, n-butanol and chloroform fractions of 80% methanol leaf extract of *A. aspera* (Amaranthaceae) respectively.

4.3 Anti-inflammatory activity

4.3.1 Carrageenan-induced paw edema

Sub-plantar injection of 0.05 ml of 1% carrageenan to the rat's left hind paw produced a progressive increment of paw thickness that reached its maximum value after 2 hr post-induction with 2% tween 80 (negative control) (Table-4). All tested doses of the chloroform fraction showed a significant inhibition of paw edema starting from 1 hr and the effect lasted until 4 hrs post-induction compared with 2% tween 80 ($p < 0.01$). Maximum anti-inflammatory effect (% inhibition) in 100, 200 and 400 mg/kg doses of CF was observed at 4 hr post induction with respective values of 31.85 %, 51.25 % and 52.5 % in dose dependent manner ($R^2= 0.891$). Comparison among doses of the CF with other fractions also showed a statistically significant effect. For instance, both the middle and the highest doses of CF showed a significantly different effect compared to 100 mg AF ($p < 0.01$) at 1, 2, 3 and 4 hrs. The 200 mg/kg and 400 mg/kg doses of AF showed statistically significant inhibition of paw edema at 2 hr and 1 hr post induction compared with 2% tween 80 respectively ($p < 0.01$). The effect in both dose levels then persisted significantly at 3rd and 4th hrs post-induction compared with the 2%tween 80 and 100 mg/kg AF ($p < 0.01$). However, 100 mg/kg AF did not show significant inhibition of paw edema compared with the 2%tween 80 throughout the observation period except for 4th hr post-induction. Maximum percent inhibition by all tested doses of AF was observed at 4th hr post induction with respective values of 22.50%, 45.00%, and 45.83% dose-dependently ($R^2= 0.881$). Comparison among doses of the BF also showed a statistically significant anti-inflammatory effect. Hence both middle and highest doses of BF showed statistically different effect compared to 100 mg/kg AF at 3 and 1 hr post induction ($p < 0.01$) respectively and the effects persists up to 4th hr post- induction.

Significant inhibition of paw edema occurred with10 mg/kg indomethacin from the 1st hr until the 4th hr after carrageenan injection compared with 2% tween 80 ($p < 0.01$). Moreover, no difference in onset and duration of action was observed by all tested doses of CF and 200, 400 mg/kg BF as all showed significant inhibition of paw edema from the 1st hr up to the 4th hr pos- induction ($p < 0.01$). Nevertheless, throughout the observation both 200 and 400 mg/kg of CF had shown a comparable anti-inflammatory effect with 10 mg/kg of indomethacin. CF was the most active fraction which is evidenced by the higher percent edema inhibition (%) values of all tested doses of CF throughout the observation period compared to the equivalent doses of the BF and AF (Table-4). Maximum percent inhibition in all tested doses of BF was also observed at 4 hr post-induction with respective values of 39.16%, 42.50%, and 45.0% in dose-dependent manner ($R^2= 0.997$).

Table 4:Anti-inflammatory effect of the solvent fractions of 80 % methanol leaf extract of *A. aspera* on carrageenan-induced paw edema in rats

Treatment group	Basal volume (ml)	Change in paw volume (mean ±S.E.M) (ml) [percent inhibition (%)]			
		1 hour	2 hours	3 hours	4 hours
2%Tween10ml/kg	0.22±0.01	0.42 ± 01	0.43 ± 0.01	0.41 ± 0.01	0.40 ± 0.01
Indo-10mg/kg	0.22±0.1	0.29±0.01(24.79) ^{*ab}	0.26±0.20(39.99) ^{*ab}	0.22±0.01(47.18) ^{*ab}	0.18±0.01(53.33) ^{*abc}
100mg/kg AF	0.18± 0.02	0.38 ± 0.15(3.85)	0.37 ± 0.02(14.67)	0.35 ± 0.01(14.92)	0.31 ± 0.03(22.50) ^{*a}
200mg/kg AF	0.22± 0.01	0.36± 0.01(8.55)	0.31± 0.02(27.41) ^{*a}	0.24±0.01(37.09)* ^{ab}	0.22± 0.01(45.00) ^{*ab}
400mg/kg AF	0.23± 0.00	0.31±0.02(20.94) ^{*a}	0.34±0.029(29.34) ^{*a}	0.28±0.03(42.33) ^{*ab}	0.24±0.01(45.83) ^{*ab}
100mg/kg BF	0.22± 0.01	0.35 ±0.02(11.11)	0.34 ±0.02(21.24) ^{*a}	0.28±0.03(31.85) ^{*a}	0.24 ±0.01(39.16) ^{*a}
200mg/kg BF	0.23± 0.00	0.32±0.02(16.23) ^{*a}	0.33 ±0.00(22.78) ^{*a}	0.26±0,01(37.10) ^{*ab}	0.23 ±0.00(42.5) ^{*ab}
400mg/kg BF	0.22± 0.01	0.30±0.00(24.36) ^{*ab}	0.27±0.01(36.67) ^{*ab}	0.24±0.00(42.74) ^{*ab}	0.22 ±0.01(45.0) ^{*ab}
100mg/kgCF	0.21 ±0.02	0.33±0.02(13.68) ^{*a}	0.33±0.01(22.78) ^{*a}	0.28±0.01(31.85) ^{*a}	0.27 ±0.02(31.25) ^{*a}
200mg/kgCF	0.15± 0.02	0.30±0.02(24.36) ^{*ab}	0.26 ± 0.02(37.12)* ^{ab}	0.23±0.02(42.75) ^{*ab}	0.12± 0.03(51.25) ^{*abc}
400mg/kgCF	0.20 ±0.01	0.30±0.02(23.93) ^{*ab}	0.28 ± 0.02(35.91) ^{*ab}	0.22±0.01(44.76) ^{*ab}	0.19±0090(52.50) ^{*abc}

Values are expressed as mean ± SEM (n=6 rats in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; ^{a} compared with 2% tween 80, ^{b} compared with 100 mg/kg aqueous fraction, ^{c} compared with 100 mg/kg chloroform fraction; * = $p<0.01$; Indo = Indomethacin; AF, BF &CF are aqueous, n-butanol and chloroform fractions of 80 % methanol leaf extract of *A. aspera* (Amaranthaceae) respectively.

4.3.2 Cotton pellet induced granuloma

Chloroform fraction (CF) and n- Butanol fraction (BF), at all tested doses significantly inhibited the formation of inflammatory exudate and granuloma mass ($p < 0.01$) compared with 2% tween 80. Comparisons among doses of the CF with other groups revealed a statistically significant different effect at 400 mg verses 100 & 200 mg/kg AF and BF ($p < 0.01$) in both exudate and granuloma inhibition. Chloroform fraction also revealed statistically significant anti-inflammatory activity even at the lowest and middle doses compared with 100 and 200 mg/kg AF ($p < 0.01$) in both exudate and granuloma inhibition). Furthermore, the anti-inflammatory effect of the CF was found to increase in dose-dependent manner ($R^2 = 1$ for exudates inhibition; $R^2 = 0.994$ for granuloma inhibition). Maximum percentage inhibition of exudate and granuloma formation was indicated by 400 mg/kg of CF (37.50 and 52.81%), respectively as compared with all other doses of the CF, BF, and AF.

The n-butanol fraction showed comparable effect at all tested doses in inhibiting cotton pellet induced exudate and granuloma tissue formation. Intergroup comparisons among doses of the BF revealed a statistically significant different inhibition against exudate and granuloma formation in 400 mg versus 100 mg/kg AF, BF and CF ($p < 0.01$). In addition, the anti-inflammatory effect of the BF was ascertained to increase in dose-dependent manner ($R^2 = 0.988$ for exudates inhibition; $R^2 = 0.981$ for granuloma inhibition).

Only the middle and highest doses of the aqueous fraction (AF) significantly inhibited the formation of both inflammatory exudate and granuloma mass as compared with 2% tween 80 ($p < 0.01$). The dose-dependent activity also observed in AF ($R^2 = 0.972$ for exudates inhibition; $R^2 = 0.993$ for granuloma inhibition). However, the lowest dose of AF failed to show statistically significant inhibition of exudate formation.

The standard drug, 10 mg/kg of indomethacin, significantly inhibited the formation of both exudates and granuloma (39.31 and 53.10%), respectively compared with 2% tween 80. A statistically significant difference was noted when all doses of the three fractions were compared with 2% tween 80 in terms of granuloma inhibition ($P<0.01$). The 400 mg/kg of both the CF and BF showed a comparable inhibition of exudates formation with the standard drug. But the chloroform fraction was the most active fraction in inhibiting the formation of exudate and granuloma mass as evidenced by the higher percentage of inhibition (Table -5).

Table 5: Effects of solvent fractions of 80 % methanol leaf extract of *A. aspera* on cotton pellet induced granuloma in rats

Treatment group	Mean weight of exudates in mg **(**mean ±S.E.M)	% inhibition of exudation	Mean weight of granuloma in (mg) **(**mean ±S.E.M)	% inhibition of granulation
2%Tween80 10ml/kg	**123.33 ± 0.54**	**-**	**143.70 ±0.77**	**-**
Indo 10mg/kg	**74.85 ± 2.01**^{*ab1b2c1d1}	**39.31**	**67.39± 0.83**$^{*a\ b1b2c1d1}$	**53.10**
AF 100mg/kg	**121.83 ± 0.97**	**1.22**	**132.54 ± 2.45**^{*a}	**7.76**
AF 200mg/kg	**95.33 ± 2.64**^{*ab1}	**22.71**	**98.15 ± 3.17**$^{*a\ b1}$	**31.69**
AF 400mg/kg	**84.56 ± 0.72**^{*ab1c1d1}	**31.44**	**75.42 ± 0.55**^{*ab1b2}	**47.51**
BF 100mg/kg	**100.85 ± 0.61**$^{*\ ab1}$	**18.47**	**80.04 ± 1.37**$^{*a\ b1}$	**44.30**
BF 200mg/kg	**91.37 ± 2.27**^{*ab1}	**25.92**	**76.50 ±0.93**^{*ab1b2}	**46.75**
BF 400mg/kg	**85.92 ± 1.94**^{*ab1c1d1}	**30.33**	**69.36 ± 1.06**^{*ab1b2c1d1}	**51.73**
CF 100mg/kg	**98.54 ± 1.26**^{*ab1b2}	**20.10**	**79.71± 1.4**^{*ab1b2}	**44.53**
CF 200mg/kg	**88.32 ± 1.81**^{*ab1b2}	**28.39**	**74.87 ± 0.87**^{*ab1b2}	**47.90**
CF 400mg/kg	**77.06 ± 2.77**^{*ab1b2c1c2d1}	**37.52**	**67.81 ± 0.67**^{*ab1b2c1c2d1}	**52.81**

Values are expressed as mean ± SEM (n=6 rats in each group) and analyzed by one way ANOVA followed by Post Hoc Tuckey test; ^{a} compared with 2% tween 80, ^{b1} compared with 100 mg/kg aqueous fraction, ^{b2} compared with 200 mg/kg aqueous fraction, ^{c1} 100 mg/kg n-butanol fraction, ^{c2} 200 mg/kg n-butanol fraction, ^{d1} compared with 100 mg/kg chloroform fraction ; * = $p<0.01$; Indo= indomethacin; AF, BF & CF are aqueous ,n-butanol and chloroform fractions of 80 % methanol leaf extract of *A. aspera* Amaranthaceae) respectively.

4.4 Phytochemical screening

Table 6: Preliminary phytochemical screening of solvent fractions of 80 % methanol leaf extract of *A. aspera*

Secondary metabolite	Aqueous fraction	n-Butanol fraction	Chloroform fraction
Saponin	+	+	-
Terpinoids	+	+	+
Tanins	-	-	+
Flavonoid	+	+	+
Glycosides	+	+	+
Steroids	-	+	+
Alkaloids	+	+	+

(**+,** Present); (-, Absent)

5. Discussion

Herbal medicines have been shown to be beneficial in wound care. Medicinal plants promote the rate of wound healing with minimal pain, discomfort, and scarring to the patient (Schultz *et al.*, 2003). Ointment formulations of medicinal plants could achieve wound healing (Zeng *et al*., 2016). The ointments prepared from the crude extract showed rapid wound contraction (Barua *et al.*, 2012). This enhanced wound contraction by crude extract ointments might be related to the ability of plant extracts to promote the proliferation of epithelial cells (Gebrehiwot, 2015). Additionally, the wound contraction effect of ointments of the fraction may be associated with their mitogenic activity which enhances fibroblast motility and its cellular proliferation as well as sequential transformation to myofibroblast that facilitates wound contraction during wound healing (Thakur *et al.*, 2011; Ayal *et al.*, 2019).

The reduced period of epithelialization produced by the ointments could be due to rapid wound contraction as contraction shortens the distance for migrating keratinocytes (Yadav *et al.*, 2016). The shorter period of epithelialization in the groups treated with fraction ointments might be related to the antibacterial activity of the leaf extracts of the *A. aspera* (Begum *et al*, 2002; Jebashree *et al*., 2011; Kaur *et al*. 2005; Ndhlala *et al*., 2015; Rao *et al.*, 2018). Ointments prepared from solvent fractions had different wound healing activities in an excision wound model. Fast contraction rate (higher percentage of contraction) and decreased period of epithelialization was observed in rats treated with the chloroform fraction ointment.

The findings of this study on wound healing activity of the CF and n-BF of 80% methanol leaf extract of *A. aspera* showed a statistically significant increased rate of wound contraction in most post wound days ($p<0.01$) and decreased period of epithelialization ($p<0.01$). This may be possibly due to similar content of phytochemicals present in the fraction and this finding was comparable with the finding of other studies This might be attributed to secondary metabolites (Krishnaveni & Thaakur, 2006; Mekonnen *et al.*, 2013). Secondary metabolites could facilitate wound healing either individually or through their additive effects (Agyare *et al.*, 2013). Tannins enhance wound healing through improving regeneration and organization of the new tissue through their astringent and antioxidant properties (Agyare *et al.*,2013; Ayal *et al.*, 2019). Flavonoids reduce lipid peroxidation by preventing or slowing the onset of cell necrosis and improving vascularity and have astringent and antimicrobial properties (Fikru *et al*., 2012; Talukder *et al*., 2012). Phenolic compounds have antioxidant, anti-inflammatory, and antimicrobial activities (Gautam *et al.*, 2014; Ghosh *et al*., 2011).

During the process of wound healing, an infection mostly from *S. aureus* and anaerobic bacteria may extend the inflammatory phase of the wound and thus leads to the failure of wound healing (Bowler & Davies 1999; Abo *et al*., 2004). I*n-vitro* study form chloroform and methanol root and shoot extracts of *A. aspera* showed signifi-

cant activity against *Klebsiella* which could support the findings of the current study (*Kaur et al.*, 2005). In line with this, tannins found in chloroform extract of *A. aspera* were reported to inhibit bacterial growth (Noor-Ul-Amin *et al.*, 2012).

Triterpinoidal saponins induced bacterial cell membrane disruption (Netala *et al.*, 2015). Hence, increased wound contraction and reduced period of epithelialization in groups treated with CF and n-BF compared with AF and SO (simple ointment) treated groups. Flavonoids could also promote wound healing through their astringent and antibacterial activities (Nayak & Pereira, 2006).

Previous studies reported that crude extracts and fractions of the leaf of *A. aspera* had antibacterial activities against common wound infecting bacteria such as *S. aureus*, *E.coli* and *P.aeruginosa* (Begum *et al*, 2002; Jebashree *et al*., 2011; Kaur *et al*. 2005; Ndhlala *et al*., 2015; Rao *et al*., 2018). This antimicrobial activity may partly be contribute to the wound healing effect of *A. aspera* by eliminating infection and allowing initiation of the natural tissue repair process.

In the group treated with SO, the prolonged period of epithelial restructuring and wound closure was observed. But solvent fractions of 80 % methanol leaf extract of *A. aspera* and NF 0.2% treated wounds were clean with healthy tissues. This might be as a result of the occurrence of microorganisms and their metabolites in the SO treated group, which inhibit wound contraction and weaken the wound healing activity.

An increase in tensile strength in the incision model indicated enhanced collagen maturation. Collagen, the major protein of the extracellular matrix, is the component that gives strength, support, and integrity to the wound matrix. Breakdown of collagen liberates free hydroxyproline and its peptides. A healing tissue synthesizes collagen, which is a constituent of growing cells (Lodhi *et al., 2*016). The increased tensile strength may be due to collagen synthesis, maturation, angiogenesis and stabilization of fibers (Murti & Kumar, 2012).

Abundant ROS damage extracellular structure proteins, lipids, and DNA and stimulate signal transduction pathways to prolong the inflammatory phase of wound healing (Upadhyay *et al.*, 2014). The existences of ROS and microbes at the wound area have synergistic effects causing a delay in wound healing. Therefore, elimination of ROS could be an important strategy in the healing of wounds. *A. aspera* var. porphyrostchya leaf contains vitamin C and reported to be 46.876 mg/100 g (Raut, 2013). Nutritional deficiencies can slow down wound healing. Hence, vitamin C is crucial for collagen formation, proper immune function, and a tissue antioxidant which could enable fractions in inhibiting reactive oxygen species in the wound (Mackay & Miller, 2003). Furthermore, any agent that inhibits lipid peroxidation is believed to augment the viability of collagen fibrils by increasing the strength of collagen fibers, by mounting the circulation, by preventing the cell damage

and promoting DNA synthesis (Arun *et al.*, 2016). Ethanol stem and leaf extracts of *A. aspera* showed *in-vitro* free radical scavenging and lipid peroxidation inhibitory activity (Krishnakumari & Priya, 2007). Thus, the fractions might have roles in collagen synthesis, maturation, and stabilization. Hence, antioxidant and antimicrobial properties of phytochemicals found in the fractions could fasten the wound healing process. For instance, flavonoids are potent antioxidants and free radical scavengers which prevent oxidative cell damage (Arun *et al*, 2016; Raut, 2013). The hydroxylation and alkoxylation pattern of flavonoids play great importance in determining their activity as antioxidants (Sharma *et al.*, 2014)

Previous study reported that total flavonoids found in chloroform extract were higher compared with ethanol and aqueous extracts which is 0.102, 0.012, 0.002 mg/g respectively. Hence, a higher percentage of tensile strength may be due to the higher concentration of flavonoids in the chloroform fraction when compared with n-butanol and aqueous fraction ointments. Ndhlala *et al.* (2015) reported that flavonoids; rutin, chlorogenic acid, and genistein; found in *A. aspera* have an antimicrobial activity which could promote wound healing.

The normal physiology of inflammation in an acute wound is to get ready the wound bed for healing by removing necrotic tissue, debris, and bacterial contaminants as well as recruiting and activating fibroblasts. Under normal conditions, inflammation is a self-limiting process. On the other hand, extreme inflammation is a major causal factor to the persistence of chronic non-healing wounds, which are "stuck" in the inflammatory phase of healing and fail to re-epithelialise (Enoch & Price, 2004; Rohl *et al.*, 2015).

The delayed period in the inflammatory phase results in retardation of healing, the anti-inflammatory activity of test agent is essential for the wound healing process (Akkol *et al.*, 2011). Infection may extend the inflammatory phase of the wound and thus leads to the failure of wound healing (Abo *et al.*, 2004). This may occur due to liberation of bacterial enzymes and MMPs that can degrade collagen fibers as well as wound growth factors and impaired wound healing process (Stadelmann *et al.*, 1998). The increase in free radical production and diminished antioxidant activity may worsen the condition and account for the delay in healing (Adly, 2010).

The previous study reported that saponin rich n-BF of the whole plant of *A. aspera* shown statistical significant inhibition of pro-inflammatory cells and improved in oxidative stress possibly due to direct antioxidant effect of saponin rich fraction coupled with an improvement in the body's natural antioxidant enzyme mediated defense (Kothavade *et al.*, 2015).

Alkaloids present in chloroform fraction showed wound healing through inhibition of inflammation by blocking the metabolic pathway of arachidonic acid (Gangopadhyay *et al.*, 2014; Krishnaveni & Thaakur, 2006). The chloroform fraction could promote wound healing which is in agreement with the study done by Khuda *et al.*,

(2014) and reported the highest *in-vitro* lipoxygenase inhibition was observed in chloroform fraction of *A.aspera* compared with aqueous and n-butanol fractions.

Prolonged inflammation could also increase the level of neutrophil-derived MMPs that can degrade the wound ECM (Armstrong & Jude, 2002). Moreover, Noor-Ul-Amin *et al*. (2012) reported that the chloroform extract of seeds of *A. aspera* exhibited antibacterial activity because of the presence of alkaloids and terpenoids. The promising activity of chloroform fraction ointments could be due to upregulation of inhibitory MMPs (Barua *et al.,* 2012) Additionally, prostaglandins are involved in chemotaxis of leukocytes mainly neutrophils that contribute to inflammatory response by producing oxygen-derived free radicals that damage wound tissue (Serhan *et al.,* 2008). Thus, prostaglandin inhibition benefit wound healing by reducing the damaging effect of neutrophils. Moreover, Previous studies reported that crude extracts of *A. aspera* showed promising wound healing and anti-inflammatory activities (Barua *et al.,* 2012; Edwin *et al.,* 2008; Fikru *et al.,* 2012; Ghosh *et al.,* 2011; Mondal *et al.,* 2016; Staden, 2015).

Although the aqueous fraction was screened for the presence of flavonoids, it could not produce significant wound healing effects compared with standard and chloroform fractions. This might be attributed to either suboptimal concentrations or loss of additive effect with other secondary metabolites.

The present *in-vivo* study showed that solvent fractions of 80 % methanol leaf extract *of A. aspera* possess anti-inflammatory activity in carrageenan-induced paw edema model in rats. 100 mg/kg of AF did not show significant anti-inflammatory activity at 1, 2 and 3 hr post carrageenan injection but percent inhibition of inflammation was highest at 4 hr after inflammation was induced. All test doses of chloroform fraction showed significant anti-inflammatory activity in all-time points in different percentages of inhibition ($p < 0.01$) but the highest percentage edema inhibition was observed with 400 mg/kg (52.50%) at 4 hr post-induction ($p < 0.01$). This is in line with *in-vitro* anti-inflammatory activity of chloroform fraction of leaf of *A. aspera* (Khuda *et al*., 2014). Lower doses, 100 mg/kg AF and BF also exhibit edema inhibition but incapable to reach a significant level. This may be due to an insufficient concentration of an active constituent in a lower dose of fractions. The observed edema inhibition was prominent in the later phase of inflammation, which was similar to the effect of nonsteroidal anti-inflammatory drugs such as indomethacin, indicating that the antiedematogenic activity is possibly mediated through a cyclooxygenase enzyme inhibitory pathway (Burke *et al.,* 2006). Medium and high doses of n-butanol fractions showed significant inhibition of inflammation after 2 hr of carrageenan injection ($p < 0.01$). This may be due to the inhibition of inflammatory mediators released at the later phase. Additionally, previous studies has reported anti-inflammatory activity of *A. aspera* (Bhosale *et al*., 2012; Vetrichelvan & Jegadeesan,2003).

The anti-inflammatory effect of the fractions may possibly be associated with the activities of secondary metabolites. The previous study has shown that saponin rich n- butanol fractions possess anti-inflammatory activity (Kothavade *et al.,* 2014; Kothavade *et al*., 2015). Previously triterpenoid saponins were isolated and block arachidonic acid metabolism in the later phase of inflammation possibly due to inhibition of phospholipase A2 (Gunter, 2000; Ullah *et al.,* 2014).

Flavonoids can significantly inhibit a number of inflammatory mediators and prevent the synthesis of prostaglandins (Sowemimo *et al.,* 2013). The previous study showed that polyphenols found in *A. aspera* possibly inhibit inflammation (Sharma *et al.,* 2014).

Achyranthes aspera also contain alkaloid and showed anti-inflammatory activity (Goyal *et al.,* 2008). Alkaloids prevent inflammation by blocking the metabolic pathway of arachidonic acid. It is evidenced by antioxidant and anticarcinogenic effect of alkaloid fraction of leaf of *A. aspera* (Tahiliani & Kar, 2000). These could be the possible reason for highest percentage inhibition of inflammation by chloroform fraction in carrageenan-induced hind paw edema model.

In the cotton pellet induced granuloma model, all tested doses of the solvent fractions of 80% methanol leaf extract of *A. aspera* showed statistically significant inhibition of both exudates and granuloma formation. In this model, all tested doses of the CF showed a statistically significant inhibition granuloma formation. The significant inhibitory effect of *A. aspera* on formation of exudates ($p < 0.01$) (Table -5) substantiates the finding of carrageenan-induced acute model, i.e. both results strengthen the effectiveness of the CF in inhibiting the exudative and proliferative component of inflammation. The statistical significant inhibition of granuloma formation ($p < 0.01$), on the other hand, rationalizes the effectiveness of this fraction in inhibiting the proliferative phase of inflammation evidenced by highest percentage of granuloma inhibition (52.81%).

Phytochemical screening for secondary metabolites revealed the presence of alkaloids, glycoside, flavonoids, tannins and steroids, and terpenoids in the chloroform fraction. The anti-inflammatory activity of this fraction may possibly be derived from the presence of these secondary metabolites whose protection against inflammation is reported somewhere else; terpenoids (Kothavade *et al.,* 2014; Monterio *et al*.2014; Zhang, 2009), alkaloids (Chen *et al.,* 2012), flavonoids (Chen *et al.,* 2019; Liu *et al.,* 2014; Moscatelli *et al.,* 2006; Sakat *et al.,* 2010; Narayan & Kumar, 2013). The overall order of efficacy in inhibiting the exudative phase of acute inflammation, as evidenced by the percentage inhibition of carrageenan-induced rat paw edema in the acute model and the cellular response of proliferative phase of inflammation as evidenced by the percentage of exudate and granuloma inhibition in the chronic model, was found to be CF > BF > AF. Hence the number of phyto-

chemicals in the CF could be the highest and most effective in inhibiting acute and chronic phases of inflammation.

From the overall results of this study, it can be deduced that *A. aspera* to be rich in anti-inflammatory secondary metabolites which act by different mechanisms to inhibit acute and chronic inflammatory conditions. Moreover, the analogous anti-inflammatory activity of the solvent fractions 80% methanol leaf extract of *A. aspera* in this study compared to the standard drug indomethacin, perhaps due to cumulative effects of different active constituents in reducing the synthesis, release and/or action of different inflammatory cytokines, chemokines, and mediators such as histamine, serotonin, prostaglandins, and leukotrienes. There were differences in point times at which peak inhibition of edema as well as proliferation occurred, which might possibly be due to the metabolic rate differences and/or nature of phytoconstituents' available in the fractions. This idea is in line with the proven concept that medicinal plants possess a combination of phytoconstituents with different anti-inflammatory mechanisms, offering synergistic or additive effects

6. Conclusion

The finding of this study revealed that promising wound healing and anti-inflammatory activity effect elicited by all fractions compared with negative control which corroborates the folkloric practice of *A. aspera*. The results from this study also confirmed that chloroform fraction was found to be the most efficacious fraction in wound healing and inhibiting both exudative component of acute inflammation and the cellular response of proliferative component of chronic inflammation. Additionally, all three fractions possessed a varying degree of wound healing and anti-inflammatory activity, with the chloroform fraction being the most active fraction followed by the n-butanol fraction and then aqueous fraction in all the four models. It also confirms the presence of biologically active components, which are worth further investigation and elucidation.

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7. Recommendations

The results of this study should be taken as a basis for further investigation of the wound healing and anti-inflammatory action of *A. aspera*. Hence, further studies are warranted on;

- Determination of the sub-acute and chronic toxicities to assess the long-term effect of each fraction
- Isolation and identification of pharmacologically active anti-inflammatory compounds of the chloroform fraction
- Determination of the exact mechanism(s) of action of wound healing and anti-inflammatory activity of fractions
- Further validation of the efficacy of the fraction(s) on other wound healing and anti-inflammatory models
- Conducting other pharmacologically related activities of the fractions claimed by folklore medicine, such as its analgesic and antipyretic activities.

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