brighton and sussex medical school

EFFICACY OF ETHIOPIAN MEDICINAL PLANT EXTRACTS FOR LOWER LIMB CARE (LYMPHOEDEMA) IN *IN VITRO* MODELS

A thesis submitted to Brighton and Sussex Medical School in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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March 2022

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I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

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Dated 2 March, 2022

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ABSTRACT

Lymphoedema is a pathological process that results from damage, infection, blockage, or genetic defects in the lymphatic system. The main causes of lower limb lymphoedema in Ethiopia are lymphatic filariasis (LF) and podoconiosis. The current care of lymphoedema in Ethiopia consists of foot hygiene, skin care, compression bandaging, exercise, and antibiotic therapy for 'acute episodes' (recurrent acute adenolymphangitis). A range of endemic plant extracts have also been used for the management of pain, wound infection, and inflammation in Ethiopia boosting the interest in medicinal plants and their compounds as an alternative medicine for the management of different diseases. Our systematic review revealed that there are many promising Ethiopian medicinal plant extracts and compounds for the management of wound infection and inflammation. *Lawosinia inermis L, Achyranthes aspera L, and Azadirachta indica* A. Juss were selected for the evaluation of the anti-inflammatory, wound healing, and anti-infective activities of the crude extracts in *in vitro* model. *Aeromonas hydrophila/caviae, Acinetobacter lwoffii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shewanella algae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus dysgalactiae, Staphylococcus haemolyticus, Streptococcus* agalactiae, and *Staphylococcus simulans* were the most predominant bacteria identified which contributed to wound infection in patients with lower limb lymphoedema in the study area. Among the isolated bacteria, most of them were resistant to ampicillin, cefazoline, clindamycin, erythromycin, and tetracycline. Furthermore, this study showed that the methanol extracts of *L. inermis L* exhibited significant activity and demonstrated a bactericidal effect against most of the tested bacterial strains. However, *A. Indica* A. Juss and *A. aspera L* showed low to moderate activity against most tested strains at 400mg/ml. In *in vitro* model, all the methanol extracts have shown inhibitory activity towards COX-1, COX-2, and 15-LOX enzymes at 10mg/ml. However, *L. inermis* L was superior in dual inhibition of both LOX and COX enzymes. All the tested extracts showed the best free radical scavenging activity at 10 mg/ml, which is comparable to inhibitory activity to that of ascorbic acid. Moderate antioxidant activity was observed in stimulating the multiplication of human epidermal keratinocytes. Compound isolation, and characterization from the methanol extract of the leaves of *L. inermis* L was performed. *L. inermis* L has shown significant activity against bacteria isolated from the lymphoedema patients, in *in vitro* anti-inflammatory and wound healing activities. This has led to the isolation of three compounds, namely 2-hydroxy-1,4-naphthoquinone (lawsone), 2,3,4,6-tetrahydroxyacetophenone-2β-D-glucopyranoside (lalioside) and 1,2,3,4,5,6-hexahy-droxyhexane (D-mannitol). The new findings in this research work are the micro-organisms isolated and characterized from the wound lymphoedema patients, and the three major compounds that have been isolated from the leaves of methanol extracts of *L. inermis* which is growing in Ethiopia.

ACKNOWLEDGMENTS

My special thanks to Prof. Gail Davey, Prof Eyasu Makonnen, and Dr. Belete Adefris for their joint guidance, supervision, critical evaluation of draft written works, and immediate feedback with valuable comments and suggestions throughout my work. I am very thankful to Prof. Abebaw Fekadu for the encouragement and support during the laboratory and fieldwork. My special thanks to the National Institute for Health Research (NIHR) Global Health Research Unit on NTDs and Brighton and Sussex Medical School for funding the research work. I would like to thank CDT-Africa, Addis Ababa University for the field support, for organizing and sponsoring trainings on meta-analysis and Statistical analysis software. I would like to thank Ethiopian Public Health Institute for allowing me to use its laboratories, for organizing and sponsoring trainings on systematic review and meta-analysis. Konchi Catholic Church Clinic for supporting sample collection. My special gratitude goes to Clare Callow, Grit Gansch, and Manuela McDermid for supporting the procurement of lab supplies. Special thanks should also go to Dr. Abrham Tesfaye, Dr. Yimitubezena Wolde-Amanuel, Mr. Tesfa Addis, Mr. Zeleke Ayenew, Sister Cicily Joseph Cherriakara, Mr. Abdi Samuel, and Mr. Estifanos Tsige, M/s Frehiwot Teka, Mr. Meseret W/Yohanes. Dr Kibrom Gebreheiwot, Dr. Mekonnen and Dr. Geremew Tasew for the support during sample collection and laboratory work. I would like to extend my gratitude to Dr. Getachew Addis (Herbalist) for his assistance in the collection and identification of the plants. My special thanks goes to Thedros Getachew for supporting me in data analysis.

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LIST OF ABBREVIATIONS/ ACRONYMS

ANOVA - One-way analysis of variance
ARRIVE - Animal Research: Reporting of In Vivo Experiments
AST- GN71 - Antimicrobial Susceptibility Test -gram-negative 71
AST-GP67 - Antimicrobial Susceptibility Tests-gram-positive 67
ATCC - American Type Culture Collection
BHT – Butylhydroxytoluene
BSA - Bovine Serum Albumin
CAMARADES - Collaborative approach to Meta-Analysis and Review of Animal Data from Experimental Studies
CC_{50} - Minimum dose that is toxic to 50 % of cells
CD4 - Cluster of Differentiation 4
CFU - Colony Forming Unit
Chi^2 test – Chi square test
CI - Confidence Interval
CLSI - The Clinical and Laboratory Standards Institute
CoNs - Coagulase-Negative
COX - Cyclooxygenase
CRIS - Checklist for Reporting *In vitro* Studies
$CHCl_3$- Dichloromethane
DEPT - Distortions Enhancement by Polarization Transfer
DLA – Dermatolymphangioadenitis
DMSO - Di-methyl-Sulfoxide
DNA – Deoxyribonucleic Acid
DPPH - 2,2-Diphenyl-1-picrylhydrazyl
ED_{50} - Minimum dose that is effective to 50% of the population
ELISA - Enzyme-Linked Immunosorbent Assay
EMBASE- Excerpta Medical data BASE
ENAO - Ethiopian National Accreditation Office
ERK - Extracellular-signal-Regulated Kinase
ESBL- Extended-Spectrum Beta-Lactamases
GAS - Group A streptococcus
GBD - Global Burden of Disease
GIVIMP - Guidance Document on Good In-Vitro Method Practices
GLP - Good Laboratory Practice
Gov't - Government
GRADE - Grading of Recommendations, Assessment, Development and Evaluation

HDF3CGF- Primary human neonatal fibroblasts
HEK293 - Human Embryonic Kidney 293
HMBC - Heteronuclear Multiple Bond Coherence
HMGB1- High mobility group box 1 protein
HMQC - Heteronuclear Multiple Quantum Coherence
HSQC - Heteronuclear Single Quantum Correlation
I2 test - I square test statistics
IC50 - Half minimum inhibitory concertation
IFN-γ – Interferon gamma
IL1 – Interleukin 1
IL10 - Interleukin 10
IL13 - Interleukin 13
IL1β – Interleukin one beta
IL4 - Interleukin 4
IL6 - Interleukin 6
IL8 – Interleukin 8
iNOS - Inducible Nitric Oxide Synthase
IP-10 - Interferon gamma-induced Protein 10
I-TAC - Interferon-inducible T-cell chemoattractant
ITN - Iodonitrotetrazolium Chloride
JNK – Jun N-terminal Kinase
LD50 - Lethal dose in 50% of the population
LD50 – Minimum dose that is lethal to 50% of the population
LDL - Low-Density Lipoproteins
LF - Lymphatic Filariasis
LOX - Lipoxygenase
LPS- Lipopolysaccharides
M2 - Macrophage type 2
MeOH - Methanol
MBC - Minimum Bactericidal Concentration
MD – Mean Difference
MDR – Multiple Drug Resistance
MeSH - Medical Subject Heading
MFC – Minimum Fungicidal Concentration
mg/ml – Milli gram per ml
MHA - Mueller Hinton Agar
MHB – Muller Hinton Broth
MIC- Minimum Inhibitory Concentration

MIG - Monokine Induced Gamma Interferon
MLD - Manual Lymphatic Drainage
mRNA -Messenger Ribonucleic Acid
MRSA – Methicillin Resistance *Staphylococcus aureus*
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDGA - Nordihydroguaiaretic acid
NF-kB p65 – Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NMR - Nuclear Magnetic Resonance
NO - Nitric Oxide
NSAIDs - Non-Steroidal Anti-inflammatory Drugs
NTD – Neglected Tropical Disease
OECD - Organization for Economic Cooperation and Development
PAMPs - Pathogen- Associated Molecular Patterns
PBS- Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PDR - Pan Drug Resistance
PICO – Population, Intervention, Comparator, Out-come
PRISMA-P - Preferred Reporting Items for Systematic Review And Meta-analysis Protocols
PROSPERO - International Prospective Register Of Systematic Reviews
PUBMED/MEDLINE- National Library of Medicine
RoB – Risk of Bias
SCOY - COrrelated SpectroscopY
SD – Standard Deviation
SI - Selectivity Index
SPSS – Statistical Package for Social Science
TGF-B1- Transforming Growth Factor Beta-1
TLC - Thin Layer Chromatography
TLRs - Toll Receptors
TM - Traditional Medicine
TNF-alpha – Tumour Necrosis Factor Alpha
VCAM-1 - Vascular Cell Adhesion Molecule 1
WHO - World Health Organization
XDR – Extensive Drug Resistance
ZI – Zone of Inhibition

CHAPTER ONE

1.INTRODUCTION

1.1. Background

The lymphatic system is an important part of the circulatory system that maintains homeostasis used for the movement and carriage of white blood cells, which regulates inflammatory responses and enables nutrient intake. The lymphatic vessels begin from the capillaries of the lymphatic system and transport the interstitial fluid back to the heart (1). It helps the removal of extracellular fluids (1-2L), soluble proteins (50 -80%) and passage of lymphocytes and antigen presenting cells to lymph nodes (2). The lymphatic system is important in immune surveillance in the body where circulating antigens and antigen-presenting cells are transported to the lymphnodes to activate immune cells (3).

Lymphoedema is a pathological process that results from damage, infection, blockage, or genetic defects in the lymphatic system. Characteristic hallmarks of lymphoedema include lack of lymphatic flow, chronic inflammation, fibro-adipose tissue accumulation, and tissue degeneration. The decrease in oxygen tension due to the obstruction of the lymphatic system results in chronic inflammation and tissue fibrosis (3).These pathological changes over time lead to lymphoedema (1). Initially, it is manifested as soft pitting oedema and later harder skin. These result in disfigurement of the affected area and can lead to disability (Figure 1.1). Lymphoedema may predispose to recurrent cellulitis and lymphangitis, which can further damage the lymphatic system (4).

Lymphoedema is categorized as primary and secondary lymphoedema. The former is caused by genetic disorders such as Milroy disease and Meige disease or by unknown pathology, whereas secondary lymphoedema is the result of pathological changes to the lymphatic system due to lymphatic vessel infection, surgical removal of the lymph node or radiotherapy in patients with cancer (1). Filarial helminths (round worms) such as *Wuchereria bancrofti* (responsible for most of the cases), *Brugia malayi, Brugia timori* are the most common causative agents of secondary lymphoedema globally. They occupy the lymphatic vessel and block the flow of lymph from the extremities (1,2). The causative larvae of lymphatic filariasis (LF) are transmitted by mosquitoes in areas where the disease is common (Figure 1.2) (5).

Podoconiosis is the most common cause of tropical lymphoedema next to lymphatic filariasis globally (3) (less common than LF), but thirty times more common than LF within Ethiopia (6). It affects genetically predisposed people who are barefooted for a long period of time on volcanic clays (Figure 1.1). As a result there is an uptake of irritant components of clay particles which appear to cause lymphatic inflammation (5,7) followed by lymphatic oedema and blockade of the lymphatic lumen (3).

Similarly, cellulitis can result in lymphoedema, and the attacks are variable depending on severity, and may differ from classical cellulitis (8). Cellulitis is an acute dispersing inflammation of the skin and subcutaneous tissues characterized by pain, warmth, swelling and erythema. Various risk factors have been linked with cellulitis, and bacterial cellulitis associated with lymphoedema is aggressive with severe symptoms and morbidity. Bacteria may also cause recurrent cellulitis in the setting of lymphoedema resulting in progression of swelling and fever (8). Staphylococcus aureus and group A streptococcus (GAS) are the most common known causes of cellulitis (9).

There are separate staging systems for podoconiosis (five stages) and LF (seven stages), but currently there is a move to simplify and unify these into 3 stages classifications of lymphoedema. These are mild, moderate and severe stage (10).

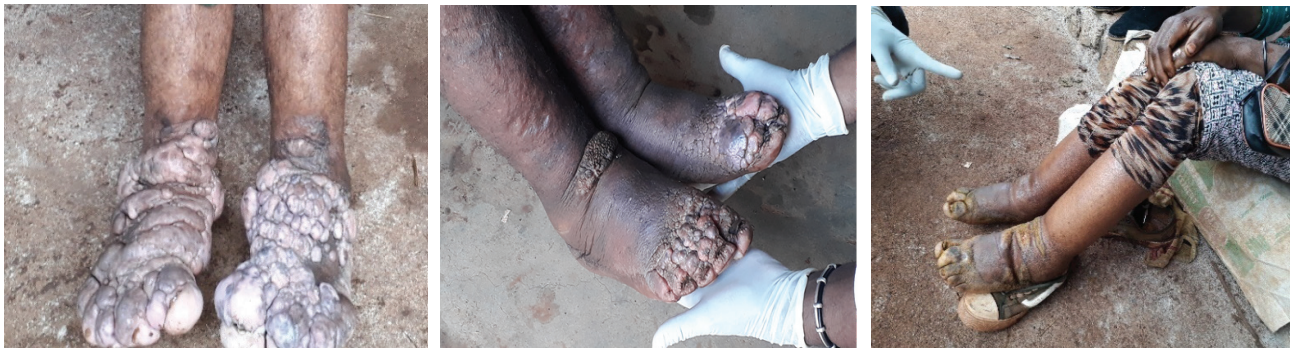


Figure 1.1: Lower limb lymphoedema – pictures taken during wound swab collection from lymphoedema patients, East Wollega, Ethiopia

An estimated 120 million people in 73 countries around the world are currently infected with filarial worms. Near 40 million individuals are disfigured or incapacitated due to LF-induced lymphoedema (3). An estimated 1.4 billion people live in areas where filariasis is common, predominantly in South-East Asia and Africa (5). The general spreading of LF in Ethiopia is not well-known. However, about 30 million individuals are assumed to be at risk, and among the East-African countries, Ethiopia shares 6-9% LF burden (11). Podoconiosis is highly prevalent in countries such as Uganda, Tanzania, Kenya, Rwanda, Burundi, Sudan, and Ethiopia (12). In Ethiopia, podoconiosis is endemic in 345 districts; 1,537,963 adults were estimated to live with podoconiosis in 2015 (7) and almost 49 million people are at a high risk of this disease (13).

At global level, skin disorders are the fourth leading cause of non-fatal disease burden. As said by previous Global Burden of Disease (GBD) reports, 66,500 annual deaths related to skin disease were due to bacterial infection such as cellulitis (14). Skin infections are the most important causes of skin disorders in children and adolescents in Ethiopia (14).

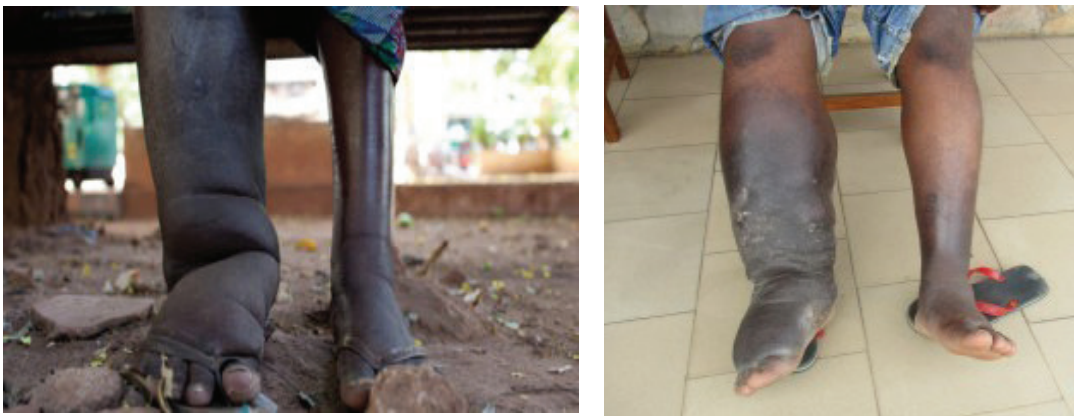


Figure 1.2: Lymphoedema due to lymphatic filariasis (Source : End NTD in Africa - https://end.org/ntds-in-focus/lymphatic-filariasis/, and https://endinafrica.org/news/africas-first-generation-free-from-lymphatic-filariasis-togos-triumph-over-an-infectious-disease/

Lymphoedema management includes several approaches such as physical therapy, drug therapy, surgical therapy and psychosocial rehabilitation. In most countries, lymphoedema management is approached through non-drug treatment such as exercise, compression through bandaging or stockings, manual lymphatic drainage (MLD) in the form of massaging the affected limb, and pneumatic compression therapy (8). Ideally, the treatment of lymphoedema aims to restore the normal functional and structural appearance of the limbs. But using current therapeutics, it is not possible to achieve these aims (15). Current therapeutic approaches include lifestyle modification (exercise), intermittent pneumatic compression, pharmacological interventions such as antibacterial to control infections, molecular modifications including growth factors and cellular therapies, and surgical interventions. All have been shown to have an advantage in improving function and quality of life (16).

The main intentions of lymphoedema treatment are to improve lymphatic fluid flow, decrease recurrent infections and to reduce 'acute attacks' (acute dermatolymphangioadenitis). Acute attack comprises inflammation of local skin, lymph nodes, and lymphatic vessels and is characterized by fever, confusion, headache, drowsiness, vomiting, fever, shaking, chills and soreness of the lymph glands. This may occur several times each year and results in severe pain, oedema, and exacerbation of physical disability (17). Penicillins and cephems are recommended for the management of acute attacks (18).

Pharmacological therapies include diuretics, benzopyrones, antimicrobial agents, and traditional medicines. Diuretics enhance the excretion of body fluids as urine but are effective only in some patients. Oral benzopyrones may help in dissolving tissue protein and facilitating lymph transport (18,19). Lymphatic filariasis is treated with anti-filarial drugs including diethylcarbamazine, ivermectin or albendazole (20,21). The treatment approach for podoconiosis lymphoedema involves foot hygiene, regular use of emollient and/or antifungal agents, and use of shoes and socks are recommended (22). Furthermore, a recent study finding outlines washing with 2% glycerine in soaking water as an alternative

treatment (23).

In earlier experiments, the benzopyrones were shown to be very useful in the treatment of chronic and acute lymphoedema by reducing high protein oedema, enhancing the lysis and removal of abnormal accumulated proteins from the affected parts and increasing glucose uptake by cells to keep them viable in severe conditions (24,25).

1.2. Overview of inflammation in lymphoedema

Inflammation is a local defensive response of cells/tissues to substances that cause allergic or body irritation due to chemicals, injury, and/or infections which are characterized by painful area, hyperthermia, reddish skin colour, swelling, and none- functioning cells/ tissues. These features are due to blood vessels dilations and resulting in the flow of white blood cells and micro nutrients into the inflamed areas (26). A localized inflammatory response typically happens in response to tissue damages which take in production of local mediators such as kinins and arachidonic acid metabolites, and release of histamine from mast cell. These local mediators are responsible for capillary permeability, fluid accumulation in the tissue and increased local immune cells access to the area. Another intracellular activator of inflammation is high mobility group box 1 protein (HMGB1), due to dead tissue and are responsible for the attraction of neutrophils and macrophages to the damaged area, and increases blood flow (27).

The lymphatic and immune systems are closely interconnected; and the lymphatic vessels are active structures that reasonably react to inflammatory stimuli. During the acute inflammation, the lymphatic drainage increases to compensate the fluid leakages from blood vessels (28). When the collateral lymphatic system is unable to balance the fluid leakages, there will be persistence of fluid accumulation in the interstitial space that ultimately leads to the abnormal functional change which result in lymphoedema (29).

In lymphoedema, chronic interstitial fluid accumulation due to damage of the lymphatic system results in inflammatory cascades and adipose cell differentiation activation which leads to progressive inflammation, and this change is the most important in the pathophysiology of lymphoedema. Formation of fibrosis and chronic inflammation are the hallmarks of lymphoedema. According to recent studies, most inflammatory cells that present in lymphoedematous tissues express the cell surface receptor CD4 which contains many different mature cell types such as T-helper cells, natural killer cells, and T-regulatory cells. T-helper-1 cells reactions occurs in response to acute inflammation and helps in preventing microbial infections by producing cytokines such as interferon-gamma while T-helper-2 cells have an vital role in response to parasite infection and shown to promote collagen deposition and fibrosis by secreting profibrotic cytokines such as interleukin 4 (IL-

4), interleukin 13 (IL-13), and transforming growth factor beta-1 (TGF-B1) in lymphoedematous limbs (Figure 1.3) (29,30).

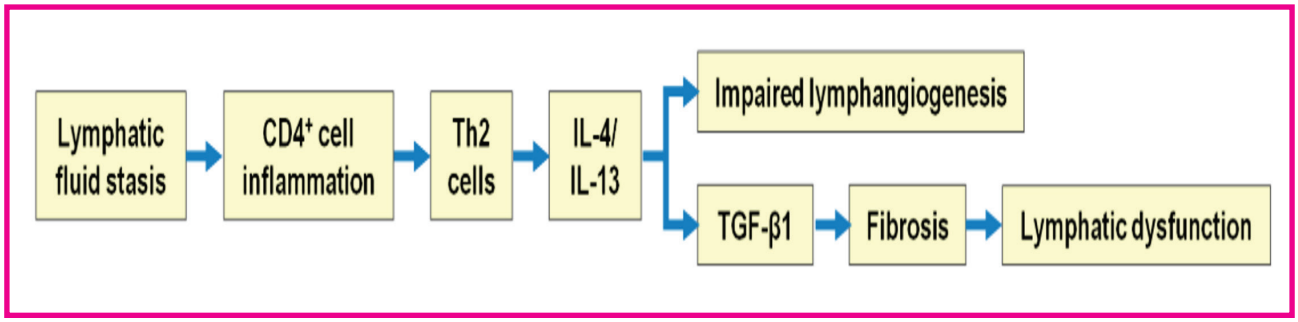


Figure 1.3. Pathophysiologic sequence following CD4+ cell inflammation in lymphoedema (29).

In lymphatic injury, there is an accumulation of macrophages and favoured differentiation of macrophage type 2 (M-2) in response to IL-4 and IL-13 secretion which have significant importance in inflammation and fibrosis. In different studies, macrophages are shown in production and activation of TGF-β1 and the depletion of macrophages in mice tail model study significantly increases fibrosis (29). Macrophages regulate CD4+ accumulation and subsequent T-helper-2 differentiation directly or indirectly, which shows that macrophages as the opposing purposes of fibrosis. Macrophages are also sources of IL-6 cytokine which have an important role in chronic inflammation and adipose tissue accumulation. Increase in cellular response of IL-6 in mouse models of lymphedema shown to reduce adipose accumulation. In addition, macrophages help expression of inducible nitric oxide synthase (iNOS) which play a role in decreasing lymphatic vessel constriction in inflammation and supports lymphatic vessel pumping (29,31).

1.3. Wound infection overview in lymphoedema

Wound is the damage of cellular and anatomical function of a tissue with or without infection (32).Wound healing is a usual physiological response to an injury and starts healing from the time of tissue damage and continues for some periods of time depending on the degree of the wounds (Figure 1.4). It is an orchestration of complex biochemical process of interrelated actions mediated in a closely organized process by different chemically organized cellular and hormonal processes. Wounds are classified as open and closed based on the causes of wound creation, and acute and chronic based on the physiology of wound healing (33).

Generally, regardless of the cause of the wound, the wound healing involves homeostasis, inflammation, tissue formation, and tissue remodelling phases. Homeostasis promotes vasodilation by production of nitric oxide and histamine to facilitate entry of inflammatory cells to the wound area, and blood clotting to prevent further blood loss. The inflammatory phase involves vasoconstriction, platelet aggregation and subsequent vasodilation and phagocytosis. It starts immediately after the injury and usually lasts from one to two days, and sometimes beyond this up to two weeks. The proliferative phase lasts from two days to two weeks and involves granulation, contraction, and epithelization. The remodelling phase might last for three weeks to two years. It involves the formation of new collagen and increased tissue tensile strength (34). However, factors such as uncontrolled physiological processes in response to environmental stimuli, the environment itself, and patient's health status can change the healing process of wounds. Poor venous drainage, inadequate blood supply, increased skin tension and infection are some of the local factors that impair wound healing (35).

Fig 1. 4 - Contaminated wound infection of lymphoedematous limb – picture taken during wound swab collection from East Wollega, Ethiopia

Patients with lymphoedematous legs have a high chance of getting microbial infections due to skin break downs. As a result, the subcutaneous tissue is exposed to microorganisms to create and becomes conducive to micro-organisms multiplication. Furthermore, wet patches in the interdigital spaces and fissures are usually associated with micro-organisms. There is also slow removal of micro-organisms from these areas due to non-functional lymphatic system and results in recurrent infections (36). There are several different types of infection that a lymphoedema patient may experience. These are bacterial and fungal infections. Lesions of the skin of lymphoedematous limb favour the entry of micro-organisms into the tissues. Micro-organisms may invade the epidermis and subcutaneous tissues and results in swelling, erythema, and severe pain. Patients with secondary lymphoedema are more prone to develop cellulitis (37).

Micro-organism infection occurs when there is an invasion of soft tissue through small abrasions on the skin surfaces. Cellulitis is the most frequently found bacterial infection of the skin that is associated with inflammation and affects the skin surface and/or the subcutaneous tissues. Most microbial infections are due to different groups of streptococci (A, C, or G) and *S. aureus* (38). In addition, Streptococci species, Staphylococci species, Pseudomonas species, and Bacteroides species are known to cause chronic wound infection and cellulitis (39). Fungal infections are also reported due to moist skin development in between the skin folds which leads to infection in the lacerated regions (40).

1.4. Treatment of wound inflammation and infection in lymphoedema

In order to protect wounds from contamination and to accelerate wound healing, wound dressing is recommended in open wound infections. Synthetic and natural materials have been utilized with different formulations such as sponges, hydrogels, hydrocolloids, hydro-fiber mats to create a favourable environment and facilitate wound healing. In addition to this, systemic antimicrobial agents are recommended to remove spreading of infections and for treating uninfected wound beds (41).

Antibacterial agents are also given usually for overlaid infections such as cellulitis and lymphangitis. They are usually recommended during acute attacks of cellulitis and should be prescribed after laboratory-based diagnosis is made. Prophylactic broad-spectrum antibacterial agents are prescribed for recurrent attacks. Similarly, prophylactic antifungal therapy is recommended to prevent fungal infection (42).There are many classes of antibacterial drugs used for the management of wound infections. However, quinolones, tetracyclines, aminoglycosides and cephalosporins are preferred for treatment. These classes of antibacterial drugs act by either inhibiting bacterial cell wall synthesis, blocking key metabolic pathways, or interfering with protein or nucleic acids synthesis. However, most bacterial species are resistant to at least one commonly used antibacterial drugs (41).

Oral amoxicillin (500 mg) every eight hours is the first line of treatment for the management of the acute attack of cellulitis. Erythromycin (500 mg) every six hours or clarithromycin (500 mg) every twelve hours is recommended for patients allergic to penicillin. If there is no response to the first-line agents, clindamycin 300mg four times a day is recommended as a second-line oral treatment. Antibacterial therapy shouldn't be interrupted until all symptoms of acute inflammation are removed. Furthermore, paracetamol is prescribed as an analgesic agent for the treatment of inflammation (43).

Prophylactic antibacterial drugs are prescribed for those who have repeated attacks of cellulitis. Penicillin (250 mg) twice a day for two years is the first choice for prophylaxis, and for those hypersensitive to penicillin, erythromycin (250 mg) twice a day is recommended. If this is not tolerable clarithromycin 250 mg per day is an alternate treatment. Decision in

the use of prophylaxis antibacterial drugs for patients at risk of repeated infection should consult a physician, microbiologist, and pharmacologist to reduce the emergence of antimicrobial resistance (44).

1.5. Herbal Medicines in the management of inflammation and wound infection

Traditional medicines (TM) are widely used all over the world. In Africa, greater than 80% of people use folk medicines for their health care needs (45). In Ethiopia, the trend is similar, where traditional medicine has been in use since ancient time and is culturally integrated in all societies (46). In Ethiopia, it is assumed that about 80% of the rural community and around 90% of the livestock relay on folk medicine to meet their primary healthcare needs (47). Ethiopia has a long and various history of traditional medicine practices. Ethiopia is a home of many plant species (>7000 higher plant species), and about 12% are endemic plant species, making it a rich source of useful plant compounds for human health (48).

Medicinal plants have attracted global attention in the search for effective bioactive agents that can be used for the management of many diseases including for wounds care and limb swelling. Plant materials have historically been used as teas and tonics for the treatment of ailments by indigenous societies (49). Nowadays, most medicinally active compounds are initially isolated from natural products and become the basis for synthetic drugs, so most clinically used drugs have originated directly or indirectly from plants (50).

Use of medicinal plants, with known pharmacological activities, can be of great importance in the treatment of infections and inflammations associated with wounds. In the last decade, many studies have been done to show the efficacy of medicinal plants in a wide range of countries. Because of the emergence of resistance from micro-organisms, health care workers have gone back to investigate ancient healing methods with traditional and alternative medicines (51,52).

Synthetic medicines used for the management of pain and inflammation have a variable range of toxicity and side effects and are expensive and time-consuming to develop. Medicinal plant constituents have been shown to have anti-pain and anti-inflammatory activities (53). Thus, there is a great need to use medicinal plants extracts and compounds from natural sources as alternative medicine since they have been used for centuries in the treatment of pain and inflammation. Most of them are acting in a similar mode to non-steroidal anti-inflammatory drugs (NSAIDs) on the inflammatory pathways (54).

Likewise, there is an evidence for the use of medicinal plants for wound healing which suggests that they may be cost-effective substitutes for wound healing (55). Herbal medicines are known to make the wound area moist to facilitate healing. As modern health care systems cannot cover all the health requirements of the entire population, traditional

therapy and herbal medicines may offer an option for the treatment of wounds and other conditions (56).

As a result, these medicinal plants will have immense importance in limb care (lymphoedema). The following are some of the Ethiopian medicinal plants that have anti-inflammatory, wound healing, and anti-infective effects.

1.5.1. *Cymbopogon citrates* (Poaceae)

Cymbopogon citrates (Figure 1.5) is a native plant in tropical areas of Asia and cultivated in South and Central America, Africa, and other tropical countries. It belongs to the family of Poaceae. It is usually called lemongrass and in Ethiopia locally named as 'Tej-sar'. It grows to the height of 1.8 meters and about 1.2 meters in width and has a short rhizome. It is an aromatic perennial and most important medicinal plant. It has been used extensively as a medicinal, cosmetics and nutrition for centuries. Studies showed that it has been used as antimicrobial, anti-inflammatory, anti-oxidant, anti-cancer, anti-pain and analgesic remedies (57). Pharmacologically, it is reported to have anti-inflammatory, antimicrobial, antioxidant, anti-diarrheal, anti-mutagenic, anti-malarial, anti-nociceptive, hepatoprotective effects (58), to increase lymphatic circulation, detoxification and blood circulation and to activate T-lymphocytes (59). In animal studies, a similar genus of *C. Citratus, Cymbopogon aurantifolia* essential oil exhibited anti-inflammatory effects and significantly modulated expression of genes that are important for inflammation and tissue remodeling processes (59,60).

Figure 1.5: Cymbopogon Citiratus - Photo taken during collection of the plants at Joy Tech Ethiopia herbal farm in Bishofitu

1.5.2. *Trachyspermum ammi*

Trachyspermum ammi L. (Apiaceae) known as 'nech azmude' in Ethiopia is an important medicinal plant, which is originally from Egypt and found in different regions of the world (61). It is one of the most useful herbal plants for the management of human diseases in Ayurveda medicine. It has been used for flavouring foods, as preservatives, in medicine and perfumery. It has been shown to have anti-inflammatory, anti-pain, antimicrobial, anti-filarial, and hepatoprotective activities. The major essential oil constituents are carvone (46%), limonene (38%), and dillapiole (9%) which are known for the pharmacological activities of the medicinal plant (62).

1.5.3. *Croton macrostachyus* (local name 'Bisana')

Croton macrostachyus (Euphorbiaceae) is a useful medicinal plant in Ethiopia, Kenya, and Tanzania. It has diverse medicinal properties for humans and animals. The pharmacological activities of this plant include anthelmintic, antibacterial, anticonvulsant and sedative, antifungal, anti-inflammatory, antileishmanial, antioxidant, and larvicidal activities (63). Leaves and fruit extracts of *Croton macrostachyus* have activity against most bacteria (63). Methanol extracts from the stem bark of *C. macrostachyus* have anti-inflammatory and anti-nociceptive activity (63).

1.5.4. *Achyranthes aspera*

Achyranthes aspera (Figure 1.6) known as 'telenje' in Ethiopia is one of the most traditionally used medicinal plant in Ethiopia and India. It has been studied for its remedial characteristics and reported to have immunostimulatory properties, wound healing, antioxidant, anti-inflammatory, antibacterial and antifungal activities (64). It belongs to the family of Amaranthaceae, genus – achyranthes, species – Aspera, and grows to about 1-2 meters high. The plant grows in moist soil and prefers medium sandy, loamy and clay soils (65). Traditionally it is used to treat asthma, bleeding, boils, bronchitis, colds, gastrointestinal problems, debility, dropsy, dog bite, dysentery, ear complications, headache, leucoderma, renal complications, scorpion and snake bites, skin diseases (13). Similarly, the leaf extract of *Achyranthes aspera* is traditionally used in Ethiopia for the management of skin conditions and for bleeding during injury. It has been also reported that *A. aspera*, had antibacterial activities against *E. coli, P. aeruginosa, S. aureus, S. boydii* and *S. typhi* and it was safe to laboratory animals at doses up to 2000 mg/kg (66).

Figure 1.6 - Achyranthes Aspera - Photo taken during collection

1.5.5. *Lawsonia inermis* (Lythraceae)

Lawsonia inermis (Figure 1.7) is widely distributed in Africa, including Ethiopia. It is a well-known plant which has been used for cosmetics and as a medicinal plant for over 9,000 years. *Lawsonia inermis* usually called Henna and belongs to the Lythraceae family. It grows up to 2–6 meters with spine-tipped branchlets. Henna grows in a semi-arid, frost-free zones and poor soil types (67). The plant is well known worldwide as a cosmetic agent. It is stated to contain phytoconstituents such as Lawsone, Esculetin, Fraxetin, Isoplumbagin, Scopoletin, Betulin, Betulinic acid, Hennadiol, Lupeol, Lacoumarin, Laxanthone, Flavone glycosides and two pentacytic triterpenes (68). The constituents of the plant have been shown to have analgesic, hypoglycemic, hepatoprotective, immunostimulant, anti-inflammatory, antimicrobial, antiparasitic, antioxidant, antifertility, tuberculostatic and anticancer activities (68).

Figure 1.7 - Lawsonia inermis - tree and leaves – picture taken during collection.

1.5.6. *Azadirachta indica* (Meliaceae)

Azadirachta indica, (Figure 1.8) is a family of Meliaceae and usually known as neem. It grows in tropical and semi-tropical regions of the world, and its different parts are widely

used for different purposes (69). *A. Indica* A. Juss has complex and different phytoconstituents including nimbin, nimbidin, nimbolide, and limonoids which are important in disease management. The first flavonoid isolated from the leaves of *A. Indica* A. Juss are Quercetin and ß-sitosterol which have antimicrobial activities (70). *A. Indica* A. Juss has been shown to have different pharmacological actions including analgesic, anthelminthic, antibacterial, anti-yeast, antiulcer, antifertility, anti-filarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, anti-nematode, antipyretic, antispasmodic, insecticidal, antitumor, anti-hypercholesteraemic, hypoglycaemic and immunomodulatory effects (71). Microbial cell wall breakdown, inhibition of pro-inflammatory enzyme, cyclooxygenase (COX) and lipoxygenase (LOX) enzymes are suggested mechanism of action of *A. Indica A. Juss* (72).

Figure 1.8 - Azadirachta Indica – Photo taken during collection

1.5.7. *Moringa oleifera* (Moringaceae)

Moringa oleifera (Figure 1.9) is a deciduous tree which grows up to 10 meters height and usually smaller, pale feathery foliage. It is called Aleko (Konsogna), Shiferaw (Amargna) and Kalan'gi (Hamer-Bena) in Ethiopia. It is originating from India & Arabia and was introduced to Ethiopia a long time ago. The tree is now growing in many parts of southern Ethiopia. It is the most abundant plant in Ethiopia and distributed worldwide. It has been used for food, medicine, cosmetics, and animal feed. It has been studied for a wide range of disease conditions and shown to have antioxidant, anti-inflammatory, hepatoprotective, antibacterial, for management of hypercholesterolemia and cancer. Vitamins (A and C), flavonoids (myrecytin, quercetin and kaempferol), phenolic acids (gallic acid, chlorogenic acid, caffeic acids), alkaloids, glucosinolates, isothiocyanates, tannins and saponins are the main constituents of *Moringa oleifera.* Flavonoids, tannins and phenolic acids are plant constituents play major role for the major pharmacological activity of the plant (73).

Figure 1.9 - Moringa oleifera tree, tree leaves, and leaves powder (73)

1.5.8. *Thymus vulgaris* (Lamiaceae)

Thymus vulgaris (Figure 1.10) also known as 'tosegne' in Ethiopia, is in the family of Lamiaceae, native to southern Europe. It is cultivated in warm areas and planted in the spring (74). The phytochemical components of these plants are phenols, terpenoids, thymol, eugenol, and saponins. The major constituent essential oil identified from the plant was thymol while the amount of all other constituents were less than 19% (75). *T. Vulgaris* essential oil is used in the food, pharmaceutical and perfumery industries as a flavoring agent. It has also been reported to have anti-inflammatory, anti-nociceptive, antimicrobial, antioxidant, and anti-spasmodic activities. In addition, it is shown to have insecticidal activity and beneficial effects in behavioral disorders (75). Formulated as a semi-solid, it has shown antibacterial activity against major pathogenic microorganisms causing skin diseases (76,77). In addition, *Thymus vulgaris* essential oil was reported to be effective for topical skin infections in dogs (78).

Figure 1.10: Thymus Vulgaris – Photo taken during the visit of Joy tech Ethiopia herbal farm in Bishofitu.

1.5.9. *Aloe vera* (Liliaceae)

Aloe vera (Liliaceae) is known as 'eret' in Ethiopia. It grows usually in the dry parts of all regions. It has been used for medicinal purpose in different cultures. The gel of *Aloe vera*

reported to heal ulcer and burn wounds by forming protective cover on the affected area, and the various constituents of *Aloe vera* promotes wound healing and have anti-inflammatory activity. Anthraquinones (aloin and emodin) are components of the gel of *Aloe vera* which possess analgesic, antibacterial, antifungal, and antiviral activities. Tannic acid and polysaccharides which are the constituents of the plant material helps wound healing by reducing the inflammatory phase and promotes maturation of granulation tissue which finally promotes healing. The wound healing property was attributable to indoles, and alkaloids. Whereas, the antibacterial property of *Aloe vera* was due to its constituents, phytosterols and indoles (79). Besides, *Aloe vera* inhibits the COX-1 and COX-2 enzyme pathway and reduces prostaglandin E2 release from arachidonic acid. C-glucosyl chromone, a novel anti-inflammatory compound was isolated from gel extracts (80).

1.5.10. *Aloe Trigonantha*

Aloe Trigonantha (Figure 1.11) belongs to a group of Aloes that often have secondary branching and are usually stemless (81). Locally it is called 'Eret' and it is one of the Aloe species which is endemic to Ethiopia. The species grow on dry stony ground near roads and along field margins between 1900 to 2100 meters above sea level in Gonder and Gojam regions (82). Like other species of Aloe, people are using it extensively for the management of infectious and inflammatory diseases (83).

Figure 1.11: Aloe trigonantha – Photo taken during collection of the plant

1.5.11. *Acokanthera schimperi*

Acokanthera schimperi (Apocynaceae) (Figure 1.12) is called "merenz" in Ethiopia. It is a well-known East African arrow poison plant (84). It is a shrub up to 9 meters height, densely branched; young branchlets glabrous or pubescent, angular and ribbed (85). It usually grows at 1,100 - 2,400 meters above sea level (86). The bark, wood and roots are the parts of the plants used for arrow poison, and they are also used for suicide and homicide (87). In Ethiopia, the leaves and bark are used to treat skin disorders, and leaves were used to treat tonsillitis. In Kenya, Samburu women drink a bark decoction to stop menstrual

bleeding. It is used to treat sexually transmitted diseases, and as an aphrodisiac in Kenya and Tanzania (85). The dried, pulverized leaves are taken with honey as an antifertility medicine. A mixture made from the leaves, bark and butter is used for gall-bladder problems. In addition to these, *Acokanthera Schimperi* is indicated for headache, epilepsy, amnesia, eye disease, syphilis, rheumatism, elephantiasis, scabies and leprosy traditionally in Ethiopia (84).

Figure 1.12: Acokanthera Schimperi – photo taken during collection and drying the leaves of the plant

Based on the finding of the systematic review (Chapter Three), three medicinal plants namely, *Lawosinia inermis, Achyranthes aspera* and *Azadirachta indica* were selected to evaluate the anti-inflammatory, wound healing, and anti-infective activity of the crude extracts in *in vitro* model. These medicinal plants are well known ethnopharmacological names which is an implication of their traditional use in the management of different aliments.

The general hypothesis is that bioactive components from Ethiopian medicinal plants can lower the risks of lymphoedematous limbs complication by promoting wound healing, through inhibition of microbial growth and multiplication, and via inhibition of inflammation.

1.6. Aim and Objectives

The aim of this study was to investigate the potential role of Ethiopian medicinal plants extracts for lymphoedema care. The first phase of the research work was to identify plants with potential topical anti-infective, anti-inflammatory, and wound healing activities by conducting a systematic literature review, presented in Chapter Two. The aim of the review was to evaluate Ethiopian medicinal plants found to have anti-infective, anti-inflammatory, or wound-healing properties in *in vitro* and *in vivo* studies.

The second activity of the study was to evaluate the pharmacological activity of selected plant extracts using different *in vitro* models. These are cytotoxic, anti-inflammatory, wound healing, and anti-bacterial activities of selected Ethiopian plant extracts in *in vitro* models.

Based on the systematic review findings, three medicinal plants were selected for pharmacological investigation. These plants were selected based on prior reports of their activity against multiple conditions of lymphoedema and their traditional utilization for inflammation and wound infection.

Under the second activity there are three specific objectives. The first objective was to identify micro-organisms associated with lower limb lymphoedema and evaluate the antibacterial activity of selected medicinal plants against the most important ones, which is presented in Chapter Three. The second objective was to investigate *in vitro* cytotoxicity assay of the extracts, *in vitro* anti-inflammatory, and wound healing activity of selected Ethiopian medicinal plant extracts, which is presented in Chapter Four. The last activity was to conduct compound isolation and characterization. Extracts which demonstrated good activity and safety were further subjected to fractionation and compound isolation to characterize their biomolecules, presented in Chapter Five.

CHAPTER TWO

2. ETHIOPIAN MEDICINAL PLANTS USED FOR THEIR ANTI-INFLAMMATORY, WOUND HEALING AND ANTI-INFECTIVE ACTIVITIES – A SYSTEMATIC LITERATURE REVIEW

2.1. Background

Herbal medicines are commonly used worldwide as alternative and complementary treatments for a range of conditions; and are widely accepted across various cultures and socio-economic levels. They have immense importance to the well-being of individuals and societies and their medicinal values arise from the active substances produced by the plants as secondary metabolites that produce physiological changes in the human body (53). Scientific interest in medicinal plants is currently high due to searches for 'lead' molecules from plants. Most of the drugs used against different disease conditions are derived from natural products or structures suggested by natural products ('leads') (88).

In search of improved, safe, effective, and affordable drugs, plant-derived products represent an attractive option (89). Herbal products are relatively safe, chemically complex mixtures composed of a range of constituents with multiple potential targets and different mechanisms of action (90). Phytochemicals found in certain herbal extracts are reported to have analgesic and anti-inflammatory properties (53). Most of them act by a similar mechanism to non-steroidal anti-inflammatory drugs (NSAIDs) on inflammatory pathways (54). Similarly, there is evidence for use of medicinal plants for wound healing which suggests that they may be cost-effective promoters of wound healing (55).

In Ethiopia, the use of folk medicine has been in place in rural areas for many years, and many Ethiopian medicinal plants are claimed to have anti-inflammatory and wound healing activities (56). Phytochemical and pharmacological investigations of endemic plant extracts for the care of wounds and swelling caused by bacterial infections have shown anti-inflammatory and diuretic activities in different models (51,52).

Thus, there is a need to investigate the potential therapeutic merits of Ethiopian endemic plants in different disease conditions. One of these is lymphoedema management, which includes limb care, and is assumed to reduce acute attacks of lymphoedema, to treat infections and associated wounds. Lymphoedema is a condition due to damage of the lymphatic drainage leading to accumulation of protein-rich fluid in the interstitial spaces. It is classified as primary or secondary lymphedema. Primary lymphoedema arises from genetic disorders while secondary lymphoedema from damage to the lymphatic system, due to lymphatic vessel infestation, lymphadenectomy, or radiotherapy in cancer patients

(2,91). Filariasis is the main cause of tropical lymphoedema, followed by podoconiosis (non-filarial elephantiasis) (91). Recurrent bacterial cellulitis can also result in lymphoedema (8).

The treatment of lymphoedema aims to improve lymph drainage and reduce 'acute attacks' (acute dermatolymphangioadenitis) which can be achieved through a range of approaches. An acute attack is an inflammation of local skin, lymph nodes, and lymphatic vessels characterized by very high fever, confusion, headache, drowsiness or vomiting, fever, shaking, chills, and soreness of the lymph glands (17). A pharmacological approach is one option to treat acute inflammations and infections. Traditional medicines are widely used in the management of pain and infections (89).

Many *in vitro* and *in vivo* studies have been conducted on the safety and efficacy of Ethiopian medicinal plants against inflammation, and infection to facilitates wound healing. However, data on the efficacy and safety of these medicinal plants in the management of limb conditions have never been summarized. This systematic review will, therefore, draw together up-to-date information on Ethiopian medicinal plants used as anti-inflammatory, wound healing, and anti-infective agents that might potentially be employed for limb care (lymphoedema and associated wounds).

This systematic literature review aims to evaluate Ethiopian medicinal plants found to have anti-infective, anti-inflammatory, or wound-healing properties in *in vitro* and *in vivo* studies, by answering the following questions:

- In *in vitro* and *in vivo* (animal) studies, do Ethiopian medicinal plants have anti-infective, anti-inflammatory or wound healing activities compared to conventional treatments or placebo?
- Which medicinal plants secondary metabolites have been studied for anti-infective, anti-inflammatory and wound healing activities?
- What experimental models are usually used to study the efficacy of medicinal plants and their compounds?

In the context of this review terms are defined as follow:

'Ethiopian medicinal plants' refers to plants found in Ethiopia and have been utilized traditionally for medicinal purposes by societies in Ethiopia and elsewhere.

'In vitro studies' refers to studies conducted by inducing disease conditions such as inflammation or scratch wounds in commercially available human and animal cell lines in a controlled environment. Similarly, *in vitro* anti-infective activity tests involve direct culture of

the micro-organisms in media and application of plant extracts to the media to evaluate their activity.

'In vivo studies' refers to studies conducted using laboratory animals such as mice and rats in an experimentally controlled environment. In this review, *in vivo* studies will not include human studies (clinical trial studies).

'Anti-infective agents' refers to agents (medicinal plant extracts, fractions and/or compounds) that act against infective agents (bacteria, fungi and others) either by inhibiting the agent's growth or by killing it.

'Anti-inflammatory agents' refers to agents (medicinal plant extracts, fractions and/or compounds) that reduce inflammation (redness, swelling, and pain) and/or decrease the production of inflammatory and pro-inflammatory cells in experimentally-induced inflammation in laboratory animals and/or cell lines, respectively.

'Wound healing agents' refers to agents (medicinal plant extracts, fractions and/or compounds) that promote wound contraction, reduce time of epithelization and increase wound tissue tensile strength in experimentally-induced incision and excision wounds in laboratory animals and/or agents that promote cell migration and proliferation in experimentally-induced wounds in cell lines.

'Cell lines' refers to a cell culture selected for uniformity from a cell population derived from a usually homogeneous tissue source that can grow in a suitable nutrient culture media in a controlled laboratory environment.

'Laboratory animals' - in this review, laboratory animals refer to mice and rats. Laboratory mice are small mammals of the species of *Mus musculus*, order *Rodentia* which are in-breeds and used for scientific research. Similarly, rats are belonging to the species *Rattus norvegicus* domestica, which are in-breeds kept for scientific research.

2.2. Methods

Reporting Items for Systematic Reviews and Meta-analysis guidelines were followed for conducting and reporting this systematic literature review (92). The protocol for this review has been registered on PROSPERO with a registration number of CRD42019127471.

2.2.1. Study design

This review considered all controlled *in vivo* and *in vitro* studies conducted on anti-inflammatory and wound healing activities; as well as *in vitro* anti-infective studies evaluating the efficacy and safety of Ethiopian medicinal plants. The components population, exposure (intervention), comparator and outcome (PICO) of this review are as follows:

Study subjects: Laboratory animals (test and control groups) used for experimentally induced inflammation and wounds; cell lines used in *in vitro* model for anti-inflammatory and wound healing assays; and micro-organisms (bacteria and fungi) which were used for anti-infective efficacy tests of medicinal plants.

Intervention: Medicinal plants as whole plants or their adjuncts: seed, root, flower, bud and leaf extracts used in the experimental groups; and conventional drugs and placebo used in control groups. Medicinal plants used were manufactured single or complex medicinal plants, plant extracts, and plant preparations used regardless of their preparation (extracts, decoctions, tablets, capsules, pills, powders, injections or other types of preparations), but not synthesized compounds. There was no restriction on dosage form, concentration, frequency of administration, dose, intensity or duration of medicinal plants used.

Comparator: Placebo (no intervention) and/or conventional (reference) drugs used for treatment of controls.

Outcomes: The primary outcomes were the rate of response to treatment, such as changes in frequency and intensity of symptoms in the intervention and placebo groups. The primary outcomes analysed were the efficacy of medicinal plants (at safe concentrations) in reducing symptoms of inflammation (in experimental animals), in wound contraction and healing (in experimental animals), and in inhibiting or killing microbial growth (in culture media) in comparison with conventional drugs. In addition, in *in vitro* studies, downregulation of inflammatory and pro-inflammatory cells; cell proliferation and migration rate (wound healing) in µg/ml and mg/ml; minimum effective dose in 50% of the population (ED50), and minimum lethal dose in 50% of the population (LD50). Secondary outcomes were data such as liver function tests, organ histopathology results (as a surrogate indicator for long-term toxicity), and mortality.

2.2.2. Eligibility criteria

Inclusion criteria: Published works including thesis, articles and proceedings which deal with efficacy evaluation of antibacterial, antifungal, anti-inflammatory and wound healing activities in *in vivo* and *in vitro* studies.

Exclusion criteria: Newspapers and reviews

2.2.3. Information sources

Searching was conducted in electronic databases using a combination of free text keywords and Medical Subject Heading (MeSH) terms related to Ethiopian medicinal plants investigated to have anti-inflammatory, anti-infective and wound healing activities. Scopus, EMBASE, PUBMED/MEDLINE, and Google Scholar were used as sources of information for the search. Grey literature such as theses, technical reports, working papers, evaluation reports,

conference proceedings, patents, preprints was also included in the review.

2.2.4. Search strategy

The search strategy included all articles containing the descriptors published until June 28, 2019. Only articles written in English were used for this study. The search strategy included all articles containing the descriptors. Structured search strategies were developed using the vocabulary terms of each database and targeting the "title" and "abstract" fields. We also manually searched using the references of previously published works and databases of ongoing trials. The following search terms were used: Ethiopia, medicinal plants, Ethiopian medicinal plants, herbal products, care, management, therapeutic, lymphoedema, lymphedema, swelling, podoconiosis, elephantiasis, wound, wound healing, inflammation, anti-inflammatory, bacteria, anti-bacterial, fungi, anti-infective, antimicrobial, anti-fungal and other related words or phrases.

2.2.5. Selection of studies

After electronic searching, the records were uploaded to Mendeley. Some studies were piloted before undertaking full study selection. All studies were screened independently by two investigators (DNW and TBT) by scanning the titles and abstracts of the articles based on the inclusion criteria. For the documents that fit the inclusion criteria, the investigators read the entire article to confirm if it met the criteria and prepared to extract relevant information. Disagreements were resolved by discussion between the two investigators.

2.2.6. Data extraction

Two reviewers (DNW and TBT) independently extracted data using a data extraction form and summarized experimental works including study types. We did a calibration exercise before starting the review to ensure consistency across the reviewers. The following data were extracted: title, author, year of publication, type of study (*in vivo* or *in vitro*), statistical methods used, type of animals used, age and weight of animals, study duration, number of animals used for the study; route and time of administration; dose/kg, type of micro-organisms used for the study (clinical isolated or reference strains), reference drugs used, minimum inhibitory concentration (MIC) of extracts/fractions (µg/ml, mg/ml), zone of inhibition (mm) techniques used to induce inflammation or wounds, concentration at which inflammatory cells were down regulated or inhibit paw oedema formation, concentrations that inhibit microbial growth of the extracts/fractions, minimum concentration that are toxic to 50% of cells (CC_{50}), type of solvent extracts and fractions used for activity and safety, parts of plant used, extraction type, sources of the plants, place of collection, traditional use, scientific names of the plants, local names of the plants, voucher numbers, types and number of compounds isolated (if any). When individual studies have multiple treatment groups we combined the groups to avoid the possibility of introducing bias caused by

multiple statistical comparisons with one control group (92).

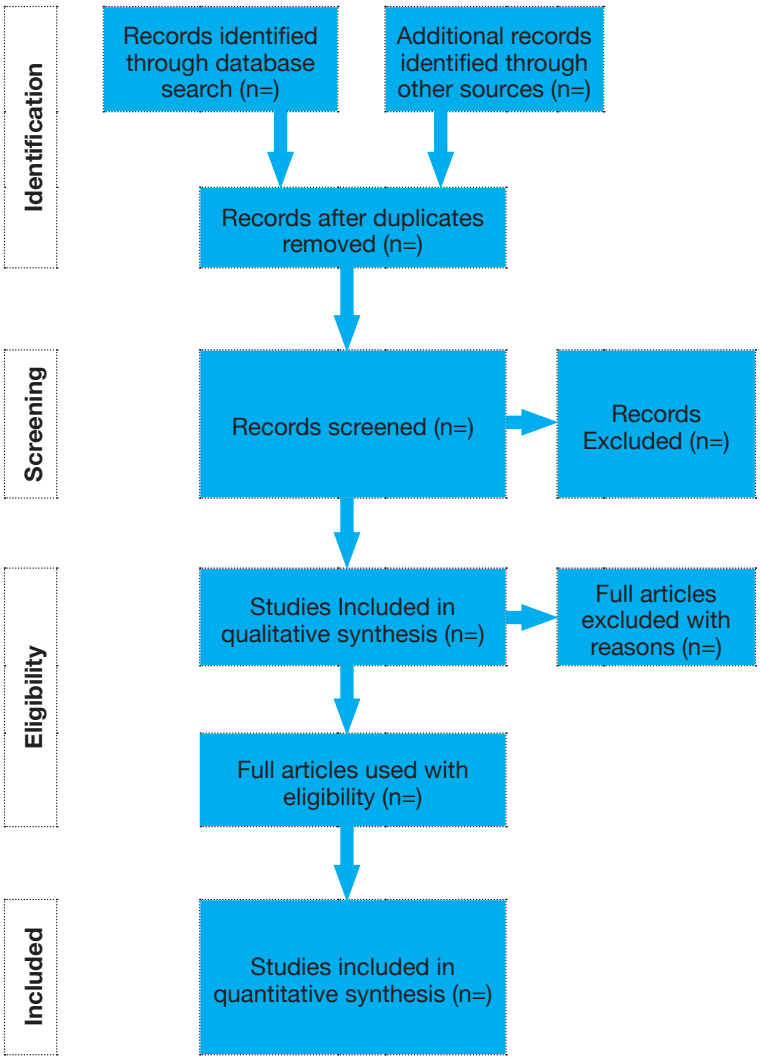


Figure 2.1: Flow diagram of study selection process (92)

2.2.7. Outcomes measured

For the *in vivo* studies of anti-inflammatory activity, the main outcomes were the number of animals responding to treatment with Ethiopian medicinal plants, defined as percent inhibition of carrageenan-induced oedema and/or percent inhibition of the weight of granuloma tissue formation relative to the controls. In *in vitro* studies of anti-inflammatory activity, the primary outcomes were percent inhibition of inflammatory cells and pro-inflammatory cells, defined as percent inhibition of the enzyme lipoxygenase, % inhibition of protein denaturation, levels of inflammatory cytokines (TNFα, IL6, IL10, and IFN-γ) secreted by cells, level of anti–COX-2 antibody and concentration of nitric oxide (NO) in inflammation-induced cell lines after treated by plant extracts. The type of data (variables) extracted for anti-inflammatory activity, wound healing and anti-infective activities were

continuous (percentage and pg/ml, µg/ml).

In *in vivo* wound healing assays, the main outcomes were percent wound contraction, period of epithelization and percent tensile strength in experimentally induced wounds in laboratory animals; whereas for the *in vitro* wound healing assays, relative cell spreading and migration, percent cell proliferation and viability were the main outcomes. In *in vitro* studies of anti-infective activity, percent of inhibition of growth of micro-organisms, minimum inhibitory concentration, the concentration that inhibits 50% percent of the growth of micro-organisms (IC_{50}) will be the main outcomes. Secondary outcomes will be long term toxicity, death of animals and experiment dropouts.

2.2.8. Assessment of risk of bias

Two review authors (DN and TBT) independently assessed the risk of bias for each study included. The critical appraisal process for *in vivo* anti-inflammatory activity and wound healing was performed using the Risk of Bias tool for animal intervention studies (SYRCLE's RoB tool) (93) and Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines to assess the internal validity of the studies (94). These tools were used to assess the methodological quality of studies to generate reliable information and create transparency. Studies with highly unacceptable levels of bias were excluded. A highly unacceptable level of bias occurs when studies have serious errors in conducting, analysis, or reporting, have large amounts of missing information or discrepancies in reporting. For instance, in the randomization of experimental animals, if there is direct evidence that animals were allocated to study groups using a non-random method, studies were categorized as 'definitely a high risk of bias'. The judgment of bias will be categorized as yes, no, or unclear. A "yes" judgement indicates a low risk of bias; a "no" judgment indicates a high risk of bias; the judgement will be "unclear" if insufficient details are reported to assess the risk of bias properly. Studies were evaluated for their internal and external validity. Reviewers were judged the risk of bias for individual elements from five domains of bias (selection, performance, attrition, reporting, and other) using the SYRCLE's risk of bias tool for animal studies -Appendix D and decide the inclusion and exclusion of the studies (93).

The following criteria were used to assess the quality of individual *in vivo* studies:

- Systematic differences between study groups at the start of an experiment (selection bias) -

  - ✓ Did the investigators describe a random component in the sequence generation process (e.g., methods used for randomization of the animals)?
  - ✓ Balanced distribution of relevant baseline characteristics for the intervention and

control groups (e.g., age, sex, the weight of the animals).

- ✓ If relevant, did the investigators adequately adjust for unequal distribution of some relevant baseline characteristics in the analysis?

- Systematic differences occur in how the groups are handled during a study (performance bias)-

  - ✓ Adequacy of the timing of disease induction in both the test and control groups.
  - ✓ Experimental animals random housing to test and control group; and feeding conditions.
  - ✓ Was the allocation to the different groups adequately concealed during the study such as blinding of the caregivers and/or investigators from knowing which intervention each animal received during the experiment (labelling the cages and drug containers with codes).

- Circumstances during the experiment in both experimental and control groups

  - ✓ Timing of administration of the placebo and experimental extracts,
  - ✓ Instruments used to conduct experiments differ between experimental and control groups.

- Systematic differences occur between groups in how outcomes are ascertained, diagnosed, or verified (detection bias)

  - ✓ Was the outcome assessor blinded? If not blind, do review authors judge that the outcome is not likely to be influenced by lack of blinding?
  - ✓ Did the investigators randomly pick an animal during outcome assessment, or did they use a random component in the sequence generation for outcome assessment?

- Whether all animals receiving the same intervention are caged together, but the analysis was conducted as if every single animal was one experimental unit.
- Incomplete data (attrition bias)

  - ✓ Were all animals included in the analysis?
  - ✓ Were missing outcome data imputed using appropriate methods?

- ✓ Were missing outcome data balanced in numbers across intervention groups, with similar reasons for missing data across groups?

- Selective reporting (reporting bias)

  - ✓ Was the study protocol available and were all of the study's pre-specified primary and secondary outcomes reported in the current manuscript?
  - ✓ Was the study protocol not available but was it clear that the published report included all expected outcomes (i.e., comparing methods and results section).

- Other biases - unit of analysis errors, the inappropriate influence of funders and adding new lab animals to replace dropouts from the original population, etc.

The critical appraisal process for *in vitro* anti-inflammatory activity and wound healing was performed using the Guidance Document on Good *In vitro* Method Practices (GIVIMP) (95), and the Checklist for Reporting *In vitro* Studies guidelines (96). For the *in vitro* antibacterial studies, checklists for good practice for pharmaceutical microbiology laboratories (WHO) was customised and used for quality assessment (97,98). The following key criteria were used to assess the quality of individual *in vitro* studies:

- Assurance of the quality of all materials and methods, and of their use and application, to maintain the integrity, validity, and reproducibility of the laboratory work conducted.

  - ✓ Test definition (including purpose, need and scientific basis)
  - ✓ Laboratory reproducibility, validity, and international acceptance of the in vitro method (s)
  - ✓ Clearly written and well documented *in vitro* method description, and related Standard Operating Procedure(s)
  - ✓ Did the *in vitro* method(s) include all relevant and reliable positive and negative controls, including acceptance criteria?
  - ✓ SOP/ guidelines for cell culture maintenance, and safety practices for use and disposal of the test system, including transport and containments.
  - ✓ Relevant documentation of proof of sterility, date of arrival, expiry dates and batch numbers (as the suitability and acceptability) of laboratory consumables (materials)
  - ✓ Evidence of provision of relevant and adequate education and training for all

personnel, to promote high quality work and safety

- Are the *in vitro* cell and tissue culture facilities fit for purpose? Evidence of quality laboratory management maintained:

  - ✓ Was facility designed or adapted to minimise the risk of errors (e.g., mix-ups) and to avoid (cross-contamination) which may adversely affect the quality of the work performed
  - ✓ Was appropriate environment maintained for the type of work conducted in the laboratory (appropriate biosafety level)
  - ✓ Was there an appropriate documented procedure for disinfection of work surfaces, safety cabinets and equipment?
  - ✓ Any establishment and maintenance of adequate measures to protect individuals and the environment from any potential hazards

- Was equipment regularly maintained, monitored, and calibrated?
- Compliance of laboratory suppliers with good laboratory practice principles; whether test system providers were adhered to a formal quality system, such as International Standard (GMP, GLP, ISO standards).
- Evidence that the cell lines were free from any contaminants, indicate functionality, genetic stability, and identity; reference data to assess the relevance of *in vitro* methods; Dose the media and serum used precisely specified (source, batch number, expiry date, components) and meet the required specifications; Was the maximum acceptable levels of serum components, such as immunoglobulins and haemoglobin were defined well.
- Were reference and control items described well, such as negative and positive controls? suitability of reference and control items and justification for the selection of the reference item(s), stability and solubility of the reference and controls items.
- Were applicability domain of the *in vitro* method described well, as well as any limitations or exceptions.
- Was concentration of solvent(s) used without interfering with the *in vitro* method? Compatibility and toxicity of the solvent with the test system assessed, to select the appropriate solvent at an acceptable final concentration in the *in vitro* method medium.
- Was the number of replicates for each testing condition, including concentration level(s) used for the reference and control item(s), and test items etc., specified?

- Was there evidence that cell seeding, treatment and measurement is performed uniformly across the whole plate (well-to-well), between plates and across multiple runs (minimise any potential systematic effects).
- Statistical method used for data analysis and interpretation.

Studies with a low and moderate risk of bias were reported, whereas a high risk of bias studies was omitted from the analysis.

2.2.9. Data synthesis

All included studies for data synthesis were classified into six different experimental models according to the type and purpose of the studies. These were *in vivo* and *in vitro* anti-inflammatory studies, *in vivo* and *in vitro* wound healing studies, antibacterial and antifungal activity studies. Heterogenicity was evaluated descriptively from the narrative synthesized data, and potential reasons for heterogeneity were found by examining an individual study and subgroup characteristics. As a result, there was an interventional, methodological, and statistical heterogeneity among the studies, and a lack of similarity between the included studies. Consequently, statistical pooling of studies was not possible to perform a meta-analysis.

Instead, a narrative (qualitative) overview of the studies was conducted using a textual description of studies, grouping, and tabulation. Then, a description of characteristics of studies compared the effect of each plant extract relative to controls, the main parameters measured/analysed, quality of included studies and the risk of bias of all studies were described.

2.3. Results

2.3.1. Literature search results and description of study characteristics

A total of 3627 relevant articles were independently identified by two reviewers for preliminary review from electronic and manual searches. Of these, 1824 were from PubMed/Medline, 884 from EMBASE, 107 from Scopus, 9 from Google scholar, and 825 from other sources. After the removal of duplicates by reviewing relevant titles and abstracts, a total of 440 articles were retrieved for full-text review. After a detailed review of each article, 271 articles were excluded and a total of 159 articles were retrieved of which 31 *in vivo* anti-inflammatory,15 *in vitro* anti-inflammatory, 15 *in vivo* wound healing, and 2 *in vitro* wound healing studies, 79 anti-bacterial activity and 17 anti-fungal activity (Figure 2.2).

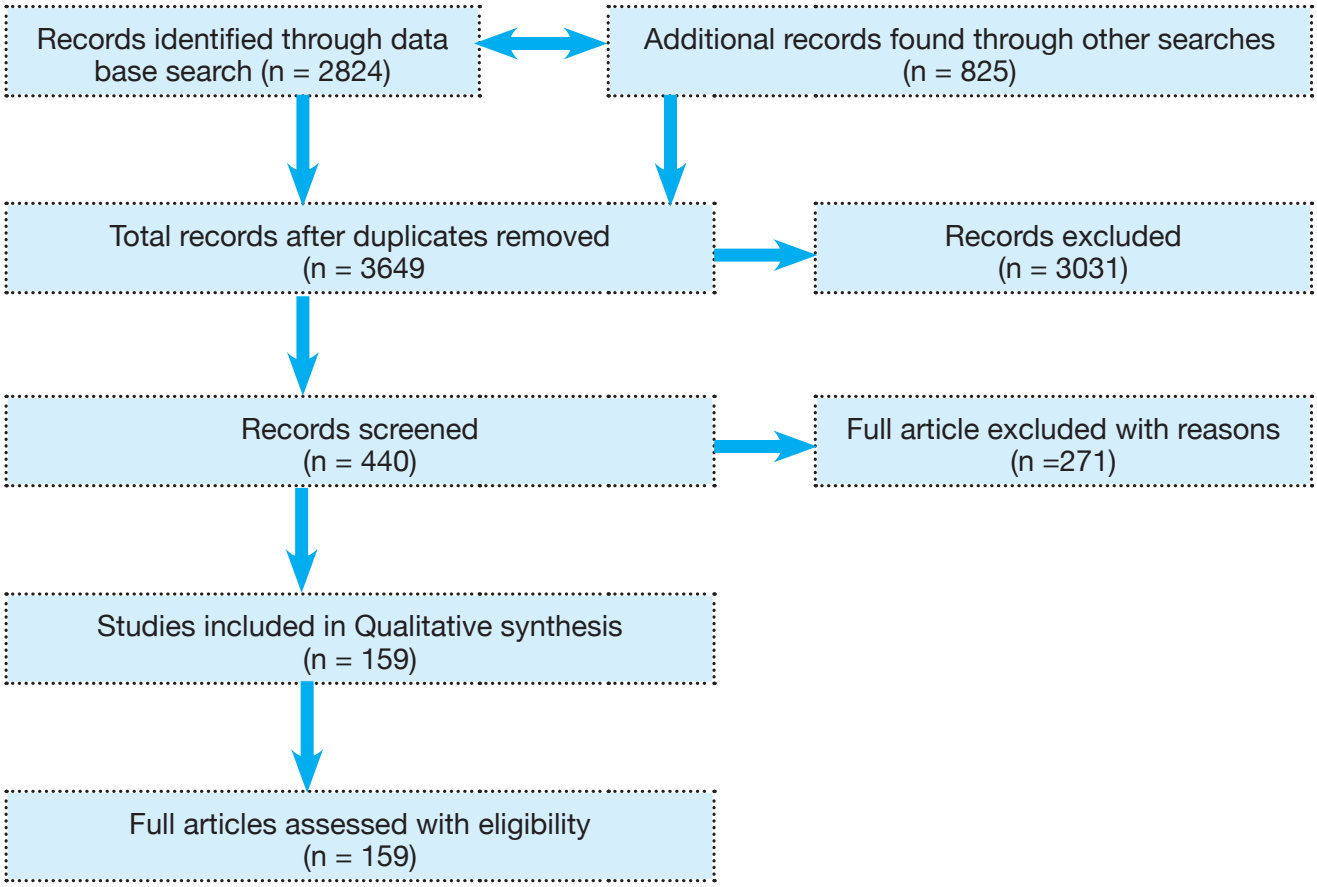


Figure 2.2: Flow Diagram of the literature search results

2.3.2. Excluded studies: There were many studies conducted in these areas. However, most of the articles were not eligible for inclusion. The reasons for exclusion were given as follow:

- Incomplete information:

  - ✓ The concentration of plant extracts used for activity and the number of experimental duplicates were not reported,
  - ✓ Method of outcome measurement was not reported.
  - ✓ Negative and positive controls used were not reported.
  - ✓ Sources of micro-organisms, quality control of micro-organism were not reported.
  - ✓ Time at which the outcomes were measured, sources of the cell lines, statistical methods used for data analysis were not reported.

- Not relevant studies:

- ✓ Clinical trials.
- ✓ Studies conducted on medicinal plants not growing in Ethiopia,
- ✓ Activity was not conducted for human pathogens (animal and plant pathogens).

2.3.3. Included studies

All the studies included were classified in to *in vivo* and *in vitro* anti-inflammatory studies, *in vivo* and *in vitro* wound healing studies, anti-bacterial and anti-fungal activity studies.

2.3.3.1. *In vivo* anti-inflammatory activity studies

i. Characteristics of the studies

Thirty-one studies met the review criteria, and the year of publication of the studies ranged from 1994 to 2019. Thirty were peer-reviewed full articles, and one was an MSc thesis. All study designs met the criteria for inclusion and followed internationally accepted guidelines. Studies were conducted in eight different countries. These were Ethiopia (n=13), Austria (n=1), Costa Rica (n=1), Egypt (n=3), India (n=5), Iran (n=1), Nigeria (n=4) and Tunisia (n=2). Thirty-five (35) plant species were investigated for anti-inflammatory activity in animal models (Table 1). All the medicinal plants investigated were found in Ethiopia, and they were investigated for anti-inflammatory, analgesic, antioxidant, anti-microbial, wound healing, anti-ulcer, and/or anti-arthritic activities in animal models.

The following plant families were reported in the *in vivo* anti-inflammatory studies. These were *Oleaceae, Lythraceae, Amaranthaceae, Meliaceae, Lamiaceae, Lauraceae, Asteraceae, Malvaceae, Myrtaceae, Apiaceae, Anunculaceae, Fabaceae, Zingiberaceae, Combretaceae, Acanthaceae, Polygonate, Salicaceae, Adianthaceae, Anacardiaceae, Rosaceae, Xanthorrhoeaceae, Cucurbitaceae* and *Moringaceae* (Annex 2.1). All the plants investigated were authenticated by botanists and were given voucher numbers. The most studied plant parts were leaves (n=16), followed by aerial parts (8), roots (n=3), rhizomes (3), fruits (n=3), stem bark (n=2) and whole plants (n=2). The most frequently used techniques for the extraction of the plant parts were maceration (n=20), followed by Soxhlet (n=6) and decoction (n=5) methods. The rest of the investigators used other methods of extraction and two combined techniques (maceration and Soxhlet) together. Methanol (n=11) was the most frequently used solvent for crude extraction, followed by water (n=7), ethanol (n=6), and acetone (n=1).

Most studies used both sexes of Swiss albino mice (n=15) and Wistar rats (n=16). The age range of Swiss albino mice and Wistar rats used for the experiments were 6-8weeks and 12 -13 weeks, respectively.

There was a wide range of variation in the concentration of plant extracts/fractions administered to the animals. Dose selection was made based on acute toxicity tests and pilot experiments. All concentrations used were safe doses to the laboratory animals and most extracts were administered orally to the animals. However, Naik *et al.* (99), Bhosale *et al.* (100) Mohammed *et al.* (101), Hosseinzadeh *et al.* (102), and Badilla *et al.* (103) used intra-peritoneal and subcutaneous routes to administer the extracts to the animals, while Khedir *et al.* (104) used topical route of application (Annex 2.1).

For the carrageenan-induced mice paw oedema model (acute inflammation) Tadiwos *et al.* (105), Abdissa (106), Wolde-Mariam *et al.* (107), Ayal *et al.* (108), Ching *et al.* (109) and Alemu *et al.* (110) used three different doses (100, 200, and 400 mg/kg) of different plant extracts. In another study, Hamad *et al* (111) and Gebremeskel *et al.* (112) used 200, 400 and 600 mg/kg doses for different plant extracts.

However, Yonathan *et al* (113) used 400mg/kg dose; Dilebo *et al.* (114) used 300 and 500mg/kg doses; Mulisa *et a.l* (115) used 250, 500 and 750 mg/kg doses; 50, 150 and 350 mg/kg doses. Three studies, Adedapo *et al* (116). Masresha *et al.* (117) and Mequanint *et al.* (118) used 400, 600, and 800 mg/kg doses. However, Sewuye *et al.* (119) used 200mg/kg dose (Annex 2.1).

Similarly, cotton pellet induced granuloma method in mice (chronic inflammation) Umar *et al* (120) and Alemu *et al* (110) used 100, 200 & 400 mg/kg) doses of 80% methanol extracts. However, Hosseinzadeh *et al* (102) used 5, 15, 30, 100, and 200 mg/kg aqueous extract and 0.05, 0.15, and 0.35 g/kg ethanolic extracts for chronic inflammation. Vijayaraj and Kumaran (121), and Sharma *et al.* (122) used 100 and 200 mg/kg doses of ethanol and water extracts (Annex 2.1).

Aspirin, diclofenac, indomethacin, and dexamethasone were used as standard drugs. The number of animal groups for anti-inflammatory tests depended on the number of different concentrations tested. The number of groups ranged from 3 to 5, where six animals were randomly assigned to each group.

For the carrageenan-induced paw oedema method, the duration of exposure of the animals to the extracts (treatment) was 4-6hrs, and the time of measurement of end points was after 1, 2, 3, 4, 5, and 6 hrs. of exposure.

At each end point the volume of paw oedema of the test and control groups was measured, and the percentage inhibition of oedema formation was calculated. For the cotton pellet-induced granuloma method (chronic inflammation), animals were exposed to 7 to 14 days of treatment and end points were measured on the 8^{th} and 15^{th} days; weight of cotton pellet (gm), granulation tissue formation (gm) and percent inhibition of tissue granulation were the end points measured. Seventeen studies used one-way analysis of variance (ANOVA)

followed by Tukey's post-hoc test, and six used the student t-test.

ii. Main parameters analysed

The main parameter used to analyse the activity of plant extracts for carrageenan-induced paw oedema (for acute inflammation) was the paw volume. After oral administration of the plant extracts or fractions to the test, reference and control groups, the volume of injected paws was measured before and after the injection of the inflammation-inducing agent (carrageenan) by measuring the linear paw circumference of oedematous legs using a plethysmometer or digital calibre. Alternatively, the volume of oedema was measured using water displacement method, where the volume of water displaced corresponded to the volume of oedema. Then, the anti-inflammatory activities of plant materials were expressed as percentage and calculated using the following formula:

$$\% \text{ Inflammation (I)} = \frac{Vf\text{-}Vi}{Vi} \text{ x100 (105)}$$

V_i is the volume of paw before carrageenan injection and V_f is the volume of paw after carrageenan injection at a given time.

$$\text{Percent of anti-inflammatory} = \frac{\%Ic\text{-}\%Ie}{\%Ic} \text{ x100}$$

where %Ic and %Ie are the mean inflammation values reached in control and experimental groups, respectively.

For sub-acute or chronic inflammation, autoclaved cotton pellets were aseptically implanted subcutaneously in the axillary region of rats anesthetized with diethyl ether. Extracts were administered once daily for 7 days. On day 8, the animals were anesthetized, and cotton pellets were removed surgically, freed from extraneous tissue, and dried in an oven overnight at 60 °C. The dried pellets were weighed and the mean weight of granuloma tissue around each pellet was determined. The percent inhibition of granuloma tissue development was calculated using the following formula: (Tc –Tt)/Tc ×100, where Tc = weight of granuloma tissue of control groups Tt = weight of granuloma tissue of treated groups (122).

Most studies used carrageenan-induced paw oedema (n=23) to study the anti-inflammatory activity of the plant materials in laboratory animals, while some of them used other methods such as topical croton oil-induced or xylene-induced ear oedema method, histamine-induced paw oedema, and serotonin-induced paw oedema.

Except for Dilebo *et al.* (114), all studies showed at least one safe concentration of the plant material with significant inhibition of inflammation in animals in a dose-dependent manner. The maximum inhibition of oedema at a low dose of plant extracts was reported by Tamrat

et al. (123) which showed 64.71%, 76.47% and 82.35% inhibition, respectively, at 50, 100, and 200 mg/kg of the methanol extract of *Jasminum abyssinicum* Hochst. ex DC 2h after carrageenan injection (105).

Aqueous extract of *Achyranthes aspera L.* exhibited higher inhibition of paw oedema formation at a dose of 800 mg/kg compared with vehicle control in mice in 4h (100). *Azadiractha Indica A. Juss* chloroform extract was shown to have 53.25% inhibition of oedema in rats, comparable to the control drug indomethacin (46.9%) (120). Fraction-2 from *Azadiractha Indica A. Juss* showed higher inhibition of granuloma tissue formation at doses of 200 and 400 mg/kg in rats. The activity of a flavonoid isolated from *Lawsonia inermis* L. Similarly reported by Manivannan *et al* (124) to have significant anti-inflammatory activity at a dose of 100 and 200 mg/kg in rats after induction of inflammation in 3 hrs.

Acetone extract of *Vernonia amygdalina* (Delile) Sch.Bip. (Delile) *(Gerawa)* reported by Adedapo *et al* (116), showed the fastest action at low concentration. They reported 100 mg/kg and 200 mg/kg doses of the extract reduced inflammation after 1 hr, 2 hrs and 3 hrs incrementally compared to the controls. The 200 mg/kg dose gave the most pronounced inhibition of oedema after 3hrs which was greater than that of the standard drug, indomethacin (116). Dilebo *et al*, reported that methanol extract of *Ferula communis L.* was devoid of mouse paw oedema inhibition (114). The highest dose of plant extract used was reported by Adeyem *et al.* (125), which showed that aqueous extract of *Persea americana var. guatemalensis (L.O. Williams) Scora* (Avocado), significantly ($p < 0.05$) inhibited carrageenan-induced rat paw oedema at 1600 mg/kg, almost similar to that produced by 100 mg/kg of acetylsalicylic acid with percent inhibition of 57.2% and 58.0%, respectively (125).

In another study reported by Marzouk *et al* (126), the aqueous extracts of different parts of *Citrullus colocynthis (L.) Schrad.* (Roots, stems, fruits, and seeds) injected inter-peritoneal at a dose of 1mg/kg and 4mg/kg showed moderate to high activity. In this study, immature fruits (88.33%) and seeds (98.06 %) of *Citrullus colocynthis (L.) Schrad.* showed better activity than ripe ones (65.00% to 97.90%). The percent inhibition of aqueous extracts of root and stem of *Citrullus colocynthis (L.) Schrad.* was 90.00% and 98.84%, respectively showing better activity compared to the standard drug, acetylsalicylate of lysine (74.40% after 4hs) (126).

For the chronic inflammation (cotton-pellet) test, three studies were included. The first study was by Alemu *et al* (110), and used 100, 200 & 400 mg/kg doses (oral) of *Leonotiso cymifolia (Burm.F)* 80% methanol extract, showed a significant ($p < 0.001$) inhibition of the formation of inflammatory exudates and granuloma mass. On the other hand, 28.91%, 37.68%, and 45.91% inhibition of inflammatory exudate and 24.03%, 40.18%, and 50.65% reduction of granuloma were recorded at 100 mg/kg, 200 mg/kg, and 400 mg/kg doses, respectively, while, dexamethasone showed 52.89% and 79.54% inhibition of exudates and granuloma,

respectively (110).

The second study was reported by Hosseinzadeh *et al* (102) which used different concentrations of *Myrtus communis L.* (aerial) (0.005, 0.015, 0.03, 0.1, and 0.2 g/kg doses from aqueous extract and 0.05, 0.15, and 0.35 g/kg doses from ethanol extract). The aqueous extract exhibited significant inhibition of inflammation in a dose-dependent manner. Maximum efficacy was noted (57.9%) for the dose of 0.03 g/kg of the aqueous extract (102). The last study was on anti-inflammatory activity of ethanol and aqueous extract of *Caesalpinia pulcherrima* (L.) Sw.(aerial) on a rat model. They used 100 mg/kg and 200 mg/kg doses of ethanol and aqueous extracts orally. Both extracts showed a significant ($p<0.05$) inhibition of the growth of granuloma tissues compared to the controls. Water extract of 200 mg/kg dose (54.09±2.76%) produced significant ($p<0.05$) inhibition of granuloma tissue compared to indomethacin (53.32±9.00%). The aqueous extracts had better anti-inflammatory efficacy than ethanol extracts(122).

iii. Quality of included studies (Bias analyses)

The critical appraisal process for *in vivo* anti-inflammatory activities was performed as part of the systematic review using the Risk of Bias tool for animal intervention studies (SYRCLE's RoB tool) and ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines to assess the validity of scientific finding (94,127). Studies with a high level of bias were excluded. However, the included studies have still some weaknesses in their methodology. The external and internal validity of the studies were evaluated. In external validity evaluation using animals as models for basic biological functions, there must be an implication that the results could be extrapolated in humans (128).

We categorized the judgment of bias as yes, no, and unclear. A "yes" judgment indicates a low risk of bias; a "no" judgment indicates a high risk of bias; the judgment will be "unclear" if insufficient details have been reported to assess the risk of bias properly.

Under the internal validity, the following ten main criteria were used: (1) Adequate generation and application of the allocation sequence; (2) Similarity of the control and test groups at baseline; (3) Adequate concealment of allocation to different groups; (4) Random housing of the animals during the experiment; (5) Blinding caregivers and/or investigators from knowledge which intervention each animal received during the experiment; (6) Random selection of animals for outcome assessment; (7) Blinding the outcome assessor; (8) Adequate addressing of incomplete outcome data; (9) Outcome reports from selection bias; (10) Absence of high risk of bias results.

Generally, from the studies that were analysed, their titles were consistent with the objectives that the authors stated. Overall, the risk of performance and selection biases were low because studies adequately generated and applied the allocation sequence, and allocations

were adequately concealed. Furthermore, the baseline characteristics of the different treatment groups did not significantly differ within any of the included studies. However, eighteen studies did not report whether animals were randomly housed during the experiment (risk of performance bias) or not. Therefore, it is unclear about the description measures they used to randomize the animals during grouping in cages.

In addition, not all studies reported whether the animal caregivers or investigators were blinded during the experiment (risk of performance bias). Similarly, it is unclear whether the outcomes were assessed blindly or not (detection bias). Five studies did not report whether all animals were included in the analysis (risk of attrition bias). All studies followed study protocol and were free of selective reporting outcomes.

2.3.3.2. *In vitro* anti-inflammatory studies

i. Characteristics of the studies

For the *in vitro* anti-inflammatory activity, ten (10) studies were included for full review and data extraction. The year of publication ranged from 2011 to 2019 and all of them were peer-reviewed full articles. Like the *in vivo* anti-inflammatory studies, all the study designs met the inclusion criteria and followed internationally accepted guidelines/protocols to conduct the *in vitro* experiments. Studies were conducted in South Africa (n=1), USA (2), India (n=3), China (3), Canada (n=1), Tunisia (n=1), Morocco (n=1), Nigeria (n=1), Luxembourg (n=1), and Cameroon (n=1) but not in Ethiopia though the medicinal plants were also found in Ethiopia. However, the medicinal plants investigated are found in Ethiopia. The studies investigated the antioxidant, anti-granuloma and anti-bacterial activities, and anti-acetylcholinesterase activity.

A total of 29 medicinal plant extracts, essential oils and one compound were investigated. Two of the medicinal plants, commonly used in Chinese traditional medicine (*Zingiber officinale Roscoe* and *Artemisia annua L.*), are also known for their medicinal value in Ethiopia. Plant parts such as leaves (n=7), seeds (n=5), whole plants (n=3), roots (n=2), bark, stems (n=1), fruits (n= 1), flower (n=1) and bulbs (n=1) were investigated in these studies.

Plant families reported in this review were *Apocynaceae, Myrtaceae, Berberidaceae, Zingiberaceae, Apiaceae, Betulaceae, Meliaceae, Amaranthaceae, Brassicaceae, Cucurbitaceae, Rosaceae, Anacardiaceae, Lythraceae, Lamiaceae, Myrtaceae, Solanaceae, Fabaceae, Poaceae, Verbenaceae, Rutaceae, Liliaceae, Euphorbiaceae and Capparidaceae*.

For the *in vitro* anti-inflammatory assays, commercially available cell lines and mononuclear cells (macrophages) isolated from laboratory animals were used. These were*;* Primary human neonatal fibroblasts (HDF3CGF assays/ELSEA), RAW264.7 cells lines (for nitric

oxide assay), RAW 264.7 cell lines (cytokines assay, Real time PCR and Western blotting), peritoneal mice macrophages (cytokines assay, western blotting), HP-1 cells lines (TNF-alpha assay), Murine macrophages RAW264.7 cells, mouse splenocytes, and human embryonic kidney 293 (HEK293) cell lines, primary human neonatal fibroblasts (gene expression assay) and kits for colorimetric *in vitro* anti-inflammatory assay were used.

ii. Main parameters analysed

Many methods were used to measure the anti-inflammatory activity of plant material in the *in vitro* model. The main parameters measured in the included studies were percent inhibition of production of cyclooxygenase enzymes (COX-1 and COX-2), inflammatory biomarkers such as vascular cell adhesion molecule-1 (VCAM-1), interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell a chemoattractant (I-TAC), and monokine induced by interferon (MIG), nitric oxide (NO) production, IL-1β, and IL-6 mRNA expressions; quantity of pro-inflammatory cytokines (TNFα and IL-6), median inhibitory concentration (IC_{50}) values, percentage inhibition of protein denature and gene expression of inflammatory cells.

Cytotoxicity assays were conducted for all plant extracts tested in the *in vitro* models and all concentrations used were safe to the cell lines used in the experiments. The concentration of essential oils used for *in vitro* anti-inflammatory activity ranged from 0.00041- 0.011% (v/v). Han *et al* (129) tested 0.011, 0.0037, 0.0012, and 0.00041% (v/v) *Eugenia caryophyllata* Thunb. essential oil which significantly decreased the levels of inflammatory biomarkers such as vascular cell adhesion molecule-1 (VCAM-1), interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell a chemoattractant (I-TAC), and monokine induced by c interferon (MIG). This indicated that *Eugenia caryophyllata* Thunb. essential oil may possess anti-inflammatory and pro-wound-healing properties. The effects of *Eugenia caryophyllata* Thunb. essential oil on these biomarkers were concentration-dependent (60) (Annex 2.2).

Chaibi *et al* (130) and Bouhlali *et al* (131) showed cyclo-oxygenase enzyme inhibition (IC_{50} value of 51 ± 0.23 mg/L), and inhibition of heat-induced albumin denaturation and haemolysis (IC_{50} = 170.24 µg/mL) by methanol extracts of *Lawsonia inermis* L. Similarly, *Rosa damascena* f. trigintipetala (Dieck) R.Keller flowers extract exhibited the highest inhibition of haemolysis (IC50 = 125.02 µg/mL). Fraction-2 (F-2) extracted from the chloroform of *Azadiractha Indica A. Juss* inhibited the production of IL-1 and TNF-ά in a dose-dependent manner. Fractions 1, 2 and 3 had significant activity on COX-1 and COX-2 at 200µg/ml, comparable to indomethacin.

In another study, 0.01% (v/v) essential oil of *Cuminum cyminum* L. inhibited iNOS and COX-2 mRNA levels in Lipopolysaccharide (LPS)-stimulated cells; and reduction of proinflammatory cytokines was observed in a dose-dependent manner. *Cuminum*

cyminum L. essential oil (0.01%) reduced IL-1β and IL-6 mRNA expressions to 30.2% and 1.3% in LPS-stimulated cells (132). Furthermore, 0.01% *Cuminum cyminum L.* essential oil produced 52% inhibition of nuclear NF-kB p65 levels in LPS-stimulated cells, which exhibited anti-inflammatory effects partially by blocking NF-kB activation; it also resulted in 45% and 53% inhibition of LPS-induced JNK and ERK phosphorylation which was partially attributed to regulating JNK and ERK pathways (132) (Annex 2.2).

Essential oils of *Allium sativum* L., *Allium cepa* L., *Drypetes gossweileri* S.Moore, *Pentadiplandra brazzeana* Baill. were also tested and inhibited the denaturation of protein (albumin) in a concentration dependent manner (3.125 to 125 µg/ml) (133). In addition, essential oil of *Cymbopogon flexuosus* (lemon grass) significantly decreased production of several inflammatory biomarkers, including vascular cell adhesion molecule 1 (VCAM-1), interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell alpha chemoattractant (I-TAC), and monokine induced by gamma interferon (MIG) (60) (Annex 2.2).

The time of measuring the outcomes for the essential oils varied significantly across the experiments, i.e., 24h (n=4), 15 minutes (n=1) 10 minutes (n=1) and 30 minutes (n=1).

The concentration of plant extracts reported ranged from 0.25 µg/ml to 100 µg/ml. The minimum concentration reported for *in vitro* anti-inflammatory activity was 0.25 µg/ml for dichloromethane and ethanol extracts of *Huernia hystrix N.E.Br.* In this study, petroleum ether extract of roots, all the petroleum ether and dichloromethane extracts showed good activity by inhibiting cyclo-oxygenase enzyme 1 and 2 (COX-1 and COX-2) by more than 70% (134). The highest concentration of methanol extract of *Alnus nepalensis* D.Don, used for the test was 100 *µg*/ml (135). The *in vitro* anti-inflammatory activity of the leaf extract of *Alnus nepalensis* D. Don against pro-inflammatory cytokines (TNF-α and IL-6) were assessed using ELISA technique in LPS-induced inflammation macrophage cells at concentrations of 10, 30 and 100 *µ*g/ml. *Alnus nepalensis* D.Don butanol fraction significantly inhibited the production of pro-inflammatory cytokines (TNF- α and IL-6) in a dose-dependent manner (135).

In another study, anti-inflammatory activity of twelve different medicinal plants was investigated. *Amaranthus dubius* Mart. ex Thell. was reported to be the most potent extract with IC_{50} of 9 *µ*g/ml, followed by *Ocimum americanum* L., *Vigna unguiculata* (L.) Walp., and *Zanthoxylum chalybeum* Engl. with IC_{50} values of 16, 27, and 47 *µ*g/ml, respectively. However, *Brassica oleracea* L., *Ocimum gratissimum* L., *Azadiractha Indica A. Juss* and *Mangifera indica* L. showed weaker responses (136).

iii. Quality of included studies (Bias analyses)

The critical appraisal process for *in vitro* anti-inflammatory activity was performed using

Guidance Document on Good In vitro Method Practices (GIVIMP) (95), Checklist for Reporting *in vitro* Studies (CRIS) guidelines (96), and guidance document on the good *in vitro* method practices (GIVIMP) for the development and implementation of *in vitro* methods for regulatory use in human safety assessment (137).

For the *in vitro* studies, quality assessment was based on seven main criteria which were extracted from these guidelines: (1) quality assurance of all materials and methods, and their use and application, in order to maintain the truthfulness, soundness and reproducibility of laboratory work conducted; (2) appropriateness of the *in vitro* cell and tissue culture facilities for purpose and a detailed understanding of the work flow for the *in vitro* methods and related processes; (3) apparatus, materials and reagents; (4) cell lines, media and serum, (5) test substance and reference/control items, (6) performance of the experiment, and (7) data analysis and interpretation. Under each criterion there are 2-8 sub criteria to evaluate the internal validity of the studies (See Annex 2.4).

The judgment of biases was categorized as yes, no, and unclear. A "yes" judgment indicated a low risk of bias; a "no" judgment indicated a high risk of bias; "unclear" if insufficient details had been reported to assess the risk of bias properly.

Generally, all *in vitro* anti-inflammatory studies included fulfilled the test definition, which included the purpose, requirements and scientific basis of the tests. In addition, all the methods followed were valid, internationally acceptable, clearly written and documented *in vitro* method descriptions. Relevant documentation of proof of sterility, laboratory consumables suitability and acceptability for use was reported in all studies. However, none of the studies reported evidence of provision of relevant and adequate education and training for all personnel to maintain high quality work and safety.

Furthermore, no studies reported whether the *in vitro* cell and tissue culture facilities were fit for purpose, or a detailed understanding of the workflow for the *in vitro* methods, which may adversely affect the quality of the work performed. One study reported the compliance of laboratory suppliers with good laboratory practice (GLP) principles, whereas nine of them did not report this. Reference data to assess the relevance of *in vitro* methods were provided; sources, components and batch numbers of media and serum used were precisely specified; and maximum acceptable levels of serum components were defined well.

In addition, reference and control items, concentration of solvent(s) used, suitability of reference and control items, and justification for the selection of the reference item(s) were reported in all the studies. However, eight studies did not report the applicability domains of the *in vitro* methods, and the limitations of the test methods were not described well (risk of performance bias).

The number of replicates for each testing condition, the concentration level(s) used for the test and control items and the measurement of the outcomes were uniform across the whole plate (well-to-well), between plates and across multiple runs which minimised any potential systematic effects. Appropriate statistical methods were used for all *in vitro* anti-inflammatory studies.

2.3.3.3. In vivo wound healing studies

i. Characteristics of the studies

Fifteen *in vivo* wound healing studies were eligible for data extraction and the year of publication ranged from 2001 to 2019. Except for one study that investigated both anti-inflammatory and wound healing activities, all studies investigated wound healing as a health outcome category. All studies included were peer-reviewed full research articles and were conducted in three countries: Ethiopia (n=9), India (n=5), Pakistan (n=1) and Cameroon (n=1).

In this model, 14 medicinal plant species, namely *Commiphora guidottii chiov, Achyranthes aspera L., Rumex abyssinicus* Jacq*., Calpurnia aurea* (Aiton) Benth*., Croton macrostachyus* Hochst. ex Delile*s, Kalanchoe petitiana* A. Rich*., Aloe megalacantha* Baker*, Stereospermum kunthianum* Cham., *Hibiscus micranthus* L.f., *Lantana camara L., Clematis longicauda* Steud. ex A. Rich., *Clematis burgensis Engl., Lawsonia inermis* L. and *Azadiractha Indica* A. Juss, belonging to 12 families, were investigated.

All studied medicinal plant parts were given a voucher number and authenticated by a botanist. The most studied plants parts were leaves (n=9) and the rest were stem bark (n=2), rhizome (n=1), leaf latex (n=1) and one plant constituent, oleo-gum-resin. Eleven studies used excision and incision wound models to investigate wound contraction and the period of epithelization and wound tensile strength, respectively, while three of them used only excision wound models. Swiss albino rats and mice of both sexes were used for both incision and excision models.

The age range of mice used for the studies was 6 – 11 weeks and 12 – 35 weeks for rats. All studies reported positive and negative controls: they used 0.2 % (w/v) nitrofurazone ointment and 1% silver sulfadiazine cream as positive control and simple ointment BP as negative control. Ten studies used one-way analysis of variance (ANOVA) followed by Dunnett's test for experimental data analysis, and one study used the Student t-test. All studies considered a 95% confidence interval and $p < 0.05$ value as statistically significant.

ii. Main parameters analysed

The main parameters measured in the *in vivo* wound healing studies were percentage wound contraction, histopathological studies (collagen formation, fibroblast proliferation

and angiogenesis), percent tensile wound strength (skin breaking strength), period of epithelization (number of days required for complete falling of scab without any residual raw wound) after treatment of incised and excised wounds with plant extracts and standard drugs. The parameters were calculated using the following formula:

$$\% \text{ Wound contraction} = \frac{\textit{Healed area (mm2)}}{\textit{Original wound area}} \text{ x100}$$

where, healed area = original wound area – present wound area.

$$\textit{\% tensil strengeth (TS) of test sample} = \frac{\textit{(TS of test sample- TS so)}}{\textit{TS so}} \text{ x100}$$

$$\textit{\% Tensile strength (TS) reference} = \frac{\textit{(TS of reference-TS so)}}{\textit{TS so}} \text{ x100}$$

$$\textit{\% Tensile strength (TS) of so} = \frac{\textit{(TS so-TS lu)}}{\textit{TS lu}} \text{ x100}$$

where, s.o and l.u stands for simple ointment treated and left untreated groups, respectively.

both sexes were used for both incision and excision models.

The age range of mice used for the studies was 6 – 11 weeks and 12 – 35 weeks for rats. All studies reported positive and negative controls: they used 0.2 % (w/v) nitrofurazone ointment and 1% silver sulfadiazine cream as positive control and simple ointment BP as negative control. Ten studies used one-way analysis of variance (ANOVA) followed by Dunnett's test for experimental data analysis, and one study used the Student t-test. All studies considered a 95% confidence interval and $p < 0.05$ value as statistically significant.

iii. Main parameters analysed

The main parameters measured in the *in vivo* wound healing studies were percentage.

2.3.3.4. *In vitro* wound healing

i. Characteristics of the studies

Two *in vitro* wound healing studies were included. These were conducted in Ethiopia and Denmark. Both were peer-reviewed full articles, met the inclusion criteria, and were conducted according to internationally acceptable guidelines/protocols. The assay methods used for these in vitro tests were wound healing scratch assays, and antioxidant and radical scavenging activity assays, which were correlated with wound healing activity.

A total of eighteen Ethiopian medicinal plants were studied in the included two studies:

Aframomum melegueta K.Schum., *Allophylus spicatus* (Poir.) Radlk., *Annona senegalensis* Pers, *Folium cissusqua* drangularis, *Herba gymnanthemum* coloratum, *Folium cum* Flos Radix, *Jasminum dichotomum* Vahl, *Folium Leonotis* nepetifolia, *Melanthera scandens* (Schumach. & Thonn) Roberty, *Herba Millettia* thonningii, *Ocimum gratissimum* L., *Philenoptera cyanescens* (Schum. & Thonn.) Roberty, *Fructus Rourea* coccinea, *Folium radix* Thonningia, *Herba trichilia* monadelpha, *Triumfetta acracantha Hochr., Uvaria ovata* (Vahl ex DC.) Hook.f. & Benth. and *Aloe harlana Reynolds.* These belong to 14 plant families. All the plants were authenticated by a botanist and had voucher numbers.

Stamen, radix, aerial parts, leaves, cortex, leaf latex, stems and flowers bulbs were parts of the plants included in these *in vitro* studies; and leaf latex of aloe, aqueous, methanol, 70% ethanol, petroleum ether, ethyl acetate and methanol extracts were the types of plant extracts used in these studies.

ii. Main parameters analysed

The main parameters measured in the *in vitro* wound healing assay were cell migration and proliferation and IC_{50} values for radical scavenging activity. Cytotoxicity assays were conducted for all medicinal plants, and all concentrations used for activity were safe to the cell lines used for the tests. In the first study, aqueous extracts of seventeen medicinal (17) plants were studied using a wound healing scratch assay in 3T3 fibroblasts cell lines. The outcome measured after 21h of exposure to test and reference substances was cell migration and proliferation using a Leica DMLS microscope at 4x magnification before and after incubation to estimate the proliferation and migration of cells. Then, cell proliferation/ migration rate was calculated as percent closure of the linear scratch made within 21h, as shown below:

$$\frac{\textit{Cell migration}}{\textit{Cell prolifiration}} = \frac{\textit{(gap distance t0 - gap distance t21)}}{\textit{gap distance t0}}\ \textit{x100}$$

Seventeen medicinal plants were evaluated at a concentration of 10 μg/ml for the *in vitro* wound healing. Only five of the 17 plant species tested increased proliferation or migration of fibroblasts in the scratch assay. These were *Allophylus spicatus* (Poir.) Radlk. (Warm and cold extracts of herbals), *Philenoptera cyanescens* (Schum. & Thonn.) Roberty (warm extract), *Melanthera scandens* (Schumach. & Thonn.) Roberty (warm extract), *Ocimum gratissimum* L. (cold extract), and *Jasminum dichotomum* Vahl (warm extract). The warm water extract of folium/fructus of *Philenoptera cyanescens* (Schum. & Thonn.) Roberty was shown to have a 100% proliferation/migration rate compared to the negative control group (138) (Annex 2.4).

In the second study, the antioxidant activities of the latex and isolated compounds of *Aloe*

harlana Reynoldsa Reynolds were tested using two complementary test systems, namely DPPH and 2-deoxyribose degradation assay methods. It was shown that increasing concentrations (1000, 500, 250 and 125 µg/ml) of the latex and isolated compounds increased the percent radical scavenging rates. The IC_{50} of the latex was found to be 14.21 µg/ml, while that of ascorbic acid (used as a reference) was 4.76 µg/ml. Both the isolated compounds and latex exhibited free radical scavenging properties in a concentration-dependent manner. Similarly, a compound, 7-O-Methylaloeresin A, was shown to have much stronger radical scavenging activity (IC_{50} = 0.021 mM) than aloin. At a concentration of 1.81 mM, 7-O-methylaloeresin A was shown to have a maximum inhibitory effect of 63.4%, which was comparable to the reference material, butylhydroxytoluene (BHT) (70.7%). Aloin shown to have less activity (IC_{50} = 0.600 mM) in deoxyribose degradation assay (139) (Annex 2.4).

iii. Quality of included studies (Bias analyses)

The quality of these two studies was evaluated using the method and guidelines used *in vitro* anti-inflammatory studies. The Guidance Document on Good In vitro Method Practices (GIVIMP) (137), and the Checklist for Reporting In vitro Studies (CRIS) guidelines (96), were used for the internal quality test of the studies. As above, the quality of the studies was evaluated based on seven main criteria. The judgment of biases was categorized as 'yes', 'no' or 'unclear' under each criterion; and based on this judgment, studies were either included or excluded in data extraction.

Both *in vitro* studies fulfilled the test definition criteria, the purpose and the scientific basis of the tests. In addition, valid and internationally acceptable *in vitro* method(s), written and documented *in vitro* method description and related standard operating procedure(s) were followed. However, neither study reported evidence of provision of adequate education and training for personnel to promote high-quality work and safety. In addition, there was no evidence of minimisation of the risk of errors (e.g., mix-ups) or avoidance of cross-contamination which might adversely affect the quality of the work performed, or safety measures to avoid environmental and individual contamination. Furthermore, there was no report of equipment calibration, or compliance of laboratory suppliers with GLP principles of international standards which are recommended for formal quality systems.

Evidence showed that the cell lines were free from any contaminants, and that identity, functionality and genetic stability, batch number and sources of antioxidant test kits, media and cell lines were reported in both studies. Reference and control items were described well. However, no justification was provided for the selection of the reference item(s) or the applicability domains of the *in vitro* method, and limitations of the *in vitro* test were not well described. In addition, compatibility and toxicity of the solvent was not reported in either study.

Concentration levels of plant extracts and controls, number of replicas of experiments, evidence of uniform fashion treatment, cell seeding and reagents across the whole plate (well-to-well) were reported in both studies to minimise systematic errors.

2.3.3.5. Antibacterial activity

i. Characteristics of the studies

For the anti-bacterial studies, seventy-nine studies were eligible for data extraction. The year of publication ranged from 2003 to 2019. A total of 76 peer-reviewed full articles and 3 MSc theses were included. The seventy-nine studies were conducted in Ethiopia (48), India (eight), Kenya (seven), Iran (three), Sudan (three), Cameroon and South Africa (two each), China (one), Oman (one), Malaysia (one), Nigeria (one), Pakistan (one), The Netherlands (one) and Tunisia (one) (Annex 2.5).

All the studies designs met the inclusion criteria and tests were performed according to the procedures described in the national, regional and international guidelines. The titles of the studies met the objectives stated in the studies. Of the 79 studies, 36 used agar well diffusion techniques with micro-dilution assay (MIC and MBC), and 28 used paper disc diffusion method with micro-dilution assay (MIC and MBC). Two of the studies used agar-well diffusion alone, while 9 used microdilution methods for minimum inhibitory concentration and minimum bactericidal assays together with other methods, colorimetric assay and crystal violet assay methods.

A total of 144 plant species and 4 compounds were tested and all except two plant species were identified and authenticated by a botanist. Out of the 144 plant species 14 of them are found in Ethiopia. Leaves were the most used plant parts for anti-bacterial tests (n=82) (Annex 2.5). All essential oils were extracted by steam distillation with a Clevenger-type apparatus, while maceration and Soxhlet techniques were the most frequently used techniques to extract plant materials.

A total of 25 gram-negative and 17 gram-positive bacteria were tested in the studies. Most of the microorganisms tested were American Type Culture Collection (ATCC) reference microorganisms and some were clinical isolates from samples. Among the gram-negative bacteria, *Escherichia coli* was tested against more than 70 types of medicinal plants, followed by *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Salmonella typhi* tested, which were tested against thirty-nine, twenty-eight and twenty-two medicinal plants, respectively. Among the gram-positives, *Staphylococcus aureus* was the most tested bacteria and was tested against sixty-six medicinal plants. Others included *Bacillus subtilis* (twelve), *Streptococcus pyogenes* (ten), and *Enterococcus faecalis* (eight).

Twenty-six studies used SPSS statistical software for data analysis. One-way ANOVA was

used to test the existence of statistically significant differences between mean zones of inhibition of controls and test substances. However, 45 studies did not report the statistical method used. The rest used the unpaired Student t-test to test the differences between treatment and control arms (Annex 2.5)

ii. Main parameters analysed

For agar well and paper disc diffusion assay methods, the outcome measured at each test level was the diameter (mm) of the zone of inhibition of the control and experimental tests using a calibrated distance measuring instrument. The time of measurement for all included studies was after 24h exposure to reference and test substances. For the microdilution methods (MIC and MBC), colour change for colorimetric assays or bacterial growth for non-colorimetric methods (visually identified as clear or turbid solution in test tubes after 24h treatment) were used.

A wide range of concentrations and different types of units of measurements were used across the studies. Among the medicinal plants tested against the microorganisms, at least one plant constituent was active against bacteria. The units used to describe MIC were µg/mL, µL/mL, mg/mL, µg/disc, %(w/v) and ppm. Similarly, for the measurement of zone of inhibition of bacterial growth, millimetre (mm) and millimetre squared (mm^2) were used.

The lowest concentration (8 µg/mL) of plant material that inhibited the growth of microorganisms was reported by Chaieb *et al.* (140). Eleven human pathogenic strains were tested against thymoquinone, a constituent of the black seed of *Nigella sativa* L. It was shown to inhibit the growth of *Bacillus cereus ATCC 14579* and *S. epidermidis CIP 106510 at* 8 µg/mL and *S. aureus ATCC 25923* 16 µg/mL (140). Ameya *et al.* (141) reported the minimum inhibitory concentration of the alcoholic extract of *Echinops kebericho* Mesfin against *S. aureus, E. coli* and *E. faecalis,* which ranged from 3.12 to 12.5 µg/mL (142). Similarly, Oumer *et al*. reported the MIC values of latex of *Aloe trichosantha* Berger *,* which ranged from 10 to 100 µg/mL for bacterial species such as bacillus species, *E. coli* species, Salmonella species, Shigella species, *S. aureus* and *V. cholerae* (143) (Annex 2.5).

Minale *et al.* reported the MIC values of *Aloe sinana* Reynolds and its compounds, Microdontn, Aloin and Aloinoside ranged from 10 to 50 µg/mL for the leaf latex, 10 - 25 µg/mL for Aloinoside, 5 - 200 µg/mL for Microdontn, 10 - 200 µg/mL for Aloin against gram-negative and gram-positive bacterial species. This was shown to have a strong anti-bacterial activity in comparison to the positive control, ciprofloxacin (144). Gadissa *et al.* tested the essential oils of *Blephari scuspidata*, *Boswellia ogadensis Vollesen* and *Thymus schimperi* Ronniger (145). The MICs of *Blepharis cuspidate* against *S. aureus (*ACCT & MRSA), *E. coli* (MDR) and *K. pneumonia* (MDR) were 1.56, 12.5 and 3.12 µL/mL, respectively, which is comparable to ciprofloxacin activity (145). Similarly*, Thymus schimperi* Ronniger was

reported to have MICs of 3.12, 6.5l, 3.12 µL/mL against *S. aureus* (ATCC & MRSA*)*, *E. coli* (ACCT & MDR) and *K. pneumoniae* (ATCC & MDR), respectively, and the minimum bactericidal concentration (MBC) ranged from 3.12 to 12.5 µL/mL. In addition, *Boswellia ogadensis* Vollesen was shown to have MIC values of 3.12, 6.25, and 3.12 µL/mL against *S. aureus* (ATCC & MRSA*)*, *E. coli* (ACCT & MDR) and *K. pneumoniae* (ATCC & MDR), respectively, and MBC ranged from 6.25 to 12.5 µL/mL (145).

The MIC values of *Boswellia ogadensis* Vollesen and *Thymus schimperi* Ronniger essential oil combination against *S. aureus (*ATCC & MRSA*), E. coli (ACCT &* MDR), *K. pneumoniae (ATCC &* MDR) were 3.12, 6.25 and 1.56 µL/mL, respectively. The MBC ranged from 1.56 to 25 µL/mL. Similarly, the combination of essential oil of *T. Schimper* Ronniger and *Blepharis cuspidata* Lindau showed significant activity against *S. aureus (*ATCC *& MRSA), E. coli (ACCT &* MDR) and *K. pneumoniae (*ATCC & MDR) with MIC of 0.39, 1.56, and 0.39 µL/mL, respectively, and with MBC values ranges from 0.39to 3.12 µL/mL. The combined activities of essential oils of *B. cuspidata* Lindau and *B. ogadensis* Vollesen showed similar activity against *S. aureus (*ATCC & MRSA*), E. coli (*ACCT & MDR) and *K. pneumoniae (*ATCC & MDR) with MICs of 1.56, 6.25, and 0.78 µL/mL, respectively. The MBC ranged from 1.56 to 25 µL/mL. These essential oils were shown to have comparable activity to ciprofloxacin (145).

Habbal *et al.* reported that 50% ethanol extracts of *Lawsonia inermis L.* demonstrated antibacterial activity against a wide range of gram-negative and positive bacterial strains with the highest antibacterial activity against *P. aeruginosa* (146). Nagarajan *et al.* reported that ethanol, chloroform, hexane and methane extracts of *L. inermis L* showed nearly equal zones of inhibition against *B. subtitles, S. aurous, and E. coli* at 400mg/kg comparable to that of tetracycline (147). Ethanol, methanol, and ethyl acetate extracts of *Azadiractha Indica A. Juss* were reported by Maleki *et al.* to have a wider zone of inhibition against *P. aeruginosa, S. aureus* and *E. faecalis* at 300mg/mL. The extracts had bactericidal activity against both reference and clinical isolates of *S. aureus* and *P. aeruginosa*, and bacteriostatic activity against *E. faecalis* (148).

The degree of bacterial growth inhibition, as determined by values of diameter of inhibition zone (IZ) of the respective plant extracts, varied among the extracts and microorganisms. The widest inhibition was reported by Bacha *et al*. who showed the inhibitory zones of petroleum ether extract (500 mg/mL) of seeds of *Nigella sativa L.* to be 44 ± 0.31 mm against *Bacillus cereus* and 40 ± 2.33 mm *against B. cereus* ATCC 10987 compared to that of gentamycin (29 mm) (149). Wide zones of inhibition were recorded for the petroleum ether extract of stem of *Kosteletzkya begonifolia* Ulbr. and stem of *Leucas marthineensis* (Jacq.) R.Br. against *E. coli*, *S. typhimurium*, *S. aureus* and *P. aeruginosa* at all concentrations, comparable to ciprofloxacin (150). In another study, acetone extract of *Capsicum frutescens*

L. against ATCC *S. aureus* at a concentration of 0.1 mg/mL was reported to produce an inhibitory zone of 28 mm (151).

iii. Quality of included studies (Bias analysis)

Critical appraisal of the studies included was done using the checklist for Good *In vitro* Method Practices (OECD) and the WHO Good Practice for Microbiology Laboratory. Seven main criteria were used to evaluate the validity of methodological and reporting qualities (details are in the Methods section).

Under the main checklist there were thirty criteria to evaluate the internal validity of the studies. Studies with unacceptable levels of bias were excluded. However, the studies included still had some weaknesses in reporting the status of microbiology facilities, regular equipment, apparatus maintenance and calibration. In addition, there was lack of clarity as to whether the test methods were validated or not; and there was also lack of evidence as to whether the microbiological tests were performed and supervised by an experienced person qualified in microbiology or equivalent, and whether the opinions and interpretations of test results in reports were done by authorized personnel with suitable experience and relevant knowledge.

There was also some methodological weakness. For instance, the number of replicates for each testing condition, including concentration level(s) used for the reference and control item(s), and test items were not specified in some studies. Two studies reported evidence on whether the micro-organisms were free from any contaminants and indicated functionality, genetic stability, and identity (143,152) but the rest did not. None of the studies reported the applicability domain of the *in vitro* methods or any limitations or exceptions to the methods. Four studies did not report complete information about the degree of inhibition of bacterial growth and the concentrations by the respective medicinal plants, and one study did not mention the unit of measurement of the zone of inhibition by plant extracts.

We categorized the judgment of bias as ‘yes’, ‘no’, or ‘unclear’. A “yes” judgement indicated a low risk of bias; and a “no” judgment indicated a high risk of bias; the judgment was “unclear” if insufficient details were reported to assess the risk of bias properly.

2.3.3.6. Anti-fungal activity

i. Characteristics of the studies

Seventeen studies that evaluated anti-fungal activities of Ethiopian medicinal plants were included. The year of publication of the studies included ranged from 2000 to 2018, and studies were conducted in six different countries, Ethiopia (n=7), Kenya (n=1), India (n=5),

Colombia (n=1), Lithuanian (n=1), Republic of Korea (n=1), and Romania (n=1). Sixteen studies were peer reviewed full articles and one was an MSc thesis. Five studies used microdilution assay, two used agar well diffusion method, and twelve used both methods (Annex 2.6).

Medicinal plants claimed to have anti-fungal activities were tested against different fungal species and one species of yeast. These were *Candida albicans*, *Aspergillus* species, *Trichophyton* species, *Microscopium* species, *Penicillium* species, *Fusarium* species, *Epidermophyto*n species and *Rhodotorula rubra.* Aspergillus species (n=18) were the most-studied fungi, followed by *Trichophyton* species (n=13) and *Candida albicans* (n=10).

A total of 42 different species of medicinal plants were tested against different fungi, and all of them identified and authenticated by botanists and with voucher numbers. Out of these, 4 plant species are endemic to Ethiopia (Annex 2.6).

Six studies used the agar well diffusion (AWD) method, six the micro dilution (MID) method and five both methods. For both experimental methods, the duration of exposure of the microorganisms to the extracts ranged from 2-7 days incubation time; and outcomes were measured after this. Zone of inhibition of fungal growth, turbidity (visually) and anti-fungal activity index (%) were the outcomes measured in the included studies. All the measurements were replicated three times and the results were presented as mean ± SD. One-way ANOVA followed by Tukey's test was used to compare extraction solvents and the difference in the sensitivity of the test microorganisms.

ii. Main parameters

The antifungal activity of plant extracts was measured in a similar way as that of the anti-bacterial activity. These were zone of inhibition of fungal growth for the agar well and paper disc diffusion methods and fungal growth which distinguished clear and turbid solutions for the micro-dilution methods, measured after incubation periods.

A wide range of concentrations and units of measurement were used across the studies. For the MIC and minimum fungicidal concentration (MFC) mg/mL, µg/mL and activity index in percent (%), and mm was used for the measurements of ZI in AWD assays.

The activity index of the extracts was determined by using the following formula:

$$\textit{Activity index (AI)} = \frac{\textit{(Inhibition zone of extracts)}}{\textit{(Inhibition zone of standard)}} \textit{x100}$$

Ameya *et al.* (142) reported that the methanol extract of *Echinops kebericho* Mesfin against *Aspergillus flavus* and *Candida albicans* had MICs of 6.25 µg/mL and 3.12 µg/mL,

respectively; and the MFC of methanol extract to be 12.5 µg/mL and 6.25 µg/mL against *A. flavus* and *C. Albicans,* respectively. The ethanol extract had MICs of 12.5 µg/mL and 6.25 µg/mL against *A. flavus* and *C. albicans,* respectively with fungicidal activity of 22.92 µg/mL and 12.50 µg/mL, respectively. The zone of inhibition of the methanol extract against *C. albicans* and *A. flavus* were 18.66 ± 0.57 mm and 20.33 ± 0.57 mm, respectively. In this study, ethanol, and methanol extracts of *E. kebericho* Mesfin were shown to have comparable activity with ketoconazole (Annex 2.6).

Kasparaviciene *et al.* evaluated the activity of oleo-gels formulated with different concentrations of thyme essential oil. The MIC value of 0.25% essential oil of thyme in oleo-gels against *C. albicans* was 0.05% (153). In another study, the antifungal activity of *T. vulgaris* essential oil against dermatophytic fungi was reported by Neetu *et al*. to have a very strong antifungal activity at low concentrations. The MIC values ranged from 0.05 µL/mL to 0.1 µL/mL; and the MFC ranged from 0.05 µg/mL to 2 µg/mL against the dermatophtic fungi (154) (Annex 2.6).

The seed extracts of *Trachyspermum ammi* (L.) Sprague (0.2 mg/mL) and the leaf extract of *Cestrum nocturnum L.* (0.2 mg/mL) exhibited the widest zones of inhibition, at 38.3 mm and 31.3 mm, respectively against *C. albicans*. Similarly, the methanol extract of *E. kebericho* Mesfin exhibited ZI of 20.33 ± 0.57 mm against *C. albicans* and 18.6 mm against *A. flavus* (Annex 2.6). Salazar *et al.* showed that leaf and seed oil extracts of neem tree inhibited the growth of *Trichophyton menta, Trichophyton rubrum, Epidermophyton floccos* and *Microsporumcanis*. Whereas Simhadri *et al* reported that the aqueous extract of *Azadiractha Indica A. Juss* leaves had superior activity against *T. rubrum, M. gypseum*, *E. floccosum*, and Candida species (155).

iii. Quality of included studies (Bias analysis)

Checklists employed for antibacterial studies were also used in the antifungal studies. There were gaps in methodology as well as in reporting and interpreting the outcomes. Validation of the test methods before conducting the experiments was not reported for all included studies, and eight studies did not report the statistical methods used.

There was no evidence that the anti-fungal activity tests were performed or supervised by an experienced person qualified in microbiology or equivalent. Similarly, there was no report on whether the microbiology facilities were fit for purpose or detailed description of the workflow for the microbiology methods and related processes. Furthermore, nine studies did not report the statistical methods used, not expressed an estimate of the uncertainty of the test result on the test report, and limitations of the test were not reported clearly.

2.4. Discussion

The purpose of this review was to demonstrate anti-inflammatory, wound healing and anti-infective activities of some Ethiopian medicinal plants that might potentially be used for limb care (tropical lymphoedema and associated wounds). This systematic review identified a total of 138 articles covering six different experimental models. Of these, 25 concerned *in vivo* anti-inflammatory models, 11 *in vitro* anti-inflammatory, 11 *in vivo* wound healing, 2 *in vitro* wound healing, 74 antibacterial activity and 15 antifungal activity models. Overall, medicinal plant extracts tested for these three conditions in *in vitro* and *in vivo* models were shown to have good activity. Despite the heterogeneity of the studies, all plant extracts investigated succeeded in inhibiting bacterial and fungal growth, in reducing experimentally induced inflammation in animals, in down-regulation of inflammatory and pro-inflammatory cells, and in promoting wound healing.

Inflammation is a complex pathophysiological process mediated by different signalling molecules produced by leukocytes, macrophages and mast cells as well as by the activation of complement factors resulting in extravasations of fluid and proteins and accumulation of leukocytes at the inflammatory site (101).

Non-steroidal anti-inflammatory drugs are the most widely used medications for the management of pain and inflammation though usually associated with adverse effects such as gastrointestinal ulcer and haemorrhage (156). Long term use of steroidal antiinflammatory drugs may have severe adverse reactions leading to cardiovascular, endocrine, metabolic, musculoskeletal, and ophthalmologic problems (157). Plant materials are claimed to be an alternative remedies for the management of pain and inflammation, with fewer side-effects (156).

In *in vivo* anti-inflammatory assays, 80% methanol root extract of *Jasminum abyssinicum* Hochst. ex DC was shown to have good activity in a dose-dependent manner, possibly through secondary metabolites such as saponins, terpenoids, triterpenes, glycosides and flavonoids (105). The anti-inflammatory activity might also be attributed to polyphenols like flavonoids, which are present in the extracts and exert their activity through free radical scavenging and inhibition of inflammatory pathways (158). This agrees with reports that flavonoids inhibited the biosynthesis of prostaglandins by inhibiting COX and LOX inflammatory pathways (134,159).

Acetone extract of *Vernonia amygdalina (Delile) Sch.Bip. (Delile) Sch.Bip. (Delile) Sch.Bip.* (Gerawa) was also shown to have fast activity at low concentrations due to its inhibitory effect on the biosynthesis of prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2). It contains secondary metabolites such as tannins, potent cyclooxygenase inhibitors (116).

Tannins also act on leukocyte migration and antagonism of the phlogistic actions of mediators of inflammation (160). This report is in agreement with the finding of Mulisa *et al* (55).

In another study, aqueous extracts of the root and stem of *Citrullus colocynthis (L.) Schrad.* were shown to have good activity compared to aspirin. This activity is due to the presence of secondary metabolites (alkaloids), which have analgesic and anti-inflammatory activities and act via inhibition of pro-inflammatory cytokines (IL-6 and IL-1) and the expression of COX-2, and increasing the level of anti-inflammatory cytokine IL-4 in carrageenan-injected rat paw tissues (126). A similar mechanism of action of alkaloids was reported by others (156). The anti-inflammatory and analgesic properties of alkaloids have also been reported by other authors (157,161). For instance, colchicine is an alkaloid used for the treatment of gout (arthritis) and leukocytoclastic vasculitis (161).

The methanol extract of *Leonotiso cymifolia (Burm.F)*(110)*,* aqueous extract of *Myrtus communis L.* (102)*,* ethanol and aqueous extract of *Caesalpinia pulcherrima (L.) Sw.*(122) were evaluated for chronic inflammation and showed inhibition of formation of inflammatory exudates and granuloma mass in a dose-dependent manner compared to the standard drugs. Aqueous extract of *Caesalpinia pulcherrima (L.) Sw.*(200 mg/kg dose) showed better activity than indomethacin (122) due to its major constituent, flavonoids, known to inhibit fibroblasts and the synthesis of collagen and mucopolysaccharides during granuloma tissue formation (162).

The essential oil of *Eugenia caryophyllata Thunb.* and its main active component, eugenol (Figure 3), were shown to have anti-inflammatory and pro-wound healing activity in an *in vitro* model due to the significant inhibition of many pro-inflammatory cytokines in dermal fibroblast cells (129). In another study, eugenol was shown to have a similar anti-inflammatory activity to the COX antagonist (indomethacin) and COX-2 selective antagonist (celecoxib) in a similar model (163). 0.01% essential oil of *Cuminum cyminum L.* exhibited prominent anti-inflammatory activity by inhibiting inducible NO synthase (iNOS) and cyclooxygenase (COX-2) mRNA expression in LPS-stimulated RAW 2647 cells. It has also been found to decrease the levels of IL-1 and IL-6, which are important factors in inflammatory responses and chronic inflammatory diseases (132).

Citral, the main component of the essential oil of lemongrass, inhibits the production of TNF-ά, IL-8, VCAM-1, and ICAM-1 in human umbilical vein endothelial cells and promotes wound healing through a tissue remodelling process (164). The effects of *Allium cepa L.* and *Pentadiplandra brazzeana Baill.* essential oils were found to be 2 times greater than those of sodium diclofenac against heat denaturation of bovine serum albumin (BSA) (133).

Lawsonia inermis L. was shown to have anti-inflammatory activity in *in vivo* and *in vitro*

studies (124,130,131). Flavonoids isolated from *Lawsonia inermis* L. reduced carrageenan-induced paw oedema and inflammation due to their antioxidant activity and their ability to modulate gene expression of cytokines and adhesion molecules (165). Similarly, the methanol extract of *Lawsonia inermis* L. and two alkaloids isolated from the methanol extract inhibited lipo-oxygenase enzyme by decreasing the production of nitric oxide (NO) and pro-inflammatory cells and suppressing the biosynthesis of prostaglandins (130). Furthermore, *Lawsonia inermis* L. was reported to inhibit thermal induced tissue protein denaturation and promoted membrane stability by altering the electrostatic bond of hydrogen, and hydrophobic and disulphide bonds (166).

Similarly, *Azadiractha Indica A. Juss* was reported to have significant anti-inflammatory activity in both *in vivo* and *in vitro* studies. The chloroform extract and fractions reduced carrageenan-induced oedema and granulation tissue formation in rats more powerfully than indomethacin. In addition, the fractions inhibited pro-inflammatory cells (IL-1 and TNF-α), COX-1 and COX-2 in *in vitro* tests by inhibiting the interaction between pathogen-associated molecular patterns (PAMPs) and toll receptors (TLRs), which impair the expression and production of pro-inflammatory cells (120). Similarly, seed oil of *Azadiractha Indica* A. Juss inhibited oedema formation after carrageenan injection. The phytochemical constitutes of the oil, flavonoids, are shown to inhibit prostaglandin biosynthesis, endoperoxides, enzyme-like protein kinase and phosphodiesterase, which have key roles in inflammation (99). Bhosale *et al* reported the anti-inflammatory activity of aqueous extract of *Achyranthes aspera L.* in carrageenan-induced paw oedema mice, which was attributable to flavonoids, alkaloids, saponins, and triterpenoids phytoconstituents found in the extracts (100). Similarly, Gokhale *et al.* reported the anti-inflammatory activity of ethanolic extract of *Achyranthes aspera L.* in Wister rats due to the aforementioned plants constituents (167).

For the *in vivo* anti-inflammatory assay, the most frequently used experimental model was carrageenan-induced paw oedema. This is a highly sensitive and reproducible test for non-steroidal anti-inflammatory drugs and is established as a valid and widely used model to study new anti-inflammatory drugs and mechanisms of action (168,169). It is a useful method for detecting orally active anti-inflammatory agents and has significant predictive value for anti-inflammatory agents acting through mediators of acute inflammation (168). It induces a biphasic oedema in the mouse paw. The first phase (0-1h) is characterized by an acute and local inflammatory response which involves secretion of histamine, serotonin, and bradykinin. The second phase usually develops after 24h, and is characterized by more pronounced oedema with maximum effect in 48-72h. Prostaglandins and cytokines (IL-1ß, IL-6, IL-10, and TNF-ά) are inflammatory mediators involved in the second phase of oedema (168–170). The inflammatory response of carrageenan is quantified by measuring the circumference (size) of oedema which is maximal around 5h post-carrageenan injection

(170).

ELISA was used for the *in vitro* anti-inflammatory assay to measure inflammatory biomarkers such as cytokines, chemokines, and metalloproteinases produced in the supernatants in LPS- stimulated cell culture. ELISA uses antibodies to differentiate epitopes of cytokines and uses them to capture and quantify an analyte of interest (cytokines from a cell culture supernatant) with a remarkable sensitivity (171,172). Similarly, Western blotting and gene expression quantification were used to measure the gene responsible to produce inflammatory biomarkers.

Wound healing is a complex process that includes a harmonized interaction between different immunological and biological systems (173). It is divided into four phases. These are haemostasis, inflammation, proliferation and tissue remodelling which are overlapping processes in time (174,175). The first phase involves blood coagulation, haemostasis, and cellular events such as infiltration of leukocytes with different purposes in antimicrobial and cytokine release, which initiates the proliferative response for wound repair (174). The second phase of wound healing is inflammation where its aim is to prevent infection and involves initiation reaction of the body to the injury (175). The third phase is characterized by fibroblast migration and deposition of newly synthesized extracellular matrix, acting as a replacement for the provisional network composed of fibrin and fibronectin. Finally, the remodeling phase is responsible for the development of new epithelium and final scar tissue formation (173).

Plant extracts have been reported for wound healing activity and promoted wound healing through angiogenesis, activation of NF-κB, favouring pro-inflammatory cytokines, upregulation of iNOS and alpha-1 type-1 collagen, fibroblast proliferation, and anti-oxidant activity (32).

The wound healing studies reported in this review tested the ability of plant extracts and essential oils to contract wounds, reduce the period of epithelization, increase the tensile strength of the wound (115,176,177), influence cell migration and proliferation (138), and act through antioxidant and anti-microbial activities (139), leading to tissue repair and restoration of function.

In the study reported previously, the oil and resin of *Commiphor aguidottii* increased wound contraction in rats, enhanced the proliferation of epithelial cells in incision wounds and increased tensile strength in mice. The resins and oil were shown to increase collagen synthesis as well as aid the cross-linking of proteins (178).

In another study, incision and excision wounds of laboratory animals treated with 5% and 10% methanol extracts of *Achyranthes aspera L.* showed faster healing, increased cellular proliferation and shorter epithelization time in albino rats, which could be due to stimulation

of cellular proliferation and enhancement of collagen synthesis (179). This report is in agreement with the study reported by Barua *et al* (180) and Edwin *et al* (181) which indicated that methanol and water extracts of *A. aspera L*promoted wound healing, likely due to the secondary metabolites, triterpenes, known to promote wound healing process through their astringent and antimicrobial property. Tannins promote capillary vasoconstriction, which decrease vascular permeability and cause a local anti-inflammatory effect (181). Phytochemical screening of the extracts revealed the presence of tannins and triterpenes in the water and methanol extracts of *A. aspera L*(180,181).

Mulisa and his colleagues tested 4% and 10% (w/w) methanol extract (in ointment base) of *Rumex abyssinicus Jacq.* which significantly increased the percent wound closure in excision wounds; shortened the epithelization time and increased the hydroxyproline content (115). In another study, *Rumex abyssinicus Jacq.* was shown to possess antibacterial and anti-inflammatory activities; and the ability to regenerate epithelial cells, which might contribute to wound healing (182).

Ointments prepared from a solvent fraction of *Calpurnia aurea (Aiton) Benth.* were shown to reduce the epithelization time and increase wound contraction in mice, which might be attributed to the secondary metabolites present in the fraction, such as terpenoids, tannins, alkaloids, saponins, phenols and flavonoids that are known to promote wound healing via anti-bacterial and anti-inflammatory activities (108). In the rat excision wound model, 10% ointment of methanol extract of *Croton macrostachyus Hochst. ex Deliles* showed the fastest and most complete wound healing compared with the negative control. This activity may be due to its individual activity or the combined activity of phytoconstituents such as anthraquinones, flavonoids, phytosterols, polyphenols, saponins and tannins (183).

The wound healing effect of the latex of *Aloe megalacantha Baker* in an ointment form in excision and incision wound models in rats was greater than that of the standard drug, nitrofurazone, which might be attributable to an individual or multiple phytochemical constituents (112). This finding is consistent with other reports of wound healing activity of other species of Aloe (139,184). Similarly, the hydroalcoholic extract of *Kalanchoe petitiana A. Rich.* in an ointment base was shown to have better wound healing activity than 0.2% nitrofurazone ointment.

A delay in wound healing was observed in higher concentrations of the extracts. There was an enhanced rate of wound contraction and a reduction in healing time in animals treated with ointment containing the methanol and aqueous fractions of *Kalanchoe petitiana A. Rich.* The tensile strength of 5% and 10% ointments of the crude extract treated groups were slightly greater than those of the 0.2% nitrofurazone group. These activities might be due to the phytoconstituents, especially flavonoids, which play a great role in wound healing activity (185).

The wound healing activity of *Lawsonia inermis* L. shown that henna leaf extracts had a high healing rate due to secondary metabolites (186,187). Similar, stem bark extract of *Azadiractha Indica* A. Juss was shown to have a high level of hydroxyproline and increased total protein content, which are indicators of wound healing. In addition, it increased wound contraction, and DNA content in the scar tissue, which was a sign of cellular proliferation (188).

Allophylus spicatus (Poir.) Radlk., *Philenoptera cyanescens (Schum. & Thonn.) Roberty*, *Melanthera scanden*, *Ocimum gratissimum L.*, and *Jasminum dichotomum Vahl* were reported to increase proliferation and migration of cells in 3T3 fibroblasts cell lines (*in vitro*). The warm water extract of folium/fructus of *Philenoptera cyanescens (Schum. & Thonn.) Roberty* was shown to have the highest (100%) proliferation and migration rate compared to the negative control groups, which was attributable to the active compounds rutin (quercetin-3-O- rutinoside) and quercetin-triglycoside, isolated from *Philenoptera cyanescens (Schum. & Thonn.) Roberty* (138).

The antioxidant properties of *Aloe harlana Reynoldsa* Reynolds were evaluated using two assay methods, DPPH, and deoxyribose degradation assay. The latex and the compounds isolated from *Aloe harlana Reynoldsa* Reynolds correlated with the traditional use of the plant for the treatment of wounds, infection and inflammatory diseases (139). Antioxidants are assumed to help in controlling wound oxidative stress, which accelerates wound healing. They are important mediators in regulating the damage that is potentially incurred by biological molecules such as DNA, protein, lipids, and body tissue in the presence of reactive species (189).

For the *in vivo* wound healing model, excision and incision wound models were the most frequently used methods to test wound contraction, period of epithelization and wound tensile strength. The wound breaking strength was measured using the incision method while collagen estimation, period of epithelization and wound contraction were measured using the excision method (190). Among the *in vitro* wound assay methods, the wound scratch assay was the most frequently used method and used alongside well-developed methods to measure cell migration *in vitro*. A “wound gap” in a cell monolayer is created by scratching, and the “healing” of this gap after treatment with plant extracts such as cell migration and growth towards the center of the gap is monitored and quantified (191). This is particularly suitable for studies on the effects of cell interactions on cell migration, which mimic *in vivo* cell migration and are compatible with imaging of live cells during migration to monitor intracellular events (192).

In this review of antibacterial activity, a total of 144 medicinal plant species and four compounds were investigated against 25 gram-negative and 17 gram-positive bacteria using agar well diffusion, paper disc diffusion, broth micro/macro-dilution and agar dilution

method. A summary of plant species whose extracts and their isolated compounds were shown to have significant *in vitro* activity against bacteria is the focus for our discussion.

Chaieb *et al.* reported the MIC of thymoquinone, constituent of *N. sativa* L., which was shown to have MIC of 32 µg/mL against *V. parahaemolyticus ATCC 17802* and *E. faecalis* ATCC 29212, 16 µg/mL against *L. monocytogene ATCC 19115*, 8 µg/mL against *B. cereus ATCC 14579, S epidermidis CIP 106510, M. luteus NCIMB 8166, S. aureus ATCC 25923* and *S. epidermidis CIP 106510* in a broth microdilution assay method. Its activity was shown to be similar to the standard drugs gentamycin and erythromycin (140). This finding agrees with the report of Kokoska *et al.,* who tested the essential oil of *N. sativa* L. seed against gram-positive bacteria. Thymoquinone, the main constituent of the essential oil, was shown to have a potent bacteriostatic effect with MIC ranging from 8 to 64 µg/mL in broth microdilution method (193). However, *E. coli ATCC 35218*, *S. enterica serovar Typhimurium ATCC 14028*, and *P. aeruginosa ATCC27853* were found to be resistant to this compound (MIC > 512 µg/mL) (140).

Ameya *et al.* tested the alcoholic extract of *E. kebericho* Mesfin against *S. aureus, E. coli and E. faecalis* and demonstrated significant activity with MIC ranged from 3.12 µg/mL to 12.5µ g/mL using AWD, while *E. coli* was found to be resistant (108). Anwar *et al.* assessed the antimicrobial activity of latex of *Aloe trichosantha* A. Berger and its compounds (aloin A/B and aloin-6'-O- acetate A/B), which were effective against *E. coli, Salmonella* and *V. cholerae* strains with an average MIC value of 25 µg/ml. The activities of the test substances could be due to changes to cell wall integrity, enzymatic activity and protein inactivation in the microorganisms (143).

Minale *et al.* performed anti-bacterial activity tests on *Aloe sinana* Reynolds and its compounds (Microdontin, Aloin and Aloinoside) against 21 strains of bacteria using the disk diffusion method. The leaf latex showed high inhibitory activities against *B. pumillus 82, B. subtilis ATCC 6633 and S. aureus ML 267, E. coli K99, E. coli K88, E. coli CD/99/1, E. coli LT37, E. coli 306, E. coli 872, E. coli ROW 7/12, E. coli 3:37C, S. enterica TD 01, S. typhi Ty2, S. boydii D13629, S. dysentery 8, S. flexneri Type 6, S. soneii 1, V. cholerae 85, V. cholerae 293, V. cholerae 1313 and V. cholerae 1315* at a concentration of 200 µg/mL, which showed comparable activity to the standard drug ciprofloxacin. Similarly, compounds isolated from *Aloe sinana* Reynolds were shown to have high activity against *E. coli, S. typhi Typ 2, Shigella, S. aureus and V. cholerae*, comparable to the reference drug, ciprofloxacin (144). The leaf latex's action was due to the secondary metabolite anthraquinones, which possess a range of functional groups and have the ability to disrupt bacterial cell wall permeability and inhibit nucleic acid synthesis and then cause death of the microorganism (194,195).

According to Gadisa *et al*., combined essential oils of oregano-basil, basil-bergamot,

oregano-bergamot and oregano-perilla have significant activity against *S. aureus, E. coli, B. subtilis and S. cerevisiae,* respectively. The synergistic effect of these essential oils may be due to synergistic or additive interactions between different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons, which might act on the same target or different targets (145). This finding is consistent with Nasir *et al.* (196), who postulated that the ability of plant extracts to act synergistically with antibiotics and other plant extracts could be considered a new approach to combat antimicrobial resistance. There is low risk of bacterial resistance in plant extracts and antibiotics combinations, due to the varied modes of action of the compounds present in the extracts, to which the organism had never been exposed before (196).

Antibacterial activity of *Lawsonia inermis* L. was also reported against wide range of gram-positives and gram-negatives (146,147). This could be due to the presence of a compound, 2-hydroxy-1,4- naphthoquinone. Quinones are the main constituent in the leaves of *L. inermis* L. and are known in making complexing irreversibly with nucleophilic amino acids, leading to inactivation of the protein and loss of function in microorganisms. Cell wall adhesions, polypeptides and membrane bound enzymes are the targets in microbial cells (146).

In another anti-microbial study, the leaf and stem bark extracts of *Azadiractha Indica* A.Juss. exhibited significant antibacterial activity against a wide range of bacteria due to the tricyclic diterpenoids isolated from stem bark, and azadirachtins, quercetin and β- sitosterol isolated from the leaves (197).

Bacha *et al* tested 18 plant extracts against *E. coli K12 DMS 498, S. aureus DMS 346, B. cereus ATCC 10987, B. cereus, Lab strain* and *P. aeruginosa 1117* using AWD and MID methods. The highest ZI was recorded with petroleum ether extract of *N. Sativa* L against *B. cereus* and *B. cereus ATCC 10987;* and mature unripe fruit oil of *Aframomum corrorima (A. Braun)* P. C. M. Jansen against *S. aureus.* The activities of petroleum ether extract of seed of *N. sativa* L against both laboratory isolated and reference strain of *B. cereus* were greater than the activity of gentamycin sulphate. The oil extract of unripe fruit of *A. corrorima* (A.Braun) P.C.M.Jansen was shown to have an activity comparable to the reference drug gentamycin sulphate. *P. aeruginosa* was the most resistant to all the plant extracts tested in this study (149). The antimicrobial activities of extracts of *A. corrorima*, *Nigella sativa* L*., A. angustifolium* (Sonn.) K. Schum. and *V. amygdalina* (Delile) Sch.Bip. were due to the presence of phenol, tannin, saponin and flavonoids, flavonoids and terpenoids compounds and their combinations (149).

The antibacterial activity of flavonoids is well documented and found in almost all parts of the plants, which inhibit the energy metabolism and synthesis of nucleic acids of different microorganisms (198). Furthermore, tannins were reported to have antibacterial activity

against *S. aureus,* acting by inducing complexation with enzyme or substrates and causes toxicity; and altering the membrane of the microbes (199).

Many studies have been carried out to screen medicinal plants for their antifungal activity, and various groups of researchers have initiated antifungal programs for traditionally used plants. Classes of compounds from plant metabolites, such as terpenoids (isoprenoids), saponins, phenolic compounds, flavonoids, coumarins, alkaloids, proteins and peptides showed anti-fungal activity against different fungal species (200,201). Under this review, 15 studies were included comprising 42 species of plant extracts against 50 species of fungus using agar well diffusion, disc diffusion, macro/microdilution and agar dilution methods.

Alcoholic extracts (methanol and ethanol) of *E. kebericho* Mesfin were tested by Ameya *et al.* against *A. flavus* and *C. albicans* using disc diffusion and agar dilution methods, and shown to cause significant inhibition at low concentration, comparable to the reference drug ketoconazole. The alcoholic solvents have the ability to extract phenolic compounds such as flavonoids, anthocyanins and phenolic acids which may contribute to the antifungal activity of the extracts (142).

Kasparaviciene *et al.* tested the activity of oleo-gels, formulated with different concentrations of thyme essential oil against *C. albicans* by broth dilution method, which showed significant activity with MIC value of 0.25%. Thymol was reported the major constituent of the thyme essential oil in this study. The biological activity of thyme essential oil depends on its yield and chemical composition, and the essential oils have several chemical names depending on the main constituents they have, such as thymol, carvacrol, terpineol, and linalool (153).

Similarly, Jain *et al.* reported the antifungal activity of *T. vulgaris* essential oil against *T. mentagrophytes* MTCC 7687*, M. gypseum* MTCC 452, *M. fulvum*MTCC2837*, T. rubrum* MTCC 296, *T. soudanense* (isolate) and *T. interdigitale* (isolate) using macro-dilution method. *T. vulgaris* L. essential oil was shown to have significant activity against the dermatophytes with MIC ranges from 0.02 to 0.1µl/mL (154). These activities could be due to high content of phenolic compounds and potent vapour activity against dermatophytes (202). This finding agrees with the report of Marina *et al.,* which showed the activity of *T. vulgaris* L. essential oil against *Alternaria alternata*, *Fusarium tricinctum*, all Aspergillus species and dermatomycetes at concentration of 0.25 µL/mL and *Phomopsis helianthi* and *Cladosporium cladosporioides* at 0.125 µL/mL by macro-dilution method (203).

In another study, *T. ammi* (L.) Sprague seed extract exhibited potent antifungal efficacy, with a maximum MZ of 38.3 mm diameter against *C. albicans* using the AWD method (204). This is in agreement with the finding of Sharifzadeh *et al.,* which evaluated *T ammi* (L.) Sprague essential oil against *C. albicans*, which were fluconazole-resistant, with MIC values ranging from 300 to 400 µg/mL (205). The extracts of *A. Indica* A. Juss was also shown to

have antifungal activity, attributable to the terpenoids. The fractions of *A. Indica A. Juss* have complex mixtures of compounds reported to have synergistic and additive effect of against fungus (206).

2.5. Conclusion

From the present study it can be concluded that there are promising medicinal plant extracts and compounds traditionally employed in Ethiopia to be used as anti-infective, anti-inflammatory, and wound healing agents. Among these plants, *Lawsonia inermis* L., *Azadiractha Indica* A. Juss, *Cuminum cyminum L.* and *Achyranthes aspera L.* are the most studied plant species for the management of wound infection and inflammation, whereas *Lawsonia inermis. L.* and *Azadiractha Indica* A. Juss were the most studied for wound healing.

In terms of effectiveness, methanol extract of *Jasminium abyssinicum*, chloroform extract of *Azadiractha Indica* A. Juss, a flavonoid isolated from *Lawsonia inermis* L. and acetone extract of *Vernonia amygdalina (Delile) Sch.Bip. (Delile) Sch.Bip. (Delile) Sch.Bip.* are the most effective anti-inflammatory agents in animal models. Similarly, methanol extracts of *Lawsonia inermis* L., essential oil of *Eugenia caryophyllata Thunb.,* butanol fraction of *Alnus nepalensis D.Don, Amaranthus dubius Mart. ex Thell., Ocimum americanum L., Vigna unguiculata (L.) Walp., and Zanthoxylum chalybeum Engl.* are the most effective plant extracts in regulating inflammatory and pro-inflammatory cells in *in vitro* model. On the other hand, ethanol extract of *Lawsonia inermis L.* aqueous extract of *Azadiractha Indica A. Juss* 5% w/w ointment of *Azadiractha Indica* A. Juss , extract and fractions of *Calpurnia aurea (Aiton) Benth.* in 10% ointment base, 10% (w/w) extract of *Croton macrostachyus Hochst. ex Deliles* in ointment base, leaf latex of *Aloe megalacantha Baker* (5% and 10% in ointment base) are the most effective plant materials for the management of wounds in animal models.

Whereas *Calpurnia aurea (Aiton) Benth.* (Aiton) Benth*., Croton macrostachyus Hochst. ex Deliles* Hochst. ex Delile*, Withania somnifera* (L.) Dunal, *Achyranthes aspera L. Datura stramonium* L., *Solanum incanum* L., *Verbascum erianthum* Benth., *Nigella sativa* L., *Gymnanthemum amygdalinum* (Delile) Sch.Bip., *Olinia rochetiana* A.Juss., *Sida rhombifolia* L*, Bersama abyssinica* Fresen. and *Azadiractha Indica* A. Juss are the most studied plants species against bacteria, and *Azadiractha Indica* A. Juss and *Lawsonia inerms* L. against fungal species. Thymoquinone, a constituent of the black seed of *Nigella sativa* L., alcoholic extract of *Echinop skebericho* Mesfin, *Aloe sinana* Reynolds and its compounds (Microdontin, Aloin and Aloinoside), alcoholic extract of *Azadiractha Indica* A. Juss. and *Lawsonia inerms* L. are the most effective plant materials against gram negative and gram-positive species. In addition, *Azadiractha Indica* A. Juss and *Lawsonia inerms* L. have activity against a wide

range of gram-negative and positive bacterial strains. Similarly, methanol extract of *Echinops kebericho* Mesfin and oleo-gels formulated with different concentrations of thyme essential oil are the most effective against different fungal species.

2.6. Strength and limitations

This systematic review will provide up-to-date information on Ethiopian medicinal plants used as anti-infective agents that might potentially be used for limb care (lymphoedema and associated wounds). This information could lead to the development of more research on the investigation of the effect of medicinal plants on against infection for future therapeutic use. However, as it will summarizes studies written only in English this could be and which is considered the anticipated one of the limitations of this review. In addition, this study has considered a wide range of methodological approaches and used medicinal plants with a wide range of concentrations. .

2.7. Implications for future research and recommendations

It is vital to systematically summarize, and document medicinal plants tested against different diseases and used traditionally for treatment, and to test further their effectiveness against a range of disease-related pathology such as lymphoedema. Information about many medicinal plants is fragmented, meaning that systematic compilation and synthesis is important. This systematic review helped us in identifying medicinal plants for the planned anti-inflammatory, wound healing and anti-infective effects which aimed for the development of safe, effective, and affordable alternative for the management of tropical lymphoedema. It will also create an opportunity for future research and practice by identifying and characterizing compounds that could be developed into new standardized medicines. Medicinal plants hold much promise for treating diseases, and there is a great potential for new drug discovery and development.

CHAPTER THREE

3. ANTIBACTERIAL ACTIVITY OF SELECTED ETHIOPIAN MEDICINAL PLANTS AGAINST BACTERIA ISOLATED FROM WOUNDS OF LYMPHOEDEMA PATIENTS

3.1. Background

Lymphoedema has a marked physical and psychological impact in affected patients and significantly reduces their quality of life (3). Wound ulcer development is one of the most serious complications and often makes it impossible for patients to work (207). Patients with lymphoedema have a high risk of wound formation resulting from infection, including fungal infection in skin folds, moisture build-up and trauma (207). Skin lesions, including wounds, fissures, paronychia, and eczema, allow the penetration of bacteria and fungus into the underlying tissues. Secondary infection along with inflammation also seems to play a major role in the skin changes seen in the limbs of individuals affected by lymphoedema and the development of elephantiasis (207).

The lower limbs are more prone to infection than other parts of the skin because of exposure to the environments, which predisposes the skin to bacterial colonization. In patients with lymphoedema, the lymphatic system is partially or completely halted (208). As a result, the lymph transport is restricted and the patient predisposed to infection and chronic dermatolymphangioadenitis (DLA) (208). Risk factors for recurring infection in lymphoedema patients are minor skin injuries, venous insufficiency, nematode infection and skin diseases, such as fungal infections (209).

Since wound colonization most frequently involves microorganisms that are potentially pathogenic (poly-microbial), the wound is at risk of becoming infected (210). The most frequent micro-organisms reported to cause wound infection are *Staphylococcus aureus, Streptococcus pyogenes, Enterococci, Escherichia coli, Klebsiella pneumonia, Proteus species and Pseudomonas aeruginosa* (211). The resistance of *Staphylococcus aureus* to methicillin remains a global problem which makes wound infections more challenging. Use of broad-spectrum antimicrobial agents changes microbial genetic ecology, which results in spread of antimicrobial resistance globally (212).

Continued use of systemic and topical antimicrobial agents has provided selective pressure that has led to emergence of antibacterial-resistant strains which in turn, has driven the continued search for new agents. The increased costs of searching for effective antimicrobial agents and the decreased rate of new drug discovery has made the situation increasingly worrisome (210). On the other hand, lymphoedema and its associated wounds is a major

concern for its increasing disability, stigma, and financial impact in places where podoconiosis and lymphatic filariasis are prevalent (213).

Systemic antibiotic therapy is recommended for complicated wound infections, cellulitis and sepsis. However, there is an increase in the prevalence of antimicrobial resistance due to misuse of antimicrobial agents, which results in the insufficient control of some microbial infections. Therefore, it necessitates the development of safe and effective new antimicrobials.

Traditional medicines with herbal remedies have an important role in the management of microbial infection. Folk medicine provides an important and unexplored resources of herbal remedies for the discovery and development of potentially new medicines for microbial infections to overcome resistance and toxicity. Furthermore, use of medicinal plants plays an important role to overcome the need of developing countries (214).

In Ethiopia, ethnobotanical studies reported by different researchers showed that endemic plant extracts have been used against different aliments by traditional healers. Most of the herbal medicines are very popular and used by different societies for the management of open wound infections. However, the scientific evidence available regarding the antibacterial activity of traditionally used endemic plant extracts against bacterial pathogens that involved in wound infection are limited (152).

3.2. Objectives

To date, the organisms found in the wounds of patients with lower limb lymphoedema in Ethiopia have not been identified. Therefore, this study has two main aims. The first was to investigate the bacterial profile involved in wound infection of patients with lymphoedema, and to conduct antimicrobial susceptibility tests against commonly used antibacterial agents. Second, to test the antibacterial activity of the methanol extracts of *Lawsonia inermis*, *Azadirachta indica*, and *Achyranthus aspera* against selected bacteria isolated from the wound of patients with lymphoedema, and against standard ATCCs.

3.3. Material and Methods

3.3.1. Materials

3.3.1.1. Plant selection

Following the information obtained from the systematic review (Chapter Two), plants which were used traditionally by the traditional healers and easily available, *Lawsonia inermis, Azadirachta indica* and *Achyranthes aspera* were selected for the evaluation of antibacterial

activity against selected bacteria isolated from the wound of patients with lymphoedema and standard ATCCs.

3.3.1.2. Plant materials collection

Plants materials were collected from different parts of Ethiopia. The plant collection was carried out in consultation with a botanist from Ethiopian Public Health Institute, local people, and traditional healers in the areas. The plants were collected in two categories. The first part was used for authentication and the second for experimental use. The specimens were archived at the Herbarium of Ethiopian Public Health Institute after voucher specimen numbering. The following information was included in the voucher for each specimen: Voucher number, confirmed scientific name, local plant name, previous purpose, district of collection, Kebele, village, habitat, and date of collection. The list of three medicinal plants shown in Table 3.1 were collected from their habitat. Plants destined for extraction were transported in ice-cooled boxes and stored at -80°C until used for experiment.

S/N	Plant Scientific name	Local name	Place of collection	Plant parts collected
1	*Achyranthes aspera* (Amaranthaceae)	Telenge	In the Abay (Nile) Gorge, 210 kms northwest from Addis Ababa to Dejen, Ethiopia.	Leaves
2	*Lawsonia inermis* (Lamiaceae)	Henna	Laga Gandisa (River Gandisa), 53 kms from Dire Dawa Town on the way to Erer, Ethiopia	Leaves
3	*Azadirachta indica* (Meliaceae)	Neem, Kinin	Kurare Goti, home garden cultivated, 209 kms northwest from Addis Ababa to Dejen Town Ethiopia.	Leaves

Table 3.1: List of medicinal plants used for this study

3.3.1.3. Solvents and Chemicals

The following chemicals and solvents were used: N- hexane (LobaChemie, India), methanol (LobaChemie, India), ethanol, 1N hydrochloric acid (LobaChemie, India), anhydrous sodium sulphate (Bulex Laboratory, India), purified distilled water (EPHI), alcoholic (LobaChemie, India), Milton's reagent (Otto Chemie, India), Dragendroff's reagent (Otto Chemie, India), Wagner's reagent (Otto Chemie, India), Mayer's reagent (LobaChemie, India), 2% copper sulphate (Chemicals Udyog, India), chloroform (Loba Chmie, India), acetic anhydride (Chemicals Udyog, India), ferric chloride (Chemicals Udyog, India), sulfuric acid (Chemicals Udyog, India), sodium hydroxide (Chemicals Udyog, India), lead acetate (Chemicals Udyog, India), magnesium ribbon (Chemicals Udyog, India), Conc. Hydro-chloric acid (LobaChemie, India), phosphate buffered saline (PBS) (Chemicals Udyog, India), and Di-methyl-sulfoxide (DMSO) (Loba Chemie, India), sterile saline solution and iodonitrotetrazolium Chloride (ITN) (Fluka Biochemika, Switzerland)

3.3.1.4. Reference drugs, kits and media

Reference anti-biotics discs from Thermo-Fisher Scientific (cefixime (5µg), ciprofloxacin (5µg), cefoxitin (30µg), penicillin (10U)**),** blood agar (HiMedia laboratories, India), nutrient agar, MacConkey agar, Mueller Hinton broth (HiMedia laboratories, India), Mueller Hinton agar (HiMedia laboratories, India), Amies with charcoal transport medium (HiMedia laboratories, India), mannitol salt agar (HiMedia laboratories, India), Vitek AST-GP67 cards (BioMérieux UK Ltd, UK), VitekAST-GN71 (BioMérieux UK Ltd) cards, Vitek identification card for gram positives and gram negatives, Mueller Hinton Broth, Mueller Hinton agar and 0.85 % saline water.

3.3.1.5. Bacterial strains

The bacterial strains for this experiment were isolated from the wound of lymphoedema patients from East Wollega Zone. Standard reference bacteria were obtained from the National Referral Bacteriology and Mycology laboratory, Ethiopian Public Health Institute.

Clinical isolates- *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella Pneumonia, Pseudomonas aeruginosa* and *Shewanella alage* were used.

Reference bacteria (Ethiopian Public Health Institute, National Clinical Bacteriology and Mycology reference laboratory)–MRSA *Staphylococcus aureus* ATCC®43300™*, Staphylococcus aureus* ATCC25923*, Escherichia coli* ATCC25922*, Klebsiella Pneumonia* ATCC700603 and *Pseudomonas aeruginosa* ATCC27853 were used.

3.3.2. Methods

3.3.2.1. Study design and area

This cross-sectional study was conducted from 15 August to 5 September 2019 in East Wollega zone, Oromia Regional State, Ethiopia. Patients who visited the Konchi, Sire, Boneyya, and Bata Beseka Clinics were screened for lymphoedema with associated wounds and enrolled in the study for swab collection. All study participants were informed verbally in the local language about the objectives of the study. Written informed consent was then obtained from each participant. Ethical clearance was obtained from the Brighton and Sussex Medical School, Research Governance & Ethics Committee (Ref. ER/BSMS9DY2/1), and Institutional Review Board (Ref. 004/19/CDT), College of Health Sciences, Addis Ababa University. Demographics and socioeconomic characteristics (such as age, sex, educational background, occupation, residence), and clinical characteristics (stage of lymphoedema, type of wound, treatment received) were collected using a structured questionnaire. Lymphoedema was categorized into three stages (mild, moderate and severe) based on the International Society of Lymphology classification (215).

3.3.2.2. Swab collection and processing

Wounds were cleaned with sterile normal saline and wound swabs and discharge were obtained from all study participants aseptically using a sterile moistened cotton swab. Swabs were then immersed in a container of Amies transport medium with charcoal (Bio mark Laboratories, Pune, India). All samples were transported on ice to the Ethiopian Public Health Institute, National Referral Bacteriology and Mycology Laboratory (Ethiopian National Accreditation Office ac-credited and ranked as Five Star by American Society for Microbiology) where all laboratory tests were conducted. Swabs were used to inoculate MacConkey agar (Becton Dickinson and Company, Cockeysville, MD, USA), blood agar and mannitol salt agar (both from HiMedia Laboratories, Mumbai, India) and incubated aerobically at 37 ºC, and 5% CO2 for 24 hours. After 24 hours, plates without growth were incubated further for up to 48 hours.

3.3.2.3. Identification and antimicrobial susceptibility tests

Growth of micro-organisms was identified by examining colony morphology followed by biochemical identification using the automated VITEK® 2 COMPACT Microbial Detection System (bioMerieux, Marcy l'Etoile, France). The antibiotic susceptibility tests for each bacterial species were completed using the VITEK® 2 system susceptibility testing cards for gram-positive (antimicrobial susceptibility tests-gram-positive [AST-GP67] panel) and gram-negative (antimicrobial susceptibility tests-gram-negative [AST- GN71] panel) bacteria according to the manufacturer's instructions.

The VITEK 2 is an automated microbiology system utilizing growth-based technology. The systems accommodate a colorimetric reagent card that are incubated and interpreted automatically. The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. Inside the filling chamber the culture suspension will be inoculated into the GN and GP Cards by the help of a vacuum device then transferred in to loading chamber to be sealed and incubated in a rotating carousel at 37oC. Every 15 minutes the cards will be remove and transported to the optical system for reaction readings and returned to the carousel incubator until the next read time. Data will be captured at 15-minute intervals during the whole incubation period. Interpretation of each test will be done by the optical system using different wavelengths then after calculation are made the testing data from unknown organism are compared to the respective database to determine a quantitative value for proximity to each of the database bacterial taxonomy. Finally, the result will be displayed with the confidence level excellent (96% to 99%), very good (93% to 95%), good (89% to 92%) and acceptable (85% to 88%).

After the VITEK® 2 COMPACT automated ID/AST instrument was validated, according to the manufacturer's instructions, using the standard strains, the 24-hour bacterial cultures were tested. Aseptically, 3.0 ml of sterile saline (0.45–0.5% NaCl, pH 4.5–7.0) was transferred into a clear glass test tube (12 mm x 75 mm) and morphologically similar colonies were transferred to the saline using a sterile plastic loop. A homogenous suspension was prepared with a density equivalent to the appropriate McFarland standard (0.5 to 0.63) using the VITEK® 2 DensiCHEK Plus Instrument. A second tube of 3.0 ml saline contained 145 ml of the suspension for AST-GN or 280 ml of the suspension for AST-GP susceptibility testing cards. The time between preparation of inoculum and filling of the card was always less than 30 minutes. The tubes were then placed in the cassette with a susceptibility card. A barcode reader was used to scan the order number of identification and susceptibility cards and information from the cassette worksheet was entered in to the Maintain Virtual Cassette window on the workstation. The cassette was loaded into the filler station and transferred to the VITEK® 2COMPACT cassette loading station within 10 minutes. Results were obtained after 8–10 hours.

For *Shewanella algae,* the Kirby Bauer disk diffusion method was used for the antimicrobial susceptibility tests as it was not possible to test the antimicrobial susceptibility test for this organism using the VITEK® 2 system. For interpretation of zone of inhibitions, the Clinical and Laboratory Standards Institute guideline 2019 for Pseudomonas (Table 2B2) was used (216).

Multidrug resistance (MDR) was defined as an acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Extensive drug resistance (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. Pan drug resistance (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories (217).

3.3.2.4. Extraction and preparation of plant materials

Extraction of individual plants was done based on methods previously used with slight modifications. Each plant powder was successively extracted with three organic solvents with increasing polarity order (N-hexane, ethyl acetate, methanol and aqueous). Sequential extraction schemes give detailed information about the origin, mode of occurrence, mobility, biological and physiochemical availability of the trace elements in solid samples, and have been shown to provide a convenient means to determine the trace elements associated with principal accumulative phases in soils and sediments. Three hundred gram of each powder was soaked in 1.5 liters of petroleum ether separately and kept on by VWR DS 500 orbital shaker for 72hours. Then, extracts were filtered with WhatmanNo1 filter paper. The residue was further extracted twice with fresh petroleum ether solvent. Then all the filtrates

were combined. The resulting residues were air dried and further extracted with ethyl acetate, methanol and sterile water with similar procedure that carried out for the petroleum ether extraction. Finally, organic solvents were removed from the extracts using rotavapor and by keeping the extracts for some days in a water bath (40^0C). After complete drying, yield of each extraction was measured separately, and the extracts were stored at 2-8^0c until used for further study. Methanol extracts of the leaves of each plant was used for the pharmacological activities. Methanol is an ideal solvent because of its polarity . It can extract both hydrophilic and lipophilic molecules from plant parts. It was identified as the most effective solvent for the extraction which result in the highest extraction yield. Dried methanol extracts of *A. aspera*, *L. inermis L*, *A. Indica* A. Juss were dissolved in 10% DMSO and kept at 2-8^0c until used for activity.

3.3.2.5. Preliminary Phytochemical screening of the extracts

Phytochemical analysis of the methanol extract of *A. aspera, L. inermis* L*, A. Indica* A. Juss were performed using standard procedures to determine the constituents presented in the extracts (218).

Test for Alkaloids: Extracts from plants were dissolved in HCl and filtered for the following tests.

a. **Mayer's Test**: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Yellow precipitation indicates the presence of alkaloids in the extracts.

b. **Dragendroff's Test**: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Red precipitation indicates the presence of alkaloids in the extracts.

Test for Saponins (Foam Test): extract was shaken with 2 ml of water. If foam produced persists for ten minutes, it indicates the presence of saponins.

Phenol test: 0.5gm crude extracts was treated with a few drops of 2% $FeCl_3$ blush green or black coloration indicated presence of phenolic compound.

Test for Tannins (Ferric chloride test): each plant extract was stirred with 1ml of distilled water, after filtered, ferric chloride reagent added to the filtrate. A blue-black, green, or blue-green precipitate indicates the presence of tannins.

Test for Anthraquinones: chloroform was added to the extracts and shaken for 5 minutes. The extract was filtered and shaken with an equal volume of 100% ammonia solution. A pink, violet or red color in the ammoniacal layer (lower layer) indicated the presence of free anthraquinones.

Test for Terpenoids: each extract was dissolved in chloroform, then 3 ml of concentrated sulfuric acid was added carefully and examine and if reddish brown coloration indicates the presence of terpenoid.

Test for steroids: Chloroform 10ml was added to 2ml of all three plant extracts. To these extracts 1ml of acetic anhydride was added and then 2ml of concentrated sulphuric acid was added along the sides of the test tube. Colour formation at the junction is noted. The appearance of blue green colour indicates the presence of steroids.

Test for Flavonoids (Alkaline Reagent Test): Extracts were treated with drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, shows presence of flavonoids.

3.3.2.6. Bacterial culture and inoculum preparation

Fresh cultures of bacteria were prepared from frozen stocks and streaked on Mueller Hinton agar (MHA)plates and incubated for 24 hrs at 37^0C aerobically in incubator. After 18-24hrs incubation, a single colony of the microorganisms was picked and inoculated to 3ml sterile saline solution. Then the saline tube was vortexed to create uniform solution and turbidity was adjusted to 0.5 McFarland standard (10^8 CFU/ml).

3.3.2.7. Antibacterial activity assays

Agar wells diffusion assay – Kirby-Bauer technique according to the criteria of the Clinical Laboratory Standards Institute (CLSI) method was used to determine the antibacterial activity of the extracts (219). A total of 10 bacteria were used for this test. Mueller Hinton agar (pH 7.2 & 4mm depth) plates were inoculated by test organisms (prepared in sterile saline tube) by streaking the loop in a back-and-forth motion to ensure an even distribution of inoculum. MHA with 5% sheep blood was used for *Streptococcus pyogenes*. A circular 6mm diameter well was punched aseptically with a sterile cork borer. Then, a volume of 100µl methanol extracts of *A. aspera*, *L. inermis L*, *A. Indica* A. Juss (at concentration of 100 mg/ml, 200 mg/ml and 400 mg/ml) were dispensed into the wells. Similarly, 5% DMSO was dispensed into the control well and reference antibiotic discs were place on the surface of the plate and incubated for 24hrs at 37^0C. For *Streptococcus pyogenes,* carbon dioxide incubator was used for incubation. Each experiment was done three times. Plant extracts diffuse in the medium and inhibits the growth of bacteria.

Microdilution method– The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using p-iodonitrotetrazolium chloride (INT) colorimetric assay method (220). The test was done as per the recommendation of the Clinical Laboratory Standard Institute (CLSI) (221).

Bacteria were subculture on Mueller Hinton agar (pH 7.2) and incubated at 37^{O}C for 24hrs. Bacterial colonies were inoculated to a sterile saline solution and used before 30 minutes. The bacterial suspension was evenly mixed and diluted to meet the turbidity of 0.5 McFarland standard (1x10^{8} CFU/ml). Further dilution was performed to obtain the final concentration of inoculum (5x10^{5} CFU/ml) in each well. Stock solution of plants extracts was prepared in DMSO. Serial dilution of the working solution was prepared by diluting the extract solution in sterile Mueller Hinton Broth. The final concentration of DMSO was less than 2.8% in the solution. The test was performed in sterile 96-well plate. Methanol extracts of *A. aspera*, *L. inermis L*, *A. Indica* A. Juss were tested in triplicate in one plate for each bacterium. Mueller Hinton Broth (100µl) was dispensed to all wells. Working solution of extracts (100µl) and solvent controls (MH broth and 2.8% DMSO) were dispensed to their respective wells. Serial dilution was performed from columns one to nine and 50 µl of excess medium was discarded from column nine. The last columns served as a blank which contains only broth. Columns 10 and 11 were served as negative control which only contain medium and bacterial suspension, and media DMSO and bacteria respectively. 50µl of test bacteria were added to each well except for the last row which served as a blank. The concentration of plant extracts was ranged from 0.78 mg/ml to 200 mg/ml. The plates were sealed and incubated for 24 hours at 37°C. After 24hrs incubation, 40 µl (0.2 mg/mL) p-iodonitrotetrazolium chloride (INT) was added to all wells and incubated again at 37°C for 30 min. The MIC of samples was detected after 30 minutes of incubation. Viable bacteria reduced the yellow dye to a pink. MIC was defined as the sample concentration that prevented the colour change of the medium and exhibited complete inhibition of microbial growth. The MBC was determined by adding 50µl aliquots from the wells which did not show growth after incubation for the MIC test to 150µl broth in the well plate. Then, incubated for 48hr at 37^{0}C. Then, MBC was observed as the lowest concentration of extracts which did not produce a colour change after the addition of INT as mentioned above.

3.4. Statistical analysis

Sociodemographic data were entered and analysed using the statistical package for social science (SPSS) version 20. Descriptive analyses such as frequencies and mean were used, and results were presented using tables and charts. The statistical differences of the mean zone of inhibition of extract for individual bacterium and differences in the susceptibility of the test microorganisms were carried out by employing ANOVA followed by Tukey's Post Hoc at a significance level of $P<0.05$. MIC was analysed using descriptive statistics.

3.5. Results

A total of 103 participants with lymphoedema were screened and had swab samples

collected. Of these, 33 (32.0%) were male and 70 (68.0%) were female. Patients included in the study had a mean ± SD age of 44.86 ± 14.23 (range 19–75) years. The majority (74.8%) of study participants were farmers and 81.6% could not read or write (Table 3.2).

According to the International Society of Lymphology classification (215) 64 (62.1%) participants had advanced disease, 34 (33.0%) had moderate disease and 5 (4.9%) had mild disease (Table 3.2). In all participants, lymphoedema was confined to below the knee and no patient had hydrocele. All study participants had open, contaminated, and chronic wounds. In total 93 (90.3%) participants had bilateral lymphoedema, and the rest had just one affected leg. Patients had lived with lymphoedema for a mean ± SD of 15.8 ±11.53 (range 1– 50 years).

The majority (83, 80.6%) of patients had visited the clinic for treatment at least once previously while 20 (19.4%) were new cases. Among those who came to the clinic and took medications and herbal ointments, symptoms had partially resolved for 63 (75.9%) of the participants, relapsed for 16 (19.3%), not changed for 2 (2.4%) and worsened for 1 (1.2%).

Characteristics	Number (%) patients
Sex	
Male	33 (32.0)
Female	70 (68.0)
Age group (years)	
19–25	7 (6.8)
26–35	28 (27.2)
36–45	20 (19.4)
46–55	23 (22.3)
≥55	25 (24.3)
Occupational status	
Farmers	77 (74.8)
Daily labourers	10 (9.7)
Government employee	1 (1.0)
Housewives	8 (7.8)
Merchants	7 (6.8)
Stage of lymphoedema[a]	
Mild	5 (4.9)
Moderate	34 (33.0)
Severe	64 (62.1)
Time lived with lymphoedema (years)	
1–10	46 (44.7)
11–20	36 (35.0)
21–30	10 (9.7)
31–40	9 (8.7)
41–50	2 (1.9)
Education level	
Could not read or write	84 (81.6)
Could read and write	2 (1.9)
Completed grades 2–5	8 (7.8)
Completed grades 6–9	6 (5.8)
Completed grades ≥10	3 (2.9)

Table 3.2: Sociodemographic characteristics of participants

3.5.1. Bacterial Profile

Most (84, 81.6%) samples were culture positive, indicating wound infection; 44 (52.4%) samples showed polymicrobial growth while 40 (47.6%) grew single bacterial isolates. Among the 134 isolates, 26 gram-negative and 12 gram-positive bacterial species were isolated (Figures 3.1 and 3.2). Two species of bacterial infection was observed most frequently 37 (44.0%), followed by three species 4 (4.8%) and four species 1(1.2%).

3.5.2. Antimicrobial susceptibility pattern of isolated gram-negative bacteria

Gram-negative bacteria isolated from patient wounds were tested against 18 selected antibacterial drugs, and susceptibility varied with type of organisms and antibacterial drug employed. In a total of 76 gram-negative isolates, levels of resistance were highest against ampicillin (43 [56.6%]) followed by cefazolin (28 [36.8%]), ampicillin/sulbactam (21 [27.6%]), trimethoprim/sulfamethoxazole (10 [13.2%]) and tigecycline (7 [9.2%]). All isolates were susceptible to ertapenem and ciprofloxacin and showed low levels of resistance to amikacin (1 [1.3%]), gentamicin (1 [1.3%]), moxifloxacin (1 [1.3%]), cefepime (1 [1.3%]), tobramycin (2 [2.6%]), aztreonam (4 [5.3%]), ceftriaxone (6 [7.9%]), meropenem (7 [9.2%]) and imipenem (8 [10.5%]) (Table 3.3). MDR of 63 gram-negative samples to different antibacterial drugs is summarized in Table 3.4. Among the identified gram-negative isolates, 28 (44.4%) were MDR, while a low level of XDR 1 (1.6%) was observed (Table 3.4).

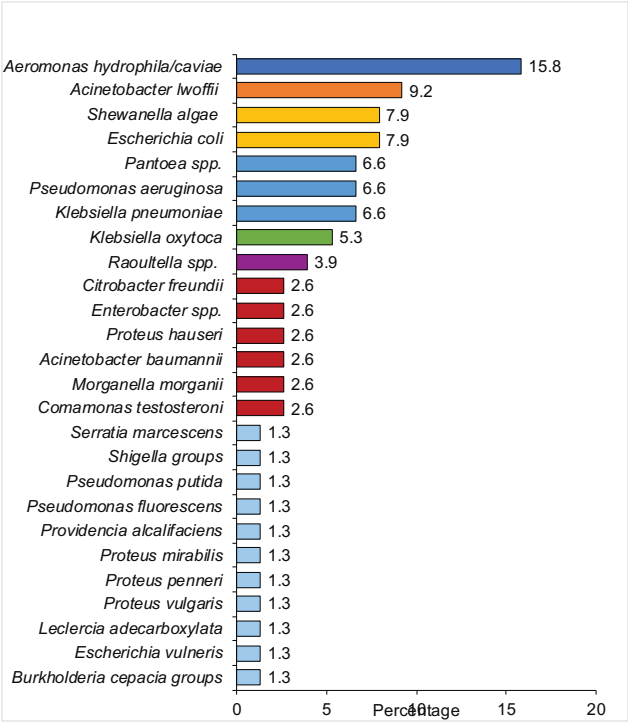


Figure 3.1: Percentage of gram-negative bacteria isolated from swab samples of lymphoedema patient with infected wounds.

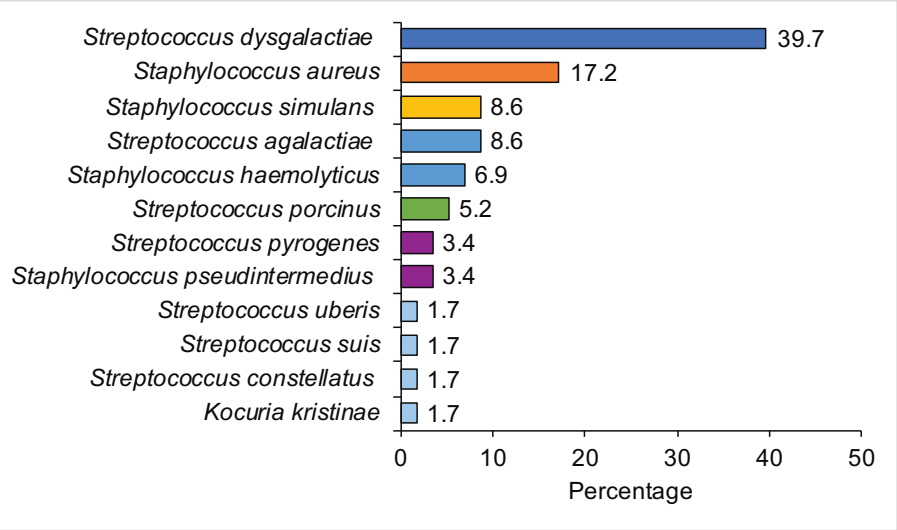


Figure 3.2: Percentage of gram-positive bacteria isolated from swab samples of lymphoedema patient with infected wounds.

3.5.3. Antimicrobial susceptibility pattern of isolated gram-positive bacteria

Gram-positive isolates were tested against 20 selected antibacterial drugs and most of the isolates were susceptible. Across 58 gram-positive isolates, the level of resistance was highest to erythromycin (21 [36.2%]) and clindamycin (18 [31.0%]), followed by tetracycline (11 [19.0%]) and penicillin (10 [17.2%]) (Table 3.5). Low levels of resistance were observed against oxacillin (2 [3.4%]), trimethoprim/sulfamethoxazole (5 [8.6%]), tigecycline (2 [3.4%]) and ciprofloxacin (1 [1.7%]). All isolates of gram-positives were susceptible to gentamicin, levofloxacin, moxifloxacin, quinupristin/dalfopristin, linezolid, vancomycin.

MDR patterns of 57 gram-positive bacterial isolates from wounds of lymphoedema patients are summarized in Table 3.6. Much lower levels of MDR (3 [5.3%]) were identified among the gram-positive compared with gram-negative isolates. Overall, 2 (3.5%) of gram-positive isolates were cefoxitin-screening (methicillin resistant staphylococcus aureus [MRSA]) positive and 7 (12.3%) showed inducible clindamycin resistance.

Microbial species isolated (n)	ESBL n (%)	Resistance pattern	Penicillins		Cephalosporin			Carbapenem				Aminoglycosides			Fluoroquinolones		Glycylcycline	Sulfonamides	Penicillins	Cephalosporin
			A	AS	CFZ	CTR	CP	AZM	E	IPM	M	AK	G	TM	CX	MXF	TGC	TS	PB	CZ
Total gram-negative isolates (n=76)	1 (1.3)		43 (56.6)	21 (27.6)	28 (36.8)	6 (7.9)	2 (1.3)	4 (5.3)	0 (0)	8 (10.5)	7 (9.2)	1 (1.3)	1 (1.3)	2 (2.6)	0 (0)	1 (1.3)	7 (9.2)	10 (13.2)	0 (0)	0 (0)
Acinetobacter baumannii (n=2)	NA	S	0 (0)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)	ND	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	2 (100)	1 (50)	2 (100)	0 (0)	0 (0)	2 (100)	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Acinetobacter lwoffii (n=7)	NA	S	5 (71.4)	6 (85.7)	6 (85.7)	6 (85.7)	7 (100)	7 (100)	ND	7 (100)	6 (85.7)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)	6 (85.7)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0)	0 (0)	ND	0 (0)	1 (14.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14.3)	1 (14.3)	ND	ND
Aeromonas hydrophila/ caviae (n=12)	NA	S	1 (8.3)	2 (16.7)	4 (33.3)	12 (100)	12 (100)	12 (100)	ND	10 (833)	7 (58.3)	12 (100)	11 (91.7)	11 (91.7)	12 (100)	12 (100)	12 (100)	11 (91.7)	ND	ND
		I	1 (8.3)	1 (8.3)	1 (8.3)	0 (0)	0 (0)	0 (0)	ND	1 (8.3)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	10 (83.3)	9 (75)	7 (58.3)	0 (0)	0 (0)	0 (0)	ND	1 (8.3)	5 (41.7)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	0 (0)	1 (8.3)	ND	ND
Burkholderia cepacia groups (n=1)	NA	S	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	1 (100)	0 (0)	1 (100)	0(0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Citrobacter freundii (n=2)	NEG	S	ND	ND	0 (0)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	ND	ND	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Enterobacter species (n=2)	NEG	S	ND	ND	0 (0)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	ND	ND	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Morganella morganii (n=2)	NA	S	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Escherichia vulneris (n=1)	NEG	S	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Escherichia coli (n=6)	NEG	S	2 (33.3)	2 (33.3)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	5 (83.3)	5 (83.3)	0 (0)	4 (66.7)	ND	ND
		I	0 (0)	2 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	0 (0)	6 (100)	0 (0)	ND	ND
		R	4 (66.7)	2 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	0 (0)	2 (33.3)	ND	ND
Klebsiella oxytoca (n=4)	NEG	S	0 (0)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Klebsiella pneumoniae (n=5)	1 (20%)	S	0 (0)	4 (80)	4 (80)	4 (80)	4 (80)	4 (80)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	4 (80)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	5 (100)	1 (20)	1 (20)	1 (20)	1 (20)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	ND	ND
Leclercia adecarboxylata (n=1)	NA	S	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Morganella morganii (n=2)	NA	S	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Pantoea species (n=5)	NA	S	ND	ND	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	ND	ND
		I	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Proteus vulgaris (n=1)	NA	S	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Proteus hauseri (n=2)	NA	S	0 (0)	0 (0)	2 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Proteus penneri (n=1)	NA	S	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	ND	ND
Proteus mirabilis (n=1)	NA	S	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	ND	ND
Providencia alcalifaciens (n=1)	NA	S	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	ND	ND
		R	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	ND	ND
Pseudomonas fluorescens (n=1)	NA	S	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND

Microbial species isolated (n)	ESBL n (%)	Resistance pattern	Penicillins		Cephalosporin			Carbapenem				Aminoglycosides			Fluoroquinolones		Glycylcycline	Sulfonamides	Penicillins	Cephalosporin
			A	AS	CFZ	CTR	CP	AZM	E	IPM	M	AK	G	TM	CX	MXF	TGC	TS	PB	CZ
Pseudomonas putida (n=1)	NA	S	0 (0)	0 (0)	0 (0)	1 (100)	ND	ND	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	1 (100)	1 (100)	1 (100)	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	ND	ND
Pseudomonas aeruginosa (n=5)	NA	S	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)	5 (100)	5 (100)	4 (80)	4 (80)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	2 (40)	2 (40)	ND	ND
		I	0 (0)	0 (0)	0 (0)	2 (40)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	5 (100)	5 (100)	5 (100)	3 (60)	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (60)	3 (60)	ND	ND
Raoultella species (n=3)	NA	S	0 (0)	1 (33.3)	2 (66.7)	2 (66.7)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	ND	ND
		I	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	3 (100)	1 (33.4)	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Shewanella algae (n=6)	NA	S	ND	ND	ND	ND	6 (100)	ND	ND	6 (100)	ND	6 (100)	6 (100)	6 (100)	6 (100)	ND	ND	ND	6 (100)	6 (100)
		I	ND	ND	ND	ND	0 (0)	ND	ND	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	ND	0 (0)	0 (0)
		R	ND	ND	ND	ND	0 (0)	ND	ND	0 (0)	ND	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	ND	0 (0)	0 (0)
Shigella groups (n=1)	NA	S	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
Serratia marcescens (n=1)	NA	S	ND	ND	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	ND	ND
		I	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND
		R	ND	ND	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND

A: ampicillin; AK: amikacin; AS: ampicillin/sulbactam; AZM: aztreonam; CFZ: aefazolin; CP: cefepime; CT, cefotaxime; CTR: ceftrixaone; CX: ciprofloxacin; CZ: ceftazidime; E: ertapenem; ESBL: extended spectrum ß-lactamase; FA, fosfomycin; G: gentamicin; I: intermediate; IPM: imipenem; M: meropenem; MXF: moxifloxacin; ND: not determined; NEG: negative; PB: piperacillin/tazobactam; R: resistant; S: susceptible; TGC: tigecycline; TM: tobramycin; TS: trimethoprim/sulfamethoxazole.
Data show the type of antibiotic tested and the number (%) of bacterial isolates that are resistant, susceptible or intermediate to that antibiotic.

Table 3.3: Antibiotic resistance profiles of gram-negative bacteria isolated from wound of lymphoedema patients

Bacterial Isolates	N (%)	Antimicrobial patterns, n (%)						Multiple-drug resistance, n (%)			ESBL
		R0	R1	R2	R3	R4	≥R5	MDR	XDR	PDR	
Gram negatives	63 (100)	9 (14.3)	19 (30.2)	9 (14.3)	7 (11.1)	11 (17.5)	8 (12.7)	28 (44.4)	1 (1.6)	0 (0)	1 (20)
Acinetobacter baumannii	2 (3.2)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	2 (100)	1 (50)	0 (0)	NA
Acinetobacter lwoffii	7 (11.1)	5 (71.4)	1 (14.3)	0 (0)	0 (0)	0 (0)	1 (14.3)	1 (14.3)	0 (0)	0 (0)	NA
Aeromonas hydrophila	12 (19.0)	1 (8.3)	1 (8.3)	4 (33.3)	4 (33.3)	1 (8.3)	1 (8.3)	8 (66.7)	0 (0)	0 (0)	NA
Burkholderiacepacia groups	1 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	NA
Citrobacter freundii	2 (3.2)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Enterobacter Species	2 (3.2)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Comamonas testosteroni	2 (3.2)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	N/A
Escherichia coli	6 (9.5)	2 (33.3)	2 (33.3)	1 (16.7)	1 (16.7)	0 (0)	0 (0)	2 (33.3)	0 (0)	0 (0)	0 (0)
Klebsiella oxytoca	4 (6.3)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Klebsiella pneumoniae	5 (7.9)	0 (0)	4 (80)	0 (0)	0 (0)	1 (20)	0 (0)	1 (20)	0 (0)	0 (0)	1 (20)
Morganella morganii	2 (3.2)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	NA
Proteus vulgaris	1 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	NA
Proteus hauseri	2 (3.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	2 (100)	0 (0)	0 (0)	NA
Proteus penneri	1 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	NA
Proteus mirabilis	1 (1.6)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Providencia alcalifaciens	1 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	NA
Pseudomonas fluorescens	1 (1.6)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Pseudomonas putida	1 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	NA
Pseudomonas aeruginosa	5 (7.9)	0 (0)	0 (0)	0 (0)	1 (20)	1 (20)	3 (60)	4 (80)	0 (0)	0 (0)	NA
Raoultella species	3 (4.8)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Shigella groups	1 (1.6)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	NA
Serratia marcescens	1 (1.6)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA

Table 3.4: Patterns of multidrug resistance of gram-negative bacteria isolated from the infected wounds of patients with lymphoedema

ESBL: extended-spectrum beta-lactamases producing; MDR: multidrug resistant – resistant to at least one agent in three or more antimicrobial classes; NA: not applicable; PDR: pan-drug resistant – resistant to all antimicrobial agents in all antimicrobial classes; R0: no antibiotic resistant; R1: resistant to one antimicrobial category; R2: resistant to two antimicrobial categories; R3: resistant to three antimicrobial categories; R4: resistant to four antimicrobial categories; ≥R5: resistant to five and more antimicrobial categories; XDR: extensive drug resistant – resistant to at least one agent in all but two or fewer antimicrobial categories

Microbial species isolated (n)	Cefoxitin screening (MRS	ICM	Resistance pattern	Penicillin			Aminoglycoside			Quinolone			Macrolides	lincosamide	Streptogramins	Glycopeptide		Tetracyclines	Glycylcycline	Sulfonamides
				PEN	A	OX	GMHL	STRH	GM	CX	LVX	MXF	ERY	CM	QDA	LNZ	VA	TE	TGC	TS
Total gram positive isolates (n=58)	2 (3.4)	8 (13.8)		10 (17.2)	0 (0)	2 (3.4)	ND	ND	0 (0)	1 (1.7)	0 (0)	0 (0)	21 (36.2)	18 (31.0)	0 (0)	0 (0)	0 (0)	11 (19.0)	2 (3.4)	5 (8.6)
Kocuria kristinae (n=1)	1 (100)	1 (100)	S	0 (0)	ND	0 (0)	ND	ND	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
			R	1 (100)	ND	1 (100)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus aureus (n=10)	0 (0)	3 (30)	S	3 (30)	ND	10 (100)	ND	ND	10 (100)	10 (100)	10 (100)	10 (100)	6 (60)	7 (70)	10 (100)	10 (100)	10 (100)	9 (90)	10 (100)	8 (80)
			R	7 (70)	ND	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	4 (40)	3 (30)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	2 (20)
Staphylococcus haemolyticus (n=4)	1 (25)	1 (25)	S	3 (75)	ND	3 (75)	ND	ND	4 (100)	3 (75)	3 (75)	4 (100)	2 (50)	3 (75)	4 (100)	4 (100)	4 (100)	3 (75)	4 (100)	1 (25)
			I	0 (0)	ND	0 (0)	ND	ND	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
			R	1 (25)	ND	1 (25)	ND	ND	0 (0)	1 (25)	0 (0)	0 (0)	2 (50)	1 (25)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	3 (75)
Staphylococcus pseudintermedius (n=2)	0 (0)	NG	S	1 (50)	ND	2 (100)	ND	ND	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
			R	1 (50)	ND	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus simulans (n=5)	0 (0)	2 (40)	S	5 (100)	ND	5 (100)	ND	ND	5(100)	5(100)	5 (100)	5 (100)	4 (60)	4 (60)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
			R	0 (0)	ND	0 (0)	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	2 (40)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus agalactiae (n=5)	0 (0)	ND	S	5 (100)	5 (100)	ND	ND	ND	ND	5 (100)	5 (100)	5 (100)	2 (40)	2 (40)	5 (100)	5 (100)	5 (100)	3 (60)	3 (60)	5 (100)
			R	0 (0)	0 (0)	ND	ND	ND	ND	0 (0)	0 (0)	0 (0)	3 (60)	3 (60)	0 (0)	0 (0)	0 (0)	2 (40)	2 (40)	0 (0)
Streptococcus constellatus (n=1)	0 (0)	ND	S	1 (100)	1 (0)	ND	ND	ND	ND	ND	1 (100)	ND	1 (100)	1 (100)	ND	1 (100)	1 (100)	1 (100)	ND	1 (100)
			R	0 (0)	2 (0)	ND	ND	ND	ND	ND	0 (0)	ND	0 (0)	0 (0)	ND	0 (0)	0 (0)	0 (0)	ND	0 (0)
Streptococcus dysgalactiae (n=23)	0 (0)	ND	S	23 (100)	3 (0)	ND	ND	ND	23 (100)	23 (100)	23 (100)	23 (100)	18 (78.3)	19 (82.6)	ND	23 (100)	23 (100)	17 (73.9)	ND	23 (100)
			R	0 (0)	4 (0)	ND	ND	ND	0 (0)	0 (0)	0 (0)	0 (0)	5 (21.7)	4 (17.4)	ND	0 (0)	0 (0)	6 (26.1)	ND	0 (0)
Streptococcus porcinus (n=3)	0 (0)	ND	S	3 (100)	5 (0)	ND	ND	ND	ND	ND	3 (100)	ND	0 (0)	0 (0)	ND	3 (100)	3 (100)	3 (100)	ND	3 (100)
			R	0 (0)	6 (0)	ND	ND	ND	ND	ND	0 (0)	ND	3 (100)	3 (100)	ND	0 (0)	0 (0)	0 (0)	ND	0 (0)
Streptococcus pyrogenes (n=2)	0 (0)	1 (50)	S	2 (100)	7 (0)	ND	ND	ND	ND	ND	2 (100)	ND	2 (100)	ND	ND	2 (100)	2 (100)	0 (0)	ND	2 (100)
			I	0 (0)	8 (0)	ND	ND	ND	ND	ND	0 (0)	ND	0 (0)	ND	ND	0 (0)	0 (0)	1 (50)	ND	0 (0)
			R	0 (0)	9 (0)	ND	ND	ND	ND	ND	0 (0)	ND	0 (0)	ND	ND	0 (0)	0 (0)	1 (50)	ND	0 (0)
Streptococcus suis (n=1)	0 (0)	0 (0)	S	1 (100)	10 (0)	ND	ND	ND	ND	ND	1 (100)	ND	0 (0)	0 (0)	ND	1 (100)	1 (100)	1 (100)	ND	1 (100)
			R	0 (0)	11 (0)	ND	ND	ND	ND	ND	0 (0)	ND	1 (100)	1 (100)	ND	0 (0)	0 (0)	0 (0)	ND	0 (0)
Streptococcus uberis (n=1)	0 (0)	0 (0)	S	1 (100)	12 (0)	ND	ND	ND	ND	ND	1 (100)	ND	0 (0)	0 (0)	ND	1 (100)	1 (100)	1 (100)	ND	1 (100)
			R	0 (0)	13 (0)	ND	ND	ND	ND	ND	0 (0)	ND	1 (100)	1 (100)	ND	0 (0)	0 (0)	0 (0)	ND	0 (0)

Data show the type of antibiotic tested and the number (%) of bacterial isolates that are resistant, susceptible or intermediate to that antibiotic.

A: ampicillin; CM: clindamycin; CX: ciprofloxacin; ERY: erythromycin; FD: nitrofurantoin; GM: gentamicin; GMHL: gentamicin high level; ICM: inducible clindamycin; I: intermediate; LNZ: linezolid; LVX: levofloxacin; MXF: moxifloxacin; ND: not determined; NEG: negative; OX: oxacillin; PEN: penicillin; POS: postitive QDA: quinupristin/dalfopristin; R: resistance; RA: rifampin; S: susceptible; STRHL: streptomycin high level; TE: tetracycline; TGC: tigecycline; TS: trimethoprim/sulfamethoxazole; VA: vancomycin.

Table 3.5: Antibiotic resistance profiles of gram-positive bacteria isolated from wounds of lymphoedema

Bacterial isolates	N (%)	Antimicrobial resistance patterns, n (%)						MDR, n (%)	Cefoxitin screening (MRSA), n (%)	Inducible clindamycin, n (%)
		R0	R1	R2	R3	R4	≥R5			
Gram-positives	57 (100)	17 (29.8)	31 (54.4)	8 (14.0)	2 (3.5)	1 (1.8)	0 (0)	3 (5.3)	2 (3.5)	7 (12.3)
Kocuriakristinae	1 (1.8)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Staphylococcus aureus	10 (17.5)	2 (20)	4 (40)	3 (30)	0 (0)	1 (10)	0 (0)	1 (10)	0 (0)	3 (30)
Staphylococcus haemolyticus	4 (7.0)	0 (0)	2 (50)	1 (25)	0 (0)	1 (25)	0 (0)	1 (25)	1 (25)	1 (25)
Staphylococcus pseudintermedius	2 (3.5)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus simulans	5 (8.8)	3 (60)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (40)
Streptococcus agalactiae	5 (8.8)	1 (20)	2 (40)	1 (20)	1 (20)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)
Streptococcus dysgalactiae	23 (40.4)	9 (39.1)	11 (47.8)	3 (13.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus porcinus	3 (5.3)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus pyogenes	2 (3.5)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)
Streptococcus suis	1 (1.8)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus uberis	1 (1.8)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3.6: Multidrug resistance patterns of gram-positive bacteria isolated from wounds of lymphoedema patients

3.5.4. Plant extracts yield and characteristics

The percent yield of the methanol extracts of *A. aspera*, *L. inermis* L, *A. Indica* A. Juss and their characteristic are given in Table 3.7. Maximum yield was obtained from *L. inermis* L (15.9%), followed by *A. aspera* L (14.7%) and *A. Indica* A. Juss (7.9%).

S/N	Plant name with parts extracted	Extract types	Appearance	Consistency	Yield (% w/w)
1	*Lawsonia inermis* (leaves)	99.8% Methanol	Brown	Semisolid	15.9%
2	*Achyranthes aspera* (leaves)	99.8% Methanol	Dark green	Semisolid	14.7%
3	*Azadirachta indica* A. Juss (leaves)	99.8% Methanol	Grey	Powder	7.9%

Table 3.7: Medicinal plant extracts with their respective yield and characteristics

3.5.5. Preliminary phytochemical screening plant extracts

The methanol extracts of *L. inermis L, A. aspera Land A. Indica A. Juss leaves* were tested positive for the phytoconstituents alkaloids, terpenoids, phenolic compounds, tannins and steroids tests. Furthermore, *L. inermis* L contains anthraquinones, whereas *A. Indica* A. Juss contains saponins and flavonoids. However, *L. inermis* L was devoid of flavonoids, and saponins and flavonoids were absent in *A. aspera*. Furthermore, *A. Indica* A. Juss were negative for anthraquinones test (Table 3.8).

S/N	Secondary metabolites	Type of Tests	L. inermis L	A. aspera	A. Indica A. Juss
1	Alkaloids	Mayer's and Dragendroff's tests	+	+	+
2	Terpenoids	Salkowski's test	+	+	+
3	Saponins	Foam test	+	-	+
4	Flavonoids	Alkaline Reagent Test	+	+	+
5	Phenols	Phenol test	+	+	+
6	Tannins	Ferric chloride test	+	+	+
7	Anthraquinones	Anthraquinone test	+	-	-
8	Steroids	Steroid test	+	+	+

+ = present, - = absent

Table 3.8: Preliminary phytochemical screening for secondary metabolites

3.5.6. Antibacterial activity

The antibacterial activity of methanol extracts of *L. inermis L, A. aspera L and A. Indica* A. Juss leaves against micro-organisms isolated from the wound of patients with lymphoedema and standard ATCCs were tested (Figure 3.3). The *in vitro* antibacterial activity was tested by the presence or absence of a zone of inhibition in diameter, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in comparison with the reference antibacterial drugs.

Generally, there has been an increased activity on bacterial growth inhibition as the concentration of the extracts increased. Pairwise comparison of ANOVA was used to test variability in susceptibility of the microorganisms toward the extracts ($p < 0.05$). Hence, among the strains, there was a significant variation in susceptibility towards the plant extracts. However, no significant difference was observed between *K. pneumonia* isolate and ATCC (p=0.91), *S. algae* isolates and *P. aeruginosa* ATCC (p=0.74), *E. coli* isolates and *K. Pneumonia* ATCC (p=0.89), *S. aureus* isolates and MRSA (P=1.0), *S. aureus* isolates and *E. coli* ATCC (p=1.0). There was a significant difference in the zone of inhibition between *L. inermis* L and the other two plant extracts, *A. aspera L*and *A. Indica* A. Juss. But

no significant growth inhibition difference was detected between *A. Indica* A. Juss and *A. aspera L*(p=0.55).

Streptococcus pyogenes isolate showed the highest susceptibility to all the extracts at all concentrations compared with the standard drugs*. K. pneumonia ATCC700603, K. pneumonia* isolates and *P. aeruginosa* isolates, showed a low level of susceptibility against all extracts.

All the three concentrations (100, 200 & 400 mg/ml) of *L. inermis* L showed significant activity against all tested bacteria species compared with standard drugs. The highest zone of inhibition was recorded by *L. inermis* L against all tested species except *K. pneumonia* ATCC700603*, P.* aeruginosa isolates and *K. pneumonia* isolates. Among the bacteria tested*, L. insermis* L showed exceptional activity against *E. coli* isolates*, S. aureus* ATCC 25923*,* and MRSA ATCC® 43300™, which was comparable to the conventional drug cefoxitin (Table 3.9).

A. Indica A. Juss was shown to have activity against all tested species at higher concentrations, and higher activity was recorded against *Streptococcus pyogenes* isolates at all concentrations (100, 200, and 400 mg/ml). However, 100 mg/ml and 200 mg/ml concentrations showed low activity against all tested strains. *A. aspera* L showed the lowest activity against all tested species, except against *Streptococcus pyogenes* isolate (10.5±0.9 to 13.3±0.6 mm ZI) compared with the reference drug cefoxitin (15 -24 mm) (Table 3.9). There were significant differences in the mean zone of inhibition between the different concentrations of *L. inermis* L, *A. aspera L*and *A. Indica* A. Juss *(*p<0.05).

Among the strains, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumonia* isolates were less susceptible to *L. inermis* L than the standard ATCCs. Similarly, multi-drug resistant *S. aureus* (MRSA) was less susceptible to all tested extracts compared to *S. aureus* isolate and standard ATCC. All the references used in the test showed the highest activity against their respective tested species. The mean inhibition zone of triplicate experiments for the three different concentrations of extracts (100, 200 and 400 mg/mL) summarized below in Table 3.9.

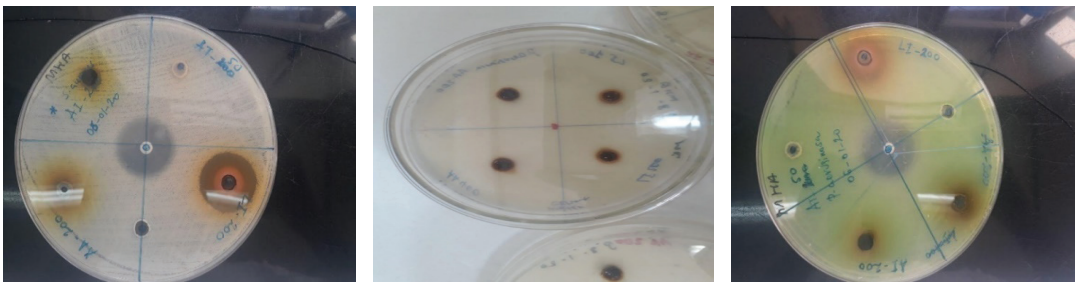


Figure 3.3: Examples of agar well diffusion before and after 24 hours incubation

Plants	Concentration	Zone of inhibition (mm) (Mean ± SD)										
		S. aureus		*E. coli*		*P. aeruginosa*		*K. pneumoniae*		MRSA	*Streptococcus pyogenes*	*Shewanella algae*
		ATCC	Isolate	ATCC	Isolate	ATCC	Isolate	ATCC	Isolate	ATCC	Isolate	Isolate
LI	100 mg/ml	33.0±1.0	15.5±0.5	15.1±0.7	8.1±0.4	20.5±0.5	12.5±0.5	8.2±0.3	7.6±0.5	15.1± 0.7	21.0±1.0	20.5±0.5
	200 mg/ml	31.0±1.0	17.5±0.5	19.3 0.6	10.3±0.8	21.2±0.7	13.8±0.8	9.6±0.5	8.8±0.7	19.3 ± 0.6	24.5±0.5	21.2±1.1
	400 mg/ml	33.0±1.0	21.3±1.5	21.2±0.3	12.1±0.6	21.5±0.9	15.6±0.5	10.5±0.9	10.8±0.8	21.2 ± 0.3	25.9±0.9	21.9±1.0
AA	100 mg/ml	7.1±0.6	6.3±0.6	6.4±0.5	6.8±0.8	6.4±0.4	6.2±0.3	6.4±0.4	6.0±0.0	6.6 ± 0.6	10.5±0.9	6.5±0.5
	200 mg/ml	9.1± 0.4	6.5±0.5	6.6±0.6	7.4±0.5	6.5±0.5	6.6±0.5	6.8±0.7	6.5±0.5	7.5 ± 0.6	12.9±1.0	6.6±0.7
	400 mg/ml	6.3±0.4	7.0±1.0	7.5±0.6	7.6±0.5	6.7±0.6	7.0±1.0	7.2±1.0	6.5±0.5	7.4 ± 0.4	13.3±0.6	6.8±0.8
AI	100 mg/ml	6.4±0.4	6.2±0.3	6.3±0.4	6.6±0.5	6.4±0.4	6.4±0.4	6.4±0.5	6.1±0.2	6.3 ± 0.4	16.7±0.6	6.3±0.6
	200 mg/ml	6.7±0.3	6.7± 0.6	6.5±0.5	7.4±0.4	6.8±0.7	6.8±0.7	6.6±0.6	6.1±0.2	6.5 ± 0.5	18.3±0.6	7.4±0.5
	400 mg/ml	7.4±0.4	8.2± 0.7	7.5±0.7	6.3±0.3	7.8±0.2	7.5±0.5	8.5±0.5	7.4±0.5	6.4 ± 0.5	21.0 ±1.0	9.5±0.5
5µg cefoxitin		27.0±0.0	27.0±0.0	24.0±0.0	24.0±0.0	29.0±0.0	29.0±0.0	24.0±0.0	24.0±0.0	15.0±0.0	----	------
5µg ciprofloxacin		--	---	---	---	---	---	---	---	---	---	24.0±0.0
10µg penicillin		--	---	---	---	---	---	---	---	---	24.0±0.0	---
5% DMSO		NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

Values are triplicate and represented as mean ± SD. AI =Azadirachta indica, LI = Lawsoni ainermis, AA = Achranthes aspera, NI = no inhibition

Table 3.9: Mean inhibition zone of bacterial growth (mm) for the leaves of methanol extracts of L. inermis L, A. aspera Land A. Indica A. Juss.

The methanol extracts of the three plants extracts showed different level of MIC against all tested bacteria. There was no inhibition of growth of bacteria in the negative controls (medium and bacterial suspension, and media DMSO and bacteria). The colorimetric broth microdilution assay showed that the methanol extract of *L. inermis* L was able to inhibit the growth of eleven tested microorganisms within the concentration ranges of 1.5±1.4 to 12.5±0.0 mg/ml. Whereas the minimum bactericidal concentration (MBC) ranges from 12.00±0.0 to 83.3±28.9 mg/ml among the strains.

The minimum bactericidal concentration (MBC) is complementary to MIC which demonstrates the lowest concentration of antimicrobial that result in microbial death. Whereas MIC is the lowest concentration agents that inhibit bacterial growth. The lowest minimum inhibitory concentration (MIC) value was recorded by *L. inermis* L against *E. coli* isolate (1.5±1.4 mg/ml) and *S. aureus* ATCC 25923 (3.1±0.0 mg/ml), and the lowest MBC against *E. coli* isolate (12.00±0.0 mg/ml). The highest MIC value of *L. inermis* L was against *K. pneumoniae* ATCC700603 and *E. coli* ATCC 25922 which was 12.0±0.0 mg/ml for both (Table 3.10).

Similarly, the MIC values of *A. Indica* A. Juss ranged from 25.0±0.0 mg/ml to 100.0±0.0 mg/ml among the tested strains, and MBC ranges from 36.7±23 to 200.0±0.0 mg/ml. The lowest MIC of *A. Indica* A. Juss was observed against *S. aureus* and *K. pneumonia* isolates, and the highest values were against *P. aeruginosa* and S. *algae* isolates (Table 3.10). The lowest MBC of *A. Indica* A. Juss was observed against *S. pyogenes* isolate (36.7±23 mg/ml) (Table 3.10). Similarly, the MIC values of methanol extracts of *A. aspera* L ranged from 50.0±0.0 – 200.0±0.0 mg/ml. The minimum bactericidal concentration for the three plant extracts were ≥ 200.0 mg/ml, except for *S. aureus* isolate and *S. pyogenes* isolate which was 100.0±0.0 mg/ml for both (Table 3.10).

S/N	Bacteria	MIC (mg/ml)			MBC (mg/ml)		
		LI	AI	AA	LI	AI	AA
1	*S. aureus* ATCC 25923	3.1±0.0	33.3±14.4	50.0±0.0	25.0±0.0	200.0±0.0	200.0±0.0
2	MRSA *S. aureus* ATCC® 43300™	4.2±2.0	33.3±14.4	42.0±14.4	50.0±0.0	200.0±0.0	>200.00
3	*E. coli* ATCC 25922	12.5±0.0	83.3±29.0	66.7±28.9	25.0±0.0	200.0±0.0	200.0±0.0
4	*P. aeruginosa* ATCC27853	4.2±1.8	50.0±0.0	200.0±0.0	18.8±10.8	200.0±0.0	200.0±0.0
5	*K. pneumoniae* ATCC700603	12.5±0.0	41.7±14.4	50.0±0.0	83.3±28.9	100.0±0.0	>200.00
6	*E. coli* isolate	1.5±1.4	83.3±28.9	100.0±0.0	12.00±0.0	100.00±0.0	200.0±0.0
7	*K. pneumoniae* isolate	7.3±4.8	25.0±0.0	50.0±0.0	50.0±0.0	100.0±0.0	>200
8	*P. aeruginosa* isolate	12.5±0.0	100.0±0.0	166.7±57.7	66.7±28.9	200.0±0.0	>200
9	*Shewanella algae* isolate	6.25±0.0	100.0±0.0	200.0±0.0	83.3±28.9	100.0±0.0	>200
10	*S. aureus* isolate	6.25±0.0	25.0±0.0	50.0±0.0	25.0±0.0	50.0±0.0	100.0±0.0
11	*Streptococcus pyogenes* isolate	6.25±0.0	33.3±14.4	83.3±28.9	41.7±14.4	36.7±23	100.0±0.0

Values are triplicate and represented as mean ± SD. MIC=Minimum inhibitory concentration, MBC=Minimum bactericidal concentration, LI = *Lawsonia inermis,* AI= *Azadirachta indica*, AA = *Achyranthes aspera.*

Table 3.10: The mean values of MIC and MBC for the leaves of methanol extracts of Lawsonia inermis, Achyranthes aspera and Azadirachta indica.

3.6. Discussion

Bacterial contamination of wounds of patients with lymphoedema is a serious problem in regions where tropical lymphoedema is common. Proper identification of pathogenic micro-organism and knowledge of their susceptibility to commonly used antibacterial drugs will help clinicians in the management of wounds in these patients. Our study characterized gram-negative and gram-positive bacteria, including the proportion of samples with MDR and XDR to antibacterial agents, from the wounds of Ethiopian patients with lymphoedema. After isolation and characterization of the micro-organisms that involved in wound infection

of patients with lymphoedema, the antibacterial activity of selected endemic plant extracts against selected isolates and standard ATCCs was conducted.

The overall bacterial isolation rate was 86.4% which was similar to previous studies of bacteria isolated from wounds in Ethiopia and Bangladesh (210,223). Most study participants with lymphoedema were farmers (74.8%). Since most farmers in rural communities do not use footwear (222), there is a high probability of injury with subsequent infection with microorganisms from the environment. Furthermore, delayed health seeking creates opportunities for bacterial contamination and multiplication.

The types of wound pathogens identified in our study, and their relative prevalence, were consistent with earlier studies (8,224). As reported previously (225), the most common gram-negative bacteria isolated from wound infections in our study, was *Aeromonas hydrophila/caviae* (15.9%). *A. hydrophila/caviae* causes mild to severe wound infection, and typically occurs on the extremities upon exposure of skin lesions to contaminated mud and river water (225).

In agreement with previous studies (226) the next most common gram-negative species isolated from the wounds of patients with lymphoedema in our study was *Acinetobacter lwoffii* (9.2%), which is known to colonize wounds and causes infections, including cellulitis, followed by *E. coli* (7.9%), *K. pneumoniae* (6.6%), and *P. aeruginosa* (6.6%). In another study from India, *E. coli* was isolated from local lesions of patients with filarial lymphoedema along with other gram-negative and gram-positive bacteria, potentially due to contamination of the wound with faeces (226).

S. algae, another frequent gram-negative isolate in our study (7.9%), is common in skin and soft tissue infections, especially in immunocompromised patients with pre-existing cutaneous ulcers and is associated with exposure to aquatic environments, as shown by Goyal *et al* (227) who reported wound infection by *S. algae* among people in Iran with a history of swimming.

Pantoea agglomerans, a gram-negative, oxidase-negative, facultatively anaerobic, fermentative, motile, and rod-shaped bacterium with the general characteristics of the family *Enterobacteriaceae*, is usually associated with plants and is not an obligate infectious agent in humans. In this study, *Pantoea* species were isolated from about 6.7% of the wounds of patients with lymphoedema (228). Wound infection with *P. agglomerans* is usually associated with piercing of the skin with a plant thorn or splinter with subsequent inoculation of the plant-residing bacteria. Infections usually occur during agricultural activities or gardening (228,229).

Streptococcus dysgalactiae, representing 39.7 % of gram-positive isolates in the present study, belongs to the group of beta-haemolytic streptococcal species and is genetically

close to *Streptococcus pyogenes*. Our data are supported by findings showing that *S. dysgalactiae* is known to cause soft tissue infection and cellulitis (230). Similar to previous findings from Ethiopia (210). *S. aureus* made up 17.8% of all gram-positive isolates in our study. Acute inflammation of the skin and tissue (cellulitis) of lower limbs of lymphoedema patients is commonly caused by *S. aureus* and *S. pyogenes* (8).

Staphylococcus simulans, which was isolated from 8.9% of wounds in the present study, is a coagulase-negative staphylococci (CoNS) species. Infection in humans predominantly occurs among patients who have contact with animals (231). Another CoNS member of the staphylococcus genus, *Staphylococcus haemolyticus*, was isolated from 5.4% of patients, in line with Czekaj *et al*, who isolated *S. haemolyticus* from toe-web swabs and lymph nodes of lymphoedematous legs of European patients (232). MDR *S. haemolyticus* was also isolated from ruminants and domestic animals; indicating that nosocomial infection and transmissions from domestic animals could be sources of wound infection (232).

S. pyogenes made up 3.6% of gram-positive bacterial isolates in the present study, supporting previously published data from Ethiopia (233). *S. pyogenes* is a member of the β-haemolytic group A streptococci, and its co-infection with other micro-organisms is the most common cause of infection in lymphoedematous limbs, causing erysipelas, particularly in the lower limbs (209).

In the present study, bacterial isolates were tested for their susceptibility to the antibacterial drugs most used for treatment in Ethiopia. Higher rates of MDR were noted among the gram-negative (44.4%) versus gram-positive (5.3%) bacteria. Among the gram-negative bacteria, all isolates of *K. pneumonia* were resistant to ampicillin whereas 20% were resistant to ampicillin/sulbactam, cefazolin, ceftriaxone, cefepime, aztreonam, and trimethoprim/sulfamethoxazole. A similar finding on *K. pneumonia* resistance to ampicillin has been reported previously in Ethiopia (210). However, all isolates of *K. pneumonia* were susceptible to carbapenems (ertapenem, imipenem, and meropenem), aminoglycosides (amikacin, gentamicin and tobramycin), fluoroquinolones (ciprofloxacin and moxifloxacin), and tigecycline, in agreement with Lin *et al* (234).

All the isolates of *Pseudomonas aeruginosa (*n=5) were resistant to ampicillin, ampicillin/sulbactam, and first-generation cephalosporins (cefazolin); whereas 3 of the isolates were not susceptible to a third-generation cephalosporin (ceftriaxone) or trimethoprim/sulfamethoxazole. Resistance to ampicillin and ceftriaxone was reported previously by Dessie et al (235). *P. aeruginosa* has intrinsic resistance to various antimicrobial agents such as beta-lactam antibacterial drugs and the carbapenem groups of antibacterial drugs because of the low permeability of its cell membrane and the presence of beta-lactamase (236). However, all isolates of *P. aeruginosa* in this study were susceptible to aminoglycosides (amikacin, gentamycin and tobramycin), fluoroquinolones (ciprofloxacin and moxifloxacin),

carbapenems (aztreonam, ertapenem and imipenem) and fourth-generation cephalosporin (cefepime).

All isolates of *A. hydrophila* showed low resistance to imipenem (8%), tobramycin (8%), and trimethoprim/sulfamethoxazole (8%). In a previous study in Ethiopia, *A. hydrophila* was resistant to ampicillin (100%), trimethoprim-sulfamethoxazole (100%), and ceftriaxone (75%) (237). However, in the present study, all isolates of *A. hydrophila* showed no resistance to the aminoglycosides (amikacin and gentamicin), fluoroquinolones (ciprofloxacin and moxifloxacin), carbapenems (aztreonam and ertapenem) or cephalosporins (ceftriaxone and cefepime).

All *Acinetobacter baumannii* isolates were resistant to ampicillin, and 50% of them to ampicillin/sulbactam. This is in line with a study from South Ethiopia which found 88.2% of isolates of *Acinetobacter* species to be resistant to ampicillin in Ethiopia (238). Manchanda *et al* reported the development of resistance of *A. baumannii* to ampicillin and first and second-generation cephalosporins since 1975, and then to ampicillin/sulbactam more recently (239).

Similarly, a low proportion (14-29%) of *A. lwoffii* isolates were resistant to ampicillin, ampicillin/sulbactam, cefazolin, ceftriaxone, tigecycline, and trimethoprim-sulfamethoxazole. A report from Kenya showed greater susceptibility of *A. lwoffii* to ampicillin, cefazolin, ceftriaxone, and trimethoprim-sulfamethoxazole (240). However, all isolates of *A. baumannii* and *A. lwoffii* were found to be susceptible to fluoroquinolones (ciprofloxacin and moxifloxacin), aminoglycosides (amikacin, gentamycin and tobramycin), carbapenems (imipenem), and cephalosporins(cefepime).

A high proportion of *E. coli* isolates showed resistance to ampicillin (66.7%), ampicillin/sulbactam (33.3%), moxifloxacin (16.7%), and trimethoprim-sulfamethoxazole (33.3%). However, all isolates were susceptible to cephalosporins (cefazolin, ceftriaxone, cefepime), carbapenems (aztreonam, ertapenem, imipenem and meropenem), and aminoglycosides (amikacin, gentamycin and tobramycin). These data suggest lower resistance level compared to the observed from the study in Gondar, Ethiopia (233).

More than 44% of gram-negative isolates were found to be MDR bacteria, of which 1.6% were XDR bacteria. *A. baumannii* (100%), *A. lwoffii* (14.3%), *A. hydrophila* (66.7%), *E. coli* (33.3%), *P. aeruginosa* (80%) and *Proteus species* (100%) were among the MDR bacteria. One isolate of *A. baumannii* showed XDR. MDR of each of these organisms has been reported previously (210,233,241). In the present study, 20% of the isolates of *K. pneumonia* were extended spectrum beta lactamase (ESBL) producing isolates. ESBL enzymes confer resistance to most beta-lactam antibacterial drugs, including penicillin, cephalosporins, and the monobactam aztreonam. Emergence of extended-spectrum beta-lactamase

producing isolates has important clinical and therapeutic implications (224).

In the present study, gram-positive isolates were most commonly resistant to erythromycin (36.2%) and clindamycin (31.0%) followed by tetracycline (19.0%) and Penicillin G (17.2%). However, all gram-positive isolates were susceptible to glycopeptides (linezolid and vancomycin), fluoroquinolones (levofloxacin, and moxifloxacin), gentamycin, and quinupristin-dalfopristin. Among the gram-positive isolates, 10% of *S. aureus* and 25% of *S. haemoliticus*, and *S. agalactiae* (group B streptococcus) isolates demonstrated MDR.

S. aureus was resistant to penicillin (70%), erythromycin (40%), clindamycin (30%), trimethoprim-sulfamethoxazole (20%) and tetracycline (10%). All isolates of *S. aureus* were susceptible to oxacillin, gentamycin, ciprofloxacin, levofloxacin, moxifloxacin, linezolid, vancomycin, quinupristin-dalfopristin, and rifampicin, in agreement with studies from other parts of Ethiopia (210,241) and elsewhere (224).

Similar to the findings of Czekaj *et al* (232), 70 % of *S. haemolyticus* isolates were resistant to trimethoprim-sulfamethoxazole; 50% to erythromycin and 25% to penicillin, oxacillin, ciprofloxacin, clindamycin, and tetracycline. However, all isolates were susceptible to glycopeptides (linezolid and vancomycin), gentamycin, quinolones (moxifloxacin and levofloxacin), quinupristin/dalfopristin and tigecycline. In the present study, *S. haemolyticus* and *Kocuria kristinae* were positive for cefoxitin screening MRSA. A similar study from Brazil reported that 91% of *S. haemolyticus* isolates were positive for MRSA screening (242).

In line with a report from Pakistan (243), 13.8% of gram-positive isolates were positive for inducible clindamycin resistance. Among these, 50% were *S. pyogenes*, 40% *S. simulans,* 30% *S. aureus* and 25% *S. haemolyticus*. A positive test indicates the presence of macrolide-induced resistance to clindamycin which induced production of methylase that alters the common ribosomal binding site for macrolides, clindamycin, and quinupristin.

Natural products are sources of antimicrobial agents due to their naturally occurring constituents. They are sources of different compounds and sought as novel antimicrobial agents since conventional antimicrobials are getting ineffective due to the emergence of resistance. The presence of plant secondary metabolites with ranges of mode of action decreases the development of resistance. Thus, it indicates the therapeutic role of traditional medicines in treating microbial disease (150).

Assessing the antibacterial activity of herbal medicines for the treatment of skin and wound infections has great importance in addressing health issues. Extracts with a zone of inhibitions that are equivalent and close to the reference drugs have high activity, and those which have half of the reference have moderate, whereas those which showed below half activity have low or mild activity (128).

Phytochemical screening and antibacterial activity of three plant extracts have been evaluated to know plant extracts and constituents that could be used for the management of wound infections in patients with lymphoedema. As a result, qualitative test for the screening of secondary metabolites in methanol extract *L. inermis* L revealed the presence of alkaloids, terpenoids, saponins, phenols, tannins, anthraquinones and steroids which are known to have pharmacological activities. This agrees with previous findings (244), except for the absence of flavonoids in this study. *L. inermis* L is a source of unique and valuable natural compounds and used for wide ranges of conditions. As a result, it is considered for the development of medicines for different diseases and cosmetics (187).

The methanol extract of *L.* inermis L had significant activity against all tested bacteria. Among the tested strains, *S. aureus* ATCC 25923, MRSA ATCC®43300TM, *E. coli* ATCC 25922, *E. coli*, and *Streptococcus pyogenes* isolates were the most susceptible bacteria, a comparable zone of inhibition with cefoxitin and penicillin at all tested concentrations. This finding supports the report of Manivannan et al (124), Kannahi et al (245), and Badoni et al (246). The plant active constituent, ß-asarones found in leaves, roots, and rhizomes of the plant *L. inermis* L were responsible for all antimicrobial activity (247).

Furthermore, the quinones which are present in *L. inermis* L (henna) found to have a high activity against all micro-organism (124). In the methanol extract of *L. inermis* L*,* alkaloid, anthraquinones, and saponins were reported to have antibacterial activity, and the highest zone of inhibition against most of the microorganisms could be due to a single or combined effect of these secondary metabolites (248).

Azadirachta indica A. Juss is the most useful medicinal plant. Every part of the tree has been used as traditional medicine and the fruits, seeds, leaves, bark, and roots contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, antiulcer and antifungal properties (249). In the present study, alkaloids, terpenoids, saponins, flavonoids, phenols, tannins, and steroids were found in methanol extract of *A. Indica* A. Juss which agrees with the previous reports (249)*.* Phytochemicals constituents such as flavonoids and saponins were responsible for the anti-inflammatory, antimicrobial, antioxidant, and antiviral of the plant (249).

A. Indica A. Juss *was* found to have a medium activity against all tested strains at 400mg/ml except for *E. coli* isolate (6.3±0.3 mm ZI) and MRSA (6.4 ± 0.5 mm ZI) which was significantly low activity. In comparison with the reference drugs, the highest activity of *A. Indica* A. Juss *was* recorded against *Streptococcus pyogenes* isolate (21.0 ±1.0 mm ZI), followed by *Shewanella algae* (9.0±0.5 mm ZI) at 400mg/ml. Clinical isolates of *E. coli*, *P. aeruginosa*, and *K. pneumonia* strains found less susceptible than the standard ATCCs at tested concentrations. *A. Indica A. Juss* at the concentration of 100mg/ml and 200mg/ml showed low activity against tested strains except for *Streptococcus pyogenes* and *S. algae*.

S. aureus isolate was more susceptible than the standard ATCC. Previous studies showed that methanol extract of *A. Indica* A. Juss (neem) found to have a high activity against standard and clinical isolated strains of *P. aeruginosa* (250). Another study indicated that ethanol extracts of *A. Indica* A. Juss (neem) leaf exhibited antibacterial activity against *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *S. aureus*, *P. aeruginosa*, *Enterococcus faecalis* at 100, 50, and 25 mg/ml (251).

Achyranthes aspera locally known as "Telegne" is one of the traditionally used medicinal plant in Ethiopia for treatment of different kind of wound infections (252). It is reported to have different pharmacological activities such as anti-bacterial, anti-inflammatory, analgesic, and antipyretic activities (65). Phytochemical screening of methanol extracts of *A. aspera* L suggested the presence of secondary metabolites such as alkaloids, terpenoids, phenols, tannins which indicated the pharmacological activity of the plant (253).

In this study, the methanol leaf extract of *A. aspera* L showed high antibacterial activity against *Streptococcus pyogenes* at 400 mg/ml, and low activity against the rest of the tested strains at tested concentrations. Next to *Streptococcus pyogenes*, *S. aureus* ATCC found more susceptible than the clinical isolate. Except for the *Streptococcus pyogenes* strain, this finding agrees with the report of Taye *et al* (52).

In this study, gram-positive bacteria were comparatively found more susceptible than the gram-negatives toward the plant extracts, which could be due to the nature of their cell membrane. Gram-negatives have phospholipid membranes carrying the structural lipopolysaccharide component that makes their cell wall impermeable to antimicrobial substances (254).

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agents that inhibited the visible growth of microorganisms after overnight incubation. The MBC is complementary to the MIC. It demonstrates the lowest level of antimicrobial agent that results in microbial death after subculture of the organism in an antibiotic-free media (255). The MIC is used to measure the antimicrobial effectiveness of new compounds/extracts by measuring the effect of decreasing the concentration of the antimicrobial agent. Antimicrobial agents with lower MIC are considered as the most effective.

In this study, MIC values of *L. inemris* L indicated that the methanol extract was more potent against *E. coli* isolate and *S. aureus* ATCC 25923, which agrees with the initial antimicrobial screening test results (agar well diffusion test). Strong antibacterial activity of *L. inermis* L was also observed against *S. aureus* ATCC 25923, MRSA ATCC® 43300TM, *P. aeruginosa* ATCC27853 at low concentrations. The results of this study agree with the previous report in Iraq (256). The difference in bacterial susceptibility could be due to the

variations of microorganism intrinsic tolerance, and the nature and combinations of phytochemicals that present in the crude extracts of the plants (256).

MBC/MIC ratio was determined for *L. inermis* L to identify the bactericidal or a bacteriostatic nature of the extract. MBC/MIC ratio greater than 4 is usually considered as a bacteriostatic agent whereas bactericidal for values less than 4 (257). *L. inermis* L has bactericidal nature against *E. coli* ATCC 25922, *P. aeruginosa* ATCC27853, *E. coli* isolate, *K. pneumonia* isolate, *S. aureus* isolate and *Streptococcus pyogenes* isolate. However, it showed bacteriostatic activity against *S. aureus* ATCC 25923, MRSA ATCC® 43300TM, *P. aeruginosa* ATCC27853, and *K. pneumonia* ATCC700603, *P. aeruginosa* isolate and *S. algae* isolate. Generally, the activity of compound/extract is significantly high when MIC is less than10 µg/mL, moderate when MIC is between 10 and100 µg/mL and low when MIC is greater than 100 µg/m (258).

A. Indica A. Juss and *A. aspera L* with MIC ranges from 25.0±0.0 mg/ml to 100.0±0.0 mg/ml, and 50.0±0.0 to 200.0±0.0 mg/ml respectively, fall in the range of low activity against the tested bacterial strains. The MBC/MIC ratio was also determined for both. Even though *A. indica* A. Juss exhibited moderate to low activity against the tested strains, it showed bactericidal activity against all tested strains. Except for *S. aureus* isolate and *Streptococcus pyogenes* isolate, the MBC values of *A, aspera* against the tested strains were ≥ 200 mg/ml and demonstrated bactericidal activity. Even though, antibacterial activity depends on the concentration of the constituents in crude plant extracts, relatively at higher concentrations plant extracts are bactericidal (259).

The three tested medicinal plants have pharmacologically active phytoconstituents in common such as alkaloids, flavonoids, tannins, phenols, steroids, and terpenoids. The presence of these bioactive phytochemical compounds accounted for the broad-spectrum antimicrobial activities observed in this study either individually or in combinations, which also reported in previous studies (260,261).

The proposed modes of action of some of the secondary metabolites could be described as follows: Tannins inactivate microbial adhesins, enzymes and cell envelope transport proteins (260); flavonoids act on the cell membranes of microbes and inhibiting energy metabolism and synthesis of nucleic acids in different microorganism (262), alkaloids inserted into the cell wall and /or Deoxyribonucleic acid (DNA); anthraquinones involve an increase in the levels of superoxide anion and/or singlet oxygen molecule (195), and diterpenes and phenolic compounds disrupt microbial cell membranes (263).

There were limitations to the present study. No attempt was made to determine the source of infection (environment or hospital acquired). The number of samples used was small and the study did not recruit patients from different parts of the country to compare the national distribution of wound infection in lymphoedematous limbs. Anaerobic micro-organisms

were not included since anaerobic transport systems and culture facilities were not available, and advanced molecular techniques were not used to characterize and identify the species. The antibacterial activity of selected medicinal plants against bacterial isolated from the wound of patients with lymphoedema and standard ATCCs tested.

3.7. Conclusions

This study has identified current bacterial species involved in colonizing wounds of lymphoedematous limbs in patients from Ethiopia and evaluated antibacterial activity of selected Ethiopian plants against selected isolates and standard ATCCs. More than 40% of wound infections were polymicrobial. The most predominant bacteria contributing to wound infections were *A. hydrophila/caviae*, *A. lwoffii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. algae*, *S. aureus*, *S. pyogenes*, *S. dysgalactiae*, *S. haemolyticus*, *S. agalactiae* and *S. simulans*. A higher rate of anti-microbial resistance was detected among the gram-negative than gram-positive isolates and MDR was also identified. We observed a high percentage of isolate resistance to ampicillin, cefazoline, clindamycin, erythromycin, and tetracycline, which are the most used antibacterial drugs for the management of bacterial infections in the study area.

Furthermore, this study has demonstrated the methanol extracts of *L. inermis* L, *A. Indica* A. Juss *and A. aspera* L exhibited antimicrobial activity against selected bacteria isolates involved in wound infections including MRSA. Among the plant extracts, antimicrobial testing and MIC values showed that the methanol extracts of *L. inermis* L exhibited significant activity and demonstrated bactericidal effect against most of the tested bacterial strains. However, *A. Indica* A. Juss *and A. aspera* L showed low to moderate activity against most tested strains at 400mg/ml. This finding supports the evidence that herbal medicines could be sources of antibacterial agents and used as alternatives treatment topically for wound infections.

3.8. Recommendations

Microbiological tests of wound infection and bacterial antibiotic susceptibility testing are recommended to guide treatment and reduce the emergence of resistant bacteria. Given the antimicrobial resistance documented, we recommend use of the fluoroquinolones (ciprofloxacin or moxifloxacin) or gentamicin for the management of both gram-negative and gram-positive wound infections in patients with lower limb lymphoedema in the study area.

These antibacterial drugs are available and affordable in these areas. In addition, carbapenems are recommended for the management of resistant gram-negative bacteria

while glycopeptides (linezolid or vancomycin) or quinupristin/dalfopristin are recommended for resistant gram-positive bacteria. XDR *A. baumannii* requires special attention and we recommend research to establish its wider prevalence and to monitor resistance. Similar research should be replicated to establish the microbial profile and antimicrobial susceptibility patterns associated with wounds in other areas where tropical lymphoedema is prevalent.

Furthermore, isolation and characterisation of chemicals constituents that are present in these plant extracts and were responsible for the observed activities against bacteria are highly recommended. More studies on the traditional use of medicinal plants will bring more important information which could help in the investigation of medicinal plants for their pharmacological activity.

CHAPTER FOUR

4. *IN VITRO* ANTI-INFLAMMATORY AND WOUND HEALING ACTIVITY OF METHANOL EXTRACTS OF THE LEAVES OF THREE MEDICINAL PLANTS

4.1. Introduction

Ethiopia has many and diversified natural product that is being used in a traditional way by people to treat different disease conditions. Herbal medicines have been used for different type of human and animals' diseases, it is estimated that 80% of humans and 90% of domesticated animals rely on medicinal plants. In Ethiopia, traditional medicines are highly in need due to the trust in the community on medicinal plants. It is associated with the societies' aesthetic, historic, social or/and spiritual values, and are relatively low in cost (264).

Herbal products have demonstrated better healing properties, recognized as important sources of therapeutics, and used to treat diversified range of conditions like swelling, burns, wounds and diseases. These have been attracted the scientific community to study the active constituents of plants that have healing properties which lead to drug development (159).

Inflammation is a local reaction/response of living tissue to pathogenic microorganisms, chemical substances and/or parasites. The purpose of the inflammatory response is to bring inflammatory mediators such as leukocytes and plasma proteins that are normally circulating in blood to the site of infection or tissue damage to eliminate the causative agent and initiate healing (157). When a cell is injured, there are cells that mediate the inflammatory response of cytokines such as tumor necrosis factor, IL-1 from leukocytes, monocytes and macrophages are released as a response to pain, thereby alerting the body to increase blood flow around the injury area. This instantiates the production of cyclooxygenase (COX), 5-lipoxygenase (5-LO), and prostaglandins and leukotrienes synthesis (159).

Generally, inflammation is beneficial to the body. However, it may cause damage to the cells, tissue and the organ if unable to remove the causative agents or inappropriately directed against host. Wounds are physical injuries that cause the skin to open or break, and wound healing is critical to restore anatomical continuity and skin function. Wound healing is the biological reaction to injury that results in the wound being reduced and closed. A complex process involving cellular and biochemical interactions and restores functional barrier (33).

4.1.1. Pathophysiology of inflammation in wound healing

Inflammation refers to a complex abnormal functioning of tissues or cells mediated by different signaling molecules in the body, which induces formation of edema due to leakage of body fluid, proteins and leukocyte accumulation at the inflammation site. (167). Prostaglandins are hormones temporarily found in some local areas in the body that are produced by all types of cells in the body at the time of injury. Once they are present in the intracellular space, they induce fever, inflammation, and pain. Bradykinin, C3a and C5a anaphylatoxins increase blood vessel leakage and allow entrance of neutrophils and monocytes to the injury area. Thromboxanes, a known hormone stimulator, controls vasodilation, platelet accumulation, and formation of blood clot to increase the response of inflammation. The cascade of inflammation pathway leads to the secretion of more cellular mediators of inflammation where its outcome is cellular/ destruction, and then restoration of tissue structure and function (54).

Response to inflammation is the beginning of different processes which happens simultaneously to promote wound healing. The main biomolecule elements to the response to inflammation in skin restoration are infiltrating leukocytes to the injury sites. They are not only the cells that combat invasive microbes, but they are also engaged in tissue breakdown and development. As a result, an abnormal entrance or activation of infiltrating leukocytes into injured tissue can have a substantial impact on cell migration, cell multiplication, in forming different types of cells, and on the quality of wound healing. Realizing the significance as well as the complicated role of the inflammatory response to wound repairs will provide approaches to have strategies of diseases known by abnormal tissue remodeling (265).

The resolution of the inflammatory response is required for successful tissue repair after injury. While knowledge of processes of inflammation and biomolecules that start and maintain the response of inflammation is continuously growing. Inhibition of chemokine production by anti-inflammatory cytokines such as interleukin-10 or transforming growth factor–beta 1, or increased expression of anti-inflammatory molecules such as interleukin-1 receptor antagonist or soluble tumor necrosis factor receptor, the cell surface receptor for hyaluronan CD44 and unresponsiveness to apoptosis receptors or down regulation by high ligand concentrations are the mechanisms of inflammation resolution by the body. Furthermore, previous research identified Nrf-2, a keratinocyte growth factor-1 target, as a novel transcription factor regulating the inflammatory response to repair (265).

The arachidonic acid pathway is a major component of the inflammation process pathway (Figure 4.1). Arachidonic acid is secreted from injured cellular and tissue membranes and converted into prostaglandins and thromboxanes by cyclooxygenase (COX) enzyme. COX has three known isomers. These are COX-1, COX-2, and the new isomer, COX-3. Inhibition

of COX-1 in the gastrointestinal mucosal layer is related to the side effects of non-steroidal anti-inflammatory drugs, and selective inhibition of COX-2 is preferable. COX-2 is stimulated in the beginning of an inflammatory in response to pro-inflammatory mediators and stimuli like endotoxins and cytokines. COX-2 favors synthesis of prostaglandins, which plays a role in inflammation, swelling, and pain, after being activated. Lipoxygenases are dioxygenase enzymes that involve in the production of leukotrienes from arachidonic acid, which act as an intermediate biomolecule of inflammatory and allergic reactions. These enzymes are responsible for catalyzing incorporation of molecular oxygen into unsaturated fatty acids like linoleic and arachidonic acids. Four main iso-enzymes of LOX enzymes have been identified based on the site of oxidation, namely, 5-LOX, 8-LOX, 12-LOX, and 15-LOX. The common substrates for LOX enzymes are linoleic acid and arachidonic acids. LOX enzyme from soybeans is usually used for the *in vitro* anti-inflammatory assay due to unavailability of human's LOX enzyme (266).

The metabolism of arachidonic acid during inflammation by the COX enzymes produce prostaglandins and thromboxane A2, or through the LOX pathway it produces hydroperoxy-eicosatetraenoic acids and leukotrienes. LOX enzyme pathway is present in leucocytes as well as in mast cells, neutrophils, eosinophils, monocytes, and basophils. Phospholipase A2 cleaves arachidonic acid from cell membrane phospholipids by phospholipase A2 and metabolizes arachidonic acids to leukotrienes. Leukotrienes act as phagocyte chemo-attractants, which attracts innate immune cells to inflammation places. Medicinal plants may thus be possible sources of COX-2 and LOX inhibitors with less side effects (159).

A multitarget inhibitors that are capable of inhibiting COX-2, and 5-LOX could be more effective anti-inflammatory drug than any currently available NSAID. They have the potential to be safer and more effective drugs for the management of inflammation. As a result, the first step toward developing an anti-inflammatory treatment alternative is to identify compounds with distinct mechanisms of action. Screening the natural world, particularly plants, is an intriguing approach to identifying biologically active compounds (266).

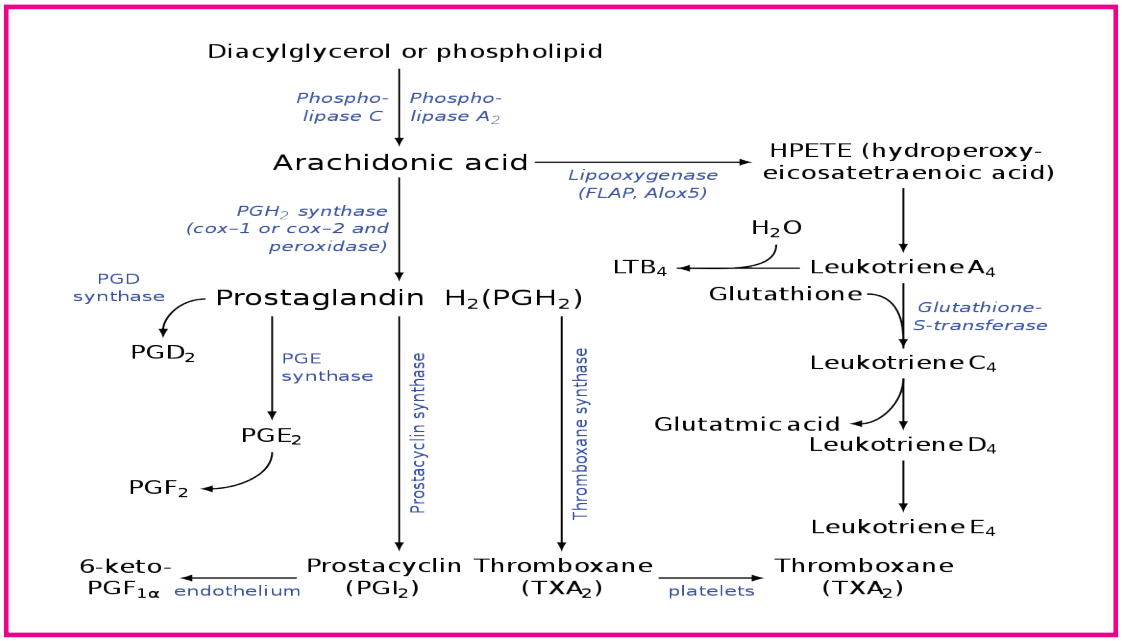


Figure 4.1: Eicosanoid synthesis (Source: https://en.wikipedia.org/wiki/Eicosanoid#/media/File:Eicosanoid_synthesis.svg)

NSAIDs are used by almost all world population to treat inflammatory conditions. These medications inhibit the release of chemical mediators associated with inducing an inflammatory reaction, such as prostaglandins. This could be accomplished by inhibiting or blocking the enzymes responsible for their production, such as cyclooxygenases and lipoxygenases. Regardless, most of these medications have different side effects. Corticosteroids, a type of anti-inflammatory drug, have side effects such as suppressing the immune system and preventing the body from fighting pathogenic microorganisms, all of which contribute to impaired healing (267).

There is an increased interest in natural product compounds, that have been used for many years for the management of pain and inflammation conditions. Most of these natural product compounds work by blocking inflammatory pathways. Furthermore, many natural compounds also inhibit nuclear factor-kB (NF-kB) inflammatory pathways. The NF-kB molecule is known in controlling DNA transcription in order to maintain the inflammatory immune response (54)

4.1.2. Plant species

Three plant species namely *L. inermis* L, *A. indica* A. Juss and *A. Aspera* L have been chosen based on the information from a previously done systematic review (268). Furthermore, because of their traditional use in the management of injuries and inflammatory conditions, the methanol extracts of their leaf parts were tested for anti-inflammatory and wound healing activities in *in vitro* model.

4.2. Methods

4.2.1. Cytotoxicity assay of plant extracts

Cell culture: Vero cells from cryopreservation were thawed and subcultured twice before use for cytotoxicity assay. Cells were sub-cultured in 10% Fetal bovine serum (FBS) medium (Sigma-Aldrich/Merck, Germany). Medium was replaced 2–3 times a week. Confluent cultures were detached using trypsin–EDTA.

Cell plating: Cells were cultured evenly in 96-wells plates at a density of $2x10^4$ cell per well and let them to attach overnight. Serial dilution of the plant extracts was prepared (0.78 mg/ml - 400mg/ml). After 24 hours of incubation, medicinal plant extracts were introduced (serial dilutions of extracts prepared in media). Then, experimental plates were incubated in cell culture incubator at 37°C, 5% CO2 for 48 hrs. Cells in media without the extract were used as controls.

Assay: After 48hrs incubation, cells were rinsed with PBS, and phenol free media were added to the cells. Then, 25 µL of MTT (5 mg/ml) (Thermo-fisher Life Technologies, USA) was added to all wells. Plates were incubated for 4 hours in a humidified cell culture incubator at 37°C. Then, medium with MTT solution was removed from the wells and 100 µL of DMSO was added to solubilize the formazan salt. The content in the plate was mixed well on an orbital shaker at room temperature, at 150 cycles/min, for 1 h. Optical density (OD) of all wells were read at a wavelength 590 nm on an ELISA plate reader (BiOTek ELISA microplate reader ELx808). Percentage of cell viability was calculated using the formula written below. Absorbance of each concentration of the extract was subtracted from the respective concentration of extract and control well.

$$\text{Cell viabilty (\%)} = \frac{\text{Average absorbance in duplicated extract well - Average blanks}}{\text{Average absorbance in controll wells}} \times 100$$

Blank wells - wells without media and cells

Control wells - wells with cell and media

4.2.2. Cyclooxygenase 1 and 2 enzyme inhibitory activity assay

Methods used for the cyclooxygenase enzyme 1 and cyclooxygenase enzyme 2 inhibitory assay was similar. The cyclooxygenase enzyme activity assay kit (Catalogue No. 701050, Cayman Chemical, Ann Arbor, MI, USA) using manufacturer's instruction with slight modification. The peroxidase activity was assayed colorimetrically at 590 nm (269).

One hundred sixty microliters of assay buffer and ten microliters of hemin was added to the background wells. Then, 150 µl of assay buffer and 10 µl of enzymes (COX-1 and COX-2)

was added to 100% initial activity wells. Plant extracts dilution was done in DMSO (Sigma-Aldrich/Merck, Germany) with a concentration from 0.78 mg/ml to 25 mg/ml. Indomethacin (Sigma-Aldrich/Merck, Germany), serially diluted (3.125 µg/ml – 50 µg/ml) was used as positive control. Ten microliters of plant extracts and indomethacin (Sigma-Aldrich/Merck, Germany) was added to the inhibitor wells and 10µl of solvent (the same solvent used for dissolving inhibitor) to the 100% Initial Activity wells and background wells. Plate contents were mixed carefully for a few seconds and incubate for five minutes at 25°C. Twenty microliters (20µl) of the colorimetric substrate solution and arachidonic acid were added to all the wells. Then, plate contents were mixed carefully and incubated for two minutes at 25°C, Finally, absorbance was measured at 590 nm using microplate reader (BiOTek ELISA microplate reader ELx808). All tests were done in three replicas. The percent (%) inhibition of COX-1 and COX-2 was determined using the following formula:

$$\text{Percent Inhibition} = \frac{(\text{AEA-AIA})}{\text{AEA}} \times 100$$

Where:

Activity enzyme test absorbance (AEA) = (Abs of enzyme test - Blank abs).

AIA (activity inhibition test absorbance) = (Abs Inhibition Test – Blank Abs). In addition, absorbance of each concentration of the extract was subtracted from the respective concentration of AEA.

$$\text{COX-2 selectivity index (SI) values were also calculated using the formula: SI} = \frac{\text{IC50 (COX-1)}}{\text{IC50 (COX-2)}}$$

4.2.3. Lipoxygenase enzyme inhibitory assay

The LOX inhibitory activity of methanol extracts was measured using 15-LOX inhibitor screening kit (Novus Biological, Bio-techne Ltd, Abingdon, UK) using manufacturer's instructions with slight modification. Plant extracts were dissolved in methanol and serially diluted from 0.78 mg/ml to 25 mg/ml. In the blank wells of the plate100 µl of 1X assay buffer; and to the 100% activity wells, 90µl 15-LOX and 10µl methanol were added. To the inhibitor wells of the plate, 90µl 15-LOX and 10µl serially diluted plant extracts and nordihydroguaiaretic acid (0.625µM-100µM) were added. Then, plates were kept for five minutes at room temperature. After incubation, reactions were initiated by adding 10 µl of linoleic acid to all wells, and plates were put on a plate shaker for 10 minutes. Then, 100µl of chromogen was added to all the wells to stop enzyme catalysis and develop the reaction, and plates were covered with a plate cover and placed on a plate shaker for five minutes. Assay was performed in duplicate on the same day. The assay plate was read at 495 nm microplate reader (BiOTek ELISA microplate reader ELx808) within 2 minutes. IC50 values were determined using GraphPad prism, and the percentage of inhibition of each extract

and NDGA (Merck Life Science UK Ltd, UK) was determined using the below equation:

$$\text{Percent Inhibition} = \frac{(\text{Initial activity} - \text{Inhibitor activity})}{\text{Intial activity}} \times 100$$

Where initial activity = The 100% activity well - Blank wells

In addition, absorbance of each concentration of the extract was subtracted from the respective concentration of extract and control well.

4.2.4. The DPPH radical scavenging assay

The free radical scavenging capacity of the methanolic extracts of *L. inermis* L, *A. aspera* L, and *A. indica* A. Juss extracts were determined using the 2,2 diphenyl 1 picrylhydrazyl (DPPH) (Sigma-Aldrich/Merck, Germany). The assay was performed in a 96-well microplate using the procedures previously mentioned (270) with minor adjustments. Hundred microliter (100 µL) of different extract concentrations in methanol (0.078 mg/ml to 10 mg/mL) were added to 100 µL of 0.04% methanolic DPPH solution. Plates were kept for 30 min in the dark place at ambient temperature and the optical density was measured at 540 nm using ELISA plate reader (BiOTek ELISA microplate reader ELx808). Ascorbic acid (Sigma-Aldrich/Merck, Germany) (0.078 mg/ml to 10 mg/mL) and Trolox (Sigma-Aldrich/Merck, Germany) (0.5 mg/ml - 0.25 µg/ml) were used as positive controls. Whereas methanol as negative control and extract without DPPH as blank. To evaluate the anti-radical scavenging efficacy, the IC_{50} (defined as the concentration of plant extracts that resulted in 50% reduction of the DPPH colour) was determined.

The DPPH radical scavenging activity (%) was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{(Ac - As)}{Ac} \times 100$$

Where the Ac = absorbance of control = [DPPH +Methanol without sample] and,

As = the absorbance of sample = [DPPH + Sample (extract or standard)].

4.2.5. Cell proliferation assay

DNA synthesis is a very popular and widely used method to study cell proliferation. Determining the synthesis of new DNA is a precise way to assess cell multiplication in individual cells or in cell populations. Direct measurement of new DNA synthesis using BrdU proliferation ELISA generally involves the incorporation of a labelled nucleoside into genomic DNA.

Human Epidermal Keratinocytes obtained from Sigma-Aldrich/Merck, Germany ($2x10^5$ cells per well) were plated together with methanolic extracts of *L. inermis* L, *A. aspera* L, and *A indica* A. Juss extracts (78 µg/ml – 10 mg/ml) in a 96-well plate in a final volume of 200 µl/well and incubate in a humidified atmosphere at 37°C. Twenty microliter per well (20 µl/well) BrdU labelling solution (Sigma-Aldrich/Merck, Germany) was added and cells were re-incubated for additional 2 hrs at 37°C. Labelling medium was withdrawn, and 200 µl/well FixDenat (Sigma-Aldrich/Merck, Germany) was added to the cells to fix it, and incubated for 30 min at 25°C. FixDenat solution was removed thoroughly, and 100 µl/well anti-BrdU-POD (Sigma-Aldrich/Merck, Germany) working solution was added and incubated for 90 min at 25°C. Antibody conjugate was removed, and plate wells were rinsed three times with washing solution, and removed by tapping. 100 µl/well substrate solution was added after removal of washing solution, and plates were incubated at 25°C (15 min). Then, 25 µl (1M H_2SO4) (Sigma-Aldrich/Merck, Germany) was added to all wells and incubated for 1 min on a plate shaker (300 rpm). Absorbance of the wells were measured in an ELISA reader (BiOTek ELISA microplate reader ELx808) at 450 nm within 5 minutes. Data analysis and interpretation of the absorbance (OD) reading. IC_{50} was determined using GraphPad prism and percent cell replication (DNA replication) was calculated using the following formula:

$$\text{Percent cell replication} = \frac{(\text{Initial activity} - \text{stimulation activity})}{\text{Intial activity}} \times 100$$

Where initial activity = The 100% activity well - Blank wells

4.3. Statistical analysis

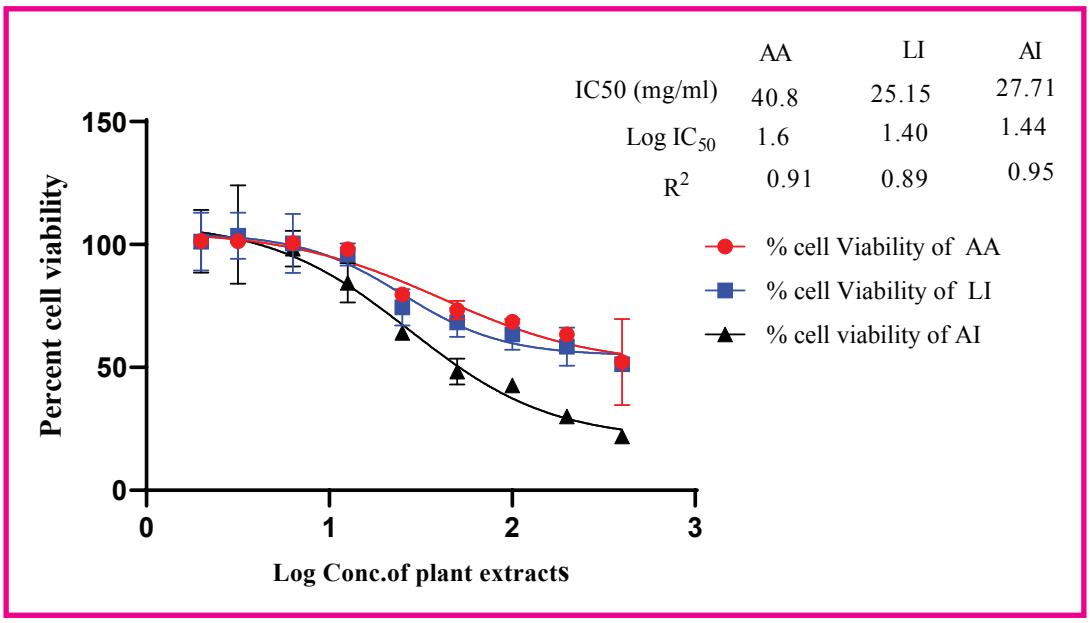
Graphs of quantitative data and values were calculated using GraphPad Prism (GraphPad Prism version 8.4.1(676)). IC_{50} and EC_{50} for the assay parameters (COX-1, COX-2, 15-LOX, radical scavenging activity, cell proliferation and cytotoxicity) were determined using the 'log concentration vs. response' of the 'non-linear regression analysis function. The samples were tested at five to seven different concentrations in a duplicate and triplicate manner. For the cyclooxygenases and lipoxygenase inhibitor assay, data of three separate experiments were used (n = 3), while for cytotoxicity, DPPH assay and cell proliferation assays, data of two separate experiments were used (n=2). In addition, percent inhibition and multiplication were calculated for inhibitory and stimulatory activities of plant extracts.

4.4. Results

4.4.1. Cytotoxicity activity

MTT assay was performed using Vero cells by treating the cells with different concentrations of methanol extracts. Vero cell lines were obtained from National Veterinary Institute (NVI),

Bishofitu, Ethiopia. The assay was based on the reduction of yellow tetrazolium MTT to insoluble formazan-blue crystals by mitochondrial succinate dehydrogenase enzymes. Only viable cells with active mitochondria reduce significant amounts of MTT, and the plate reader's absorbance value is directly proportional to cell viability. The assay was performed as a percentage of relative cell viability against various concentrations of the plant extracts (271). *A. aspera* L and *A. indica* A. Juss were less toxic at higher concentration with IC_{50} of 40.8 mg/ml and 27.7 mg/ml respectively. Whereas *L. inermis* L showed higher toxicity with IC_{50} = 25.15 mg/ml. More than 80% the cells were viable for the three methanol extracts at a concentration of 12.5mg/ml (Figure 4.2).



AA = Acyranthus asper, AI= Azadiractha Indica A. Juss , LI = Lawsonia inermis

Figure 4.2: Cytotoxicity assay of methanol extract of L. inermis, A indica and A. aspera against Vero cell line

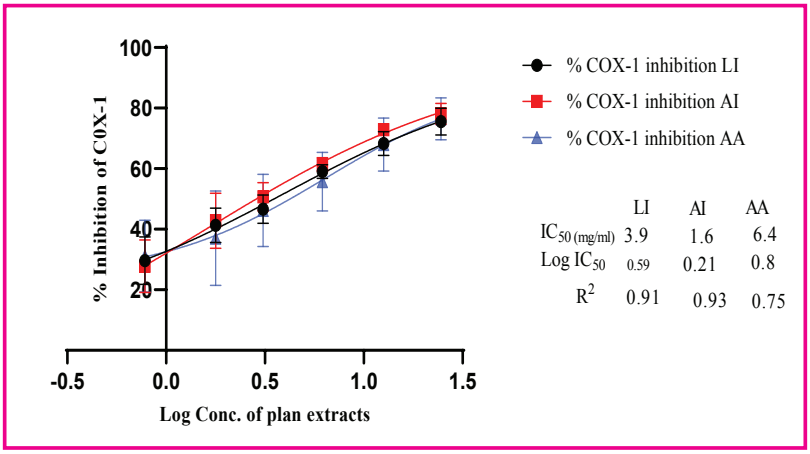
4.4.2. Anti-inflammatory activity

4.4.2.1. Cyclooxygenase 1 and 2 enzyme inhibitory assay

Methanol extracts of three medicinal plants were tested for COX-1 and COX-2 enzyme inhibitory activity and dose dependent inhibition was observed for all the extracts. IC_{50} was calculated using GraphPad prism for the crude plant extracts based on dilution series. The IC_{50} value of indomethacin (Sigma-Aldrich/Merck, Germany) was determined by testing at different concentrations (3.125 µg/ml - 50 µg/ml), and 50 µg/ml was used as a positive control in the assay.

Compared to indomethacin, *A. indica* A. Juss (82%) and *L. inerms* L (87.6%) showed significant inhibition against COX-1 at 25 mg/ml, with IC_{50} of 1.6 mg/ml and 3.9 mg/ml,

respectively. While *A. aspera* L (91.7%) and *A. indica* (95%) were effective in inhibiting COX-2 (IC_{50} = 4.86 mg/ml and 5.67 mg/ml) at 25 mg/ml. Similarly, *L. inermis* L inhibited 88.8% of COX-2 at the same concentration. Indomethacin (50 µg/ml) showed 92% and 95% of inhibition against COX-1 and COX-2, respectively (Figure 4.4 and 4.5). Regrading selectivity index of COX-2, *A. aspera* and *L. inermis* have higher values compared to indomethacin.



AA = Acyranthus asper, AI= Azadiractha Indica A. Juss , LI = Lawsonia inermis, COX-1 = cyclooxygenase enzyeme-1

Figure 4.3: Percent inhibition of methanol extract of L. inermis, A. indica and A. aspera against COX-1 enzyme

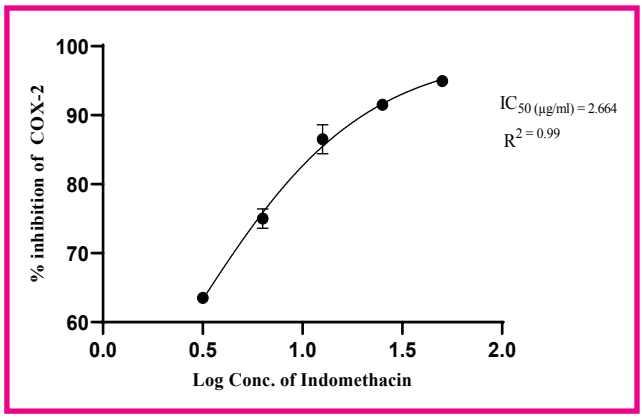


Figure 4.4: Percent inhibition of indomethacin against COX-2 enzyme

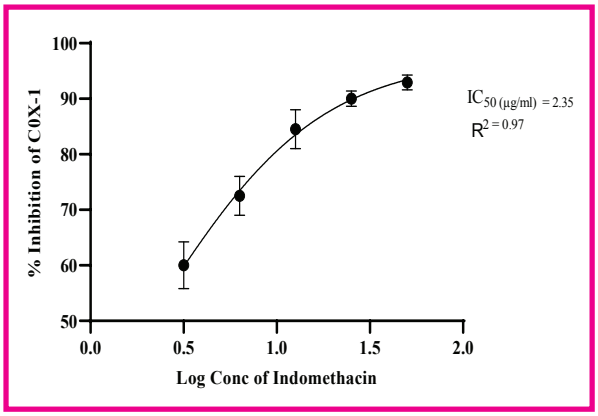
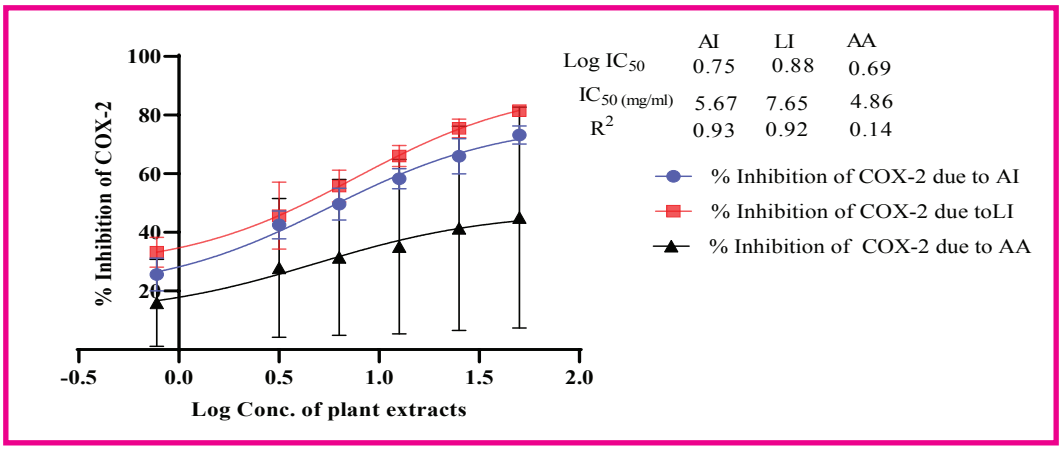


Figure 4. 5: Percent inhibition of indomethacin against COX-1

Methanol extracts of leaves plant species	IC_{50}		COX-1/COX-2 (SI)
	COX-1	COX-2	
Achyranthus aspera L (mg/ml)	6.4	4.8	1.33
Lawsonisa Inermis L ((mg/ml)	3.9	7.69	0.5
Azadirachta indica A.Juss (mg/ml)	1.6	5.67	0.28
Indomethacin (µg/ml)	2.53	2.66	0.95

Table 4.1: Selectivity index of methanol extract leaves of plant species and indomethacin

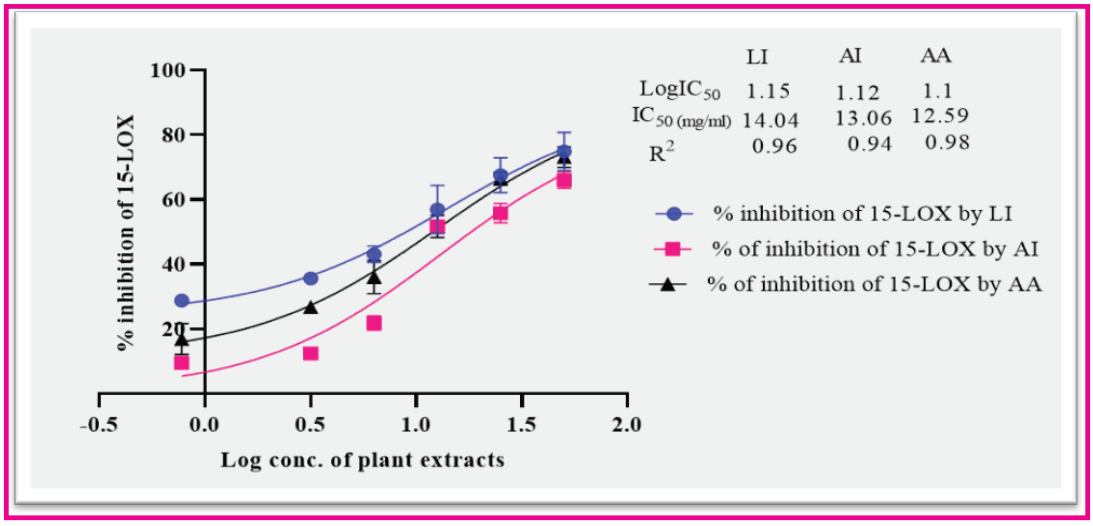


COX-2 = cyclooxygenase enzyeme-2 AA = Acyranthus asper, AI= Azadiractha Indica A. Juss , LI = Lawsonia inermis

Figure 4. 6: Percent inhibition of methanol extracts of L. inermis, A. aspera, and A indica against COX-2

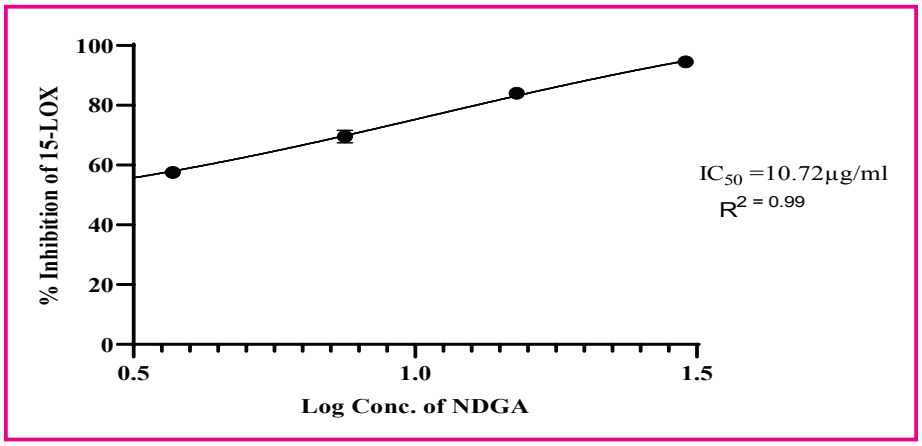
4.4.2.2. 15-Lipoxygenase inhibitory activity

The result described in Figure 4.7 showed that methanol extracts of *L. inermis. A. indica, and A. aspera* demonstrated moderate inhibition against 15-lypoxygenase enzyme. Among the three plant extracts, the methanol extracts of leaves of *L. inermis* (74.8%) had the highest inhibitory activity which followed by *A. asper* (73.1%) at 25mg/ml. *A. indica* showed the least activity against 15-LOX which demonstrated 66 % inhibition at the same concertation. Nordihydroguaiaretic acid (NDGA) used as standard drug exhibited 95.6% inhibition of 15-LOX at 30µg/ml (Figure 4.8).



AA = Acyranthus asper, AI= Azadiractha indica, LI = Lawsonia inermis, 15-LOX = 15-lipooxygenase enzyme

Figure 4.7: 15-Lipoxygenase inhibitory activity of methanol extracts of L. inermis, A. indica and A. aspera



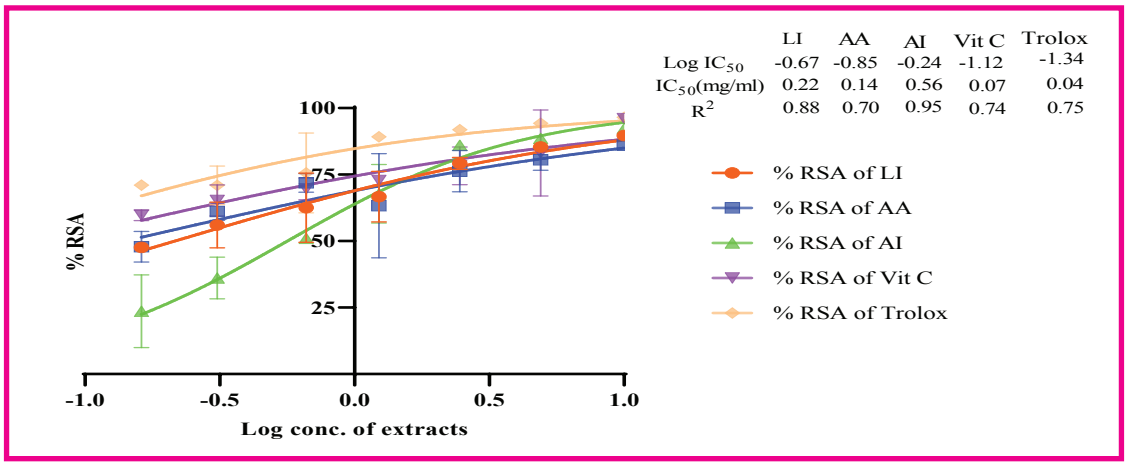
15-LOX = 15-lipooxygenase enzyme, NDGA = Nordihydroguaiaretic acid

Figure 4.8: Nordihydroguaiaretic acid inhibition of 15-LOX at different concentrations

4.4.3. Wound healing activity

4.4.3.1. DPPH radical scavenging activity

Antioxidant activity of the leaves of methanol extracts *L. inermis* L, *A. aspera* L, and *A. indica* A. Juss were conducted using a colorimetric method, DPPH radical scavenging activity. The mixture loses its purple colour that absorbs at this wavelength when accepting an electron or a free radical species, which results in a yellow colour. All the tested extracts showed the best free radical scavenging activity at 10 mg/ml as indicated by their percent inhibition (93.8%, 95%, 93.4%, respectively) and IC_{50} values, which are comparable inhibitory activity to that of ascorbic acid and Trolox, used as standard in this study (Figure 9). *L. inermis* L and *A. aspera* L had the best antioxidant activity with IC_{50} values of 0.22mg/ml and 0.14mg/ml, respectively. *A. indica* had the lowest radical scavenging activity at low concentrations, while showed highest activity at higher concentration (10mg/ml). Ascorbic acid and Trolox are known potent antioxidant and had the highest DPPH scavenging activity with IC_{50} value of 0.07mg/ml and 0.04 mg/ml respectively.

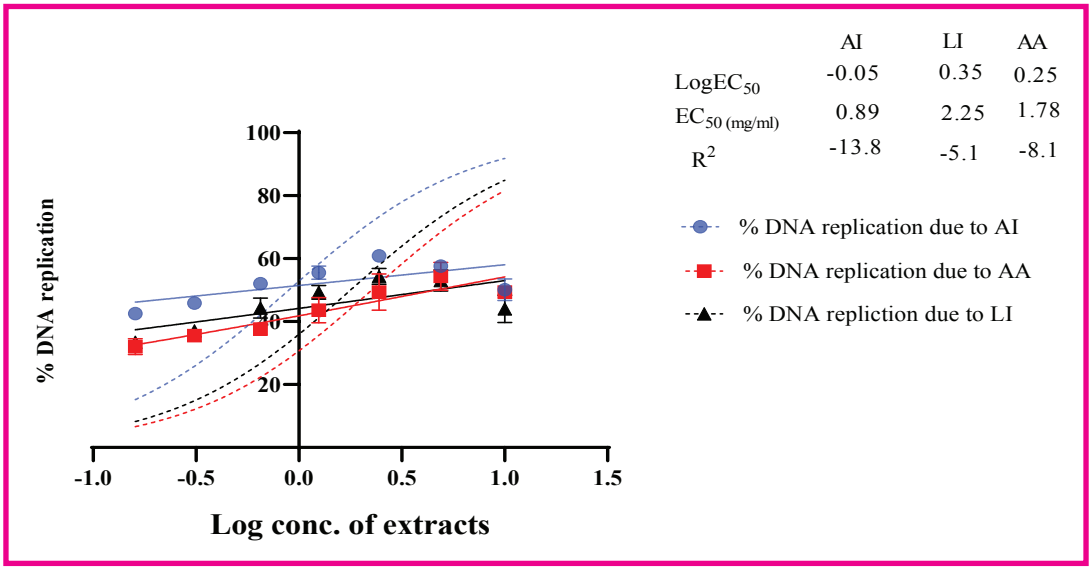


RSA = Radical scavenging activity, AA = Acyranthus asper, AI= Azadiractha Indica A. Juss, LI = Lawsonia inermis, Vit C= vitamin C

Figure 4. 9: Antioxidant activity of leaves of methanol extracts L. inermis L, A. aspera L, and A. indica A Juss

4.4.3.2. Cell proliferation activity

Quantification of cell multiplication, which based on the measurement of BrdU incorporation during DNA synthesis was used to measure percent of DNA/cell proliferation. The stimulating effect of the methanol extracts of plants on Human Epidermal Keratinocytes (HEK) were dose dependent. At higher concentrations, cell multiplication inhibitory activities of the methanol extracts were observed. *A. indica* A. Juss and *L. inerms* L have shown better stimulation of human epidermal keratinocytes, while optimal stimulation was observed between 2.5 mg/ml and 5 mg/ml (Figure 4.10). At 10 mg/ml, *A. indica* showed 50.1% increase in human epidermal keratinocytes multiplication followed by *L. inermis* L and *A. aspera* L, 49.3% and 44% respectively. The least EC_{50} value was determined for *A. indica* A. Juss (0.89 mg/ml), and *A. aspera* (1.78 mg/ml) while EC_{50} value of *L. inermis* L was 2.25 mg/ml.



DNA = Deoxyribonucleic acid, AA = Acyranthus asper, AI= Azadiractha Indica A. Juss , LI = Lawsonia inermis

Figure 4.10: Percent DNA replication activity versus Log con. of extracts of Methanol extract of leaves of L. inermis L, A. indica A.Juss, and A. aspera L.

4.5. Discussion

Cytotoxicity assays were conducted for the three methanol extracts tested in the *in vitro* models and all concentrations used were safe to the cell lines used in these experiments. However, all plant extracts showed varying level of toxicity to Vero cells, likely attributable to their differences in phytochemical constituents (274). According to our results, *A. aspera* L and *A, indica* A. Juss had the lowest toxicity on Vero Monkey kidney cell lines (Figure 4.2), while *L. inermis* L was more toxic which might be related to the level of secondary metabolites such as alkaloids and saponins in the extracts (273).

The anti-inflammatory activity of the plant extracts was determined using two parameters, cyclooxygenase, and 15-lipoxygenase inhibition assay. Both inhibition of LOX and COX have an importance in the management of chronic inflammatory conditions. Metabolism of arachidonic acid by COX enzymes lead to the secretion of prostaglandins and thromboxane that mediate pain and edema related to inflammation. The LOX pathway utilizes arachidonic acid to produce leukotriene, including the leukocyte chemoattractant LTB4 (275). All methanol extracts of *A. aspera, A. indica* and *L. inermis* shown to inhibit both cyclooxygenase and 15-lypoxygenase enzymes.

According to the result, all the tested methanol extracts were effective in inhibiting COX-2 which is a key enzyme in catalysing the secretion of prostaglandins, thromboxane, and levuloglandins. Prostaglandins are known to have an effect against almost all known physiological and pathological processes through reversible interactions with G protein-

coupled membrane receptors. Levuloglandins are a new category of products that seem to function through irreversible covalent bonding with a variety of proteins (276). *L. inermis* and *A. indica* were effective in inhibiting COX-1. Similarly, Jacob *et al* (2015) report that *L. inermis* has a dual inhibition of both LOX and COX enzymes, which is important in the reduction of chronic inflammatory conditions (277), which agrees with the current finding. In addition, ethanol, and methanol extracts of *L. inermis* were shown to inhibit inflammation in carrageenan induced rat paw oedema model (121,124). It has been noted that alkaloids, terpenoids, saponins, anthraquinones, tannins, phenols, steroids and flavonoids from plant origin were the major bioactive secondary metabolites isolated from the three methanol extracts (presented in chapter three) which have been described to have significant anti-inflammatory activity (278). Flavonoids, which have tested positive in *A. indica* A. Juss and *L. inermis* L have been reported to have a dual inhibitory activity of cyclooxygenase and lipoxygenase that know to inhibit the biosynthesis of prostaglandins, a secondary messengers in various immunologic responses (278).

In another study, Attiq *et al* (2018) reported, alkaloids and terpenoids which are present in *L. inermis* L and *A. aspera* L have shown to inhibit the COX-1 and COX-2 mediated PGE2 secretion in *in vitro* models. Further, alkaloids and terpenoids shown to inhibit PG2 and COX-2 through inhibition of cellular nuclear factor Kappa B (NF-kB) activity. In a similar study, terpenoids and flavonoids are reported to have the ability of reducing the secretion of pro-inflammatory cytokines such as IL-1beta, IL-6 and TNF-alpha (279). *A. aspera* was more selective to COX-2 than COX-1 with a higher value (1.33), which was comparable to indomethacin. Selective COX-2 inhibitors such as Coxibs are preferred to decrease the incidence of gastrointestinal hemorrhage and ulceration upon long-time intake (280). The anti-inflammatory compounds with better COX-2 selectivity index have shown to have low side effects which is the requirement for compounds to be COX inhibitors (281).

Many inflammatory diseases are due to the lipoxygenase enzyme families such as 5-LOX, 8-LOX, 12-LOX, and 15-LOX enzymes. The isomeric enzyme 15-LOX is a major enzyme implicated in the synthesis of leukotrienes from arachidonic acids. Bio-active leukotrienes act as promoter for numerous pro-inflammatory and allergic reactions, therefore inhibition of leukotriene synthesis by 15-LOX is considered as one of the treatment approaches to regulate inflammation and important for the management of different inflammatory diseases (282).

Anti-LOX enzyme activity of the plant extracts was also measured by the inhibition of linoleic acid which is a substrate for soybean lipoxygenase (15-LOX) enzyme. According to previously reported finding, methanol extract of *L. inermis* L and *A. aspera* L shown to have moderate activity (66-74.8 % inhibition) in the inhibition of 15-LOX (269). In another study, leaves of methanol extracts of *A. aspera* showed 70% inhibition of lipoxygenase enzyme,

which agrees with the current finding (283). Mzindle (2017) reported in his finding that the aqueous and methanol extracts of *A. aspera* L were shown to control the release of various mediators in both the early and late stage of inflammation, and observed to have a wound healing potential (284). In addition, methanol extract of *A. aspera*, shown significant activity in Carrageenan-induced paw edema in rat model (285,286). There is a direct relationship of anti-LOX activity and presence of plant secondary metabolites such as phenols and flavonoids in plant extracts. Furthermore, different studies have implicated that oxygen free radicals blocks the process of arachnoid acid metabolism by inhibiting lipoxygenase (LOX) enzyme activity (287).

Chaibi *et al* (2017) described the anti-inflammatory activity of methanol extract of *L. inermis* L, which showed the methanol extract was superior in inhibiting the LOX family, 5-LOX with IC_{50} value of 51 ± 0.23 mg/L (130). Schumacher *et al* (2011) reported that the methanol extract of *A. indica* shown to inhibit the TNF-alpha-activated NF-kB pathway at 240 µg/ml , which indicated the anti-inflammatory activity of the crude extract(288). Plants with antioxidant properties can have also anti-inflammatory activities, because lipoxygenase is reported to be sensitive to antioxidants due to its inhibition of substrate (lipid hydrogen peroxide) formation that required for lipoxygenase catalysis (289).

Wound healing is a complex and ongoing process which includes homeostasis, re-epithelization, granulation, tissue reformation, and remodeling of the extracellular matrix. Even though, the wound healing can take place by the body and doesn't much help external factors such as wound infection and delay in healing has brought an interest in wound healing (181). Many medicinal plants have been claimed to be useful for wound healing in Ethiopian traditional medicine, and some of the traditional medicines in use have been used for the management of dermatological disorders (268). In this study the antioxidant activity and cell proliferation tests of the methanol extracts of *L. inermis* L, *A. aspera* L and *A. indica* A. Juss were tested to understand their roles in wound healing.

All methanol extracts exhibited dose dependent DPPH antioxidant activity and their free radical scavenging activity was correlated to the content of flavonoids, phenols and terpenoids (290). Methanol extract of leaves of *A. aspera* was effective in scavenging DPPH free radicals, which is in agreement with those previously reported findings (180,181,252,291). Further, Fikru *et al* (2012) reported, the considerable antioxidant and antimicrobial activity of methanol extract of *A. aspera*, and its wound healing properties. The wound healing activity could be due to its role in promoting fibroblast adhesion and/or reduce xenobiotic-induced leukocyte hyperactivity and inflammatory damage (179). Similarly, *L. inermis* L have been shown high antioxidant and wound healing potential, and it is in agreement with previously reported findings (131,292,293). In another study, Alzohairy (2016) reported the significant antioxidant activity of *A. indica* A. Juss, and it is revealed that azadirachtin and

nimbolide are the main compounds that have radical scavenging activity and reductive potential (294).

Oxidative stress and free radical have been an implication in impaired wound healing (295). Different excessive reactive oxygen species and its degradation product are generated during the healing of cutaneous wound which cause oxidation of biomolecules and ability to damage numerous molecules in the cell membrane (296,297). In addition, high level reactive oxygen species have the capacity of inflicting peroxidation of membrane lipids, aggression of tissue membranes and proteins, or harm to DNA and enzyme via oxidation of low-density lipoproteins (LDL) (296). Thus, decrease of antioxidant ability results in redox imbalance, which is a major cause of nonhealing wounds (298).

A previously established *in vitro* cell proliferation assay method was used to measure percent human epidermal keratinocytes in a monolayer cell model. Cell multiplication and migration are the characteristics of cell division. DNA multiplication is the key stage in cell division, and it is regulated by different stages: G1/0, G2/M, and S phases of cell cycle. As described on Figure 10, the methanol extracts of leaves of *L. inermis* L, *A. aspera* L and *A. indica* A. Juss demonstrated moderate activity in stimulating proliferation of human epidermal keratinocytes. The optima concentration that showed optima stimulation was between 2.5mg/ml to 5 mg/ml. Plant secondary metabolites, triterpene, are known to stimulate cell proliferation, which positively influence the wound healing effect of the methanol extracts of leaves of *L. inermis* L*, A. aspera* L*, A. indica* A. Juss (299).

Fikru *et al* (2012) reported 5% and 10% ointment base methanol extracts of *A. aspera* which demonstrated high DNA and protein content of granulation tissue in animal model, implies cellular multiplication and suggests an increase in the synthesis of collagen, a predominant tissue in wound healing (300). In another study reported by Rekik *et al* (2019), the *L. inermis* L oil promoted wound healing via cell proliferation in animal model (292). Furthermore, methanol extract of *L. inermis* L has also antibacterial and anti-oxidant activity as observed in our study (301), which could contribute to wound healing. In another animal study, steam bark of *A. indica* has shown to increase the tissue DNA content of plant extracts treated wounds which indicates cell proliferation. In addition, there was considerable increase in the protein and hydroxyproline content of plant extract treated wound tissues which is an indication of fibroblast cells and epithelial cells migration, and synthesis of extracellular matrix in *A. indica* extracts treated mice (302).

4.6. Conclusion

Plant extracts or compounds that inhibit these enzymatic inflammatory activities may contain potential drivers or templates for the development of effective anti-inflammatory

drugs. More work needs to be done to correctly characterize the compounds in charge for anti-inflammatory principles in these plant species and understand their mechanisms of action. The results provided in this section finally confirmed the presence of biologically active molecules in these plant extracts, which can inhibit both the LOX and COX enzymes involved in the biosynthesis of pro-inflammatory leukotrienes and prostaglandins. Oxidative stress must be considered in the inflammatory process of wound healing and chronic wound treatment. Antioxidants that maintain non-toxic ROS levels in wound tissue can improve wound healing.

CHAPTER FIVE

5. ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM METHANOL EXTRACTS OF LEAVES OF *LAWSONIA INERMIS* L.

5.1. Background

Natural products, especially plants, have been used as sources of traditional remedies for a range of ailments for decades. Due to their chemical diversity, they have been sources of a large number of new drug discoveries (303), and have provided new lead compounds relevant to a wide range of health issues. Natural flora have become a very useful source of health improvement and even cure for diseases across a range of human communities in many parts of the world (304).

Ethiopia is known for its biodiversity and is the center of a significant number of plants, domestic animals, and their wild relatives. It is the home of around 6,000 species of vascular plants due to its geological history, spread over different altitudes and immense mountain ranges. This diversity has led to the development of habitats favorable to the evolution and survival of different plant and animal species, contributing to the overall biodiversity of the country (48).

Biodiversity represents an unlimited supply of novel chemical entities (NCEs) that are potential sources of drug leads. These NCEs are secondary metabolites, synthesized by parts of the plant in an attempt to protect the plant from herbivores and pathogens or to enhance the activities of pollinating agents (305). Most currently available drugs used for the treatment of human and animal diseases are derived from natural products, particularly medicinal plants. Herbalists have observed the medicinal use of a specific plant or its parts (leaves, roots, barks, fruits, seeds, or the whole plant) and then isolated bioactive compounds from the plant or part of the plant that was traditionally used for the treatment of illnesses (306). Polyphenol and flavonoid compounds are abundantly found in food of plant origin, and have beneficial health effects due to their potential antioxidant, anti-inflammatory and cancer preventive activities (307).

Current drug discovery from plants depends on bio-assay guided compound isolation methods. Plants consist of a complex mixture of compounds which are biologically active but whose properties are not precisely known. The phytochemical analysis established through ethnopharmacology is considered an efficient approach in NCE discovery, but detailed information on the character of secondary metabolites is lacking. Thus, all the extraction, purification and separation processes tend to be performed following the pharmacologic assays with the aim of isolation and establishing the bio-active compounds (Figure 5.1) (305).

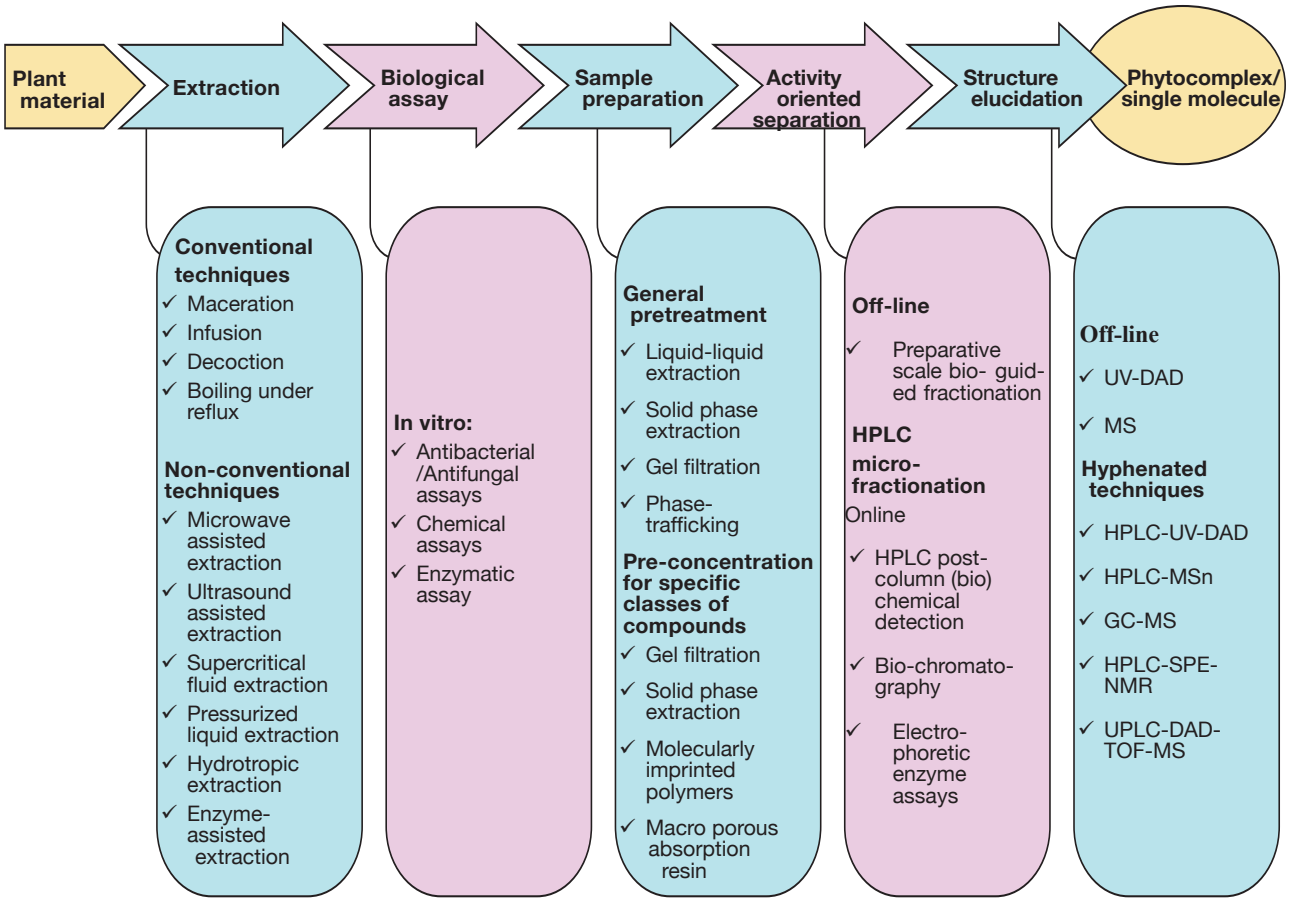


Figure 5.1: Methodologies involved in the ethnopharmacology approach (4)

However, despite a resurgence of interest in the investigation of plants as a source of natural products, the numbers of plant-derived secondary metabolites are still far from exhausted. Though hundreds of thousands of medicinal plants species are available, only a few plant species have been investigated for their active compounds and fewer species have been screened for their biological activities, showing that many more uninvestigated candidates which need to be investigated remain untouched (308).

Lawsonia inermis (Lythraceae), commonly known as 'Henna' is one of the most popular medicinal plants used traditionally. It is native to North Africa and Southeast Asia, and often cultivated as an ornamental plant throughout India, Persia, and among African countries (309). The leaves of *L. inermis* provide an important aesthetic colorant. They have been widely used for centuries in the Middle East, Far East and North Africa as a dye for nails, hands, hair, and textiles. Henna is also used in the treatment of skin problems, headaches, jaundice, filariasis, and enlarged spleen. The whole plant, roots, fruits, stems, leaves, bark, inflorescences, rhizomes, bulbs, latex, seeds, flowers, and oils have been used for treatment of various diseases (310).

L. inermis L is a pharmacologically important plant species with significant biological activities in *in vitro* and *in vivo* studies. Although different pharmacological activities have

been documented, the antioxidant, anti-inflammatory and antibacterial activities are the most investigated ones (246). Hundreds of phytochemical constituents representing many different types, have been identified in all parts of *L. inermis.* Phenolic compounds, coumarins, flavonoids, and naphthoquinones are the most identified constituents of *L. inermis* crude extracts (246).

Several studies have been conducted to reveal the pharmacological activities of *L. inermis* L. Although several active compounds have been identified from the different types of crude extracts, there are questions that remain to be answered about the nature of the active compounds responsible for the observed pharmacological activities in some of the crude extracts in biological systems.

Therefore, this chapter focuses on the isolation and characterization of the methanol extract of the leaves of *L. inermis* L which has shown significant activity against bacteria isolated from the lymphoedema patients, and *in vitro* anti-inflammatory as well as wound healing activities, described in Chapters three and four. This will add knowledge in the development of new drugs from this plant**.**

5.2. Objectives

The objective of this study was to isolate and characterize bioactive compounds from the methanol extract of leaves of *L. inermis* L, which showed important pharmacological activities in antibacterial, anti-inflammatory, and wound healing assays (Chapters Three and Four).

5.3. Material and Methods

5.3.1. Extraction and Compound Isolation

The air-dried and powdered leaves of *L. inermis* (600g) were defatted by soaking with petroleum ether for 24 h, at room temperature. The residue was soaked twice in methanol for 24 h, at room temperature. The extract was concentrated under reduced pressure to give a brown residue (58g). About 22g of the methanol extract was applied to column chromatography over silica gel 60 and eluted with petroleum ether/ethyl acetate and then ethyl acetate/methanol mixtures of increasing polarities. A total of 52 fractions of 200 mL each were collected and concentrated using a rotary evaporator (Heidolph Instruments GmbH & O.KG, Germany) under reduced pressure at 40^{O}c. All fractions were analyzed using thin layer chromatography (TLC), and separated components were initially visualized under UV-vis light, and then the plates were sprayed with vanillin-sulfuric acid, dried and heated using a plate heater. Fractions with the same TLC profile were combined and concentrated.

Based on TLC analysis, 30-36 fractions (obtained by gradient elution with 100% Ethyl acetate and then to 30% methanol in Ethyl acetate) were combined and further purified on a silica gel column by elution with chloroform/Ethyl acetate (100% CF, 90% CF: 10% EA, 80% CF: 20% EA, 50% CF: 50% EA, 48.75% CF: 48.75% EA) and then ethyl acetate/ methanol (5% MeOH, 45% CF: 45% EA: 10% MeOH, and 40%CF: 40%EA: 20% MeOH) mixtures of increasing polarities. The fractions were grouped into 13 fractions (A-M) based on TLC profile. Fractions B and C (obtained by elution with chloroform/ethyl acetate, 80:20) were combined, and represented compound **1**. Fraction I (obtained by elution with chloroform / ethyl acetate / methanol, 20:20:10) was made to pass through Sephadex LH-20 by elution with $CHCl_3$–MeOH (1:1) to yield compound **2**. Compound **3** was crystalized as a white powder from fraction 40 which was obtained with 40% methanol in ethyl acetate.

5.4. Results and discussion

The methanol extracts of the leaves of *Lawsonia inermis* on repeated column chromatography and further purification by Sephadex LH-20 yielded three compounds, namely, lawsone (**1**), lalioside (**2**) and D-mannitol (**3**) (Fig. 5.1). The three compounds were identified by interpretating the NMR spectra and comparing to literature data (311–313).

The assignment of the NMR spectra is usually performed with the help of NMR charts or diagrams that facilitate the identification of the NMR signals. The COSY (COrrelated SpectroscopY) spectra display peaks that correlate pairs of nuclei that are separated by a maximum of three chemical bonds. This correlation arises from the interaction between nuclear spins through scalar coupling. The 1 H Nuclear Magnetic Resonance (NMR) structure give the information of on the number of signals gives the number of non-equivalent hydrogens. Chemical shifts show differences in the hydrogens' chemical environments. The 13C NMR is directly about the carbon skeleton not just the proton attached to it. The number of signals tell us how many different carbons or set of equivalent carbons. The splitting of a signal tells us how many hydrogens are attached to each carbon. DEPT experiments are used for distinguishing between a CH3 group (methyl), a CH2 group (methylene), and a CH group (methine). The proton pulse is set at 45°, 90°, or 135° in the three separate experiments. The different pulses depend on the number of protons attached to a carbon atom. HSQC determines the correlations between two different types of nuclei (commonly 1H with 13C or 15N), which are separated by one bond. The HSQC spectrum coordinates the chemical shifts of two bonded nuclei where only one peak will be obtained per pair of coupled atoms. Thus, HSQC offers a very informative approach for signal assignments. An HMBC spectrum looks very similar to an HMQC spectrum, except that it shows 2-bond, 3-bond or sometimes even 4-bond coupling (not HC, but HCC or HCCC or even HCCCC).

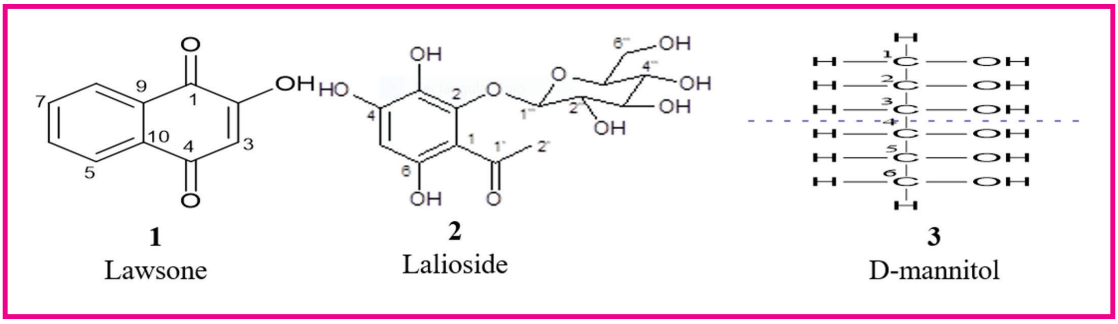
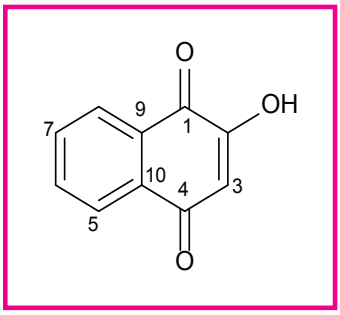


Figure 5.2: Structures of the isolated compounds from Lawsonia inermis

Compound **1** was isolated as a brown powder. The ^{13}C NMR (Table 5.1), DEPT-135 and HMQC spectra confirmed that compound **1** has 10 carbon atoms. The presence of two ketone functionalities was recognized by signals at δ_C 182.35 (C-1) and 185.51 (C-4). The ^{1}H-NMR spectrum exhibited five aromatic protons at δ_H 8.09 (1H, dd, *J* = 7.2, 1.0 Hz, H-8), 8.05 (1H, dd, *J* = 7.6, 1.5 Hz, H-5), 7.89 (1H, td, *J* = 7.6, 1.5 Hz, H-6), 7.84 (1H, td, *J* = 7.6, 1.5 Hz, H-7), and 8.10 (1H, dd, *J* = 7.6, 1.5 Hz, H-8), indicating an ortho-disubstituted aromatic ring. The other singlet signal was at δ_H 6.25 (1H, H-3) attached to δ_C 111.60 (HSQC evident). HMBC analysis indicated that the protons at δ_H 8.05 (H-5) and 8.10 (H-8) had HMBC correlations with carbonyl carbons at δ_C 185.51 (C-4) and 182.35 (C-1), indicating the positions of the carbonyl on the aromatic system. Furthermore, the singlet proton signal at δ_H 6.25 (H-3) showed HMBC correlations with carbons at δ_C 184.43 (C-4) and 131.83 (C-10) indicating that the position of this proton was adjacent to the carbonyl carbon at δ_C 185.51 (C-4). In addition, this proton exhibited HMBC correlations with carbons at δ_C 182.35 (C-1) and 159.26 (C-2), suggesting a 1,4-napthoquinon structure (311). The remaining deshielded quaternary carbon δ_C 159.26 was assigned C-2 based on HMBC correlations, suggesting a hydroxyl substitution at this carbon. Comparison of its NMR data with reported values revealed that NMR of compound **1** is identical to those of lawsone (311). Thus, compound **1** was identified as 2-hydroxy-1,4-naphthoquinone (common name lawsone). The compound was previously isolated from *Lawsonia inermis* grown in different countries (311,314,315).

C	δ_C [a]	δ_H (J = Hz) [b]	HMBC
1	182.35	-	
2	159.26	-	
3	111.60	6.25 s	C-1, 3, 4, 10
4	185.51	-	
5	126.64	8.05 dd (7.6, 1.5)	C-4, 6, 9
6	135.53	7.89 td (7.6, 1.5)	C-5, 7, 10
7	133.98	7.84 td (7.6, 1.5)	C-6, 83, 9
8	126.83	8.10 dd (7.6, 1.5)	C-1, 7, 9, 10
9	133.52	-	
10	131.32	-	



[a] *100 MHz,* [b] *400 MHz*

Table 5.1: NMR data of 1 (lawsone) measured in acetone-d_6

Compound **2** was isolated as a brown residue. The ^{1}H-NMR (Table 5.2) showed a typical acetyl group at δ_H 2.64 (3H, s, H-2') and a singlet aromatic proton signal at δ_H 6.15 (1H, s, H-5) indicating the presence of a penta-substituted benzene ring. Three broad singlets at δ_H 8.65, 10.42 and 12.66 attributable to the phenolic protons 3-OH, 4-OH and 6-OH, respectively. An anomeric proton peak at δ_H 4.81(H-1") and peaks for the sugar moiety (δ_H 3.12 – 3.62) were also observed. ^{13}C NMR (Table 5.2), DEPT and HMQC spectra confirmed the presence of 14 carbon atoms. Of these, the signal for a ketone functionality for the acyl group was confirmed from δ_C 203.88 (C-1') and 32.90 (C-2'). The carbon peaks resonating δ_C 100.0–156.6 were attributed to aromatic carbons. The ^{1}H NMR and the HSQC spectra revealed an anomeric signal (δ_H 4.81, d, J = 8.0 Hz, H1" and δ_C 105.3, C1") together with a set of signals between δ_H 3.12 – 3.62 in ^{1}H NMR and δ_C 61.1 – 77.8 in ^{13}C NMR, firmly established the presence of a β-D-glucopyranose (316). The stereochemistry of the glycosidic linkage was determined as β, judged from the coupling constant (J=8.0 Hz) of the anomeric proton signal at δ_H 4.81 (316). The significant HMBC correlation between the anomeric proton H1" (δ_H 4.81) and C2 (δ_C 145.4) indicated that the glucopyranose must be linked to the C-2 of the aromatic ring. Similarly, the observed HMBC correlation of the methyl protons H2' (δ_H 2.64) with C1' (δ_C 203.88) indicated the position of the acyl group to be C1. Furthermore, the deshielded proton signal at δ_H 12.66 for a chelated OH showed the presence of a hydrogen bond between the 6-OH group and the carbonyl group, confirming that the 6-OH and the acyl group were *ortho* to each other. Full assignments of the ^{1}H and ^{13}C NMR spectra were consolidated using COSY, HSQC and HMBC experiments (Table 5.2 and Annex 5). Comparison of NMR data with reported values revealed that compound **2** was identical to lalioside, previously isolated from *Lawsonia inermis* L (312). Therefore, compound **2** was identified as 2,3,4,6-tetrahydroxyacetophenone-2β-D-glucopyranoside with common name lalioside.

C	δ_C [a]	δ_H (J = Hz) [b]	HMBC
1	109.52	-	
2	145.44	-	
3	131.29	-	
4	154.45	-	
5	99.99	6.14 s	C-1,3, 4,6
6	156.57	-	
1'	203.88	-	
2'	32.90	2.64 s	C-1, 1'
1"	105.31	4.81 d (8.0)	C-2'
2"	74.39	3.31 t (8.7)	
3"	77.81	3.13 m	
4"	69.99	3.13 m	
5"	76.56	3.23	
6"	61.12	3.60 m 3.44 d (12.0)	
6-OH		12.66 s	

[a] *100 MHz,* [b] *400 MHz*

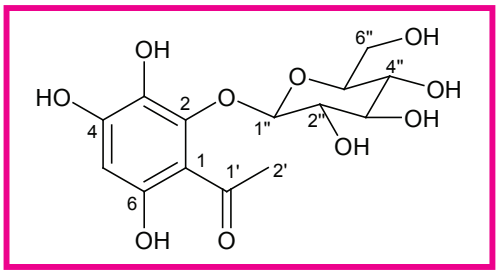
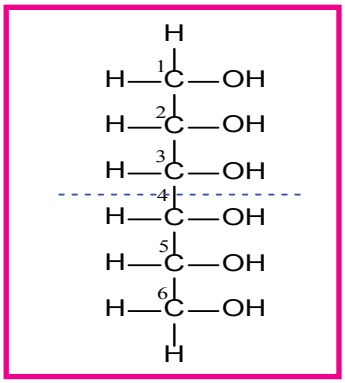


Table 5.2: NMR data of 2 (lalioside) measured in DMSO-d_6

Compound **3** (D-Mannitol) was isolated as a white powder. The ^{1}H NMR spectrum (Table 5.3) showed four hydrogen peaks δ_H 3.37 – 3.62 and three hydroxy signals δ_H 4.14 – 4.42 attributed to a polyhydroxy compound. However, absence of signals for anomeric proton around δ_H 5.0 suggested an open chain structure. The ^{13}C NMR (Table 5.3) spectra also showed polyhydroxy carbons at δ_C 64.31 (C-1), 71.75 (C-2) and 70.10 (C-3). The peak at δ_C 64.31 (C-1) was a CH_2OH group confirmed from DEPT spectra. The presence of only one -CH_2OH peak and the absence of a methyl (CH_3) peak in the ^{13}C NMR spectra suggested a symmetrical structure for Compound **3**. There was no open chain molecule with 3 carbon atoms containing one -CH_2OH without a -CH_3 group. The connectivity of C1 to C2 and C3 was supported by H-H COSY correlation of H_2-1 with H-2 and H2-with H-3. The positions of the hydroxy groups were also established using H-H COSY correlation. Thus, the structure of compound 3 was identified as 1,2,3,4,5,6-hexahydroxyhexane, whose spectrum is similar to D-mannitol (313).

C	δ_C [a]	δ_H (J = Hz) [b]	COSY
1,6	64.31	3.66 m 3.46 m	H-2
2,5	71.75	3.45 m	H-1, H-3
3,4	70.10	3.54 t (7.8)	H-2
1,6-OH		4.34 m	H-1
2,5-OH		4.41 d (5.28)	H-2
3,4-OH		4.14 d (7.08)	H-3



[a] *100 MHz,* [b] *400 MHz*

Table 5.3: NMR data of 3 (D-mannitol) measured in DMSO-d_6

5.5. Conclusion

This study identified and isolated three compounds namely, lawsone (**1**), lalioside (**2**) and D-mannitol (**3**) from methanol extract of leaves of *Lawsonia inermis* using chromatographic methods, and the structures of these compounds were elucidated by Nuclear Magnetic Resonance (NMR) technique. The amounts of Compounds **1** and **2** isolated from the plant were higher than that of compound **3**. The pharmacological activity of methanol extract of *L. inermis* observed in this study could be due to either one of these compounds or the combination of the three and other minor not isolated compounds in the crude extract.

CHAPTER SIX

6. CONTRIBUTION TO THE FIELD, LESSON LEARNT FROM THE THESIS WORK, AND FUTURE PROSPECTS

6.1. Contribution to the field and lesson learnt

There is huge information on the traditional use of medicinal plants in Ethiopia. Understanding the traditional use of herbal medicines can help us in identifying plant material with potential phytochemical which are also applicable to modern medicine. We have tested the methanol extracts of three plant extracts against three broad different disease conditions in *in vitro* method, and a valuable information has been generated to be utilized by other researchers.

This study has come up with additional information of three medicinal plants which were selected based on a systematic review and tested in *in vitro* model for the management of lymphoedema associated conditions such as inflammation and infection. Most drug candidates were emerged from early screening of the extracts and compounds. Our research work is one of the strategies for the search and discovery of new chemical entities which are active compounds for the therapeutic purpose of inflammation and infected wounds. This information can be used also by other researchers for further research on these medicinal plants, which avoids duplication of effort and resource in conducting preliminary research works on them.

Our research work investigated the cytotoxic, anti-inflammatory, wound healing, and anti-bacterial activities of the plant extracts in *in-vitro* models, which has an eminent scientific output. As a result, the conducted *in vitro* assay is important in identifying and characterize the plant extract in terms of safety, efficacy, and major compounds responsible for the pharmacological activities. The health care system of most developing countries including Ethiopia depends on the primary health care system strategy. As a result, the finding of this work can be used to integrate the use of medicinal plants into all components of primary health care system.

Inflammation is considered as the root cause of almost all diseases including cancer at molecular level. Thus, one of our objectives was to screen potential compounds from natural products that needs more attention such as inflammation, and our study have a contribution for such diseases.

This work is one of the efforts that measures the effectiveness, safety, efficacy of medicinal plants in *in vitro* method. Even though, it is difficult to use the *in vitro* study finding to directly extrapolate to human, sometimes it is possible to use these medicinal plants topically since they have been in use traditionally for many years. So that, this could be

used as base line information to integrate herbal medicines to national and local health policies and programs.

Furthermore, our study also isolated and characterized the micro-organisms that involved in wound infection of patients with lymphoedema in the places where tropical lymphoedema was common in Ethiopia. Then, antimicrobial susceptibility test was done using currently in use antibiotics in the area against the isolates. Based on our finding we recommended the appropriate treatment regimen change to those antimicrobials that the resistant bacteria are susceptible, affordable, and available in the area. This has many contributions to reduce the danger of resistant bacteria, and in reducing economic and disease burden of the community.

The antibacterial activity of selected endemic plant extracts against selected isolates and standard ATCCs was conducted. One of the medicinal plants tested in our study was the leaves of *Azadiractha indica* A. Juss that has been in use by patients of lymphoedema associated with chronic wound infection in our study area (rural areas of East Wollega). As a result, our *in vitro* test revealed that it has antibacterial, anti-inflammatory and has some benefits in wound healing, which the finding of this study has a benefit for the society in using this medicinal plant.

During these three years of study, I have conscientiously strived to gain as much research experience as possible with the guide of my supervisors. I have known different *in vitro* techniques for the pre-clinical development of natural products which I have not been in touch before. I have gained experience with research work in natural products pharmacology, research project management, critical thinking, and collaboration with others. I also gained much experience of writing and editing skill and improved my language. Supportive and committed supervisors with constructive feedbacks during the journey makes me to succeed this way.

6.2. Future Prospects

As we briefly describe in this thesis, the work reported in this study focused on the identification of potential Ethiopian medicinal plants and the investigation of their pharmacological activity in the management of inflammation, microbial infection, and wound healing in *in vitro* models. These results have shown preliminary evidence on the wound healing, anti-inflammatory, and antimicrobial activities of the plant extracts.

As a result, further research will be required to isolate, characterize, and assess the pharmacological activities of the compounds in *in vitro* and in *in vivo* models to develop cheaper and less toxic topical agents for the management of lymphoedema. Second, the mechanism of action of the compounds i.e., how they affect the target receptors of

inflammation and wound healing should be investigated. After completing preclinical trials, human trials will be necessary to evaluate the therapeutic efficacy and safety of the compounds.

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Annex 2.1: List of Included Studies for *in vivo* anti-inflammatory

S/N	studies	Lab animal used	Scientific name of the plant(s) and part used	Family of the plants	Types of plants extracts/ fractions /compounds	Model used
1	Tadiwos et al, 2017	Swiss albino mice	*Jasminum abyssinicum (R)*	Oleaceae	80% methanol extract	Carrageenan induced paw oedema
2	Alemu et al, 2017	Swiss albino mice	*Leonotis ocymifolia (L)*	Lamiaceae	80% Methanol extract	Carrageenan Induced Mice Paw Oedema and Cotton Pellet Induced Granuloma
3	Adeyem et al, 2002	Wistar rats	*Persea americana (L)*	Lauraceae	Aqueous extract	Carrageenan induced rat paw oedema
4	Dilebo et al, 2010	Albino mice	*Bidens Pilosa (L), Malva verticillate (L), Syzygium guineens (L), Ferula communis (Rh), Ranunculus multifidus (Ae)*	Asteraceae,Malvaceae, Myrtaceae, Apiaceae and Ranunculaceae	80% Methanol extract	Carrageenan induced mice paw oedema
5	Ching et al, 2009	Wistar rats	*Stereospermum kunthianum (SB)*	Bignoniaceae	Aqueous extract	Carrageenan induced rat paw oedema; Carrageenan-induced leucocytes migration
6	Asres et al, 2004	Sprague-Dawley rats	*Melilotus elegans (L)*	Fabaceae	Hexane, methylene chloride, acetone, methanol, compound (azuki saponin V (1))	Carrageenan-induced rat paw oedema
7	Badilla et al, 1994	Sprague-Dawley rats (Rattus norvegicus); (Mus musculus)	*Urera baccifera (L)*	Urticaceae	Aqueous extract, hexane, ethyl acetate, and butanol fractions from the aqueous; aqueous fraction (FA)	Carrageenan induced rat paw oedema; pleurisy induced by carrageenan; Ear oedema induced by topical croton oil; Tail-flick test;
8	Mohamed et al, 2013	Sprague Dawley rats	*Curcuma longa (Rh), Cinnamomum xylanicum (B), Thyme vulgaris (Ae)*	Zingiberaceae, Lauraceae and Lamiaceae	Ethanol and petroleum ether extracts	Induction of rheumatoid arthritis in rats using adjuvant

9	Hamad et al, 2019	rats, albino mice	*Combretum aculeatum (Ae)*	Combretaceae	70% Ethanol - fractionated to petroleum ether, methylene chloride, ethyl acetate, and n-butanol	Carrageenan-induced rat hind paws oedema method
10	Hosseinzadeh et al, 2011	albino mice	*Myrtus communis Ae)*	Myrtaceae	Aqueous, & 85% ethanolic extract	Xylene-induced ear oedema method, Cotton-pellet granuloma
11	Adedapo et al, 2014	Wister strain albino rats	*Vernonia amygdalina (L)*	Asteraceae	acetone extract	Histamine induced paw oedema in rats, Carrageenan induced paw oedema in rats
12	El-Shanawany et al, 2014	albino rats	*Anisotes trisulcus (Ae)*	Acanthaceae	Methanol --then, fractionated to n-hexane, CHCl3, EtOAc, and n-BuOH fractions	Carrageenan-induced rat hind paw oedema
13	Ayal et al, 2018	Swiss albino mice	*Calpurnia aurea(L)*	Fabaceae	80% Methanol extract, then chloroform, ethyl acetate and water fractions	Carrageenan induced mice hind paw oedema
14	Sharma et al, 2011	albino Wistar rats o	*Caesalpinia pulcherrima (Ae)*	Fabaceae	90% ethanol, aqueous	Cotton Pellet Granuloma Mode
15	Wolde-Maraiam et al, 2013	Swiss Albino mice	*Dicliptera laxata (Ae)*	Acanthaceae	Aqueous extract--then fractionated to Chloroform, chloroform: Methanol (3:1), and water fractions	Carrageenaninduced mouse hind paw oedema
16	Mulisa et al, 2015	Swiss albino mice	*Rumex abyssinicus (Rh)*	Polygonaceae	80 % methanol	Carrageenaninduced mouse hind paw oedema
17	Abdissa et al, 2011	Swiss albino mice	*Dovyalis abyssinica (F)*	Salicaceae	80% methanol	Carrageenaninduced mouse hind paw oedema

18	Mequanint et al, 2010	Swiss Albino mice	Ocimum lamiifolium (L)	Labiatae	70% Ethanol and aqueous extract. Aqueous extract was further fractionated to water, petroleum ether, butanol, and chloroform	Carrageenan-induced paw oedema; Serotonin induced paw oedema; histamine induced paw oedema
19	Yonathan et al, 2006	Wistar rats	Cheilanthes farinosa Ae)	Adianthaceae	Petroleum ether, chloroform, acetone and methanol extracts. Then, MeOH extract fractionated to---F0, F1, F2, F4, F5	Carrageenan-induced rat hind paw oedema
20	Khedir et al, 2016	Wistar rats	Pistacia lentiscus (F)	Anacardiaceae	fruit oil	Carrageenan-Induced Paw oedema
21	Masresha et al, 2012	albino mice	Ocimum suave (L)	not mentioned	70% Ethanol	Carrageenan induce paw oedema; Histamine-induced paw oedema; serotonin induced paw oedema
22	Sewuye et al, 2008	Swiss Albino mice	Rosa abyssinica (R) and Salvia nilotica (L)	Rosaceae, Lamiaceae	80 % Methanol extracts; chloroform, acetone and methanol fraction.	Carrageenan-induced mouse hind paw oedema
23	Gebremeskel et al, 2018	Swiss albino mice	Aloe megalacantha (L)	Xanthorrhoeaceae	Leaf latex	Carrageenan-induced mouse hind paw oedema
24	Marzouk et al, 2009	adult Wistar rats	Citrullus colocynthis (R, S, F)	Cucurbitaceae	Aqueous extract	Carrageenan induced paw oedema,
25	Tamrat et al., 2017	Swiss albino mice	Moringa stenopetala (L)	Moringaceae	Chloroform, methanol and aqueous fractions	Carrageenan induced paw oedema

26	Vijayaraj and Kumaran, 2018	adult Wistar rats	Lawsonia inermis	Lythraceae	Ethanol extract; 8 compounds (compounds (methyl salicylate, propanoic acid, ethyl (dimethyl) silyl ester 2, 1, 3-benzothiadiazole, diethyl phthalate, ethanol, 2-bromo, dibutyl phthalate, phytol, and disooctyl phthalate)	Carrageenan induced paw oedema,
27	Manivannan et al, 2015	adult Wistar rats	Lawsonia inermis	Lythraceae	90% MeOH, aqueous, Petroleum ether, Ethanol, compounds (Apigenin, 5, 7, 4-trihydroxy-6, 3, 5-trimethoxyflavone, Pectolinarigenin, Apigenin-7-O-β-D-glucoside, Pectolinarin	Carrageenan induced paw oedema,
28	Pereira et al, 2012	Wistar rats	Azadirachta indica (L)	Meliaceae	Petroleum ether, chloroform, methanol, and water	Carrageenan-induced rat paw 0edema
29	Naik et al,2014	Albino rats	Azadirachta indica (L)	Meliaceae	seed oil	Carrageenan-induced rat paw oedema
30	Gokhale et al,2002	Swiss albino mice	Achyranthes aspera (L)	Amaranthaceae	95% Ethanol	Carrageenan-induced paw oedema, Carrageenan-induced peritonitis
31	Bhosale et al, 2018	Swiss albino mice	Achyranthes aspera (L)	Amaranthaceae	aqueous	Carrageenan induced paw oedema

Annex 2.2: List of included studies for the *in vitro* anti-inflammatory studies

S/N	Studies	Scientific name of the plant(s)/ compounds & part used	Family of the plants	Types of plants extracts/ fractions /compounds	Model used
1	Amoo et al., 2012	*Huernia hystrix (S, R, W)*	Apocynaceae	Petroleum ether (PE), dichloromethane (DCM) and 80% ethanol (EtOH)	Cyclooxygenase enzyme inhibition assays
2	Han et al, 2017	*Eugenia caryophyllata (S)*	Myrtaceae	Essential oil	Enzyme-linked immunosorbent assay (ELISA), Microarray analysis of genome-wide gene expression
3	Chen et al., 2014	*Zingiber officinale Rosc (W), Artemisia annua (W) - from Chinese traditional medicines*	Zingiberaceae, Asteraceae,	70% Ethanol	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blotting technique, Dot-blot Analysis (for TNF-ά)
4	Wei et al, 2015	*Cuminum cyminum (S)*	Umbellifers	Essential oil	Measurement of Nitrite Production.
5	Saxen et al, 2018	*Alnus nepalensis (L)*	Betulaceae	Methanol extract - fractionated to hexane, chloroform and butanol fractions	Gene Expression Quantification, Western Blot
6	Tufts et al, 2015	*Azadirachta indica (L), Amaranthus dubius (L), Brassica oleracea, Cucurbita maxima (L), Eriobotrya japonica (F), Mangifera indica (L), Ocimum americanum (F), Ocimum gratissimum (F), Psidium guajava (L), Solanum scabrum (F), Vigna unguiculata, Vitex payos (F), Zanthoxylum chalybeum (L)*	Meliaceae, Amaranthaceae, Brassicaceae, Cucurbitaceae, Rosaceae, Anacardiaceae, Lamiaceae, Myrtaceae, Solanaceae,Fabaceae, Verbenaceae, Rutaceae	80% ethanol	ELISA (TNF-ά assay)
7	Ndoye Foe et a,l 2016	*Allium sativum L. (Bu) Allium cepa L (Bu)., Drypetes gossweileri (SB), Pentadiplandra brazzeana Baill (R),*	Liliaceae, Euphorbiaceae, Capparidaceae	Essential oil	Inhibition of albumin denaturation

8	Tomy et al, 2014	*Cuminum cyminum (S)*	Apiaceae	Methanol	Lipoxygenase Inhibition Assay
9	Yu et al, 2017	*Ginsenosides*	NA	NA	Immunoblotting (Kinase assay)
10	Han et al, 2017	*Lemongrass (Cymbopogon flexuosus) (W)*	Poaceae	Essential oil	Enzyme-linked immunosorbent assay (ELISA), Microarray analysis for genome-wide gene expression
11	Chaibi et al, 2017	*Lawsonia inermis*	Lythraceae	Hexane, chloroform and methanol	Lipoxygenase Inhibition Assay
12	Bouhlali et al, 2016	*Lawsonia inermis (Leave), Rosa damascena (flower), Cuminum cyminum (seed)*	Lythraceae, Rosaceae, Apiaceae	75% Methanol	Inhibition of albumin denaturation, membrane stabilization potential
13	Annavarapu et al, 2016	*Azadirachta indica, Lawsonia inermis*	Lythraceae, Meliaceae	Ethanol extract of each plant and combination of both	Inhibition of albumin denaturation, membrane stabilization potential
14	Umar et al, 2012	*Azadirachta Indica*	Meliaceae	Petroleum ether, chloroform, and methanol	Cyclooxygenase enzyme inhibition assays
15	Schumacher et al, 2011	*Azadirachta Indica*	Meliaceae	Compounds from Neem leaf extract (quercetin, gallic acid catechin, epicatechin, nimbin, and salannin)	Luciferase assay, Electrophoretic mobility shift assay (EMSA), Western blot analysis, IKK kinase activity, nuclear fragmentation assay

Leaves = L, root = R, Stem Bark =SB, Fruits =F, Bark =B, Aerial = Ae, Flower = Fl, Stem =St, Rhizome = Rh, Bulbs = Bu, Seed = S, Berries = Be, Whole =W

Annex 2.3: List of included studies for the *in vivo* wound studies

S/N	Studies	Lab animals used	Scientific name of the plant(s) with their parts used	Family of the plants	Types of plants extracts/fractions /compounds	Model used	Outcome measured *
1	Gebnrehiwot et al., 2015	Swiss albino mice & Wistar albino rats	*Commiphoraguidottii Chiov*	Burseraceae	oleo-gum-resin	Excision wound model; Incision wound model; hydroxyproline assay	Percent wound contraction, & percent tensile strengths
2	Fikru et al., 2012	Wistar Albino rats	*Achyranthes aspera (L)*	Amarenthaceae	80% Methanol	Circular excision wound model; Linear incision wound model	Percent wound contraction, histological changes, total amount of DNA, braking strength
3	Mulisa et al., 2015	Swiss albino mice	*Rumex abyssinicus (Rh)*	Polygonaceae	% Methanol	Excision wound model; incision wound model	Wound contraction, Epithelization period, hydroxyproline content & Percent Tensile strength
4	Ayal et al., 2019	Swiss albino mice	*Calpurnia aurea (L)*	Fabaceae	% Methanol extract; chloroform, ethyl acetate and aqueous fractions	Excision wound model; incision wound model	Wound contraction, Epithelization period & Percent tensile strength
5	Fikru et al., 2016	Swis albino rats	*Croton macrostachyus (L)*	Euphorbiaceae	% Methanol	Circular excision wound model,	Wound contraction, epithelization period & percent tensile strength
6	Mekonnen et al, 2012	Swiss albino mice	*Kalanchoe petitiana (L)*	Crassulaceae	80% Methanol extract; and chloroform, methanol and distilled water fraction	Excision wound model, Incision wound model, dead space model.	Wound contraction, epithelization period & percent tensile strength
7	Gebremeskel et al, 2018	Swiss albino mice	*Aloe megalacantha (L)*	Xanthorrhoeaceae	Leaf latex	Excision wound model; Incision wound model	Wound contraction, epithelization period & percent tensile strength

8	Tsala et al, 2016	Albino-Wistar rats	*Stereospermum kunthianum (SB)*	Bignonias	Aqueous	Excisional wound model & incisional wound model	Tensile strength, % wound contraction, epithelialization time,
9	Begashew et al, 2018	Wistar albino rats	*Hibiscus micranthus (L)*	Malvaceae	% Methanol	excision wound model	Percent wound contraction
10	Dash et al, 2001	Wistar albino rats	*Lantana camara (L)*	Verbenas	Aqueous, 50% Methanol	Excision wound model	Percent wound contraction
11	Hawaze et al, 2013	Wistar albino rats	*Clematis longicauda (L), Clematis burgensis (L)*	Ranunculaceae	Methanol	Excision Wound Model and incisions wound model	Tensile strength, % wound contraction, epithelialization time,
12	Khaliq et al, 2018	Wistar albino rats	*Lawsonia Inermis (L)*	Lythraceae	Commercial Henna	Incision wound model	Tensile strength, epithelialization time,
13	Shivananda et al, 2007	Dawley Wister rats	*Lawsonia Inermis (L)*	Lythraceae	Ethanol	Incision, excision and dead space wound models	wound closure and epithelialization time, skin-breaking strength (in grams), weight of granulation tissue
14	Maan et al, 2017	Swiss Albino mic	*Azadirachta Indica (L)*	Meliaceae	Water, ethanol and ethanol-water (1:1)	Excision and incision wound model,	Tensile strength, % wound contration, epithelialization time,
15	Nagesh et al, 2015	Wister albino rats	*Azadirachta Indica (L)*	Meliaceae	Methanol	Excision and incision wound model,	Tensile strength, % wound contraction, epithelialization time,

Annex 2. 4: List of included studies for the *in vitro* wound studies

Title	Scientific name of the plant(s)/compounds	Family of the plants	Types of plants extracts/ fractions / compounds	Model used	Outcome measured at a population level or individual level
Freiesleben et al, 2017	*Aframomum melegueta (Semen), Allophylus spicatus (Radix), Annona senegalensis Pers (folium), Folium Cissus quadrangularis L, (Herba) Gymnanthemum coloratum (Folium, cum Flos Radix). Jasminum dichotomum (Folium), Leonotis nepetifolia (Herba), Melanthera scandens (Herba), Millettia thonningii (Cortex) Ocimum gratissimum L (Heba), Philenoptera cyanescens (Folium cum Fructus), Thonningia sanguinea Vahl (Herba), Trichilia monadelpha Cortex), Triumfetta rhomboidea (Radix) and Uvaria ovata Cortex & Radix) .*	Zingiberaceae, Sapindaceae, Annonaceae, Vitaceae, Asteraceae, Fabaceae, Oleaceae, Lamiaceae, Asteraceae, Fabaceae, Lamiaceae, Connaraceae, Balanophoraceae, Meliaceae, Malvaceae Annonaceae	Aqueous	Wound healing scratch assay	Cell migration and proliferation
Asamenew et al, 2011	Aloe harlana (L)	Aloaceae	Leaf latex	In vitro antioxidant assay (DPPH & 2-Deoxyribose degradation assay)	IC_{50} values

** = measured at a population level or individual level; (L) = Leaves; (B) = Bark; (R) = Root; (SB) = Stem bark; (F) = Fruits; (Ae) = Aerial; (W) = Whole plant; (C) = Clove; (Bu) = Bulbs; Fl = Flower*

Annex 2.5: List of included studies for antibacterial activities

S/N	Authors	Name of the assay (method)	Name of microorganism (references & isolates) tested against medicinal plants activity conducted	Scientific name of the plant(s)/ compounds with parts used	Family of the plants	Types of plants extracts/ fractions / compounds used for activity	Outcome measured *
1	Oumer et al, 2014	DD	*Bacillus pumillus 82,* *B. Subtilis ATCC 6633,* *S. aureus ML 267,* *E. coli (K99, K88, CD/99/1, LT37, 306, 872, 7/12, 3:37C),* *Salmonella enteric TD 01, S. typhi Ty2,* *Shigella boydii D13629,* *S. dysentery 8,* *S. flexneri Type 6,* *S. soneii 1,* *Vibrio cholerae (293, 1313 and 1315)*	*Aloe trichosantha A.Berger (L)*	Aloaceae	Leaf latex; compounds (Aloin A/B (1), Aloin-6'-O-acetae A/B (2)	ZI
2	Amoo et al, 2012	MiD bioassay	*Bacillus subtilis ATCC 6051* *E. coli ATCC 11775 and* *K. pneumoniae ATCC 13883* *S. aureus ATCC 12600;*	*Huernia hystrix (Hook.f.) N.E.Br. (W)*	Apocynaceae	Petroleum ether, dichloromethane and 80% ethanol	MIC & MBC
3	Techana et al, 2012	MiD (colorimetric) assay	*E. coli (ATCC8739, AG100A),* *E. aerogenes (ATCC13048, CM64),* *K. pneumoniae (ATCC11296, Kp55),* *Providencia stuartii (ATCC29916, NAE16)*	*Entada abyssinica Steud. ex A. Rich. (L, R),* *Entada Africana Guill. & Perr. (B),* *Carapa procera DC. (B),* *Carica papaya L. (seed),* *Persea americana Mill. (Stones)*	Fabaceae, Fabaceae, Méliaceae Caricaceae Lauraceae	CH2Cl2/MeOH (1:1) ethyl acetate, Methanol	colour changes, MIC & MBC)

4	Romha et al, 2017	AWD and AD	*S. aureus 25923,* *P. aeruginosa 27853, and* *E. coli 20922*	*Calpurnia aurea (Aiton) Benth. (Air.),* *Croton macrostachyus Hochst. ex Delile (L),* *Withania somnifera (L.) Dunal (L.),*	Fabaceae, Fabaceae, Euphorbiacea, Solanaceae, Fabaceae, & Solanaceae	Methanol (99.8%) and chloroform (95%)	ZI & Bacterial growth (MIC & MBC)
5	Taye et al, 20111	AWD & MiD	*S. aureus, (ATCC 25923),* *S. pyogens, (ATCC 19615)* *E. coli, (ATCC 25922),* *P. aeruginosa, (27853),* *Proteus vulgaris and (PROVU-01).*	*Achyranthes aspera L.(L),* *Brucea antidysenterica J.F.Mill. (R),* *Datura stramonium L.(L),* *Croton macrostachyus Hochst. ex Delile (L),* *Acokanthera schimperi (A.DC.) Schweinf. (L),* *Phytolacca dodecandra L'Hér. (R),* *Millettia ferruginea (Hochst.) Hochst. ex Baker (L), and* *Solanum incanum L. (L).*	Amaranthaceae, Simaroubaceae, Solanaceae, Euphorbiaceae, Apocynaceae, Phytolaccaceae, Fabaceae & Solanaceae	Methanol & aqueous	ZI and bacterial growth (MIC & MBC)
6	Belal et al, 2017	Agar DD	*E. coli,* *Salmonella Typhi,* *Proteus vulgaris,* *Klebsiella pneumonae,* *Enterococcus faecalis and Staphylococcus aureus.*	*Cumin cyminum L. (S)*	Apiaceae	Essential oil	ZI
7	Baynesagne et al, 2017	MAD	*E. coli (ATCC 25922),* *S. aureus (ATCC 25923),* *Streptococcus pneumoniae (ATCC 63),* *E. coli (isolate),* *Klebsiella pneumoniae (isolate) and* *S. pneumoniae (isolate)*	*Datura stramonium L. (L)*	Solanaceae	Ethanol, methanol, acetone, chloroform and distilled water	Growth of microorganisms (MIC & MBC)

8	Vazirian et al, 2016	MiD & DD	*S. aureus ATCC 29213,* *E. coli ATCC 25922,* *Pseudomonas aeruginosa ATCC 27853, Enterococcus faecalis ATCC 29212,* *Salmonella typhimurium (Isolate) and* *E. coli (Isolate)*	*Trachyspermum ammi (L.) Sprague (S)*	Apiaceae	Essential oil	ZI & bacterial growth (MIC & MBC)
9	Yeabyo et al, 2018	AWD Assay	*E. coli (ATCC 25922),* *Enterobacter aerogenes (ATCC 13048),* *Klebsiella pneumoniae, (ATCC 700603),* *Vibrio cholera. (ATCC 39315),* *Bacillus subtilis (ATCC 3915),* *Enterococcus faecalis (ATCC 29212)*	*Verbascum sinaiticum Benth. (R)*	Scrophulariaceae	Diethyl ether, chloroform, acetone, and ethanol	ZI
10	Begashawu et al, 2016	AWD & BMD assay	*S. aureus DSM 7246, Salmonella typhimurium ATCC 13311, Aeruginosa DSM 1117 and E. coli ATCC 25722*	*Kosteletzkya begonifolia (Ulbr.) Ulbr (L)* *Leucas martinicensis (Jacq.) R.Br.(L) and* *Ranunculus multifidusForssk. (L)*	Ranunculaceae, Lamiaceae & Malvaceae respectively	Petroleum ether, Chloroform, methanol for all the three plants	ZI and Bacterial growth (MIC & MBC))
11	Burt et al, 2003	DD assay, MiD (Colorimetric)	*E. coli O157:H7*	*Pimenta racemose (Mill.) J.W.Moore (L),* *Eugenia caryophyllata,Thunberg (L)* *Origanum vulgare L. (L) and* *Thymus vulgaris L. (L)*	Myrtaceae, Myrtaceae, Lamiaceae & Respectively	Essential oil	ZI & bacterial growth (MIC & MBC)

12	Chaieb et al, 2011	MID & Crystal Violet assay	*E. coli ATCC 35218, Salmonella enterica serovar Typhimurium ATCC 14028, P. aeruginosa ATCC 27853, Vibrio alginolyticus ATCC 33787, Vibrio parahaemolyticus ATCC 17802, Bacillus cereus ATCC 14579, Listeria monocytogene ATCC 19115, Enterococcus faecalis ATCC 29212, Micrococcus luteus NCIMB 8166, S. aureus ATCC 25923 & S. epidermidis CIP 106510*	*Thymoquinone (compound) obtained from Nigella sativa L. (S)*	Ranunculaceae	Compound		Bacterial growth & Colour changes (MIC & MBC)
13	Bisht et al, 2014	AWD test	*S. aureus (MRSA and MSSA), Enterococcus spp. and Streptococcus spp*	*Withania somnifera (L.) Dunal (L)*	Solanaceae	Methanol	ZI	
14	Djeussi et al, 2016	MiD (colorimetric)	*E. coli (ATCC8739, ATCC10536, AG100, AG100A, AG100ATet, AG102, MC4100 W3110), Enterobacter aerogenes (ATCC13048, CM64, EA27, EA289, EA298, EA294), Klebsiella pneumoniae (ATCC11296, KP55, KP63, K24, K2), Enterobacter cloacae (ECCI69, BM47, BM67), P. aeruginosa (PA01, PA124) and Providencia stuartii (ATCC29916, NEA16, PS2636, PS299645)*	*Anthocleista schweinfurthii Gilg (B, F &L), Nauclea latifolia Sm (B, F & L), Boehmeria platyphylla Buch. -Ham. ex D. Don (W), Caucalis melanantha (Steud. ex Hochst.) Benth. & Hook. ex Hiern (W), Erigeron floribundus (Kunth) Sch.Bip. (W) and Zehneria scobra (L.f.) Sond. (W)*	Loganiaceae, Gentianaceae, Apiaceae	Maceration		Bacterial growth (colour changes, MIC and MBC)

15	Asres et al, 2006	MAD & DD	*E. coli (K99, K88, 306, LT37, 872, ROW 7/12, 3:37C, CD/99/1),* *Salmonella typhi Ty2,* *Shigella dysentery 1, Dysentery 8, S. soneii 1, S. boydii D13629 and S. flexneri Type 6,* *Vibrio cholerae 1313, 293, 1315 and 85,* *S. aureus ML 267,* *Bacillus pumilus 82 and* *B. subtilis ATCC 6633*	*Combretum molle R.Br. ex G.Don (B)*	Combretaceae	Petroleum ether, dichloromethane, acetone and methanol	MIC & MBC and ZI
16	Sileshi et al, 2008	AD & DD	*S. aureus (Isolate & ATCC 25923), Streptococcus pyogenes (isolate),* *E. coli (ATCC 2590),* *P. aeruginosa (isolate & ATCC 27853)*	*Clerodendrum myricoides (Hochst.) Steane & Mabb. (L),* *Ficus plamata Forssk. (L),* *Grewia ferruginea Hochst. ex A. Rich. (L)* *Periploca linerifolia Quart. -Dill. & A.Rich. (aerial)*	Lamiaceae, Moraceae, Tiliaceae, Asclepediaceae	80% Methanol extract, petroleum ether, chloroform, acetone and methanol fractions	ZI
17	Habtamu et al, 2018	Agar DD	*E. coli,* *K. pneumoniae,* *Proteus mirabili,* *S. aureus and bacillus*	*Vernonia amygdalina (Delile) Sch.Bip. (F)*	Asteraceae	Hexane, chloroform, and acetone	ZI
18	Adedapo et al, 2008	MD & DD	*Bacillus cereus,* *S. epidermidis,* *S. aureus,* *Micrococcus kristinae, and* *Streptococcus pyogens,* *E. coli,* *Salmonella pooni,* *Serratia marcescens,* *P. aeruginosa, and* *K. pneumoniae*	*Calpurnia aurea (Aiton) Benth. (S, L)*	Legumes	methanol	ZI & bacterial growth (MIC & MBC)

19	Habtamu et al, 2017	AD & Agar DD	*S. aureus,* *E. coli,* *P. aeruginosa,* *S. boydii and* *S. typhi*	*Clematis hirsuta Guill. &* *Perr. (L)*	Ranunculaceae	80% methanol and chloroform	ZI and (MIC &MBC)
20	Dua et al, 2013	AD and Agar DD	*E. coli (MTCC96),* *P. aeruginosa (MTCC741),* *S. aureus (MTCC96),* *Bacillus Pumilus (MTCC7411),*	*Cuminum Cyminum L. (S)*	Umbellifers	80% methanol	ZI and (MIC & MBC)
21	Umer et al, 2013	AWD	*Salmonella spp. (Typhi, Paratyphi,* *Typhimurium)* *Shigella species,* *P. aeruginosa,* *S. aureus and* *E. coli*	*Calpurnea aurea (Aiton)* *Benth. (L)*	Fabaceae	80% methanol	ZI
22	Vijayasanthi et al, 2014	DD & MAD	*Bacillus cereus MTCC 442,* *E. coli MTCC 598,* *K. pneumoniae MTCC 7407,* *P. aeruginosa MTCC 42642* *Proteus vulgaris MTCC 742* *S. aureus MTCC 3160,* *Salmonella typhi MTCC 3917,* *Shigella flexneri MTCC 1457* *Streptococcus pneumonia MTCC* *655,*	*Delonix elata (L.) Gamble* *(L)* *Spathodea campanulata* *P.Beauv. (L)*	Fabaceae Bignoniaceae	Aqueous, Methanol extracts	ZI and (MIC)

23	Tadeg et al, 2005	AWD & MiD	*S. aureus (ATCC 6538),* *E. coli (ATCC 25922) &* *P. aeruginosa (ATCC 27853)*	*Acokanthera schimperi (A.DC.) Schweinf. (L),* *Calpurnia aurea (Aiton) Benth. (L),* *Kalanchoe petitiana A. Rich. (L),* *Lippia adoensis Hochst (L),* *Olinia rochetiana A.Juss (L),* *Verbascum erianthum Benth (L),* *Phytolacca dodecandra L'Hér. (F) &* *Malva parviflora L. (R)*	Apocynaceae, Leguminosae, Crassulaceae, Verbenaceae, Malvacea, Oliniacea, Phytolaccaceae, Scrophulariaceae	80% methanol extract; petroleum ether, chloroform, acetone and methanol fractions	ZI and (MIC & MBC)
24	Mwitari et al, 2013	DD & AD	*S. aureus ATCC 25923,* *clinical isolate MRSA,* *E. coli ATCC 25922 and* *P. aeruginosa ATCC 27853.*	*Withania somnifera (L.) Dunal (Ar),* *Prunus Africana (Hook.f.) Kalkman (SB),* *Warbugia ugandensis Sprague (SB) and* *Plectranthus barbatus Andrews (SB)*	Solanaceae	Dichloromethane, ethyl acetate, methanol	ZI, MIC & MBC
25	Awino et al, 2007	DD	*Salmonella species,* *Proteus spp.,* *P. aeruginosa,* *K. pneumoniae,* *E. coli,* *Cryptococcus neoformans,* *Shigella dysentiae and* *S. aureus*	*Embelia schimperi Vatke (SB)*	Myrsinaceae	Ethyl acetate a compound	ZI, (MIC)

26	Seshathri et al, 2011	AWD	*Streptococcus pyogenes (ATCC 19615)*	*Clausenia anisate (Willd.) Hook.f. ex Benth. (S), Clematis simensis Fresen. (S), Cleodendrum myricoides (Hochst.) Steane & Mabb. (S), Juniperus procera Hochst. ex Endl. (S), Justicia schimperiana (Hochst. ex Nees) T. Anderson (S), Olea europea L. (S), Phoenix reclinata Jacq. (Petiole), Rubus apitalus Poir. (S), Sesbania sesban (L.) Merr. (S), Sida rhombifolia L. (S), Spilanthes mauritiana Delile (F), Stereospermum kunthianum cham.(S) Vernonia amygdalina (Delile) Sch.Bip. (L)*	Rutaceae, Ranunculaceae, Verbanaceae, Cuprassaceae, Acanthaceae, Oleacea, Arecaceae, Rosaceae, Fabaceae, Malvaceae, Compositae, Bignoniaceae & Asteraceae	Ethanol, aqueous	ZI
27	Obey et al, 2016	AWD	*E. coli ATCC 25922, Enterobacter, K. pneumoniae ATCC 1583380, P. aerogenes MTCC 2990 Salmonella typhi ATCC 2202,*	*Croton macrostachyus Hochst. ex Delile (SB)*	Euphorbiaceae	Methanol, ethyl acetate, n-butanol	ZI
28	Ewansiha et al, 2012	DD & MaD	*S. aureus, E. coli, Salmonella typhi*	*Cymhopogon Citratus (DC.) Stapf (L)*	Poaceae	Hexane, Chloroform and Methanol	ZI, MIC
29	Ameya et al, 2016	DD assay and AD	*E. coli (ATCC-25922) E. faecalis (ATCC-29212) S. aureus (ATCC-25923),*	*Echinops kebericho Mesfin (R)*	Asteraceae	Ethanol. methanol. aqueous	ZI, MIC
30	Singh et al, 2011	AD and DD	*A total of 1093 bacterial strains of 26 genera*	*Cymhopogon Citratus (DC.) Stapf (L)*	Poaceae	essential oil	ZI, MIC

31	Duraipandiyan et al, 2012	MiD and DD assay	*Bacillus subtilis MTCC 441, E. coli ATCC 25922, Enterococcus faecalis ATCC 29212, Erwinia sp. MTCC 2760 K. pneumoniae ATCC 15380, P. aeruginosa ATCC 27853 and Proteus vulgaris MTCC 1771, S. aureus ATCC 25923, S. epidermidis MTCC 3615,*	*Costus speciosus (J. Koenig) S.R.Dutta (Rh)*	Costaceae	Hexane, chloroform, ethyl acetate, methanol and water	ZI, MIC
32	Ameya et al, 2015	DD, AD	*S. aureus (ATCC-25923), E. faecalis (ATCC-29212) and E. coli (ATCC-25922)*	*Taverniera abyssinica A. Rich. (R)*	Fabaceae	Ethanol, methanol and distilled water	ZI, MIC
33	Hassanshahian et al, 2014	Agar DD and MiD	*S. aureus, E. coli K. pneumoniae*	*Trachyspermum ammi (L.) Sprague (S)*	Umbellifers	Essential oil	ZI, MIC

34	Lulekal et al, 2014	MiD (MIC)	*Bacillus cereus ATCC 11778*, *Bacteroides fragilis ATCC 25285*, *Candida albicans ATCC 10231*, *Clostridium perfringens DSM 11778*, *E. coli ATCC 25922*, *E. faecalis ATCC 29212*, *L. monocytogenes ATCC 7644*, *P. aeruginosa ATCC 27853*, *S. aureus ATCC 29213*, *S. epidermidis ATCC 12228*, *S. enteritidis ATCC 13076, and Streptococcus pyogenes ATCC 19615*	*Bersama abyssinica Fresen. (L)*, *Calpurnia aurea (Lam.) Benth (R)*, *Carissa spinarum L. (R)*, *Clematis hirsuta Guill. & Perr. (L)*, *Clutia abyssinica Jaub. & Spach (R)*, *Croton macrostachyus Hochst. ex Delile (L)*, *Cyathula cylindrica Moq. (R)*, *Dodonaea angustifolia (L.f.) J.G.West (L)*, *Embelia schimperi Vatek (S)*, *Jasminum abyssinicum Hochst. ex DC. (L)*, *Maesa lanceolata Forssk (L)*, *Ocimum lamiifolium Hochst. ex Benth. (L)*, *Olinia rochetiana A.Juss. (L)*, *Rubus steudneri Schwein (R)*, *Rumex nepalensis Spreng (R)*, *Thalictrum rhynchocarpum Quart-Dill. & A.Rich. (R)*, *Verbascum sinaiticum Benth. (L)*, *Vernonia amygdalina (Delile) Sch.Bip. (F)*	Melianthaceae, Fabaceae, Apocynaceae, Ranunculaceae, Euphorbiaceae, Amaranthaceae, Sapindaceae, Myrsinaceae, Oleaceae, Lamiaceae, Oliniaceae, Rosaceae, Polygonaceae, Ranunculaceae, Scrophulariaceae, Asteraceae	Methanol	ZI, MIC

35	Bacha et al, 2016	MiD & AWD	*E. coli K12, DSM 498,* *P. aeruginosa DSM 1117,* *S. aureus DSM 346,* *Bacillus cereus ATCC 10987,* *B. cereus (isolate)*	*Aframomum corrorima (A.Braun) P.C.M.Jansen (F),* *Albiza schimperiana Oliv. (R),* *Curcuma longa L. (Rh),* *Erythrinia brucei Schweinf. emend. Gillett (SB),* *Justica schimperiana (Hochst. ex Nees) T. Anderson (S),* *Nigella sativa L. (S),* *Ocimum sauve Wild. (L) &* *Vernonia amygdalina (Delile) Sch.Bip. (L),*	Zingiberaceae, Leguminosae, Zingiberaceae, Leguminosae, Acanthaceae, Ranunculaceae Lamiaceae	Petroleum ether, chloroform, methanol, water and oilo resins	ZI, MIC
36	Njeru et al, 2015	Agar DD & MiD	*S. aureus (ATTC 25923),* *MRSA strain (isolate);* *E. coli (ATTC 25922),* *K. pneumoniae (isolate),* *P. aeruginosa (ATCC 27853), Salmonella typhi (isolate),* *Shigella sonnei (isolate)*	*Premna resinosa (Hochst.) Schauer (R)*	Verbenaceae	Aqueous, methanol; petroleum ether. Then Petroleum ether was fractionated to --ethyl acetate, chloroform, methanol	ZI, MIC
37	Ngeny et al, 2013	Agar diffusion & MiD	*P. aeruginosa ATCC 27853,* *S. aureus ATCC 25923,* *E. coli ATCC 25922,* *K. pneumoniae (isolate),* *MRSA (isolate).*	*Hagenia abyssinica (Bruce) J.F.Gmel. (L & SB),* *Fuerstia africana T.C.E.Fr. (Ae),* *Ekebergia capensis Sparrm (R)* *Asparagus racemosus Wild. (SB)*	Rosaceae, Lamiaceae, Asparagaceae Meliaceae	Hexane, dichloromethane, methanol, aqueous	ZI, MIC

38	Hussien et al, 2011	AWD method	*S. aureus (ATCC 25923),* *E. coli (ATCC 25922),* *S. typhi (ATCC 83859),* *S. aeruginosa (ATCC 27853)*	*Brassica Nigra L. (S),* *Thymus shimperi Ronniger (L),* *Ocimum basilicum L. (L),* *Syzygum aromaticum (L.) Merr. & L.M.Perry (F),* *Electtaria Caradamom (L.) Maton (F) &* *Cinnamon Zeylanicum J. Presl (SB)*	Brassicaceae, Lamiaceae, Lamiaceae, Myrtaceae, Zingiberaceae & Lauraceae respectively	Hydrosol prepared from plurized plant parts	ZI, MIC
39	Debalke et al, 2018	AWD & MiD	*E. coli (ATCC-27853),* *S. typhi (ATCC -13062),* *S. aureus (ATCC- 2529),* *K. pneumonia and Citrobacter.*	*Sida rhombifolia L. (W)*	Malvaceae	80% Methanol	ZI, MIC
40	Unnithan et al, 2013	AWD	*E. coli (isolate) and* *S. aureus (isolates)*	*Ocimum basilicum L. (Ae)*	Lamiaceae	Essential oil	ZI
41	Meshesha et al, 2017	AWD	*S. aureus (ATCC 25923),* *Enterococcus faecalis (ATCC 29212),* *E. coli (ATCC 25922) and* *P. aeruginosa (ATCC 27853)*	*Kniphofia Moench (L.) Oken (L)*	Asphodelacea	Chloroform/ methanol (1:1), Ethyl acetate/water in (9:1), Ethyl acetate; Compounds: Chrysophanol, 3,5,8-trihydroxy-2methylnaphthalen-1,4-dione, asphodeline, 10-hydroxy-10,7□(chrysophanolanthrone) chrysophanol	ZI
42	Gadisa et al, 2019	MiD	*E. coli (ATCC25922),* *K. pneumoniae (ATCC700603)* *S. aureus (ATCC25923),*	*Blepharis cuspidata Lindau (L), Boswellia ogadensis Vollesen (L) and Thymus schimper Ronniger (L)*	Acanthaceae, Burseraceae and Lamiaceae	Essential oil	MIC and MBC

43	Belay et al, 2011	MaD	*Bacillus cereus, Citrobacter spp, E. coli (ATCC 25922), K. pneumonia and Listeria monocytogenes, P. aeruginosa (ATCC 27853), Proteus mirabilis. S. aureus (ATCC 25923), Salmonella paratyphi, Shigella dysenteriae, Streptococcus pyogenes,*	*Artemisia abyssinthium L. absinthium (L), Artemisia abyssinica (L), Croton macrostachyus Hochst. ex Delile (L), Echnops kebericho Mesfin (Tu) and Satureja puncatat (Benth.) R.Br. ex Briq. (Ber)*	Asteraceae, Euphorbiaceae, Asteraceae & Lamiaceae respectively	Essential oil	MIC & MBC
44	Mulat et al, 2015	AWD & MaD	*E. coli (ATCC25722), S. aureus (ATCC25903), Shigella sonei (ATCC259131) and Salmonella typhimurium (ATCC13311)*	*Ocimum sauve Willd (L), Ruta graveolens L. (L), Ocimum lamiifolium Hochst. ex Benth. (L), Nigella sativa L. (S) & (L).*	Lamiaceae, Rutaceae, Lamiaceae, Ranunculaceae & Asteraceae	Petroleum ether, Chloroform and Methanol, essential oil	ZI, MIC & MBC
45	Chalo et al, 2015	AWD, MD & DD	*P. aeruginosa, E. coli, methicillin resistant S. aureus (MRSA), Bacillus cereus*	*Schrebera alata (Hochst.) Welw. (B), Ormocarpum kirkii S.Moore (Ae), Cussonia holstii Harms ex Engl. (B) & Helichrysum forskahlii (J.F.Gmel.) Hilliard & B.L.Burtt (W)*	Oleaceae, Fabaceae, Araliaceae & Asteraceae respectively	Dichloromethane, Methanol, Aqueous	ZI, MIC
46	Habtamu et al, 2017	AD & DD assay	*S. aureus, Salmonella typhi, E. coli, P. aeruginosa and Shigella boydii*	*Achyranthes aspera L. (L)*	Amaranthaceae	Chloroform, Methanol	ZI, MIC
47	Genanew et al, 2017	AWD & MaD	*S. aureus (ATCC 25923), Streptococcus pneumonia (ATCC 49619), and Streptococcus pyogenes (ATCC 19615), E. coli (ATCC 25922), P. aeruginosa (ATCC 2706), K. pneumonia (ATCC 700603) and Salmonella typhi (ATCC 1912/R)*	*Aloe macrocarpa Tod (L).*	Aloaceae	Latex and gel	ZI, MIC

48	Hagos et al, 2017	AWD	*E. coli (MTCC 40), P. aeruginosa (MTCC 424), P. vulgaris (MTCC 742), S. aureus (MTCC 87) S. faecalis (MTCC5383)*	*Moringa stenopetala (Baker f.) Cufod. (L)*	Moringaceae	Methanol and Aqueous	ZI	
49	Ameya et al, 2018	MaD &, AWD	*E. coli, Klebsiella species and S. aureus*	*Nicotiana tabacum L. (L)*	Euphorbiaceae	Petroleum ether, chloroform, diethyl ether, ethyl acetate, acetone, dichloromethane and methanol	ZI, Mic and MBC	
50	Abew et al, 2014	AWD & MaD	*E. coli (Isolate and ATCC 25922) S. aureus (MRSA and ATCC 2923),*	*Zehneria scabra (L.f.) Sond. (L) & Ricinus communis L. (L)*	Curbitaceae, Euphorbiaceae	Benzen (1), chloroform/ acetone with ratio 1:1 (2); 70% alcohol (3) and distilled water (4)	ZI, MIC and MBC	
51	Mummed et al, 2018	AWD & MaD	*S. aureus ATCC) 25923, P. aeruginosa ATCC 27853, E. coli ATCC 25922 and K. pneumoniae ATCC 700603*	*Cissus quadrangularis L. (Ae), Commelina benghalensis L. (L), Euphorbia heterophylla L.(R), Euphorbia prostrate Aiton (W), Grewia villosa Willd. (L), Momordica schimperiana Naudin (F), Trianthema portulacastrum L. (ae), Schinus molle L. (L), and Solanum incanum L. (F).*	Vitaceae, Commelinaceae, Euphorbiaceae, Euphorbiaceae, Malvaceae, Cucurbitaceae, Aizoaceae, Anacardiaceae & Solanaceae respectively	Methanol extract	ZI, MIC & MBC	
52	Minale et al, 2014	AWD & MaD	*Bacillus pumillus 82, B. subtilis ATCC 6633 and S. aureus ML 267, E. coli (K99, K88, CD/99/1, LT37, 306, 872, ROW 7/12, 3:37C), Salmonella enterica TD 01, S. typhi Ty2, Shigella boydii D13629, S. dysentery 8, S. flexneri Type 6, S. sonеii 1, Vibrio cholerae (85, 293, 1313 and 1315)*	*Aloe sinana Reynolds (L)*	Asphodelaceae	Leave latex … compound (anthrones)	ZI, MIC & MBC	

53	Moglad et al, 2014	AWD & MaD	*Bacillus subtilus (NCTC 8236),* *E. coli ATCC 25922,* *S. aureus (ATCC 25923),* *Salmonella typhi NCTC 0650)*	*Maerua oblongifolia (Forssk.) A.Rich. (L & St)*	Capparaceae	Chloroform, Methanol,	ZI, MIC & MBC
54	Hawaze et al, 2012	Paper DD & MaD	*S. aureus ATCC 25923,* *P. aeruginosa ATCC 27853*	*Clematis longicauda Steud. ex A.Rich.* *(L) Clematis burgensis Guill. & Perr. (L)*	Ranunculaceae	Petroleum ether, Methanol	ZI, MIC & MBC
55	Regassa et al, 2012	DD & MaD	*S. aureus and* *S. agalactiae*	*Combretum molle R.Br. ex G.Don (L, B, St, S)*	Combretaceae	90% Ethanol	ZI, MIC & MBC
56	Zulfa et al, 2015	MiD & Paper DD assay	*Bacillus cereus ATCC 10987, E. coli O157:H7 ATCC 25922, K. pneumoniae ATCC 15692, Candida albicans ATCC 10231*	*Cymbopogon citratus (DC.) Stapf (L)*	Poaceae	100% (v/v) Methanol	ZI, MIC & MBC
57	Ameya et al, 2018	AWD & BMiD	*S. aureus (MRSA and ATCC® 25923™),* *Salmonella enterica subsp. enterica (ATCC® 13311™) and* *K. pneumoniae (ATCC® 700603™),* *P. aeruginosa and* *E. coli*	*Capsicum frutescens L. (F)*	Solanaceae	Distilled water, Acetone, Ethanol, Methanol, Ethyl acetate, and Chloroform	ZI, MIC & MBC

58	Teka et al, 2015	BMiD	*E. coli ATCC 25922,* *E. faecalis ATCC 29212,* *P. aeruginosa ATCC 27853,* *S. aureus (ATCC 25923, ATCC 29213, ATCC 33591, ATCC 33592, ATCC 43300, ATCC BAA 976), and* *S. epidermidis ATCC 12228.* *clinical isolates of antibiotic-sensitive S. aureus strains (SA1, SA2, SA3, SA4, SA5, SA6, SA7, SA8, SA9, SA10)*	*Apodytes dimidiata E. Mey. ex Arn. (SB);* *Asparagus africanus Lam. (L), Bersama abyssinica Fresen (S),* *Cucumis ficifolius A.Rich. (R), Gladiolous abyssinicus(Brongn. ex Lem.) N.E.Br.* *(Bu) Guizotia schimperi Sch.Bip. (L),* *Lippia adoensis Hochst (L),* *Olinia rochetiana A. Juss. (SB), Pavonia urens Cav. (L),* *Premna schimperi Engl. (L),* *Pittosporum viridiflorum Sims. (L),* *Polygala sadebeckiana Hoffmanns. & Link (R),* *Sida rhombifolia L.(R),* *Solanum incanum L. (F)*	Icacinaceae, Asparagaceae, Melianthaceae, Cucurbitaceae, Iridaceae, Asteraceae, Verbenaceae, Oliniacea, Malvacea, Lamiaceae, Pittosporaceae, Polygalaceae, Malvaceae & Solanaceae respectively	80 % Ethanol	MIC & MBC
59	Asamenew et al, 2011	DD & MiD	*Bacillus subtilis (ATCC 6633),* *B. pumillus,* *E. coli (K99, K88, CD/99/1, LT37, 306, 872, ROW 7/12, 3:37C),* *S. aureus (ML 267, NCTC 11994),* *Salmonella typhi (Ty2),* *S. typhimurium (ATCC 1255),* *Shigella boydii (D3629), S. flexneri, (Type 6), S. sonei (1), S. dysentery (1), S. dysentery,* *Vibrio cholerae (85, 293, (1313, 1315).*	*Aloe harlana Reynolds (L)*	Asphodelaceae	Latexa 7-OMA & Aloin	ZI, MIC& MBC

60	Kalayou et al, 2012	DD & MiD	*E coli,* *K. pneumonae* *S. aureus, S. intermedius, S. hicus,*	*Achyranthes aspera L.(L), Ficus caria L. (L),* *Malvi parviflora L. (L), Vernonia species (Willd.) Drake (L),* *Solanum hastifolium Hochst. ex Dunal (L), Calpurinia aurea (Aiton) Benth. (L), Nicotiana tabacum L. (L), Ziziphus spina-christi (L.) Desf. (L, S), Croton macrostachys Hochst. ex Delile (L)*	Asparagaceae, Moraceae, Malvaceae, Asteraceae, Solanaceae, Fabaceae, Solanaceae, Rhamnaceae & Euphorbiaceae respectively	70% Methanol	ZI, MIC & MBC
61	Begashaw et al, 2017	AWD & MiD	*E. coli (ATCC1925525),* *K. pneumoniae (ATCC70060),* *P. aeruginosa (ATCC27853),* *P. mirabilis (ATCC12386)* *S. aureus (ATCC2923),* *S. pneumoniae (ATCC137348),* *S. pyogenes (ATCC19615),*	*Hibiscus micranthus L. (L)*	Malvaceae	80% Methanol	ZI, MIC & MBC
62	Bekele et al, 2015	AWD & MiD	*E. coli (ATCC 25922),* *K. pneumoniae (isolate),* *S. aureus (MRSA and ATCC 25923),* *Shigella flexneri (ATCC 12022),* *S. pneumoniae (ATCC 63 and isolate)*	*Thymus schimperi Ronniger* *(L)*	Lamiaceae	Chloroform, ethanol, methanol, and aqueous	ZI & MIC & MBC
63	Palla et al, 2015	AWD	*Bacillus cereus,* *E. coli 018: K1:H7, strain RS218,* *Enterococcus faecalis (VRE), and* *P. aeruginosa.* *S. aureus (MRSA),* *Salmonella typhi (ATCC 14028)*	*Linum usitatissimum L. (S)*	Linaceae	70% Methanol	ZI
64	Albejo et al, 2015	MiD	*S. aureus*	*Vernonia auriculifera (Hiern) Isawumi (L)*	Compositae	N-hexane, chloroform, methanol, and water	MIC & MBC

65	Abdissa et al, 2015	Paper DD	*E. coli*, *Bacillus subtills* *Salmonella typhi*, *S. aureus*	*Aloe pulcherrima M.G.Gilbert & Sebsebe (L)*	Asphodelaceae	N-hexane, chloroform, acetone and methanol; three compounds)	ZI
66	Girmay et a, 2017	DD	*B. subtilis (ATCC 6633)* *E. coli (ATCC 35218)*, *P. aeruginosa (ATCC 27853) and S. aureus (ATCC 25923)*,	*Lepidium Sativium L. (S)*	Cruciferae	N-hexane, chloroform/ methanol (1:1	ZI
67	Seid, 2016	Paper DD	*E. coli* *S. aureus* *Shigella flexneri* *Streptococcus pyrogenes*,	*Foeniculum vulgare Mill. (L)*	Umbelliferae	Petroleum ether, Chloroform, Chloroform-methanol (1:1), Methanol	ZI
68	Teshome et al, 2018	AWD	*E. coli ATC25922*, *P. aeruginosa ATCC27853* *S. aureus ATCC 25923*, *S. typhi NTCC83859*,	*Clematis simensis Fresen (L)*	Ranunculaceae	Petroleum ether (60-80°C), acetone and methanol	ZI
69	Girmay et al, 2015	Paper DD	*Bacillus subtilis*, *E. coli and* *Salmonella typhi* *S. aureus*	*Datura stramonium L. (L)*	Euphorbiaceae	Chloroform, ethanol, hexane, petroleum ether, and acetone	ZI
70	Nyanchoka, 2016	Paper DD	*Bacillus subtilis* *diarrheagenic E. coli* *K. pneumoniae*, *Penicillium notatum*, *P. aeruginosa* *Salmonella typhi*, *Shigella dysentriae*, *Vibrio cholorae*,	*Bersama abyssinica Fresen. (SB)*	Melanthiaceae	N-hexane, Dichloromethane, ethyl acetate, methanol	ZI
71	Goji et al, 2006	Hole-plate assay	*E. coli (ATCC 25922)*, *P aeruginosa (Isolate, ATCC 27853)*, *S. aureus (Isolate, ATCC 25923)*, *Streptococcus pyogenes (isolate)*	*Jasminum abyssinicum Hochst. ex DC. (L)*, *Solanecio gigas (Vatke) C. Jeffrey (L)* *Lagenaria siceraria (Molina) Standl. (L, S &F)*	Oleaceae, Asteraceae, Cucurbitaceae		ZI

72	Megeressa et al, 2015	DD & MiD	*Bacillus pumilus 82,* *B. subtilis ATCC 6633,* *S. aureus ML 267,* *E. coli (3:37C, 7360, 872, CD/99/1, K 88, T37, ROW 7/12, 5933),* *Salmonella enterica TD 01, S. typhi Ty2, Shigella boydii D13629, S. dysentery 8, S. flexneri Type 6, S. soneii 1,* *Vibrio cholerae (NCTC 5596, NCTC 10732, NCTC 11501, & NCTC 4693*	*Aloe trigonantha L.C.Leach* *(L)*	Aloaceae	Aloesin (1), 8-O-Methyl-7-hydroxyaloin A/B (2), Aloin A/B (3); Aloin-6'-O-acetate A/B (4);	ZI and MIC
73	Hussien et al, 2010	AWD	*S. aureus ATCC 25923,* *E. coli ATCC 25922,* *P. aeruginosa ATCC 27853*	*Pycnostachys abyssinica Fresen (L, S, R)* *Pycnostachys eminii Gürke (L, S, R)*	Laminaceae	Essential oils; petroleum ether chloroform and methanol	ZI
74	Delelegn et al, 2018	AWD & AD	*E. coli (ATCC 2592, isolate),* *Salmonella typhi (isolate) and* *Shigella dysenteriae (isolate)*	*Moringa oleifera Lam. (S)*	Moringaceae	Methanol, acetone and aqueous	ZI, MIC & MBC
75	Habbal et al, 2011	AWD	*Aeromonas hydrophila,* *Bacillus species,* *Bacteriodes fragilis,* *Citrobacter frewndii,* *Clostridium perfringens* *Corynebacterium diphtheriae,* *Cryptococcus neoformans,* *E. coli,* *H. influenzae,* *K. pneumoniae,* *Micrococcus species,* *N. meningitides,* *P. aeruginosa,* *S. aureus, S. epidermidis, MRSA,* *Salmonella species,* *Shigella sonnei,* *Streptococcus pneumoniae,* *Streptococcus pyogenes,* *Vibrio cholerae,*	*Lawsonia inermis L. (L)*	Lythraceae	Methanol, ethanol and aqueous extracts	ZI

76	Nagarajan et al, 2013	AWD	*"Bacillus subtitles, S. aureus, and Escherichia*	*Lawsonia inermis L. (L)*	Lythraceae	Ethanol, chloroform, hexane and methane	ZI
77	Maleki et al, 2018	AWD and MTT MiD	*S. aureus ATCC 6538,* *E. faecalis ATCC 1394,* *P. aeruginosa ATCC 9027,* *E. coli, ATCC 25922*	*Azadiractha Indica A. Juss A.Juss. (L)*	Meliaceae	Ethanol, methanol, and ethyl acetate	ZI, MIC
78	Mohammed et al, 2018	ADD	*E. faecalis* *E. coli (21),* *K. pneumoniae (21),* *P. mirabilis (21),* *P. aeruginosa (12),* *S. aureus (17)*	*Azadiractha Indica A. Juss A.Juss. (L)*	Meliaceae	80 % Ethanol	ZI
79	Reddy et al, 2013	AWD and MiD	*E. feacalis,* *P. mirabilis* *P. aeruginosa* *S. aureus*	*Azadiractha Indica A. Juss A.Juss. (L, B, F)*	Meliaceae	Aqueous	ZI

** = measured at a population level or individual level; AD = Agar dilution; ADD = Agar disc diffusion; AWD = Agar well diffusion; BM = Broth microdilution; DD = Disc diffusion; MaD = Macrodilution; MIC = Minimum inhibitory concentrations; MiD = Microdilution; ZI = Zone of inhibition of bacterial growth; (L) = Leaves; (B) = Bark; (R) = Root; (SB)= Stem bark; (F) = Fruits; (Ae) = Aerial; (W) = Whole plant; (C) = Clove; (Bu) = Bulbs; (Ber) = berries; (Tu) = tuber; Fl = Flower; MRSA = Methicillin resistant S. aureus; VRE = Vancomycin resistant enterococcus.*

Annex 2.6: List of included studies for anti-fungal activities

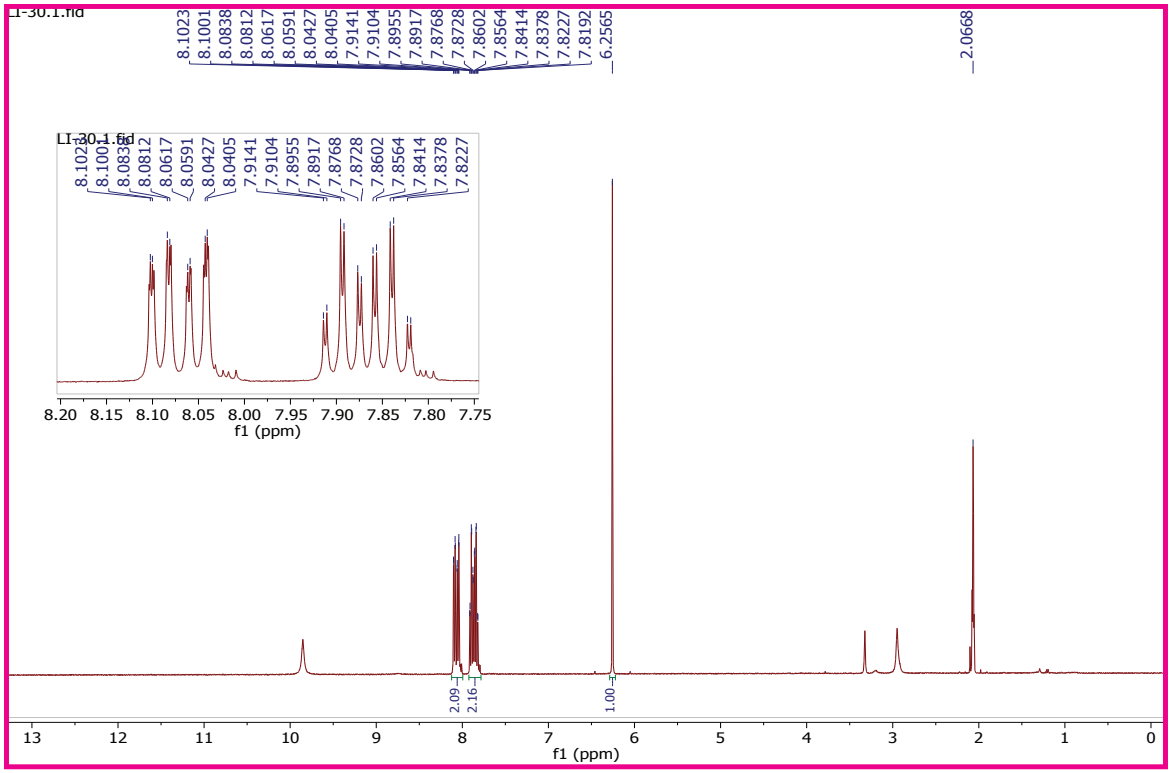
S/N	Studies	Name of the assay	Name of microorganisms *(reference & clinical isolate) that medicinal plants activity conducted*	Scientific name of the plant(s)/ compounds with their parts used	Family of the plants	Types of plants extracts/fractions / compounds	Outcome measured at a population level or individual level
1	Asres et al, 2006	Microdilution method	*Candida albicans ATCC 10231, Aspergillus niger ATCC 6275, A. terreus MTCC 1782, Penicillium notatum ATCC 11625, P. funiculosum NCTC 287* and *P.citrinum MTCC 1256*	Combretum molle R.Br. ex G.Don (SB)	Combretaceae	Dichloromethane, acetone and methanol	Fungal growth MIC & MFC)
2	Sileshi et al, 2007	Agar well diffusion & Agar dilution method	*Trychophyton mentagrophytes (ATCC 18748), Candida albicans (clinical isolate), Aspergilus niger (ATCC10535),* and *A. flavus (ATCC 1397)*	Clerodendrum myricoides (Hochst.) Steane & Mabb. (L), Ficus plamata Forssk. (L), Grewia ferruginea Hochst. ex A. Rich. (L) and Periploca linearifolia Quart. -Dill. & A. Rich. (Ae)	Laminaceae, Moraceae, Tiliaceae, Asclepeiaceace	Petroleum ether, chloroform, acetone and methanol	Zone of inhibition
3	Vaijayanthimala et al, 2000	Microdilution method	*Candida albicans*	Allium sativum L. (C), Allium schoenoprasum L. (C), Allium cepa var. cepa L (Bu), Acalypha indica L (L), Azedarach indica A. Juss. (L &S), Camellia sinensis (L.) Kuntze (L), Cassia alata L. (L), Cassia fstula L. (L), Cassia occidentalis L (L), Coffea arabica L (S), Curcuma longa L.(R), Lawsonia inermis L.(L), Murraya koenigii (L.) Spreng. (L), Ocimum sanctum L. (L), Piper betle L. (L), Psoralea corylifolia L. (S)	Liliaceae, Liliaceae, Liliaceae, Euphorbiaceae, Theaceae, Caesalpiniaceae, Caesalpiniaceae, Caesalpiniaceae, RubiaceaeZingiberaceae, Lythraceae, Rutaceae, Labiatae, Piperaceae, Papilionaceae	Aqueous, 95% ethanol	Fungal growth (MIC and MFC)

4	Bora et al, 2016	Agar well diffusion & Microdilution method	*Candida albicans*	Cinnamomum porrectum (Jack) Meisn. (S), Lippia nudiflora (L.) Greene (L), Cestrum nocturnum L. (F), Trachyspermum ammi (L.) Sprague (S), and Sida carpinifolia L.f. (F)	Lauraceae Verbenaceae, Solanaceae, Apiaceae, Malvaceae	Methanol	Zone of inhibition & Fungal growth (MIC, MFC)
5	Park et al, 2007	Agar well diffusion	*Dermatophytes Microsporum canis (KCTC 6591), Trichophyton mentagrophytes (KCTC 6077), Trichophyton rubrum (KCCM 60443), Epidermophyton floccosum (KCCM 11667),* and *Microsporum gypseum*	Leptospermum petersonii F.M.Bailey (S), Syzygium aromaticum (L.) Merr. & L.M.Perry (S)	Myrtacea, Myrtaceae	Essential oil	Zone of inhibition
6	Ameya et al, 2016	Agar diffusion & agar dilution method	*C. albicans* and *A. flavus*	Echinops kebericho Mesfin (R)	Asteraceae	Ethanol, methanol and water	Zone of inhibition and Fungal growth (MIC & MFC)
7	Ameya et al, 2015	Disc diffusion method, MIC	*clinical isolate of C. albicans* and *A. flavus*	Taverniera abyssinica A.Rich. (R)	Fabaceae	Ethanol, methanol and distilled water	Zone of inhibition, Fungal growth (MIC & MFC)
8	Gemeda et al, 2014	Agar dilution method & spore germination assay	*Aspergillus flavus (AF001, AF006, AF009, AF019, AF027, and AF037)* and *Aspergillus niger (AN002)*	Cymbopogon martinii (Roxb.) W.Watson (Ae), Foeniculum vulgar Mill. (Ae), Trachyspermum ammi (L.) Sprague (S)	Poaceae, Apiaceae & Apiaceae respectively	Essential oil	Zone of inhibition & Percent spore germination inhibition
9	Rana et al, 2011	Agar well diffusion & Micro dilution method	*Fusarium moniliforme NCIM 1100, Fusarium oxysporum MTCC 284, Aspergillus species, Mucor species, Trichophyton rubrum* and *Microsporum gypseum*	Syzygium aromaticum (L.) Merr. & L.M.Perry (L.)	Myrtaceae	Essential oil	Zone of inhibition and Fungal growth (MIC & MFC)

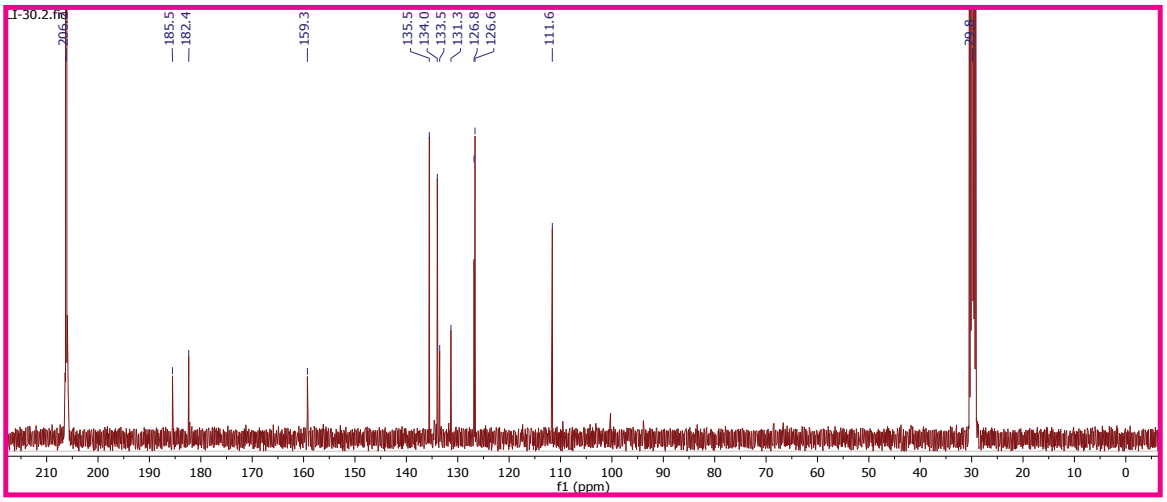
10	Getie et al, 2003	Agar well diffusion	*C. albicans, Aspergillus fumigatus* and *Trichophyton rubrum*	Dodonaea viscosa Jacq. (L); Rumex nervosus (Engl.) Dammer (R); Rumex abyssinicus Jacq. (R)	Sapindaceae Polygonaceae	80% Methanol	Zone of inhibition
11	Kasparaviciene et al, 2018	Agar dilution method	*Candida albicans, richophyton spp. (nail and scalp isolated), Microsporum spp., Aspergillus niger* and *Rhodotorula rubra*	Oleogel	Lamiaceae	Essential oil	Zone of inhibition
12	Fierascu et al, 2018	Agar well diffusion	*Aspergillus niger ATCC 15475* and *Penicillium hirsutum ATCC 52323*	Juniperus communis L. (F)	Cupressaceae	50% Ethanol	Zone of inhibition
13	Nyanchok, 2007	Agar well diffusion	*Candida albicans, Penicillium notatum*	Bersama abyssinica Fresen. (SB)	Francoaceae	N-hexane, dichlomethane, ethyl acetate and methanol	Zone of inhibition
14	Jain et al, 2017	Macro-dilution (Tube dilution) method	*Trichophyton rubrum (MTCC 296), T. mentagrophytes (MTCC 7687), Microsporum gypseum (MTCC 4524), M. fulvum(MTCC2837), T. soudanense* and *T. interdigitale*	Thymus vulgaris L. (L)	Lamiaceae	Essential oil	Fungal growth (MIC & MFC)
15	Messele et al, 2004	Agar well diffusion	*T. mentagrophytes (ATCC), A. niger (ATCC)* and *C. albicans (isol.)*	Inula confertiflora A. Rich. (L, F), Clematis simensis Fresen. (L), Zehneria scabra (L.f.) Sond. (L), Pycnostachys abyssinica Fresen. (L),	Compositae, Ranunculaceae, Cucurbitacea & Labiatae respectivelt	Petroleum ether, 80% methanol	Zone of inhibition
16	Salazar et al, 2015	Broth microdilution method	*Trichophyton menta, Trichophyton rubrum, Epidermophyton floccos, Microsporum canis*	Azadirachta indica A.Juss. (L)	Meliaceae	Oil from seed	Fungal growth (MIC)
17	Simhadri et al, 2017	Disc diffusion & Microdilution	*Trichophyton rubrum (MTCC7859), Microsporum gypseum (MTCC 4524), Epidermophyton floccosum (MTCC 7880)*	Azadirachta indica A.Juss. (L)	Meliaceae	Hexane, benzene, ethyl acetate, methanol	Zone of inhibition and Fungal growth (MIC)

Leaves = L, Bark =B, Root=R, Stem bark=SB, Fruits=F, Aerial =Ae, Whole plant=W, Clove=C, Bulbs=Bu, Minimum inhibitory concentrations = MIC, minimum fungicidal concentration =MFC.

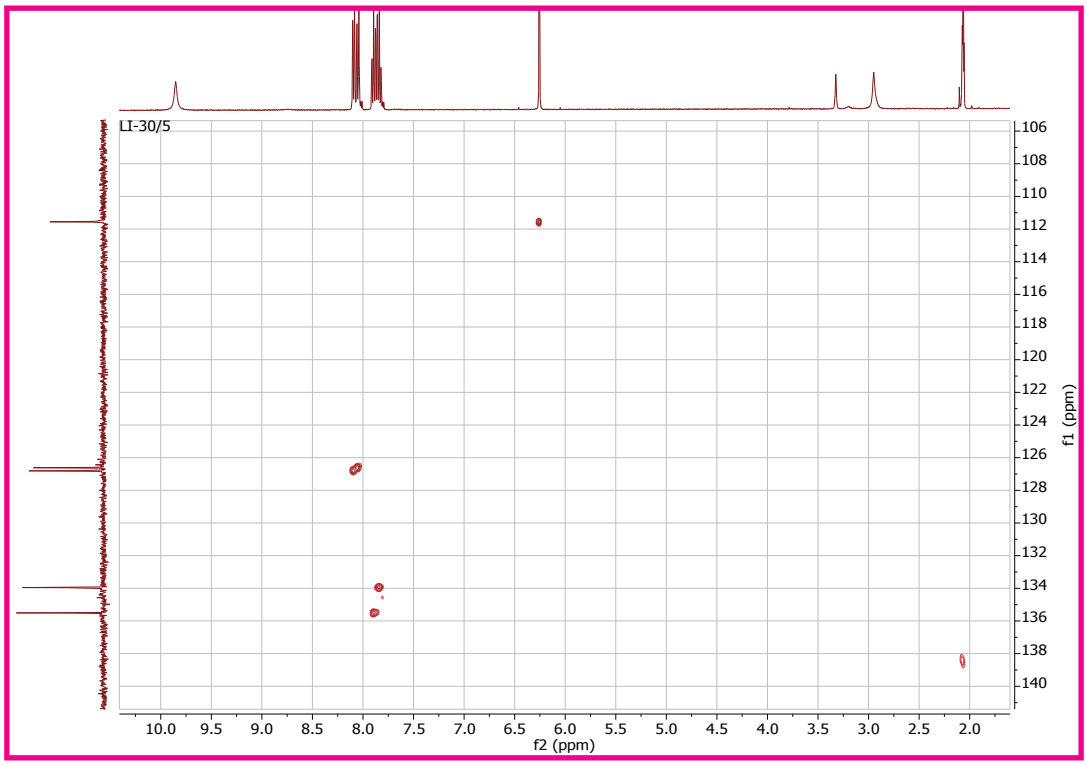
ANNEX 5.1- SPECTRUM OF COMPOUNDS ISOLATED FROM LEAVES *L. INERMIS* L.



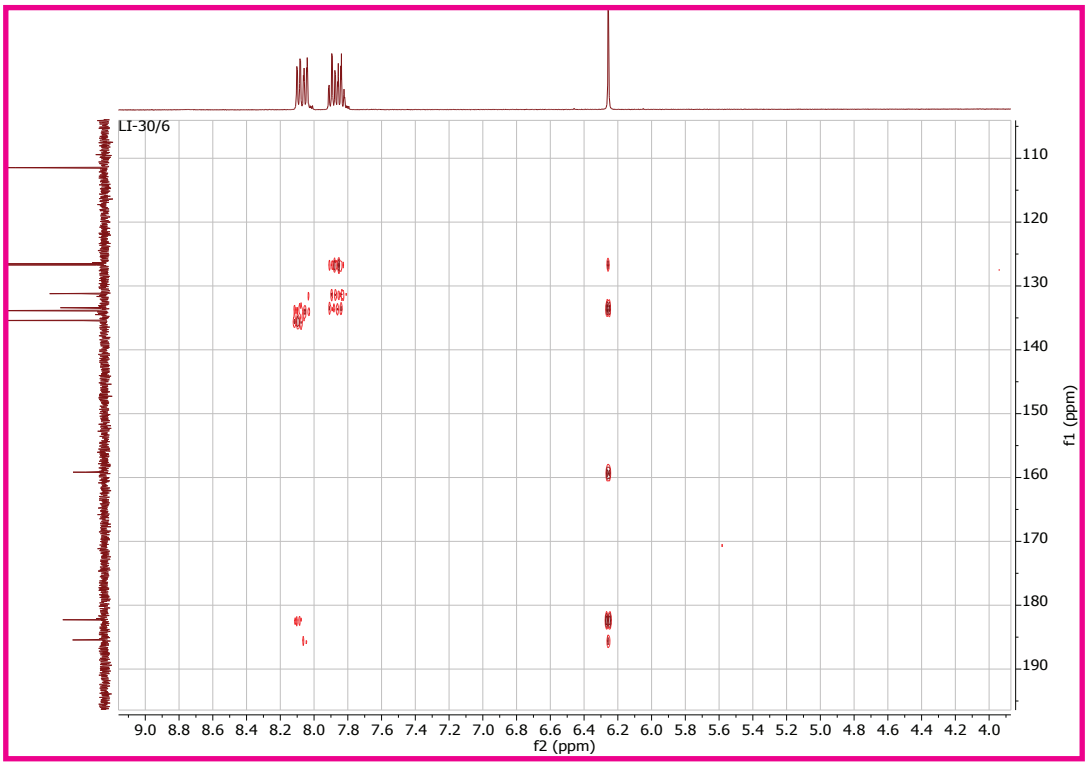
S1. ^{1}H NMR spectrum (400Hz, Acetone-d_6) of **1**



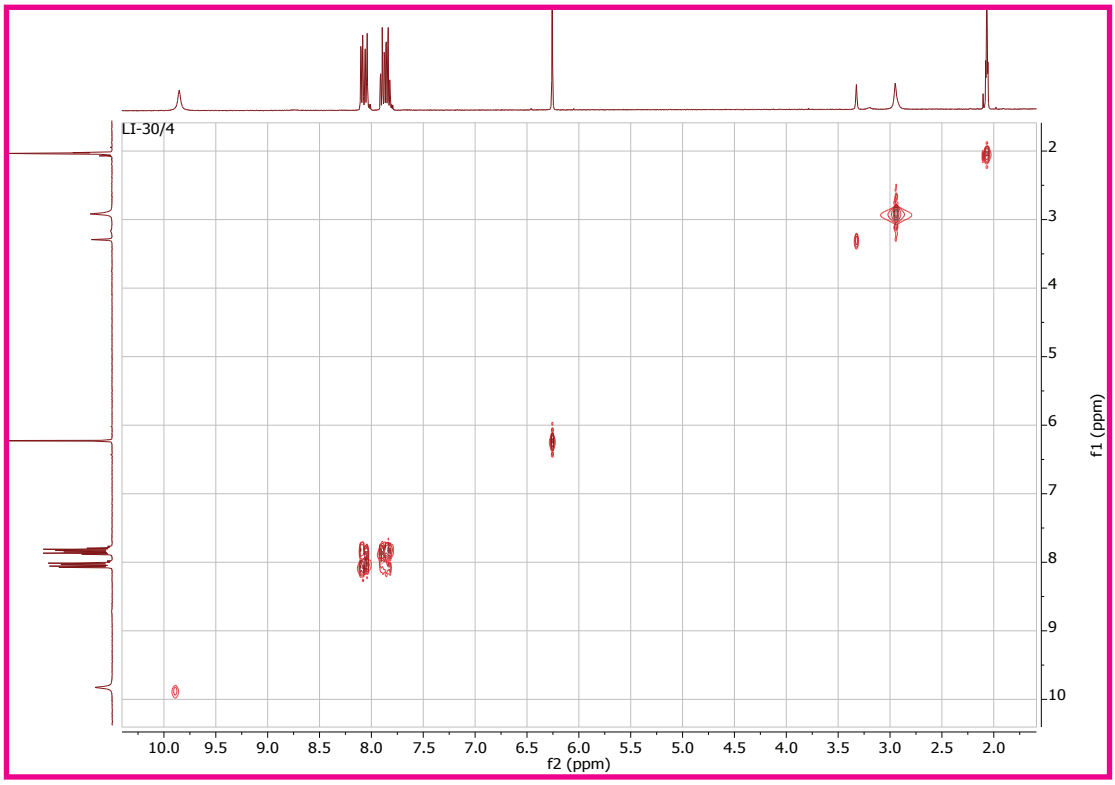
S2. ^{13}C NMR spectrum (100Hz, Acetone-d_6) of **1**



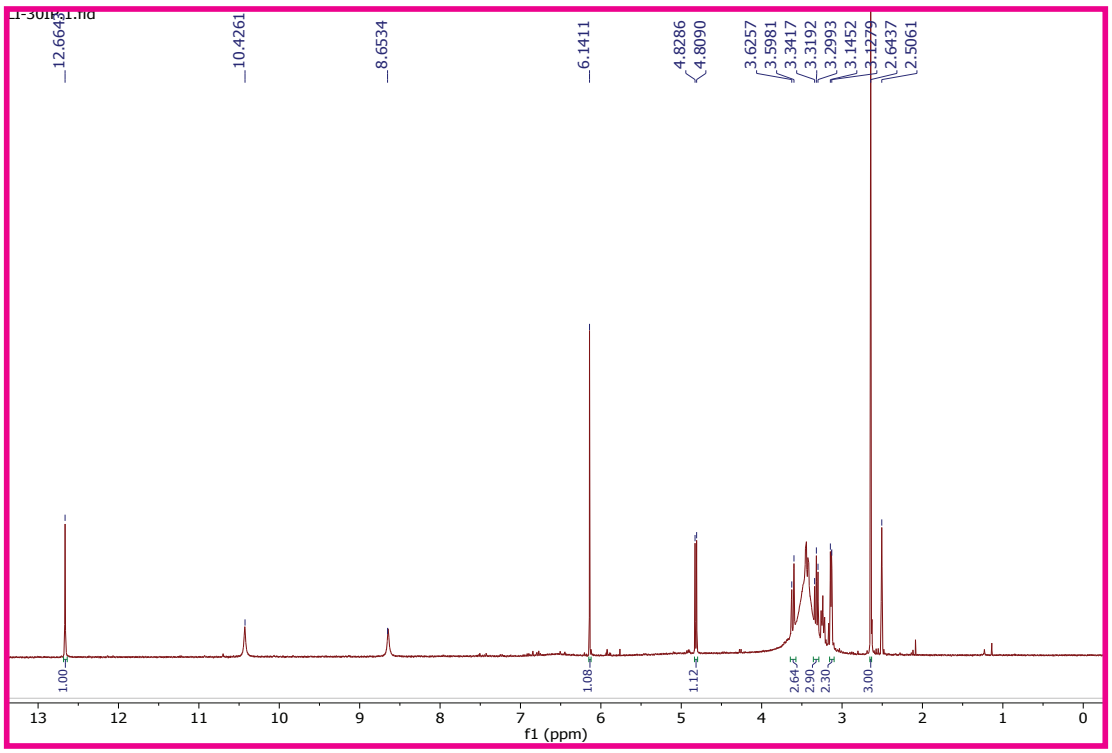
S3. HSQC spectrum of **1**



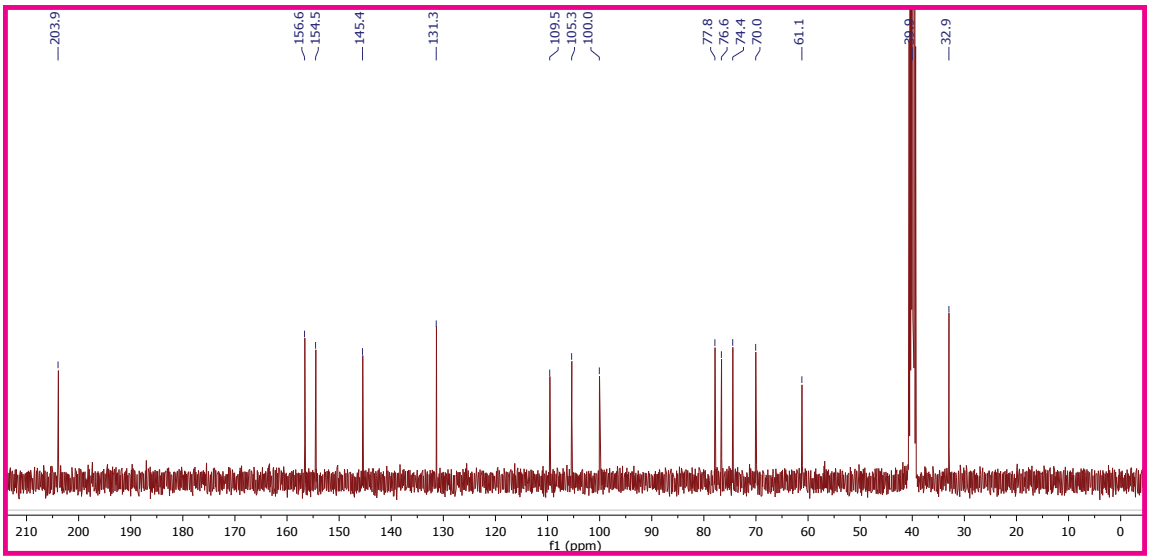
S4. HMBC spectrum of **1**



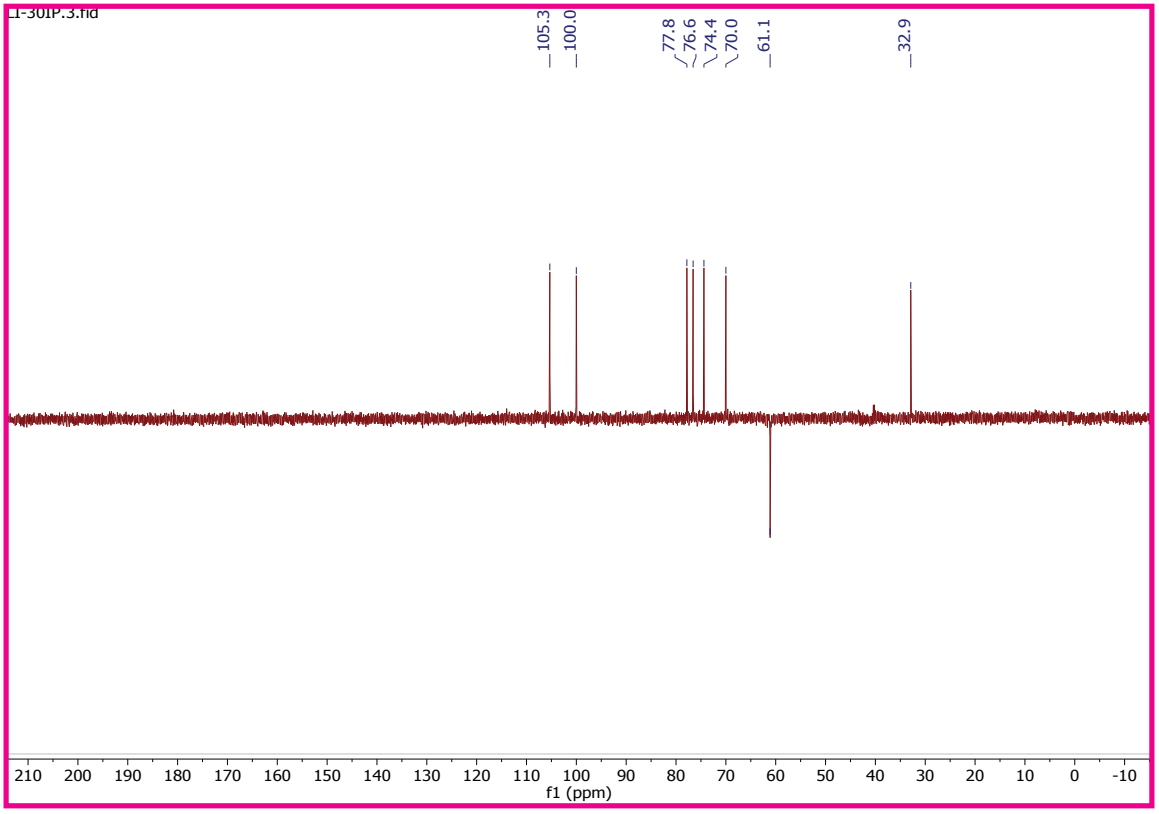
S5. COSY spectrum of 1



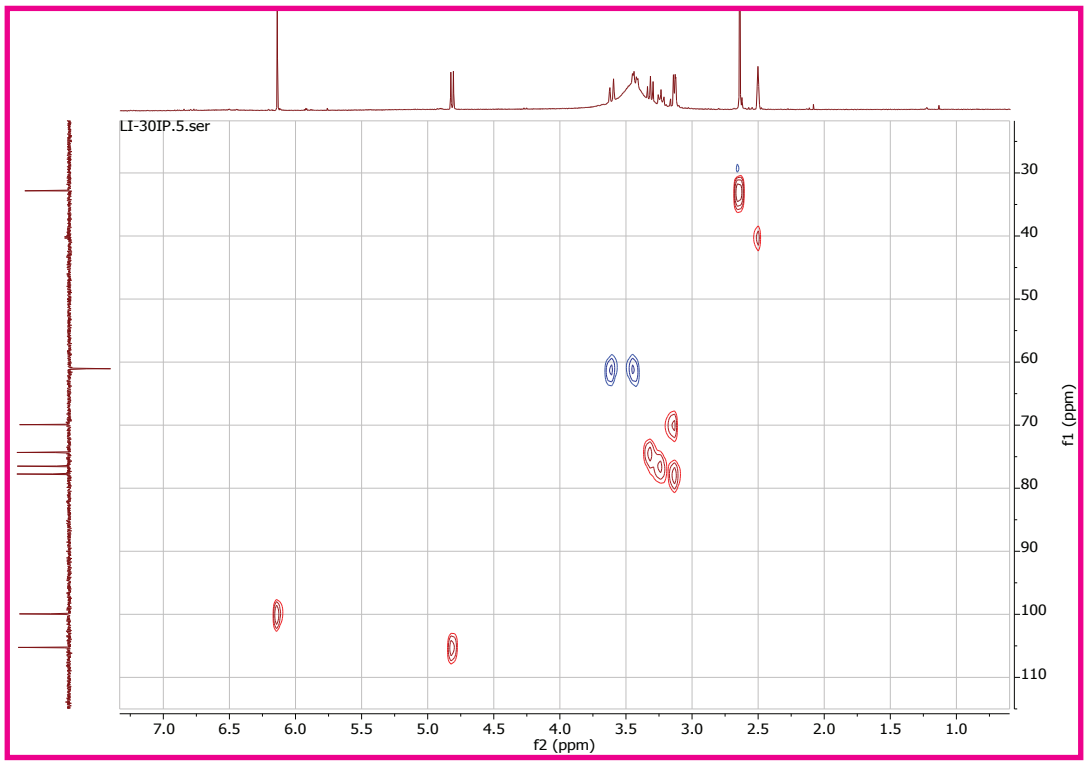
S6. ^{1}H NMR spectrum (400Hz, DMSO-d_6) of **2**



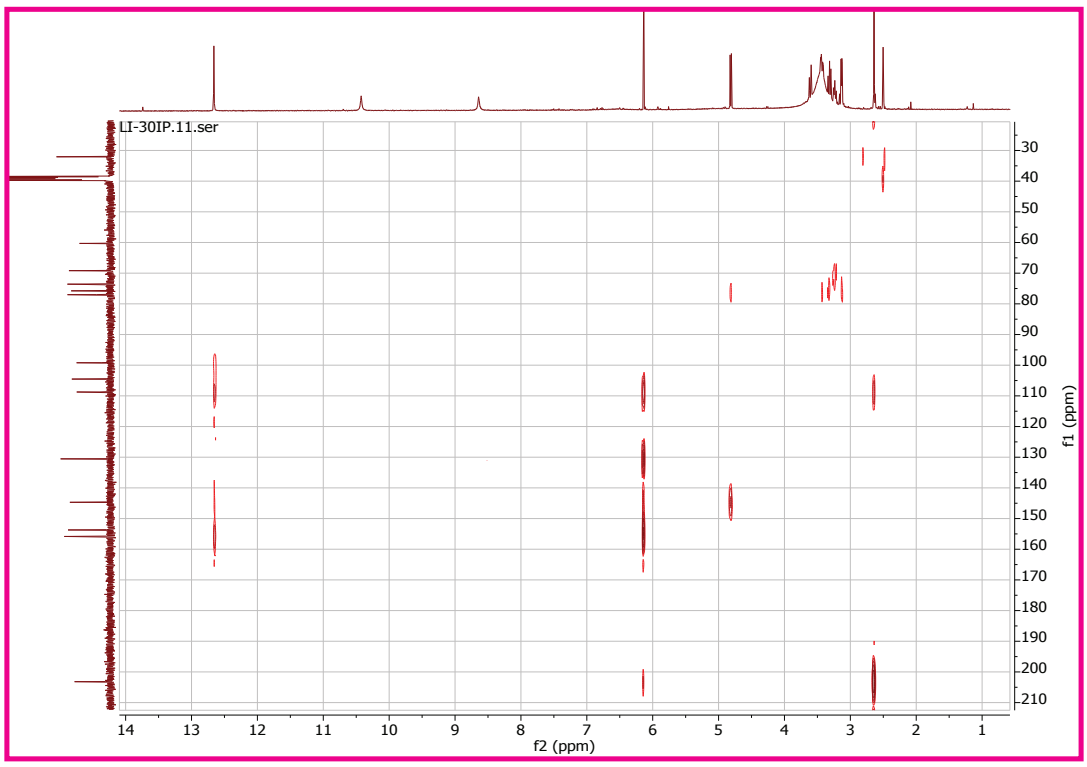
S7. ^{13}C NMR spectrum (100 Hz, DMSO-d_6) of **2**



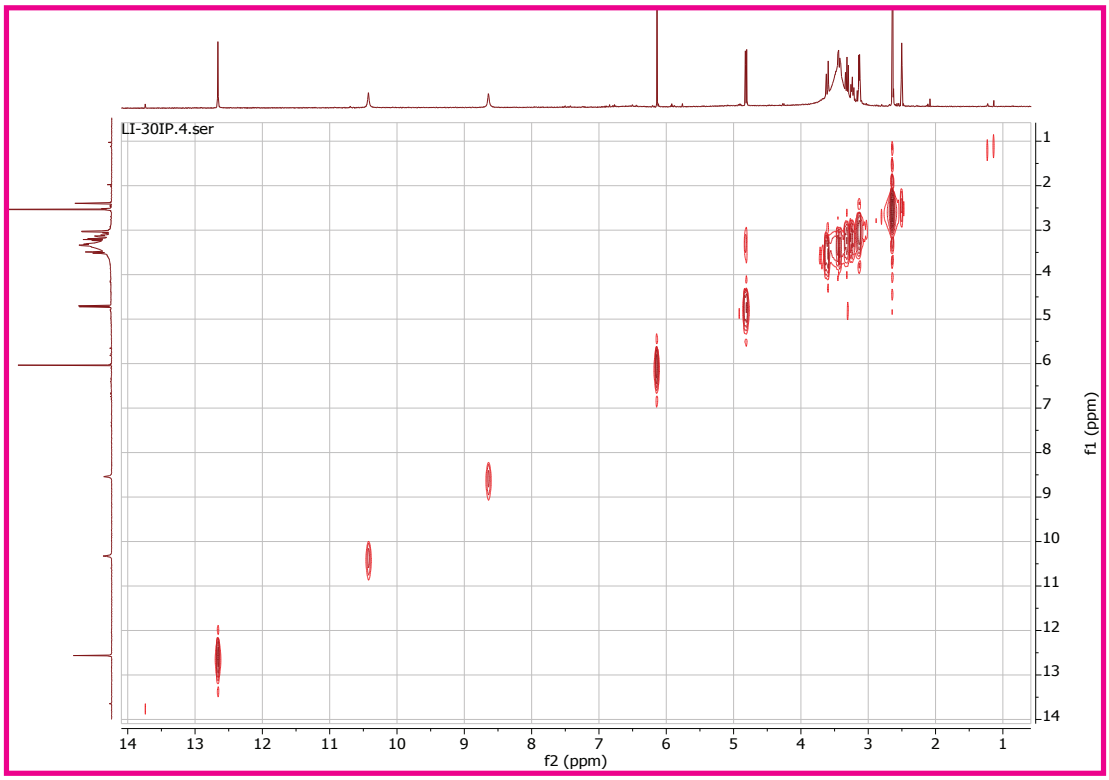
S8. DEPT-135 spectrum (100 Hz, DMSO-d_6) of **2**



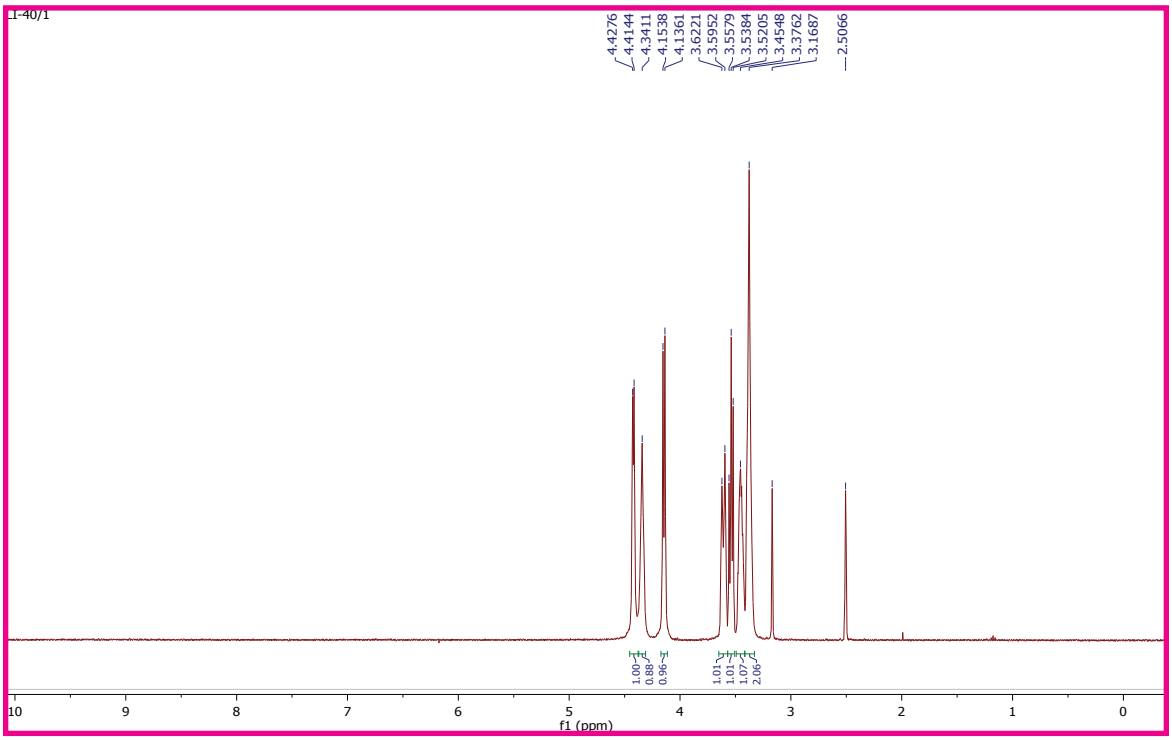
S9. HSQC spectrum of **2**



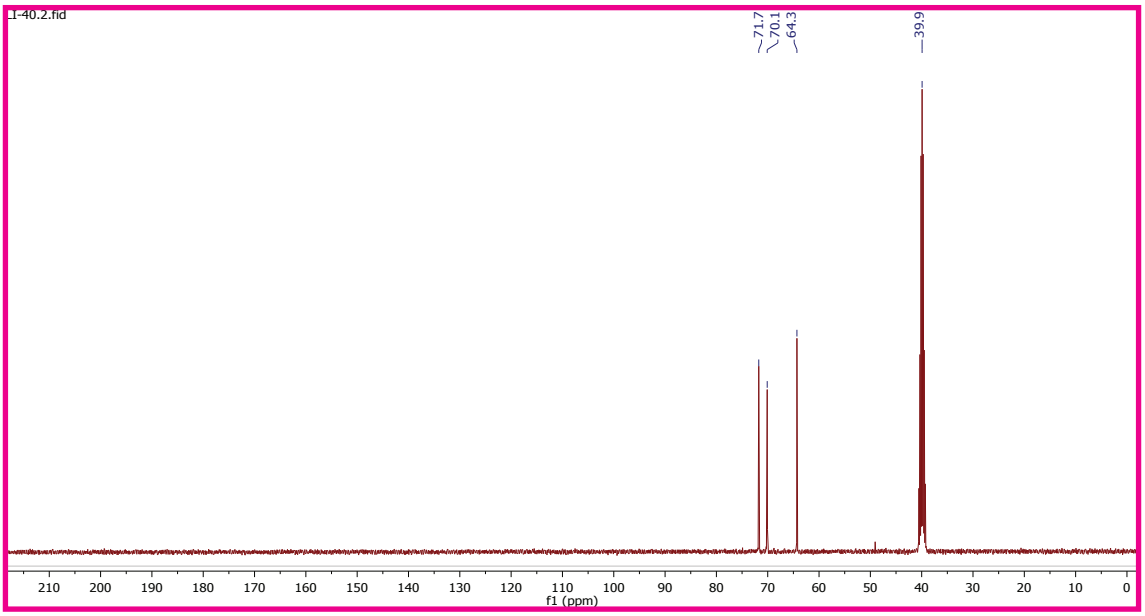
S10. HMBC spectrum of **2**



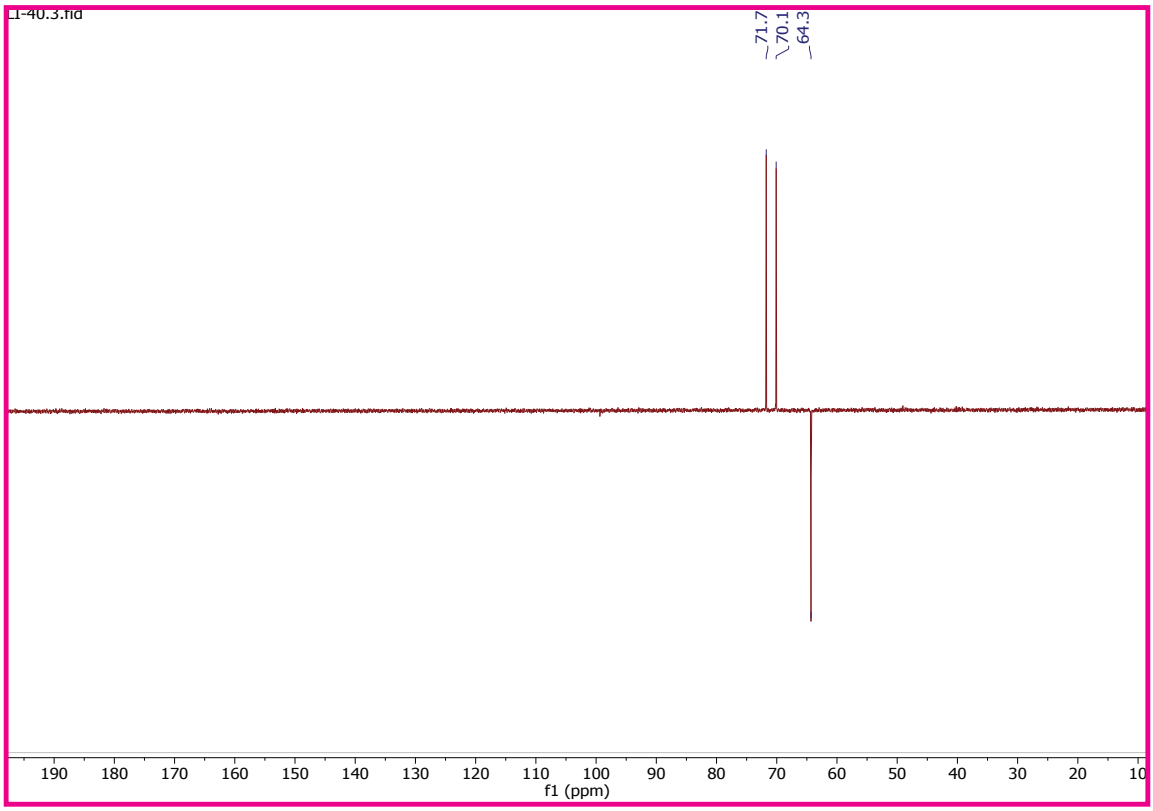
S11. COSY spectrum of **2**



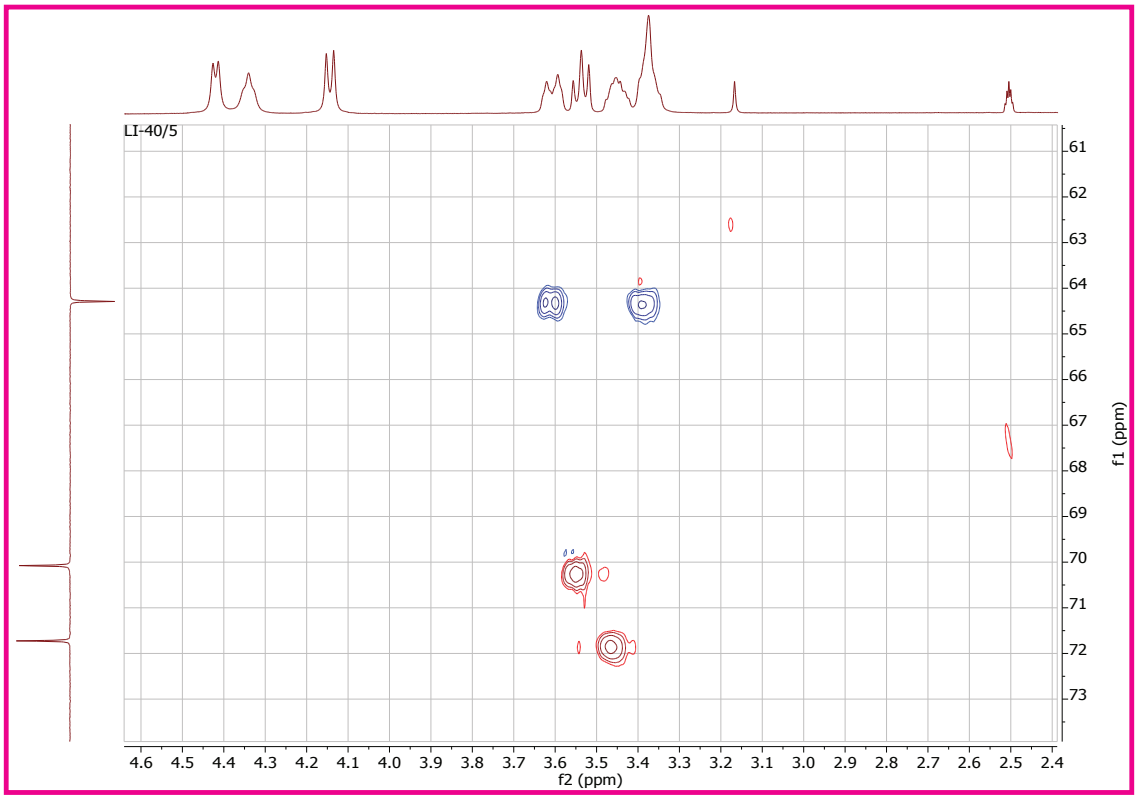
S12. ^{1}H NMR spectrum (400Hz, DMSO-d6) of **3**



S13. ^{13}C NMR spectrum (100 Hz, DMSO-d_6) of **3**



S8. DEPT-135 spectrum (100 Hz, DMSO-d_6) of **3**



S9. HSQC spectrum of **2**

ANNEX 7– LIST OF PUBLICATIONS

Open access Protocol

BMJ Open Science

Ethiopian medicinal plants used for their anti-inflammatory, wound healing or anti-infective activities: protocol for systematic literature review and meta-analysis

Dereje Nigussie ,[1,2] Belete Adefris Legesse,[1] Gail Davey,[2,3] Abebaw Fekadu,[1,2] Eyasu Makonnen[1,4]

This article has received OSF badge for Pre-registration.

To cite: Nigussie D, Legesse BA, Davey G, *et al*. Ethiopian medicinal plants used for their anti-inflammatory, wound healing or anti-infective activities: protocol for systematic literature review and meta-analysis. *BMJ Open Science* 2020;**4**:e100064. doi:10.1136/bmjos-2020-100064

► Prepublication history for this paper is available online. To view these files, please visit the journal online (http://dx.doi.org/10.1136/bmjos-2020-100064)

Received 25 February 2020
Revised 27 May 2020
Accepted 29 June 2020

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ABSTRACT

Objectives Medicinal plants are used globally as alternative medicines in the management of a range of disease conditions and are widely accepted across differing societies. Ethiopia hosts a large number of plant species (>7000 higher plant species), of which around 12% are thought to be endemic, making it a rich source of plant extracts potentially useful for human health. The aim of this review is to evaluate Ethiopian medicinal plants for their anti-inflammatory, wound healing, antifungal or antibacterial activities.

Methods and analysis The guidance of the Preferred Reporting Items for Systematic Review and Meta-analysis Protocols (PRISMA-P) statement will be used. This review will consider all controlled studies of anti-inflammatory and wound healing properties (both in vivo and in vitro) and in vitro anti-infective properties of medicinal plants found in Ethiopia. Data sources will be EMBASE, PubMed/Medline, Scopus and Google Scholar. Guidance documents on good in vitro methods and checklists for reporting in vitro studies will be used for quality assessment of in vitro studies. The risk of bias tool for animal intervention studies (the SYRCLE RoB tool) will be used to assess the validity of studies. The main outcomes will be percent inhibition of inflammation, time of epithelisation and tissue tensile strength in wounds and microbial growth inhibition.

Ethics and dissemination The findings of this systematic review will be disseminated by publishing in a peer-reviewed journal and via conference presentations. Ethical clearance was obtained from the Brighton and Sussex Medical School, Research Governance & Ethics Committee (RGEC) and Addis Ababa University, College of Health Science, Institutional Review Board.

PROSPERO registration number This systematic literature review has been registered with PROSPERO (registration number CRD42019127471).

Strengths and limitations of this study

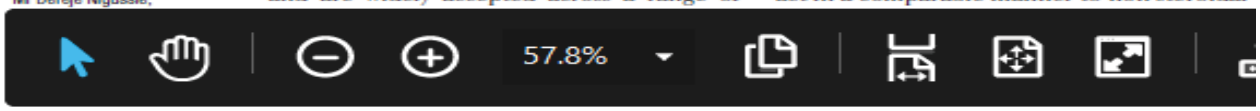
- This is the first systematic review to assess Ethiopian medicinal plants for inflammation, wound healing and anti-infection properties.
- The review includes both in vivo and in vitro studies.
- Study screening and data extraction will be conducted independently by two authors.
- This systematic review only considers studies written in English.
- It will consider a wide range of methodological approaches and uses of different types of interventions which may result in heterogeneous data. As a result of this heterogeneity, it may be impossible to conduct meta-analysis.

INTRODUCTION

Herbal medicines are used worldwide as alternative treatments for a range of conditions and are widely accepted across a range of

arise from the active substances produced by the plants as secondary metabolites, which themselves produce physiological changes in the human body.[1] Most of the drugs currently used against infectious agents are derived from natural products or from structures suggested by natural product 'leads'—chemical compounds with pharmacological or biological activity likely to be therapeutically useful, but which require modification to fit better to the target.[2]

In the search for improved, safe, effective and affordable drugs for treatment of tropical lymphoedema, plant-derived products represent an attractive option.[3] Herbal products are relatively safe, chemically complex mixtures composed of a range of constituents with multiple potential targets and different mechanisms of action.[4] Phytochemicals found in certain herbal extracts are reported to demonstrate analgesic and anti-inflammatory properties.[1] Most of these act in a comparable manner to non-steroidal



Journal of Ethnopharmacology 276 (2021) 114179

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Systematic review of Ethiopian medicinal plants used for their anti-inflammatory and wound healing activities

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ARTICLE INFO

Keywords:
Ethiopian
Medicinal plants
Anti-inflammatory
Wound healing
Systematic review

ABSTRACT

Ethnopharmacological relevance: Plant materials are used worldwide as complementary and alternative therapeutics for the treatment of various illnesses. In Ethiopia, folk medicines are utilized across a wide range of cultures and settings. Ethiopia has numerous plant species of which around 12% are endemic, making it a rich source of medicinal plants that are potentially important for human wellbeing.

Aim of the study: The aim of this study was to assess Ethiopian medicinal plants with anti-inflammatory or wound healing activities, in an attempt to compile the information required for further investigation of their potential role in the management of lymphoedema.

Methods: A systematic review protocol was developed according to the preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) statement. The protocol for this review was registered on PROSPERO with registration number CRD42019127471. This review considers all controlled *in vivo* and *in vitro* anti-inflammatory and wound healing studies evaluating the efficacy and safety of Ethiopian medicinal plants. The search strategy included all articles containing descriptors such as Ethiopia, medicinal plants, herbal products, care, management, lymphoedema, lymphedema, swelling, podoconiosis, elephantiasis, wound, wound healing, inflammation, an anti-inflammatory that were published until June 28, 2019. Outcomes were measured as the percentage of inflammatory and pro-inflammatory cell inhibition, as the percentage of carrageenan-induced oedema (anti-inflammation) inhibition, and the percentage of cell migration and proliferation (wound healing). For quality assessment of individual animal studies, the Risk of Bias tool for animal intervention studies (SYRCLE's RoB tool) criteria were used. For quality assessment of individual *in vitro* studies, the OECD guidelines and the WHO Good Laboratory Practice (GLP) handbook were used.

Results: A total of 46 articles on anti-inflammatory and 17 articles on wound healing properties were reviewed. For the *in vivo* studies, Swiss albino mice and Wistar rats were used, and the concentration of plant extracts or fractions administered to the lab animals varied considerably. Acetone extract of *Vernonia amygdalina* showed the fastest anti-inflammatory activity at lower concentrations in carrageenan-induced paw oedema.

Conclusion: Lawsonia inermis, Azadirachta indica, Achyranthes aspera, and *Cuminum cyminum* are the most studied plant species in terms of anti-inflammatory activity, while *Lawsonia inermis* and *Azadirachta indica* are the most studied ones for wound healing. The most common *in vivo* techniques used for the anti-inflammatory and the wound healing assays were carrageenan-induced paw oedema, and excision and incision wound models, respectively.

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https://doi.org/10.1016/j.jep.2021.114179
Received 14 December 2020; Received in revised form 16 April 2021; Accepted 27 April 2021
Available online 11 May 2021

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 01 June 2021
doi: 10.3389/fphar.2021.633921

Antibacterial and Antifungal Activities of Ethiopian Medicinal Plants: A Systematic Review

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OPEN ACCESS

Edited by:
Alessandra Durazzo, Council for Agricultural Research and Economics, Italy

Reviewed by:
Vivekananda Mandal, Guru Ghasidas Vishwavidyalaya, India
Wondimeneh Shiferaw, Debre Berhan University, Ethiopia

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Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 26 November 2020
Accepted: 14 May 2021
Published: 01 June 2021

Citation:
Nigussie D, Davey G, Tufa TB, Brewster M, Legesse BA, Fekadu A and Makonnen E (2021) Antibacterial and Antifungal Activities of Ethiopian Medicinal Plants: A Systematic Review. Front. Pharmacol. 12:633921. doi: 10.3389/fphar.2021.633921

Background: Podoconiosis and lymphatic filariasis are the most common causes of lower limb lymphoedema in the tropics. Many sufferers experience frequent painful episodes of acute bacterial infection. Plant based traditional medicines are used to treat infections in many countries and are culturally established in Ethiopia. Ethiopian medicinal plants found to have antibacterial and antifungal activities were reviewed with the aim of increasing information about the treatment of wound infections in patients with lymphoedema.

Methods: This study collates data from published articles on medicinal plants with antibacterial and antifungal activities in Ethiopia. A systematic search of Scopus, EMBASE, PUBMED/MEDLINE and Google Scholar was undertaken. The Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines were followed. The protocol was registered on PROSPERO with registration number CRD42019127471. All controlled studies of *in vitro* antibacterial and antifungal activities were considered. All articles containing the descriptors published until June 28, 2019 were included. The outcome was measured as percent inhibition of microbial growth. For quality assessment of individual *in vitro* studies, OECD guidelines and the WHO-Good Laboratory Practice (GLP) handbook were used.

Results: Seventy-nine studies met the inclusion criteria. A total of 150 plant species and three compounds had been tested against 42 species of bacteria, while 43 plant species had been tested against 22 species of fungus.

Conclusion: Materials derived from several Ethiopian medicinal plants have been shown to have promising activity against a variety of bacteria and fungi. Those derived from *Azadiractha indica* A. Juss. and *Lawsonia inerms* L. are the most extensively studied against a wide range of gram-negative and positive bacteria, and fungal species.

Keywords: systematic literature review, antifungal, antibacterial, Ethiopia, medicinal plants

Trans R Soc Trop Med Hyg 2020; **114**: 962–973
doi:10.1093/trstmh/traa143 Advance Access publication 28 November 2020

Antimicrobial susceptibility of bacteria isolated from the infected wounds of patients with lymphoedema in East Wollega, Ethiopia

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Received 23 June 2020; revised 23 October 2020; editorial decision 3 November 2020; accepted 7 November 2020

Background: Lymphoedema is caused by dysfunction of the lymphatic system resulting in accumulation of high-protein content fluid in the interstitial space. To date, the bacteria associated with wound infections of patients with lower limb lymphoedema in Ethiopia have not been studied. This study identified pathogenic bacteria involved in wound infection and assessed antimicrobial susceptibility patterns in patients with lymphoedema in Ethiopia.

Methods: Swab samples were collected from the wounds of patients with lymphoedema and cultured using standard microbiological techniques. Micro-organisms were identified by colony morphology followed by identification and antimicrobial susceptibility testing using the automated VITEK 2 COMPACT Microbial Detection System.

Results: Swabs were collected from 103 patients and 84 were culture positive: 44 (52.4%) culture-positive samples showed polymicrobial growth and 40 (47.6%) grew single bacterial isolates. In total, 134 isolates were obtained, of which 26 gram-negative and 12 gram-positive bacterial species were identified. A total of 28/63 (44.4%) gram-negative isolates and 3/57 (5.3%) gram-positive isolates were multiple drug resistant. There was no resistance to ciprofloxacin, moxifloxacin or gentamycin among gram-negative or gram-positive bacteria.

Conclusion: In this study, many infections were polymicrobial and showed multiple drug resistance. Fluoroquinolones and gentamycin, however, seemed to be effective against bacterial wound infection in this setting.

Keywords: antimicrobial drug resistance, bacteria, Ethiopia, lymphoedema, wound infection

Introduction

Lymphoedema is caused by failure of lymphatic drainage leading to the accumulation of protein-rich fluid in the interstitial space. It is classified into primary and secondary lymphoedema. The causes of primary lymphoedema are poorly described, but may arise from genetic disorders, while secondary lymphoedema is attributed to damage to the lymphatic system, resulting from lymphatic vessel infestation, lymphadenectomy or radiotherapy in cancer patients.[1] The two main causes of lymphoedema in the tropics are lymphatic filariasis followed bypodoconiosis.[2] In Africa, lymphatic filariasis is caused by *Wuchereria bancrofti* species transmitted by blood-feeding mosquitoes.[3] Podoconiosis is a form of lymphoedema arising among barefoot subsistence farmers who have contact with irritant red clay soil of volcanic origins over long periods of time.[4]

Lymphoedema has a marked physical and psychological impact in affected patients and significantly reduces their quality of life.[2] Wound ulcer development is one of the most serious complications and often makes it impossible for patients to work.[5] Patients with lymphoedema have a high risk of wound formation resulting from infection, including fungal infection in skin folds, moisture build-up and trauma.[5]

Skin lesions, including wounds, fissures, paronychia and eczema, allow the penetration of bacteria and fungus into the underlying tissues. Secondary infection along with inflammation also seems to play a major role in the skin changes seen in the

Nigussie et al. BMC Complementary Medicine and Therapies (2021) 21:2
https://doi.org/10.1186/s12906-020-03183-0

BMC Complementary Medicine and Therapies

RESEARCH ARTICLE Open Access

Antibacterial activity of methanol extracts of the leaves of three medicinal plants against selected bacteria isolated from wounds of lymphoedema patients

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Abstract

Background: Patients with lymphoedema are at high risk of getting bacterial and fungal wound infections leading to acute inflammatory episodes associated with cellulitis and erysipelas. In Ethiopia, wound infections are traditionally treated with medicinal plants.

Methods: Agar well diffusion and colorimetric microdilution methods were used to determine the antibacterial activity of methanol extracts of the three medicinal plants against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shewanella alage*, methicillin-resistant *S. aureus* ATCC®43300TM, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC700603, and *Pseudomonas aeruginosa* ATCC37853.

Results: The methanol extract of *L. inermis* leaves showed high activity against all tested bacterial species, which was comparable to the standard drugs. Similarly, the extracts of *A. indica* showed activity against all tested species though at higher concentrations, and higher activity was recorded against *Streptococcus pyogenes* isolates at all concentrations. However, the extract of *A. aspera* showed the lowest activity against all tested species except *Streptococcus pyogenes* isolates. The lowest minimum inhibitory concentration (MIC) was recorded with the extract of *L. inermis* against *E. coli* isolate and *S. aureus* ATCC 25923.

Conclusion: Methanol extracts of *L. inermis, A. indica*, and *A. aspera* leaves exhibited antimicrobial activity against selected bacterial isolates involved in wound infections, of which the methanol extracts of *L. inermis* exhibited the highest activity. The results of the present study support the traditional use of plants against microbial infections, which could potentially be exploited for the treatment of wound infections associated with lymphoedema.

Keywords: Lymphoedema, Wound infection, Bacteria, Medicinal plants, Ethiopia

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