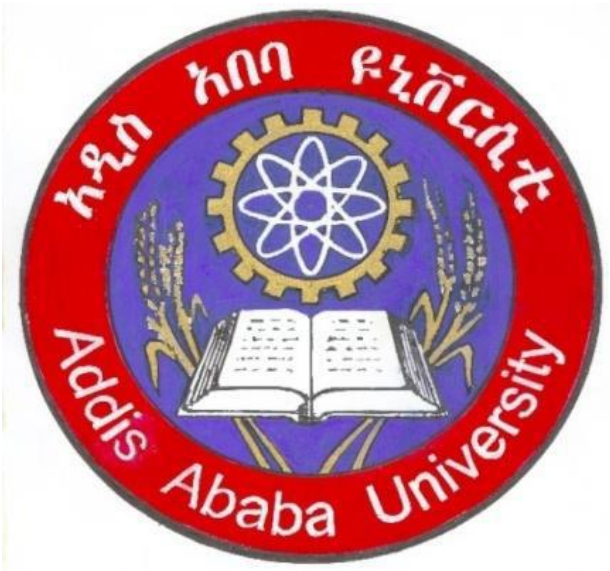

A comparative study of the leaf and root extracts of *Stephania abyssinica (Dillon & A. Rich) Walp* on wound healing activity in mice

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

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Addis Ababa University School of Graduate Studies

This is to certify that the thesis prepared by Minilu Girma, entitled "A comparative study of the leaf and root extracts of *Stephania abyssinica (Dillon & A. Rich) Walp* (*Menispermaceae)* on wound healing activity in mice," and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology, complies with the regulations of the University and meets the accepted standards concerning originality and quality.

Approved and signed by the Examining Committee:

Name	Signature	Date
Internal Examiner	_____	_____
External Examiner:	_____	_____
Advisor: Zelalem Petros (PhD)	_____	_____

(Chairman of the Department)

Abstract

A comparative study of the leaf and root extracts of *Stephania abyssinica (Dillon & A. Rich) Walp* (*Menispermaceae)* on wound healing activity in mice

Minilu Girma

Addis Ababa University, 2023

The roots and leaves of *Stephania abyssinica* are traditionally used to treat wounds in several regions of Ethiopia. The pharmacological screening for wound-healing activity of the plant was done for the crude extract and solvent fractions of the root extract. But there have been no pharmacological studies done on the wound-healing effect of the leaf extract. In this study, the wound-healing effects of both the 80% methanol extract of the leaves and the roots of *S. abyssinica* were evaluated using the excision wound model, and the results were compared. Histopathological investigations were also carried out. The antioxidant activity of both the leaf and root extracts was also assessed. In addition, preliminary phytochemical screening tests and quantification of total phenolic, flavonoid, and alkaloid contents were done for both the leaf and root extracts. Both the root and the leaf extracts significantly increased the rate of wound contraction ($p < 0.05$) and shortened the re-epithelialization period ($p < 0.01$). The root extract significantly increased the skin's tensile strength ($p < 0.001$). The quantity of secondary metabolites in the root extract, such as total phenolic, flavonoid, and alkaloid contents, was found to be higher than those of the leaf extract, and this concentration difference demonstrated a substantial difference in its wound healing activity.

Keywords: Wound healing, Antioxidant, *Stephania abyssinica*

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Table of Contents

List of Tables

List of Figures

Abbreviations

DPPH- 2,2-diphenyl-1-picrylhydrazyl

ECM- Extra Cellular Matrix

EGF- Epidermal Growth Factor

FGF- Fibroblast Growth Factor

IL-1α -Interleukin-1α

MF -Master Formula

MMPs -Matrix Metalloproteases

OECD- Organization for Economic Cooperation and Development

PDGF -Platelet-Derived Growth Factor

RF -Reduced Formula

SPSS - Statistical Package for the Social Sciences'

TGF-β- 1 Transforming growth factor beta 1

TNF-α -Tumour Necrosis Factor α

TS- Tensile Strength

VEGF- Vascular Endothelial Growth Factor

1. Introduction

1.1. Overview of Wounds

The skin is the largest organ in the human body due to its surface area, and it separates the body from the external environment. It is an important structure that protects internal tissues from harmful rays, mechanical damage, extreme temperatures, and microbial infections (Panda et al., 2011, Rodrigues et al., 2019).

Wounds are injuries that disrupt the integrity of tissues and can lead to anatomical and functional destruction. A skin wound results in a loss of epithelial continuity with or without the loss of underlying connective tissue (Ozay et al., 2018, Velnar et al., 2009). Over 80% of the estimated 14 million people who suffer from wounds and burns each year live in low- and middle-income countries (Reardon, 2014, Namunana et al., 2018). However, a wound is an everyday event that can happen to anyone at any time, and individuals don't usually seek medical attention for small wounds and burns, so this number may be underreported.

Injured tissues grow and regenerate in a specific way as a result of well-organized biochemical and cellular processes that take place during the healing of wounds. The vital biological process of wound healing involves a complex network of blood cells, cytokines, and growth factors, and it ultimately leads to the recovery of the injured skin or tissue to its pre-injury state (Bowler et al., 2001, Esimone et al., 2008).

Depending on specific classification criteria like etiology, location, type of injury or presenting symptoms, wound depth and tissue loss, or clinical appearance, there are many different types of wounds, including injuries, cuts and bites, diabetic, gastric, and duodenal ulcers (Tessema and Molla, 2021, Ugwu et al., 2020). Depending on how a wound heals, it can be classified as either acute or chronic. Acute wounds are tissue injuries that recover over a brief period of time, usually less than eight weeks, through an organized series of physiological processes that restore anatomical and functional integrity (Siddiqui and Bernstein, 2010). Conversely, chronic wounds are those that, even after three months, do not heal in a timely and ordered manner to restore anatomical and functional integrity. These wounds typically develop when a bacterial infection, metabolic abnormalities, poor circulation, unusual local pressure to the wound site, existence of neuropathy which causes loss of protective sensation, unresolved inflammation, and other severe impaired healing

processes such as lack of angiogenesis, epithelial migration, and cell proliferation, or an underlying condition impair the usual wound healing process (Agyepong et al., 2015).

In the industrialized world, the epidemiology and financial impact of chronic wounds are well documented (Järbrink et al., 2017). The clinical management of chronic wounds accounts for 2-4% of the overall health care costs in Scandinavia, which serves as evidence of this fact (Sen et al., 2009). About 3 to 6 million Americans suffer from non-healing wounds, with adults 65 years of age and older making up 85% of these occurrences. The estimated annual cost of healthcare for non-healing wounds is more than 3 billion dollars (Mathieu et al., 2006, Menke et al., 2007).

Wounds are a significant issue in developing nations because they frequently involve serious consequences and expensive therapy (Shenoy et al., 2011). In underdeveloped nations like Sub-Saharan Africa and South Asia, about 1-2% of the population endures a chronic wound at some point in their lives. According to reports, the community's prevalence of chronic wounds was 4.5 per 1,000 residents, compared to 10.5 per 1,000 residents for acute wounds. Patients who are older than 60 years old are more commonly affected by these wounds. The primary contributor to this issue is the lack of hygiene standards in some third-world nations (Siddiqui and Bernstein, 2010, Sasidharan et al., 2010).

1.2. Physiology of Wound Healing

The mending process of damaged skin and other soft tissues is known as wound healing (Nayak et al., 2007). The interaction of various cell types and their byproducts occurs during the crucial physiological process of cutaneous wound healing (Shaw and Martin, 2009). An attempt is made to repair the lesion that local aggression caused very early in the inflammatory stage. They ultimately lead to both regeneration through the processes of cell proliferation and subsequent differentiation using pre-existing tissue cells and/or stem cells as well as repair, which entails the replacement of specialized structures created as a result of the deposition of collagen. Finally, they result in regeneration via the processes of cell proliferation and subsequent differentiation using preexisting tissue cells and/or stem cells, and repair, which ultimately results in regeneration (Eming et al., 2007). Phases of homeostasis, inflammation, proliferation, and remodeling are all part of the well-organized succession series of overlapping activities that make up the healing process (Figure 1) (Diegelmann and Evans, 2004, Shedoeva et al., 2019).

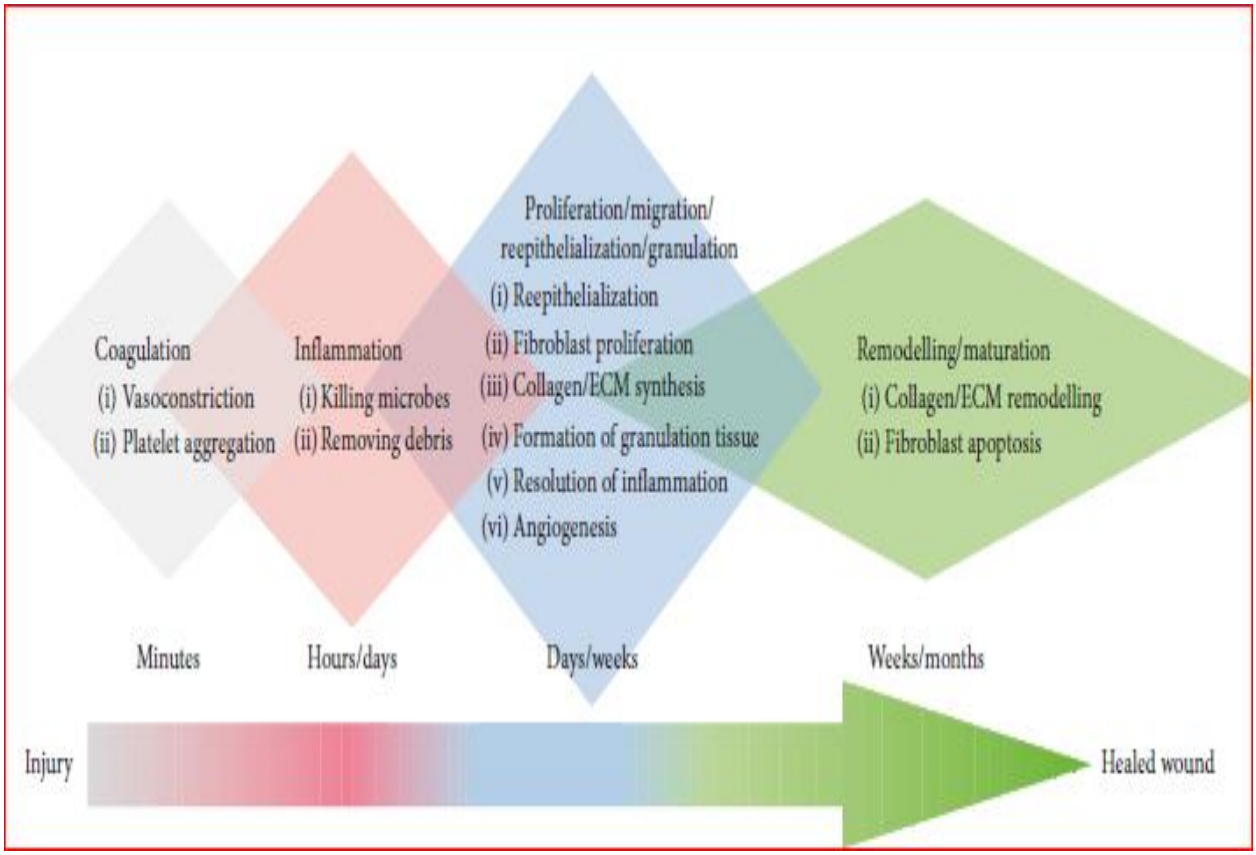


Figure 1: Wound healing cascade in human (Shedoeva et al., 2019)

1.2.1. Hemostasis Phase

Hemostasis begins with the production of clots after an injury, and is then accompanied by a variety of crucial processes, significant events, and vasoconstriction. Its distinguishing characteristics include vasoconstriction, platelet degranulation and aggregation, and fibrin deposition that leads to the production of a clot and the cessation of bleeding (Pakyari et al., 2013). During the proliferative phase, angiogenesis is stimulated by endothelial responder cells that are attracted by vasoconstriction and the hypoxia that results in the wound environment. The clotting system, in conjunction with chemical cues, initiates the plasma protein, complement, and kinin systems, which directly destroy pathogens and activate and control the inflammatory response. This is the first step in the process of starting a wound to heal (Velnar et al., 2009).

1.2.2. Inflammation

The humoral and cellular stage of inflammation is the following stage, and it is distinguished by a sharp increase in permeability of capillaries and migration of cells into the wound site. It's vital to act quickly to reduce inflammation during normal wound healing (Mathew-

Steiner et al., 2021). Typically, the goal of this phase is to create an immunological defence against invasive microorganisms (Sorg et al., 2017, Wang et al., 2018). During the initial phases of inflammation, neutrophils are the first cells to infiltrate the wound site,, followed by monocytes and lymphocytes, and they initiate the process of phagocytosis to kill and remove germs, foreign bodies, and wounded tissue (Rodrigues et al., 2019). Chemo attractant substances like platelet-derived growth factor (PDGF), transforming Growth Factor-β (TGF-β), interleukin-1 (IL-1) and other cytokines and growth factors are involved in the inflow of inflammatory cells. Within a few days, neutrophil activity gradually declines, and the neutrophils are totally eliminated through apoptosis and extrusion to the wound surface. The monocytes invade the site of injury and mature into macrophages during the later stages of inflammation. Macrophages secrete a variety of enzymes and cytokines, including collagenases, which debride the wound, ILs and TNF, which stimulate fibroblasts and encourage angiogenesis, and TGF, which promotes keratinocytes. Macrophages are crucial for wound healing.. Macrophages are crucial for wound healing (Marneros, 2013, Pandey et al., 2016). Macrophages also phagocytize bacteria, damaged tissue, and other phagocytes. The ability of the wound to heal and maintain its tensile strength will be compromised in the absence of macrophages. After skin damage, this phase typically takes 2 to 5 days to complete (He and Marneros, 2013, Naskar and Kim, 2020).

1.2.3. Proliferative Phase

In the absence of significant infection or contamination, the inflammatory phase is brief. Once the wound has been successfully cleaned of undesirable and devitalized material, the proliferative phase of healing takes over and lasts for three weeks after wounding (Schultz et al., 2011, Okur et al., 2020). Angiogenesis, granulation tissue development, collagen synthesis, and epithelialization are the major processes of this stage. The primary processes of this stage include angiogenesis, granulation tissue growth, collagen deposition, and epithelialization(Schreml et al., 2010, Sorg et al., 2017). Fibroblasts, epithelial cells, and endothelial cells are the three main cell types that are in charge of those activities (Sorg et al., 2017).

I. Angiogenesis in Skin Wound Healing

During the healing process, angiogenic capillary sprouts enter the fibrin or fibronectin-rich wound clot, and after a few days, they come together to form a microvascular network throughout the granulation tissue.. The density of blood vessels decreases when collagen

builds up in the granulation tissue to form a scar. Endothelial cells, the extracellular matrix (ECM) environment, and angiogenic cytokines like FGF, VEGF, TGF-b, angiopoietin, and mast cell tryptase interact dynamically (Tonnesen et al., 2000). Due to its basic impact from the first moment of injury until the end of the wound remodeling, neovascularization is a crucial factor in uncompromised wound healing (Demidova-Rice et al., 2012, Dulmovits and Herman, 2012).

II. Collagen Synthesis

Collagens have a significant role in every stage of the healing process for wounds. They are produced by fibroblasts and give all tissues strength and integrity. They are especially important during the proliferative and remodeling stages of healing. Collagen's biologic role as the main structural molecule binding tissues together in the body is determined by the different chemical alterations that have been made to it. There are at least 14 known collagen isoforms, each of which is produced by a different gene. Different tissues and tissue locations express different collagen isoforms (Robson et al., 2001). About 80% type I collagen and 20% type III collagen are present in the unwounded dermis. Contrarily, the granulation tissue of acute wounds expresses 30–40% type III collagen (Bailey et al., 1975)

III. Granulation

Fresh stroma, often referred to as granulation tissue, begins to enter the wound space four days after injury. The fibrin clot scaffold is replaced by new tissue that is abundant in hyaluronic acid, fibronectin, and other extracellular matrix (ECM) components, leading to granulation. The new stroma is endowed with numerous new capillaries that give it its granular look. From the surrounding tissue, fibroblasts go into the area of the wound, activate, start synthesizing collagen, and multiply. To create connective tissue, new capillaries will develop into this structure (Gross et al., 1995, Gurtner et al., 2008, Guo and Dipietro, 2010).

IV. Epithelialization

Epithelialization entails the resurfacing of the wound. The primary cell, known as a keratinocyte, is located around the edges of wounds and in the bulge region of the hair follicle. Epidermal stem cells are largely responsible for producing keratinocytes. Keratinocytes respond to signals from macrophages, neutrophils, and other elements within

hours of an injury. Later, in response to growth hormones and oxygen given by the recently created vascular network, they move forward in a sheet to rebuild the epidermis. However, because it is covering scar tissue, which lacks the same anchoring structure as normal tissue, the sheet will be significantly thinner in full-thickness wounds, rendering it more susceptible to injury from friction(Gurtner et al., 2008, Ramirez et al., 2014)

1.2.4 Remodeling

The remodeling phase of wound healing begins as the provisional ECM and type III collagen are replaced with type I collagen and the remaining cell types of the previous phases go through apoptosis (Reinke and Sorg, 2012). The maturity of the granulation tissue into mature connective tissue and/or a scar marks the end of the remodeling phase. Matrix metalloproteinases, which are released by fibroblasts, macrophages, and endothelial cells, carry out this process, which strengthens the healed tissue. As type I collagen is laid down, the wound's tensile strength significantly rises. Excess blood vessels begin to retract when granulation tissue starts to involute. The wound's ultimate appearance after healing depends on this phase, which lasts the longest. Concurrent collagen I production and collagen III lysis lead to this process, which is then followed by ECM remodeling (Caley et al., 2015). Because synthesis depends heavily on energy, any nutrient deprivation will tip the balance towards lysis and have an impact on the healing process. At this stage, excessive fibrosis causes keloid or hypertrophic scarring (Gurtner et al., 2008).

1.4. Factors Affecting Wound Healing

Numerous factors influence the healing of wounds. These elements can be broadly divided into local and systemic influences. Local factors are those that have an immediate impact on the wound's physical characteristics, whereas systemic factors are those that have an overall negative impact on a person's health or ability to recover from a condition. Depending on their source, they can also be divided into extrinsic and intrinsic categories. Intrinsic factors, on the other hand, are brought on by the patient's own physiology or condition and directly affect how well their body performs. Extrinsic variables, on the other hand, are brought on by the external environment and impede healing (Gurtner et al., 2008, Gonzalez et al., 2016). Local factors that affect wound healing include venous insufficiencies, infection, foreign bodies, and oxygenation. Age, gender, sex hormones, stress, ischemia, diseases such as diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia, and obesity, medications such as glucocorticoids, chemotherapy, alcoholism, and smoking, immune-

compromised conditions such as cancer, radiation therapy, and acquired immune deficiency syndrome (AIDS), and nutrition are some of the systemic factors (Nagori and Solanki, 2011, Vowden, 2011) .

1.5. Management of Wounds

Successful wound management calls for evaluation of the patient as a whole, not just the wound. Holistic wound management's main objective is to hasten healing while causing the patient the least amount of discomfort, pain, and scarring. According to Eming et al. healing must occur in a physiological setting that encourages repair and regeneration (Eming et al., 2014). The evaluation of the wound and the patient is the first step in wound treatment. This involves diagnosing the source of the wound, then working to improve the patient's health, particularly blood flow to the wound area. The wound must be properly debrided and dressed. The keys to a successful wound healing are the control of inflammation and infection. Drugs may be administered topically, systemically, or intravenously as part of medical wound care in an effort to speed wound healing (Lipsky and Hoey, 2009, Khan et al., 2022).

One of the most crucial techniques for wound treatment is topical antibiotic therapy. By shielding the wound from surface infection, antibiotics are said to facilitate normal healing. They are chosen because they can kill or stop the growth of harmful organisms while causing no harm to the tissue (Rodríguez et al., 2021, Odimegwu et al., 2008).

1.6. Medicinal Plants Used in the Management of Wound

Although the use of medicinal plants as a source for the treatment of illnesses has been documented by the earliest civilizations in China, India, and the Middle East over five million years ago, it is unquestionably a practice as old as humanity(Petrovska, 2012). According to estimates from the World Health Organization (WHO), 80% of people worldwide rely on herbal medicines for some part of their basic medical care. Approximately 21,000 plant species have the potential to be used as medical plants, according to the WHO (Anand et al., 2019). In addition, traditional medicine is used by 80% of people in underdeveloped Asia, Africa, and Latin American nations to satisfy their basic medical needs (Oyebode et al., 2016). Nearly 80% of the population in Ethiopia is thought to rely on traditional medicines as their primary and occasionally sole source of treatments, and 95% of traditional medicines in Ethiopia are made from herbs (Alemneh, 2021). The WHO 2014–2023 policy intends to strengthen the role of traditional medicine while highlighting the significance of promoting

and incorporating the use of medicinal plants in the health systems of its member countries (Sánchez et al., 2020).

There are numerous plants that have traditionally been used in Ethiopia to treat wounds. These include *Datura stramonium, Brucea antidysentrica, Croton macrostachyus, and Acokanthera schimperi (Taye et al., 2011); Achyranthes aspera (Fikru et al., 2012); Rumexa byssinicus (Mulisa et al., 2015), Solanum incanum, Commelin abengalensis L, and Ximenia Americana (Teklehaymanot and Giday, 2007); Acalypha volkensii Pax, and Amorphophallus gallaensi (Giday et al., 2009); Bersama abyssinica, and Cynodon dacytylon (Abera, 2014); Cordial africana, and Coffee Arabica (Regassa, 2018)* and many others have been employed to treat wounds and other illnesses in the traditional health care system of the country.

Traditional healers commonly employ the root and leaf of *Stephania abyssinica* to treat wounds (Seyoum and Zerihun, 2014, Mulugeta, 2017), but there hasn't been much scientific research on the plant's effects, and there are no studies on the differences in root and leaf activity.

1.7. Overview of the Experimental Plant

Stephania abyssinica belongs to the Menispermaceae family. It grows in grassland up to 3500 m altitude, typically in gloomy, moist areas (Semwal et al., 2010). In Stephania abyssinica, there are two recognised varieties: var. tomentella (Oliv.) Diels and var. abyssinica, both of which have a widespread range. While some plant parts in the var. tomentella are hairy, the var. abyssinica is nearly glabrous. In this study the var. abyssinica variety was studied. S. abyssinica is 2-3 m tall, with a liana wood base (Anyango, 2011). One of these plants, which is native to southern and eastern Africa, is claimed to have a number of medicinal uses. Yayit Hareg, Kib Kitel, or Etse Eyesus (in Amharic), and Hidda Kalaalaa (in Oromiffa) are the local names for *Stephania abyssinica* in Ethiopia (Teklehaymanot and Giday, 2007, Giday et al., 2009). The picture of the plant is presented in Figure 2. The hasubanan alkaloids: stephaboline, stephavamine, methaphamine, and stephabysine were discovered by phytochemical studies on *Stephania* used to treat inflammation, asthma, hyperglycemia, cancer, fever, and sleep disorders (Semwal et al., 2010). Anemia, rachitis, stomach aches, collitis, diabetes, and sterility are among the conditions that are treated in Africa with the juice from the stem or leaves of *Stephania abyssinica* (Masi et al., 2012). A root extract is used in eastern Africa to treat malaria and eliminate internal parasites. Additionally, it is

administered to treat tortoise bites and snake bites (Masi et al., 2012). S. abyssinica's roots and fruits are used to treat roundworms and as aphrodisiacs (Cousins and Huffman, 2002). The Zegie people of northern Ethiopia utilize *S. abyssinica* to treat headaches and stomachaches by taking the juice of the leaves and stems (Teklehaymanot and Giday, 2007) . Both the leaf and the root are traditionally used to cure wounds in several regions of Ethiopia. To speed up wound healing, the root of *Stephania abyssinica (*abyssinica variety *)* is crushed, combined with milk, and administered to the wound, and a small amount of the *Stephania abyssinica* leaf is pounded and applied to the wound (Seyoum and Zerihun, 2014, Mulugeta, 2017). The antihypertensive, and antimalarial, activity of the plant were confirmed by pharmacological studies (Alehegn et al.,2020, Fodem et al.,2021,). The crude extract and alkaloid and non-alkaloid fractions of the root were reported to have antimicrobial and antioxidant effect by *Chakraborty et al, and Washe and fanta* (Chakraborty et al., 2000, Washe and Fanta, 2016) . The anti-inflammatory and analgesic effects of the crude extract of the leaves were reported (Leyikun, 2015). Yiblet et al. screened the wound healing activities of the crude hydro-alcoholic extract of the root and its solvent fractions (Yiblet et al., 2022). Even though the leaves have a traditional claim for wound healing, this medicinal effect and the difference in wound healing activities with the root extract were not studied. The secondary phytochemical constituent concentration differences were also not studied.

FIGURE 2: Photograph of the *stephania abyssinica* taken from site of collection at the time of collection.

1.8. Rationale for the Study

Wounds continue to be a difficult clinical issue with frequent early and late consequences that contribute to morbidity and mortality (Chhabra et al., 2017). Particularly susceptible to infection, open wounds provide a point of entry for systemic infections, particularly those caused by bacteria.). The most serious medical issues that account for a major share of the population's illness, disability, socioeconomic crisis, and mortality are chronic or non-healing wounds (Järbrink et al., 2017).

Wounds can have an impact in three different ways: individually, on the healthcare system, and on the society. Patients suffer enormous psychosocial harm. In addition, they deal with disabilities that lower their quality of life and cause them to lose out on salaries and productivity. Furthermore, chronic wounds may progress to cellulitis, abscess growth, osteomyelitis, gangrene, sepsis, and perhaps malignant transformation (such as Marjolin's ulcer) (Menke et al., 2007).

In developing nations, people most frequently seek medical attention for skin conditions, particularly wounds. Numerous billions of dollars are spent each year on treating chronic wounds, according to medical expenses and lost productivity at work. The expensive and/or ineffective treatment regimen and its association to the recurrence rate contribute to these astounding expenditures (Dickson et al., 2010). Additionally, because controlling wound repair is a difficult and expensive program, research on medications that hasten wound healing is a growing field in contemporary biomedical science (Sengupta and Phytochemistry, 2017). Therefore, the WHO encourages researchers to study traditional medicines and/or promote traditional medicine as a source of more affordable, comprehensive medical treatment, especially in developing countries (Oyebode et al., 2016). Due to the fact that many of the common drugs used to treat wounds today are expensive and have a variety of side effects, such as toxic effects, allergies, and drug resistance (Monika et al., 2021), there is a need for less expensive, safer, and more readily available plant items that support the natural healing processes of the wound should be a concern. Currently, scientists view medicinal plants as a novel source of chemicals that heal wounds (Monika et al., 2021).

In traditional medicine, the leaf and root of *Stephania abyssinica* are often used for the treatment of various disease conditions, such as wound healing. A previous study showed that the hydroalcoholic extract of the root of *S. abyssinica* has a wound-healing effect (Yiblet et

al., 2022). To the best of our knowledge, the effect of the leaves of the plant on wound healing activity has not been investigated. There is a paucity of information on the comparative chemical composition of the leaf and the root of the plant.

An increased frequency of use of the roots of *S. abyssinica* in traditional medicine may gradually lead to a reduction in the biodiversity of the species. This can be explained by the fact that to get the roots, sometimes the whole plant is torn off, or, if the roots are partially torn off, the plant may die because of not having enough roots to support it. There are high conservation concerns when the roots of the tree are harvested (Fufa, K., 2021). Hence, there is a need to investigate the properties for justification of the use of specific parts of the plant (the root or leaves). It is important to carry out comparative studies to find out whether the use of the leaves, i.e., the renewable part of the plant, instead of its roots for wound-healing activity is more advisable. In this study, we investigated the differences in wound healing activity as well as compared the proximate composition of the phytochemical constituents of crude methanol extracts of the leaves and the roots of *S. abyssinica*. The study was based on the assumption that *S. abyssinica* contains similar secondary metabolites in the leaves and roots of the plant. The significance of this study is to promote the use of renewable parts of the plant and help preserve the plant's biodiversity.

2. Objective

2.1. General Objective

The aim of this study is to compare the wound-healing activity of 80% methanol crude extracts of *Stephania abyssinica* root and leaves in experimental mice.

2.2. Specific Objectives

- To evaluate the wound-healing activity of an 80% methanol crude extract of the leaf and the root of *Stephania abyssinica* on an excision wound healing model in mice
- To determine and compare the free radicals scavenging activity of the 80% methanol crude extract of the root and leaf of *Stephania abyssinica* using DPPH assay
- To determine and compare the quantity of phenols, flavonoids, and alkaloids of an 80% methanol crude extracts of the root and leaf of *Stephania abyssinica*

3. Materials and Methods

3. 1. Materials

3.1.1. Chemicals, Reagents and Drugs

Distilled water (Social Pharmacy and Pharmaceutics Laboratory, Addis Ababa University), white soft paraffin (Berkeley, California, USA), hard paraffin (BDH Chemicals ltd, England), cecostearyl alcohol (BDH Chemicals ltd, England) , Methanol (Carlo Erba, Italy), nitrofurazone ointment 0.2% (Shanghai General Pharmaceutical Co. LTD, China), ketamine hydrochloride (NEON Laboratories limited, India), diazepam (Roche, Switzerland), formalin (Ranchem industries, Ethiopia), indomethacin (Cadilla, Ethiopia), Normal Saline (Addis Pharmaceutical Factory, Ethiopia), hydrochloric acid (Sisco Research Lab., India), and Tween 80 (Uni-Chem, India) were used. All of the medications, chemicals, and reagents were of the required standard and analytical quality and were acquired from the proper vendors. .

3.1.2. Instruments and Apparatus

Sensitive digital weighing balance, dry oven, rotary evaporator, deep freezer, light , vacuum pump, mini orbital shaker, electrical hair clipper series 3000, water bath, mortar and pestle, ointment slab, sharp sterilized scissors, surgical threads with curved needles, forceps, surgical scalpel blade, Erlenmeyer conical flask, beaker, adhesive plaster zinc oxide, face mask, head cover, Whatman filter paper (number 1), gloves, cotton swabs, permanent markers, and graph papers made of translucent polythene were all utilized.

3.1.3. Plant Materials

The *Stephania abyssinica* roots and leaves were collected from the same plant and harvested under the same conditions in the summer from the area near Debre Linanos (104 kilometers from the capital), in the Oromia Region in central Ethiopia. At the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where the plant sample was stored with a specimen voucher number MG-001 for future reference, botanist Mr. Melaku Wendafrash performed plant identification and authentication.

3.1.4. Experimental Animals

Healthy adult Swiss albino mice of either sexes (25–30 g, and 6–8 weeks of age) were obtained from the department of Pharmacology and Clinical Pharmacy, Addis Ababa University's laboratory animal breading center. The animals were housed in a clean cage at

room conditions under 12 h light and dark cycles in a tidy cage with room temperature and 12-hour light and dark cycles. Prior to the start of the trial, the animals were given access to a regular laboratory diet, fresh water, and a 5-day acclimatization period. The animals were sacrificed at the end of the study using a high dose of ketamine and diazepam (Suckow and Gimpel, 2020). An ethical clearance was obtained from School of Pharmacy, College of Health Sciences, Addis Ababa University, ethical review committee with protocol number ERB/SOP/486/15/2023.

3.2. Methods

3.2.1. Extraction

After being removed from their natural environment, *Stephania abyssinica* roots and leaves were harvested. The fresh roots were then cleaned with tap water and broken up into smaller pieces while the fresh leaves were chosen and dried in the shade. The dried root and leaf were both ground into a coarse powder, and the resulting powder was macerated in 80% methanol for three days in a conical flask with constant stirring and shaking. The entire mixture was filtered twice through a muslin-covered funnel, and the filtrate was then allowed to flow through Whatman No. 1 filter paper. In order to thoroughly extract the plant elements and obtain additional extract, the residue was macerated twice more. The filtrates were mixed and concentrated by a rotary evaporator at 50 rpm at 40°C to remove methanol, and the concentrated extract was placed in a dry oven until dried (Belachew et al., 2020). The dried products of the extracts were stored in tight containers, and they were stored in a refrigerator until they were used for the formulation of ointments.

3.2.2.Yields of extraction

A total of 300 grams of leaves and 600 grams of roots of *S. abyssinica* were macerated with 80% methanol; residues of 45 g and 70 g were recovered, making the yields 15% and 11.6%, respectively, that were kept in the refrigerator until needed.

3.2.3. Ointment Formulation

A simple ointment made from the plant's 80% methanol extract was produced using the formula (Table 1) provided in the 1988 British Pharmacopoeia (BP, 1998). The reduced formula was used to make two root extract ointment preparations (each weighing 200 g), two leaf extract ointment preparations (each weighing 100 g), each comprising (5% and 10% w/w) of the extract, and a simple ointment without plant extract to act as a control (Deshmukh et al., 2009). The individual ingredients were melted in a beaker over a water

bath according to their descending sequence of melting points, with constant stirring until they were homogenous to make a simple ointment base. The mixture was then taken out of the water bath and stirred until it was cold. 10 g and 20 g of root extract were combined with 190 g and 180 g of the ointment base to prepare 5% and 10% medicated ointments, respectively, while 5 g and 10 g of leaf extract were blended with 95 g and 90 g of the ointment base (Ansel, 1985; Langley and Belcher, 2008). The extracted ointment was then placed in a clean container and kept until it was topically applied during the experiment.

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

Ingredients	Master formula (MF)	Reduced formula	
		Root	Leaf
Wool fat	50g	10g	5g
Hard paraffin	50g	10g	5g
Cetostearyl alcohol	50g	10g	5g
White soft paraffin	850g	170g	85g
Total	1000g	200g	100g

Dose Selection

In order to establish the dose, 2%, 5%, and 10% ointments of the root and leaf of an 80% methanol crude extract were applied to two mice per group in a pilot study utilizing an excision wound model. The two effective extracts, 5%w/w and 10%w/w, were then selected for the investigation.

3.2.4. Grouping and Dosing of Animals

Six groups of mice, each with six mice, were used for the excision model. Simple ointment was applied to the first group as a negative control. The 80% methanol leaf extract ointments were applied to the second and third groups in 5% and 10% concentrations, respectively. The fourth and fifth groups were treated with 5% and 10% ointments of the 80% root extract,

respectively. The six groups were treated with nitrofurazone (0.2 %) and served as a positive control.

3.2.5. Wound Healing Activity Test

The wound healing activity of *S. abyssinica* was evaluated by excision and incision models. In the excision wound model, various healing indicators, such as the rate of wound contraction, histopathologic analysis, and the duration of epithelialization, were monitored, whereas in the incision wound model, the skin's tensile strength was used.

Excision Wound Model

Ketamine and diazepam injections (1 ml/kg each) given subcutaneously were used to anesthetize the mice. This was followed by shaving the animals' back hair. Permanent marker was used to outline a 314 mm2 circle. Then, sharp, sterilized scissors were used to cut through the specified area's entire thickness. According to the appropriate grouping, as described in the section on grouping and dosage, equal amounts of ointments were applied to each group's wound once daily until full healing was achieved. The wound area was traced every two days until the wound had completely healed to ascertain the pace of wound closure (Charde et al., 2010). The wound was left open to the environment.

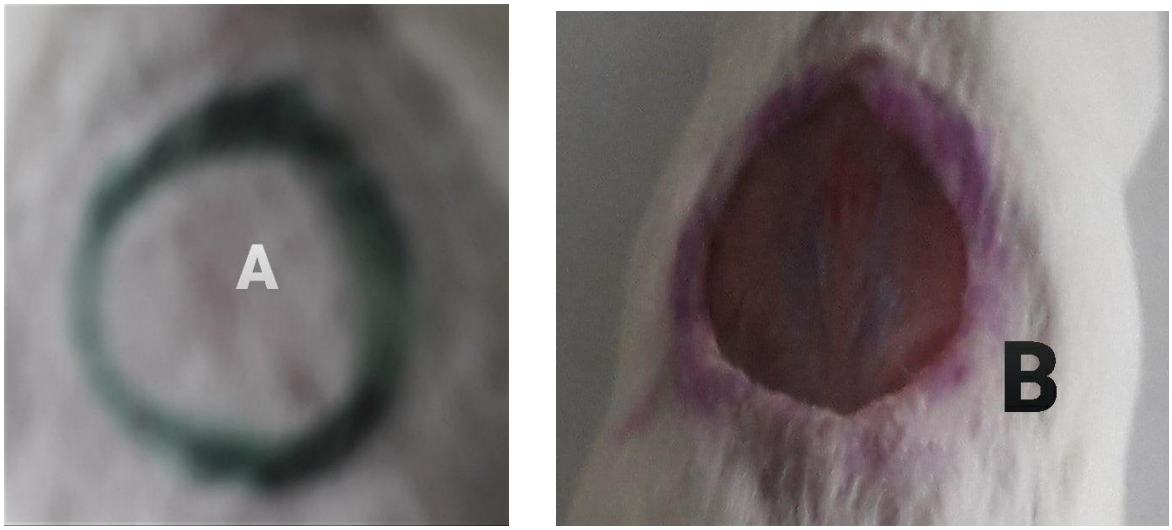


Figure 3 : Circularly marked area to be excised (A), excision wound on day zero (B)

Measurement of Wound Contraction

Using a transparent sheet and a permanent marker, the wound regions were measured in order to assess the wound healing progress. Considering that the wound's initial size (314 mm2)

was taken to be 100%, the evaluated surface was used to calculate the percentage of wound contraction, as shown below:

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

Epithelialization Time Measurement

The period of epithelialization was calculated as the number of days required for the falling off of the dead tissue remnants without any residual raw wound within twenty-three days (Wang et al., 2018).

3.2.6. Histopathological Analysis

A histopathological study was carried out according to the procedure of Kuo et al to verify the findings (Kuo et al., 2022). A senior pathologist from the Pathology Department of Addis Ababa University performed the analysis in a blind manner. After the mice were killed by an intraperitoneal injection of ketamine and diazepam (four times the anesthetic dose), the skin samples from each group were taken on the 13th day after the wounding (Beshir et al., 2016). Samples were fixed in 10% buffered formalin, treated, blocked with paraffin, sectioned into 5 micrometer sections, and stained with hematoxylin and eosin (HE) stains. A light microscope (Olympus CX41 attached to a Camera Digital Image Analyze System) was used to look at the stages of wound healing. Re-epithelization, fibroblast proliferation, collagen depositions, polymorphonuclear cells, mononuclear cells, and neovascularization were examined and rated as mild concentration (+), moderate concentration (++), and high concentration (+++) to assess the epidermal or dermal remodeling (Tumen et al., 2012).

3.2.7. Antioxidant Activity Evaluation

Free radical scavenging activity of the 80% methanol extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Paudel et al., 2021). The ability of the plant extracts to donate hydrogen atoms was assessed by the decolorization of a methanol solution of DPPH. In a methanol solution, DPPH produces a violet or purple color that, in the presence of antioxidants, fades to varying shades of yellow. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of extract in methanol at different concentrations (12.5–2000 μg/mL). The reaction mixture was completely vortexed and kept at room temperature for 30 minutes in the dark. At 517 nm, the mixture's absorbance was determined spectrophotometrically. The standard used was ascorbic

acid. The formula below was used to compute the percentage of DPPH radical scavenging activity: % DPPH radical scavenging activity = (A0− A1)/A0 × 100

Where A0 is the absorbance of the control solution containing all reagents except the extract and A1 is the absorbance of the DPPH solution containing the plant extract or the standard. Finally, percent inhibition was plotted against concentration, and from the graph, the 50% inhibitor concentration (IC50) was calculated. The absorbance of each concentration was measured three times, and mean was taken to plot the curve.

3.2.8. Phytochemical Screening

The presence of phytochemicals such steroids, anthraquinones, alkaloids, flavonoids, saponins, tannins, terpenoids, phenolic compounds, and saponins were evaluated in the crude leaf and root extracts using recognized testing methods.

Test for Anthraquinones

The extract was filtered after being vigorously shaken with 10 ml of benzene and 100 mg of the extract. After shaking, 5 ml of a 10% ammonia solution was added to the filtrate. It was deemed positive for free anthraquinones when pink, violet, or red hue appeared in the ammonia phase (Sasidharan et al., 2010).

Test for Alkaloid

One milliliter of an 80% methanol extract was divided across two test tubes. Wagner's solution, which was created by mixing 1.27g of iodine with 2g of potassium iodide, was added to one of the tubes, leaving the other tube unaffected for comparison. The presence of alkaloids was indicated by the formation of reddish-brown precipitate (Banu, 2015).

Test for Phenolic Compounds

One hundred milligrams of the extract were diluted in 1 ml of methanol, and three drops of a solution were added. This solution was created by mixing 1 ml of 1% $FeCl_3$ and 1 ml of 1% $K_4Fe(CN)_6$ right before the reaction. After then, a green-blue color was produced and seen (Prabhavathi, 2016).

Test for Flavonoids (Shinoda Reduction Test)

One-hundred milligram of the extract were dissolved in 5 ml of 50% methanol, divided into two test tubes, and then metallic magnesium and zinc were added to one test tube and five

drops of concentrated HCl to the other. When an orange or red color developed in either test tube, it was determined that flavonoids were present (Hmed et al., 2019).

Test for Tannins

After being diluted in 10 ml of distilled water, 0.25 g of the plant extract was filtered. To the filtrate, 1% aqueous iron chloride (FeCl3) solution was added. The emergence of a strong blue-green color indicated the presence of tannins in the test samples (Gilani et al., 2011).

Test for Terpenoids (Salkowski Test)

One milliliter of the extract and 2 ml of chloroform were mixed. The layer was then carefully formed by adding 3 ml of pure sulfuric acid. The presence of terpenoids was revealed by the interface's reddish-brown coloring (Gilani et al., 2011).

Test for Steroids

To create a diluted solution, the extract (0.25g) was dissolved in 0.25mL of dichloromethane. Three drops of concentrated sulfuric acid and 0.25 ml of acetic anhydride were then added to the solution. Steroids were present because a blue-green color developed (Ayoola et al., 2008).

3.2.9. Phytochemical Quantifications

Total Phenols Content Determination

The concentration of phenolic compounds in the extracts were determined spectrophotometrically by the modified Folin-Ciocalteu method (Singleton et al., 1999). For the preparation of a standard gallic acid solution, 2 mg of gallic acid was dissolved in 10 ml of methanol. Then 100, 50, 25, 12.5, and 6.25 µg/ml of gallic acid solutions were prepared through serial dilution from the original 200 µg/ml solution. To prepare the plant extract solution, 1 mg of the root and the leaf extracts were dissolved in 10 ml of methanol separately. One ml of the standard and extract solutions were poured separately into 10 ml test tubes, and 5 ml of distilled water and 0.5 mL of Folin–Ciocalteu (diluted with distilled water at a ratio of 1:10 v/v) were added to each test tube. After 5 minutes, 1.5 ml of Na_2CO_3 was added into each test tube, and the volume was adjusted to 10 ml. Finally, after 90 minutes of reaction time, the absorbance of each solution was measured at a wave length of 760 nm using UV spectrophotometry against the blank solution. The total calculated phenolic

compound content of the extracts was expressed as mg of gallic acid equivalent per gram of dry extracts. Each experiment was performed three times, and the absorbance average was calculated.

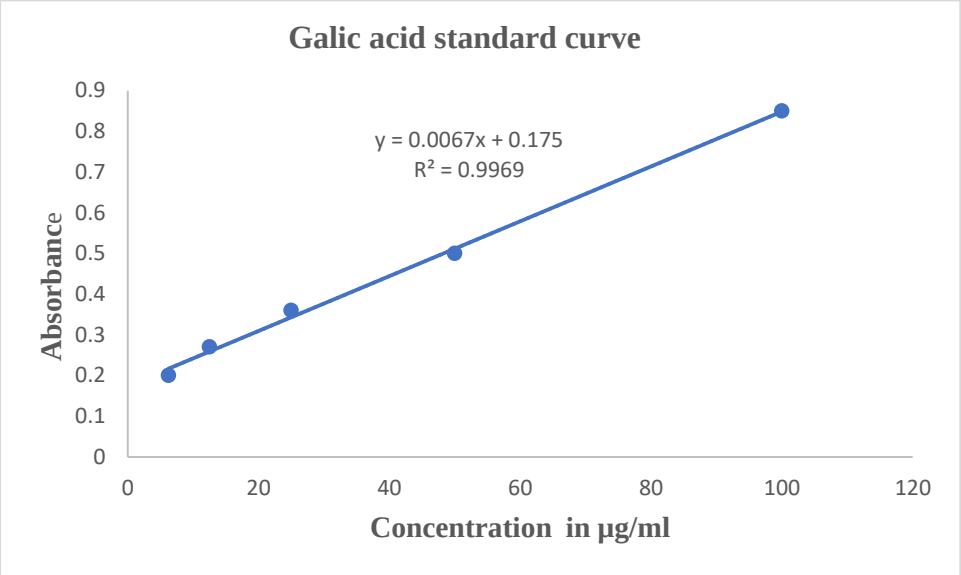


FIGURE 4 : Calibration curve of Gallic acid for total phenols content determination

Total flavonoids Content Determination

Total flavonoids content was determined based on the method previously described by (da Silva et al., 2015). For the preparation of standard quercetin solutions, 5 mg of quercetin was dissolved in 5 ml of methanol. Then 1, 0.50, 0.25, 0.125, and 0.065 mg/mL of solutions were prepared through serial dilution. One ml of the standard and extract solutions were transferred into a 10ml test tube, and 0.3 ml of 5% $NaNO_2$ was added to each test tube. Five minutes later, 0.3 ml of 10% AlCl3 was added, and after ten minutes, 2 ml of 1M NaOH was added into each test tube. Finally, after 30 minutes of reaction time, the absorbance of each solution was measured at a wave length of 510 nm using UV spectrophotometry against the blank solution. The amount of computed flavonoid compounds in each gram of dry extracts was represented as mg of quercetin equivalent. Each experiment was performed three times, and the absorbance average was calculated

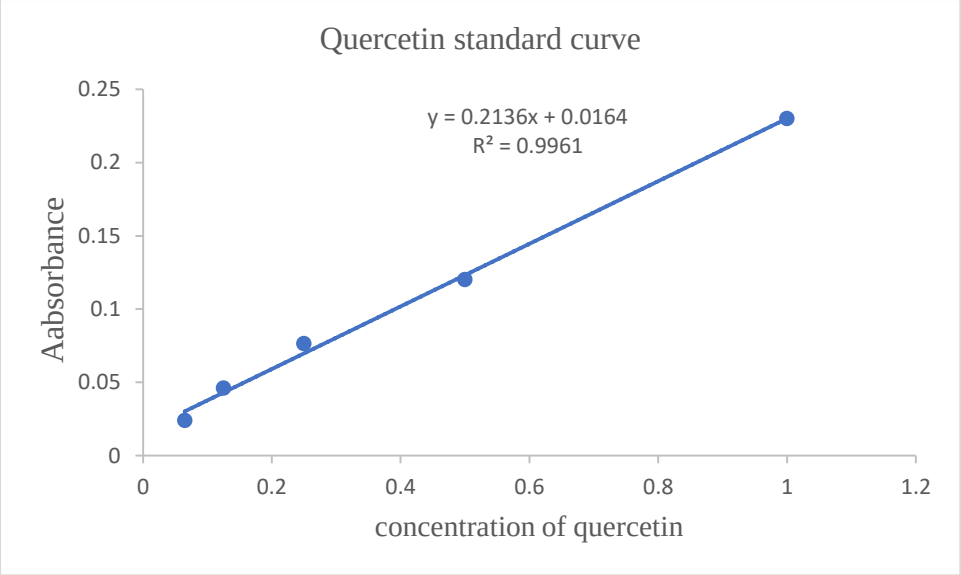


FIGURE 5: Calibration curve of Quercetin for total flavonoids content determination

Total Alkaloids Content Determination

The total alkaloid content of the 80% methanol extract of the root and leaf was determined based on the method previously described by (Ajanal et al., 2012) with minor modification. Phosphate buffer and bromocresol green (BCG) solutions were used in the experiment. The experiment made use of bromocresol green (BCG) solutions and phosphate buffer. The BCG solution was made by heating 17.45 mg of bromocresol green in 0.75 ml of 2N NaOH, then adding and thoroughly blending 1.25 ml of pure water. The final 250 ml of the mixture was then filled with distilled water.

To make the phosphate buffer, separate solutions containing 10.51 g of citric acid and 17.9 g of sodium phosphate (Na_2HPO_4) were dissolved in distilled water and then each solution's final volume was brought to 250 ml by adding more distilled water. A phosphate buffer solution (PH, 4.7) was then created by combining the two solutions. In order to separate the alkaloids, 2 ml of the extract solution (1 mg/ml), produced in methanol, were combined with 2 ml of 2N HCl solution. 1 ml of the filtrate was transferred using a separator funnel, and it was twice washed with 5 ml of chloroform. The pH of the residual solution was then adjusted to neutral by adding a 0.1 M NaOH solution after the chloroform extract was discarded. The neutralized solution was combined with 5 ml of BCG and 5 ml of buffer solution (PH, 4.7), rapidly shaken, and extracted with 4 ml of chloroform. The extract was then diluted to a final level of 10 ml and put into a 50 ml Eppendorf tube. The absorbance of the chloroform extracts was measured at a wavelength of 470 nm using a UV Spectrophotometer (Jenway

Model 6500, England). The same process was used on the methanol-based blank solution. Three copies of each procedure were completed. By deducting the average absorbance of a blank solution from the standard and extract solutions, the total alkaloid content was determined using the standard curve for atropine.

A series of serial concentrations of the standard atropine solution (120, 60, 30, 15, and 7.5 g/ml) were produced in methanol in order to create a calibration curve. After being diluted by 1 ml, the atropine solution was added to the separator funnels. The filtrates were then combined with 5 ml of phosphate buffer (PH, 4.7) and 5 ml of BCG solution, and the mixture was agitated with 4 ml of chloroform. After that, the chloroform extracts were collected in a 50 ml Eppendorf tube and its final volume was adjusted with chloroform to 10 ml. The absorbance of atropine chloroform extracts at 470 nm was measured using a spectrophotometer (Jenway Model 6500, England). The calculated total alkaloid results were expressed as mg of atropine equivalent per gm of dry extracts.

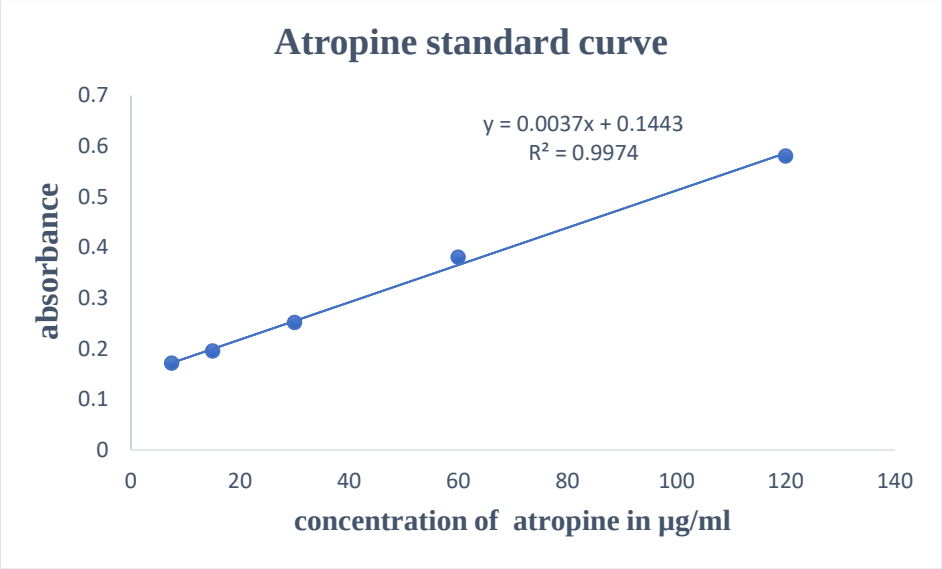


FIGURE 6: Calibration curve of Atropine for total alkaloid content determination

3.2.10. Statistical analysis

The experimental results were expressed as mean ± standard error of the mean (SEM), and the analysis was done using SPSS version 20. A test of statistical significance was carried out by employing one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test. The analysis was performed with a 95% confidence interval and $P < 0.05$ was considered statistically significant.

4. Results

4.1. Wound Healing Activity

Excision Model

Wound Contraction

When compared to the negative control, the *S. abyssinica* leaf and root extracts at 80% methanol significantly increased the percentage of wound closure (Table 2, Figure 8). In groups that received the standard medication (0.2% nitrofurazone) and 10% root extract beginning on day 4 after wounding, significant wound contraction ($p < 0.001$) was seen. Mice given the 10% (w/w) leaf extract and the 5% (w/w) root extract ointment showed considerable ($p < 0.01$) wound contraction beginning on day eight when compared to the control group. In comparison to the 5% and 10% leaf extracts, the 10% root extract significantly increased the percentage of wound contraction on days 4 and 6, as well as on days 4, 6, 14, and 16 following wounding ($p < 0.05$).

However, in terms of wound closure, there was no statistically significant difference between the 5% root extract and the 10% leaf extract. The percentage of wound closure between the standard treatment and the 10% root extract was also not statistically different. On the 10th, 12th, and 14th days, respectively, the 10% root extract experienced the highest rates of wound contraction (75.3, 92.7, and 100%). On days 12, 14, and 16, the standard medication's maximum rate of wound contraction was 92.6, 99.5%, and 100%, respectively.

TABLE 2: The effect of topically applied ointments containing an 80% methanol extract of s. abyssinica root and leaves on excision wound contraction.

Wound area in mm2 (Values in parenthesis represent percentage of wound closure)										
Group	Day2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
Simple ointment	294.44±3.03 (6.23%)	268.14±7.71 (14.61%)	245.87±3.69 (21.70%)	218.94±9.93 (30.27%)	180.24±11.81 (42.60%)	153.82±12.06 (51.27%)	86.74±8.01 (72.4%)	53.60±5.65(82.93%)	43.61±5.65 (86.11%)	21.26±3.10 (93.23%)
5%leaf extract	292.25±2.4 (6.93%)	257.36±8.06 (18.4%)	214.43±10.93 (31.71%)	162.71 ±12.67[a1] (48.18%)	122.18 ±13.10[a1] (62.36%)	86.20±15.47[a1] (72.55%)	46.93±13.23 [a1] (85.05%)	20.95±8.80[a3](93.33%)	8.83 ±5.36 [a3] (97.19%)	00 [a3c3e3] (100%)
10%leaf extract	288.50±1.73 (8.12%)	255.14±7.60 (18.75%)	206.33±9.07 (34.29%)	155.00±8.46[a1] (50.64%)	107.66±7.42[a1] (65.71%)	61.12±6.32[a2](80.54%)	31.47±3.51 [a2] (89.98%)	8.35±2.33[a2](99.26%)	00 [a2] (100%)	00 [a2] (100%)
5%root extract	287.15±1.43 (8.55%)	256.31±9.54 (18.37%)	196.90±21.89 (37.30%)	153.70 ±14.28 [a1] (51.05%)	110.83 ±17.55 [a1] (64.70%)	54.67±16.32 [a2] (82.59)	30.67±3.19[a3] (90.23%)	15.43±4.04[a3](95.09%)	2.83 ±1.83 [a3] (99.10%)	00 [a3] (100%)
10% root extract	286.93±1.00 (8.6%)	198.37±10.11 [a3c3e3f2] (36.83%)	144.94± 13.57[a3c1e1] (53.84%)	121.65±14.52 [a3] (61.26%)	77.55±12.39[a3] (75.30%)	22.92±4.39[a3e1] (92.7%)	5.42±4.00 [a3e1] (98.27%)	00[a3e1] (100%)	00[a3](100)	00 [a3] (100%)

0.2% nitrofurazone	285.95 ±1.59 (8.9%)	191.00±9.81[a3c3e3f2] (39.17%)	143.47± 15.03[a3c1e2] (54.3%)	122.27±13.87[a3] (61.06%)	82.90±12.08[a3] (73.6%)	23.33± 3.48[a3e1] (92.57%)	1.58±1.02[a3c1e3] (99.5%)	00[a3e1] (100%)	00[a3] (100%)	00[a3] (100%)

n = 6 Swiss albino mice in each group; Values are expressed as mean ± SEM, one-way ANOVA followed by Tuckey's post hoc test; [a]against negative control, [c]against 10% leaf extract, [e]against 5% leaf extract, [f]against 5% root extract $1p < 0.05$, $^2p < 0.01$, $^3p < 0.001$.

Repeated-measures ANOVA result

In the repeated-measure ANOVA, all extracts showed significant differences when compared to the simple ointment group. but a statistically significant difference was observed between 10% root extract and 10% leaf extract and 5% leaf extract at ($p < 0.01$) and ($p < 0.001$), respectively.

TABLE 3: Repeated-measures ANOVA for topically applied ointments containing an 80% methanol extract of *S. abyssinica* root and leaves on excision wound contraction.

Groups	Wound contraction
SO	156.67 ± 6.159
5%LE	121.19 ± 6.159 [a3]
10%LE	111.36 ± 6.159 [a3]
5%RE	110.85±6.159[a3]
10%RE	85.78 ± 6.159[a3c2e3f2]
NF	85.05 ± 6.159 [a3c2e3f2]

Repeated-measures ANOVA were used in the statistical analysis, and all values are expressed as mean ± SEM ; [a]against the negative control, [c]against 10% leaf extract, [e]against 5% leaf extract, [f]against 5% root extract $1p < 0.05$, $^2p < 0.01$, $^3p < 0.001$.

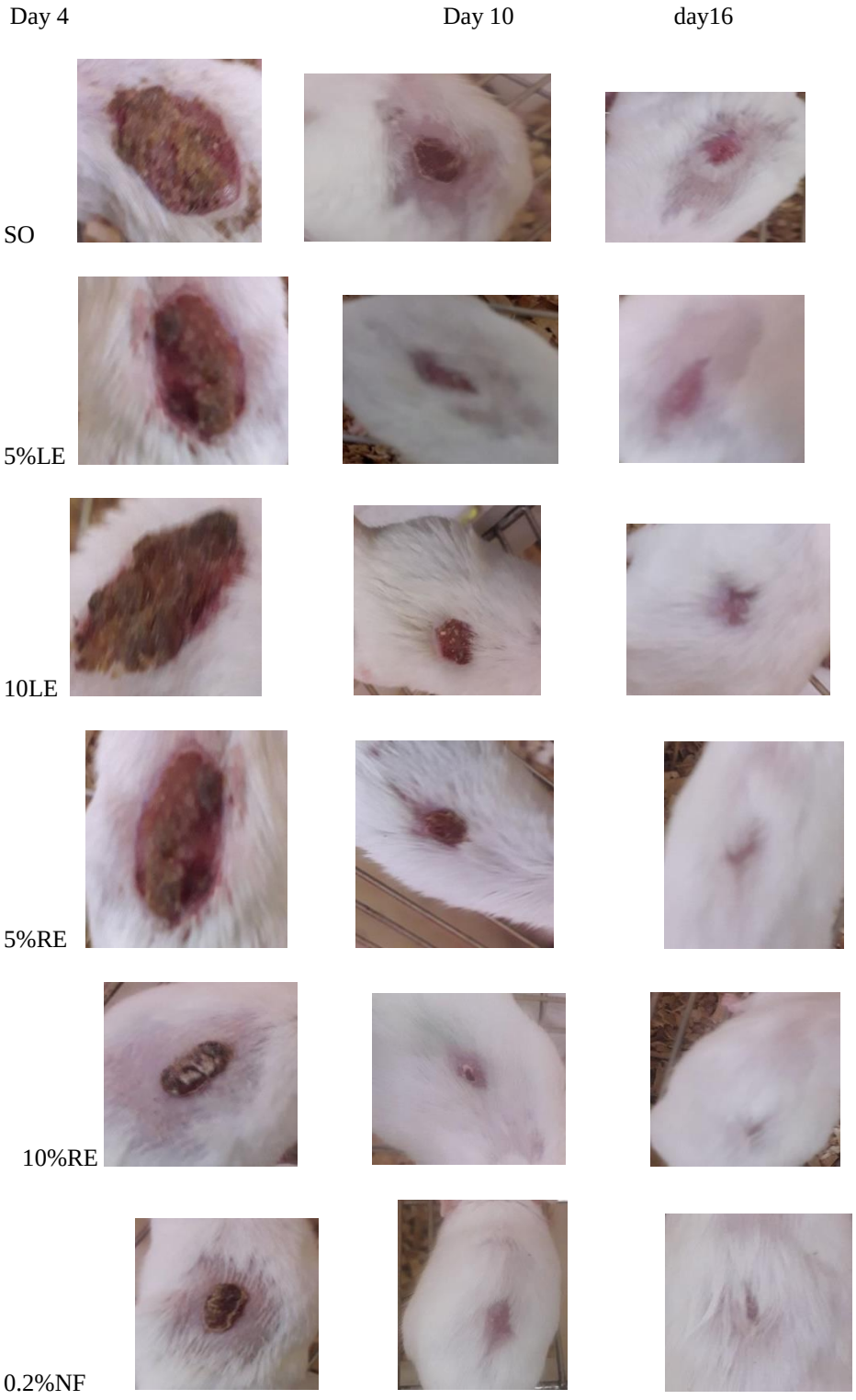


Figure7: wound healing progresses on excision wounds after application of the 80% methanol extracts of the roots and leaves of s. abyssinica.

RE: root extract, LE: leaf extract, NF: nitrofurazone, SO: simple ointment

Period of Epithelialization

Complete epithelialization time was significantly shorter ($p < 0.001$) in both the root and leaf extracts, and the standard drug treated groups as compared to the control group, but it was significantly shorter ($p < 0.001$) in the 10% root extract and standard drug as compared to both the 5% and 10 % leaf extracts and the 5% root extract (Table 3, and Fig. 8).

TABLE 4: Effect of topical application of the 80% methanol crude extract of the root and leaves of *S. abyssinica* on the period of epithelialization**.**

Groups	Complete Epithelialization period (number of days)
Simple ointment	22.00 ±0.37
5% leaf extract	18.67± 0.33 [a2]
10% leaf extract	16.83 ± 0.31 [a3e1]
5% root extract	17.33 ± 0.49 [a3]
10% root extract	13.67 ±0.42 [a3c3e3f3]
0.2% Nitrofurazone	13.83 ± 0.40 [a3c3e3f3]

n = 6 Swiss albino mice in each group; Values are expressed as mean ± SEM, one-way ANOVA followed by Tukey's post hoc test; [a]against negative control, [c]against 10% leaf extract, [e]against 5% leaf extract, [f]against 5% root extract 1p < 0.05, 2p < 0.01, 3p < 0.001**.**

Histopathological analysis

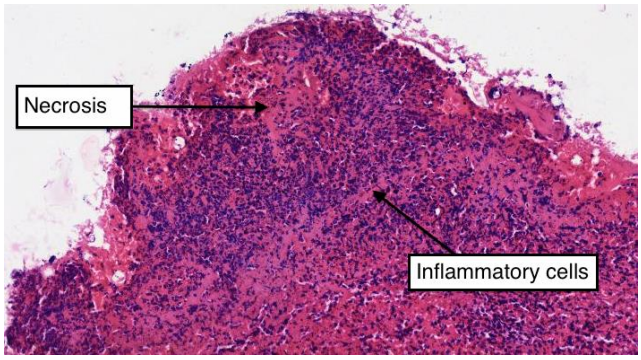
When comparing the time required for complete re-epithelialization, the group of mice that had received the 10% root extract and the standard dose of nitrofurazone ointment had comparable surface re-epithelialization. However, the group of mice that had received 10% root extract had by far the most wound remodeling during the 13-day period. As shown in Table 4 and Figure 9, this group had low inflammatory infiltrates, fewer surface blood vessels, and more organized collagen deposition. Meanwhile, the group of mice that had received 5% root extract was in the maturation phase of wound healing. On the other hand, the 10% leaf extract group did exhibit significant fibroblastic proliferation, and partial re-epithelialization with residual granulation tissue.

In contrast, the group of mice that had received either 5% leaf extract or a simple ointment did not achieve re-epithelialization. When comparing the two groups, the 5% leaf extract group had well-formed granulation tissue, hence being a step ahead of the simple ointment group that was still in the inflammatory phase of wound healing (Figure 9, Table 4).

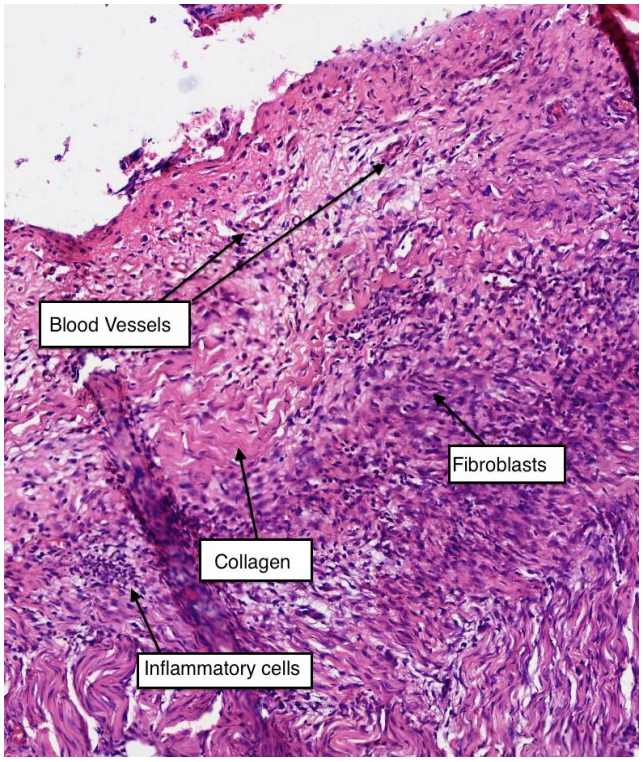
TABLE 5: Histological qualitative determination of wound healing processes and healing phases of 80% methanol crude extracts of s. abyssinica root and leaves

Groups	PMN	MN	NV	FB	CD
Simple ointment	+ + +	+ + +	++	+	+
5%w/w leaf extract	+ +	+ +	+ ++	+ ++	++
10%w/w leaf extract	+ +	+ +	+ +	+ ++	+ ++
5%w/w root extract	+	+ +	+ +	+ +	+ ++
10%w/w root extract	+	+	+	+	+ + +
0.2%w/v Nitrofurazone	++	++	+ +	+ +	+ + +

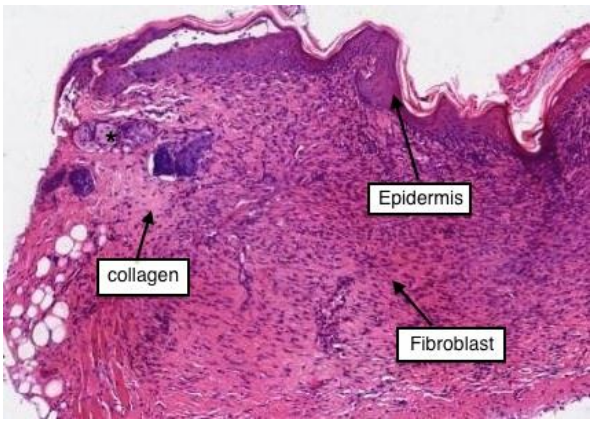
Low concentration (+), moderate concentration (++), and high concentration (+++) for epidermal and/or dermal remodeling. FP: fibroblast proliferation, CD: collagen depositions, MNC: mononuclear cells, PMN: polymorphonuclear cells, NV: neovascularization.



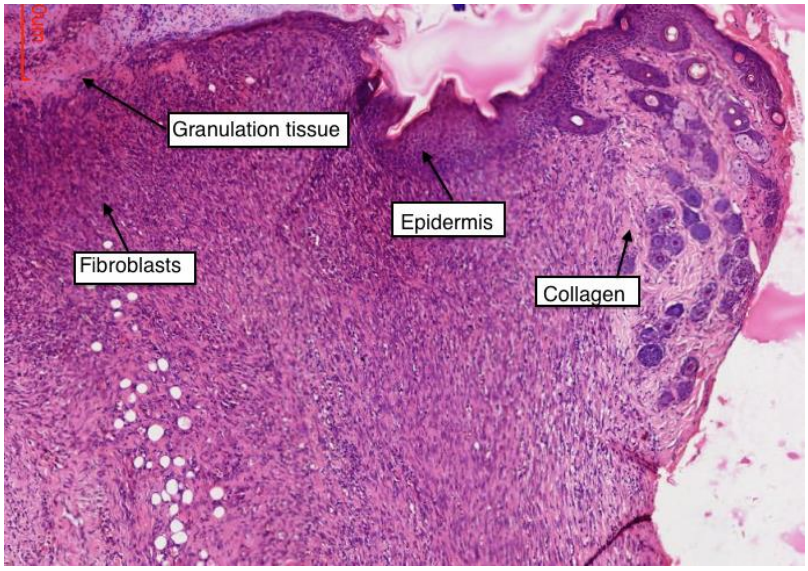
A) In this figure, fibrinoid necrosis admixed with polymorph nuclear cells and lymphocytes is seen. This is considered the inflammatory phase of wound healing, which occurs soon after injury.



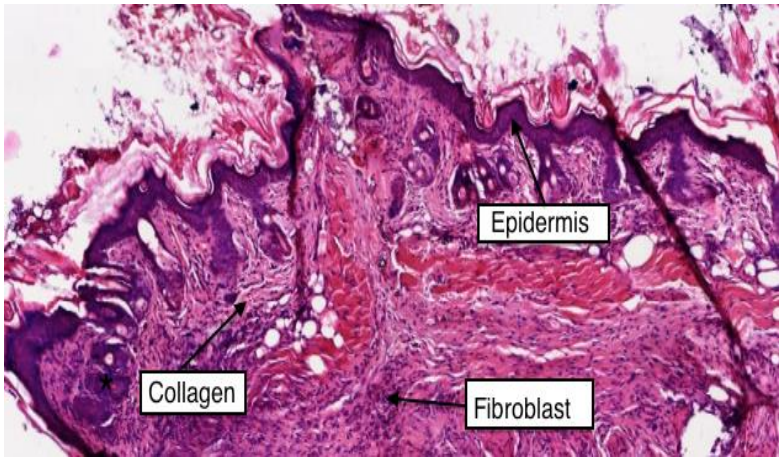
B) This figure illustrates the neovascularization of the wound bed accompanied by fibroblastic proliferation, collagen deposition, and inflammatory infiltrates (Granulation tissue). This corresponds to the proliferative phase of wound healing.



C) This figure shows the re-epithelialization of the wound bed with dense fibroblastic proliferation in the dermis. (Maturation phase)



D) In this figure, partial re-epithelialization, granulation tissue and fibroblastic proliferation can be seen (In the right upper corner of this figure, Adnexal structures and organized collagen deposition can also be seen)



E) In this figure, complete re-epithelialization of the epidermis is shown along with organized collagen deposition in the dermis. Inflammatory cells and vascular channels are significantly reduced. Dermal-based skin appendages have also regenerated. This phase of wound healing can be considered the remodeling phase. The remodeling phase of wound healing takes a prolonged period and will continue until the attainable tensile strength is achieved.

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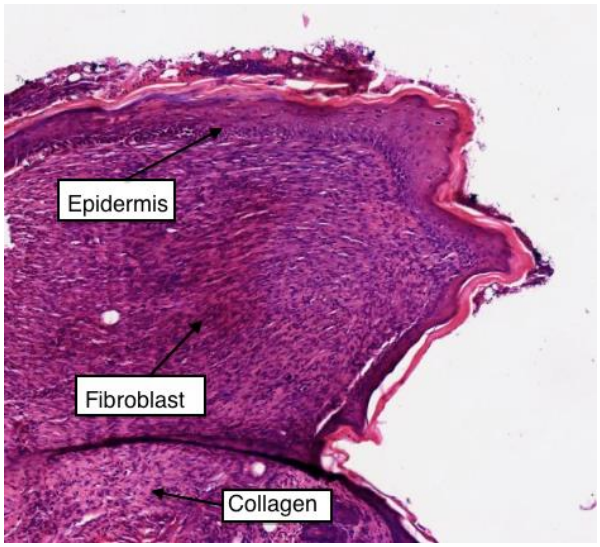

F) This figure shows the re-epithelialization of the wound bed with dense fibroblastic proliferation in the dermis. (Maturation phase)

FIGURE 8: Histological view of hematoxylin and eosin (he) stained skin sections of mice treated with: Simple ointment (A), 5% Leaf extract (B), 5 % Root extract (C), 10% Leaf extract (D), 10% Root extract (E), Nitrofurazone (F)

4.2. Antioxidant Activity

Regression equations for the extracts were determined. For the root extract, it was Y = 0.271X + 15, R2 = 0.9853; for the leaf extract, Y = 0.2027x + 7.853, R2 = 0.9967; and for the ascorbic acid, Y = 0.2444x + 33.542, R2 = 0.9915. Using these formulas, the IC_{50} values for the root extract, the leaf extract, and ascorbic acid were determined to be 129.15µg/ml, 207.93µg/ml, and 67.9 µg/ml, respectively.

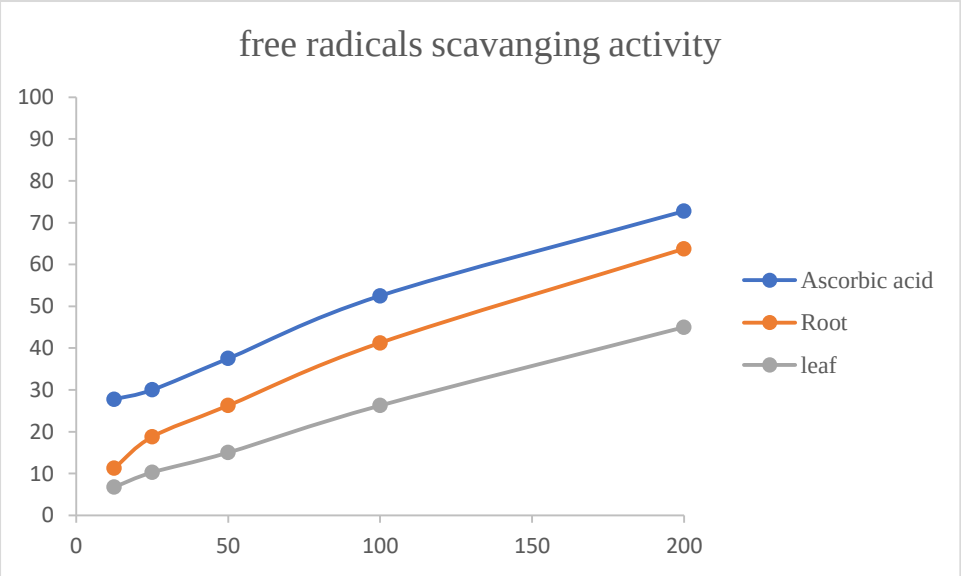

FIGURE 9: Antioxidant activity of 80% methanol extracts of the root and leaves of *S. abyssinica*

4.3. Phytochemical Screening

TABLE 6: Results of phytochemical screening of 80% methanol crude extracts of the root and leaves of *S. abyssinica*

Phytochemical components	**Results**	
	Root	**Leaf**
Alkaloids	+	+
Phenols	+	+
Flavonoids	+	+
Saponin	+	+
Tannins	-	-
Steroids	-	-
Terpenoids	-	-

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

4.4. Quantification of Total Phenol, Flavonoid, and Alkaloid Content

The preliminary phytochemical screening of 80% methanol extracts of *S. abyssinica* root and leaves showed the presence of alkaloids, phenols, flavonoids, and saponins, as shown in Table 7. Among them, the amounts of alkaloids, phenols, and flavonoids were expressed using the equation derived from the calibration curve as milligram equivalents of gallic acid, quercetin, and atropine, respectively, per gram of the sample.

In comparison to the leaf extract, the 80% methanol root extracts had higher levels of total phenolic, flavonoid, and alkaloid content.

TABLE 7: Total phenolic, flavonoid and alkaloid content of 80% methanol crude extract of s. abyssinica roots and leaves

Extracts	TPC(GAE/g)	TFC(QE/g)	TAC(AE/g)
Root	261.19	115.16	68.57
Leave	111.94	16.85	31.27

TPC: Ttotal phenolic compounds content, TFC: Total flavonoids content, TAC: Total alkaloids content, GAE: Gallic acid equivalent, QE: Quercetin equivalent, AE: Atropine equivalent

5. Discussion

For many years, both topical and oral preparations from medicinal plants have been used traditionally to aid in wound healing. It has been demonstrated that medicinal herbs can be very helpful in wound treatment, speeding up wound healing with little pain, suffering, or scarring for the patient. Some of these plants have direct effects on the processes of wound healing, while others have effects that are related to their anti-inflammatory, antibacterial, and antioxidant properties. In several of the medicinal plants used in wound treatment, a mix of these qualities is also conceivable (Sabale et al., 2012, Agize et al., 2022). One of the traditional medicinal plants used by communities in various regions of Ethiopia for its capacity to treat wounds is *Stephania abyssinica* (Seyoum and Zerihun, 2014, Mulugeta, 2017). Thus, in our study, we explored the wound healing, antioxidant, and anti-inflammatory activities of the root and leaf extracts. We found that both the root and leaf extracts of *S. abyssinica* were safe and had considerable wound healing, free radical scavenging, and anti-inflammatory activities. However, the root extract had higher activity than the leaf extract. Furthermore, the root extract had a higher concentration of secondary metabolites than the leaf extract, which might have contributed to its better activity.

The excision wound healing model is the best wound healing model for assessing epithelialization, contraction, and the creation of connective tissue in wound healing (Masson-Meyers et al., 2020). Hence, in this study, an excision wound model was used to establish the invivo wound healing potential of 80% methanol extracts of the leaves and roots of *S. abyssinica* at various phases. Epithelialization, contraction, and the formation of connective tissue are the major factors in wound healing. They are controlled by the biosynthesis and implantation of fresh collagen at the wound site. It also entails endothelial cell migration, which results in the neovascularization of connective tissues to produce ECM (Elzayat et al., 2018). Because it reduces the size of the wound and the amount of extracellular matrix required to restore damaged tissues, wound contraction speeds up the healing process. Contraction aids in the healing process by encouraging epithelialization. Epithelial cells go up from the basement membrane or from the edge of the wound to epithelialize (re-epithelialize). Contraction reduces the distance that keratinocytes must travel (Wolde et al., 2022).

In this study, 5% and 10% ointments of the 80% methanol extract of *S. abyssinic*a leaf accelerated wound contraction rates and shortened the epithelialization period in comparison

to the control group. However, the effect of the leaf extract was lower than that of the root extract and the standard drug. On day 14 after wounding, mice treated with 5% leaf extract and 10% leaf extract ointments had wound closure rates of 85.05% and 89.98%, respectively, whereas mice treated with the standard medication had wound closure rates of 99.5%. The wound contraction effect of the root extract of *S. abyssinica* was comparable to that of the standard drug. The wounds in the 10% root extract and the standard drug-treated group were completely epithelialized at 13.67 and 13.83 post-wounding days, respectively. In addition, these findings were evidenced by the histopathologic analysis of the healing wound. Less number of inflammatory cells, fibroblasts, and proliferating blood capillaries (angiogenesis) but more collagen fiber were present in the granulation tissue of the healing wound in the 10% w/w root extract treated groups compared to the 5% w/w extract. A recent study by *Yiblet et* al. showed that the crude root extract and aqueous fraction had better wound healing activity in the excision wound model (Yiblet et al., 2022). In both studies, the wounds were fully contracted in the 10% root extract-treated groups at the sixteenth day post-wounding, and the effect was almost comparable with the standard drug.

In the current study, the effect of the root extract was better than that of the leaf extract. This might be because of the difference in the phytochemical composition of the various parts of the plant. The concentration of secondary metabolites varies in different parts of the plant and depending on the location of the plants where they are collected (Wira et al., 2018, Oloo and Menge, 2020, Ghasemzadeh et al., 2018). In the present study, *S. abyssinica* root extracts contained more phenols, flavonoids, and alkaloids than leaf extracts.

Bacteria can impede the healing of wounds by secreting toxins, which often lead to local necrosis and upset the delicate balance of essential inflammatory mediators required for healing (Abeje et al., 2022). Wound infection is mostly caused by the species *Streptococci, Pseudomonas aeruginosa, and Staphylococcus aureus* (Guo and Dipietro, 2010). In a previous study, alkaloid fractions of *S. abyssinica* showed antibacterial and antifungal activity against common skin and soft tissue infection etiologic agents, including *S. aureus, P. aeruginosa, and C. tropicalis* (Chakraborty et al., 2000). The antibacterial properties of the *S. abyssinica* extract could be one of the potential pathways responsible for the wound healing effect. Alkaloids are linked to a number of pharmacological processes that may aid in wound healing, including analgesia, anti-inflammatory, antibacterial, antifungal, and vasoconstriction (Godfrey et al., 2020, Juneja et al., 2020).

Since phenolic compounds have demonstrated promising antioxidant activity in several in vivo and in vitro investigations, they have been highlighted as being among the secondary metabolites that may contribute the most to the antioxidant activity of plants (Kasote et al., 2015). Phenolic compounds have significant free radical scavenging (antioxidant) activity, which is influenced by their reactivity as hydrogen- or electron-donating agents, the stability of the radical produced as a result of the antioxidant, their reactivity with other antioxidants, and lastly, their metal-chelating properties (Eans et al., 2011, Zheng and Wang, 2001). The wound-healing activity of *S. abyssinica* could be due to the antioxidant activity of the phenolic compounds it contains.

Known flavonoid properties include the ability to scavenge free radicals, inhibit hydrolytic and oxidative enzymes, and have anti-inflammatory effects. Their ability to transfer free radical electrons, chelate metal catalysts, activate antioxidant enzymes, suppress oxidases, reduce alpha-tocopherol radicals, and transfer free radical electrons (Barku, 2019). In addition, flavonoids can scavenge nitric oxide, which, together with superoxide free radicals, creates the extremely harmful peroxynitrite (Sarkar et al., 2009). They can also block xanthine oxidase, a significant biological source of superoxide radicals that can react with hydrogen peroxide to create the even more harmful hydroxyl radical (Hirano et al., 2001, Cuyckens and Claeys, 2004). Both the root and leaf extracts displayed free radical scavenging activity in the present study, although the activity of the root extract was stronger (with an IC_{50} of 129.15 µg/ml) than the leaf extract (IC_{50} of 207.93 µg/ml). An 80% methanol root extract of *S. abyssinica* showed potential free radical scavenging action in previous investigations (Chakraborty et al., 2000) with IC_{50} values of 220 µg/ml. The difference in plant collection sites could be the reason for the disparity in IC_{50}%.

In the previous unpublished study by *Leikun T.* (Leyikun T, 2015), the 80% methanol extract of *S. abyssinica* leaf extract showed a significant anti-inflammatory effect. Inflammation typically has the role of clearing away debris, germs, and necrotic tissue from the area of an acute wound while also recruiting and activating fibroblasts in order to prepare the wound bed for healing. When inflammation is present for an extended period of time, wounds do not heal and typically progress to a pathological state (Boakye et al., 2018). The excessive and imbalanced inflammation may thus prolong the healing process. In addition, persistent inflammation, a sign of a non-healing lesion, puts the tissue at risk for developing cancer (Zhao et al., 2016, Sundaram et al., 2018). Therefore, the wound healing activity of

the 80% crude methanol extracts of *S. abyssinica* might be contributed in part by the anti-inflammatory effects of the extracts.

A study by *Yiblet et al.* showed that the root extract had a remarkable effect on the tensile strength of the skin (Yiblet et al., 2022). This increase in tensile strength might be related to factors like angiogenesis, fibroblast proliferation, increased collagen production, and fiber stability due to protein crosslinking. For effective wound regeneration, collagen deposition is crucial (Marinkovic et al., 2021). Due to the replacement of the fibrin-fibronectin clot generated during the initial stage of wound healing, the wounds strengthen and offer resistance to traumatic harm. Additionally, collagen serves as a template for the cellular components of angiogenesis and connective tissue synthesis to attach, grow, and differentiate (Sahu et al., 2021, Park et al., 2019). Therefore, based on the histopathological analysis reported (Figure 9), it could be inferred that the extract increases the collagen levels in the healing wound, which may adhere the wound edges together at the healed site and increase the wound's strength.

In this study, both the leaves and the roots showed significant wound healing differences as compared to the negative control. Even though the root extracts have a higher concentration of secondary phytochemical constituents and wound healing activity, using the leaf is better in terms of sustainable utilization and biodiversity conservation. Destructive harvesting typically leads to resource exhaustion and potentially the extinction of species of medicinal plants (Dias et al., 2012, Larsen and Olsen, 2007). Harvesting medicinal plants' roots and entire plants causes greater destruction than only taking their leaves, flowers, or buds. So that using the leaves of plants as medicine can be a safe substitute for herbal medications comprised of whole plants or roots (Wang et al., 2009).

6. Conclusion

The outcomes of this study demonstrated that the root extract has a higher wound-healing effect than the leaf extract. Furthermore, *S. abyssinica* root extract had a larger concentration of secondary metabolites than the leaf extract, and this concentration difference demonstrated a substantial difference in its wound healing ability. However, using the leaf is preferable in terms of sustainable use and conservation of biodiversity because both the root and leaf extracts have considerable wound healing effects.

7. Recommendation

The pharmacologically active component(s) underlying the wound healing and anti-inflammatory activity of the root extract have to be isolated, purified, and identified through additional studies. It is important to assess how the plant extracts work on chronic wounds, such as infectious and diabetic wounds.

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