HAWASSA UNIVERSITY

COLLEGE OF MEDICINE AND HEALTH SCIENCES

SCHOOL OF PHARMACY

EVALUATION OF WOUND HEALING ACTIVITIES OF 80% METHANOL EXTRACT OF *CLEMATIS HIRSUTA (PER* AND *GUILL)* LEAVES IN MICE.

BY: WOYESA ELEMA (B. PHARM)

OCTOBER, 2023

HAWASSA, ETHIOPIA

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A THESIS SUBMITTED TO THE SCHOOL OF PHARMACY, COLLEGE OF MEDICINE AND HEALTH SCIENCES, SCHOOL OF GRADUATE STUDIES, HAWASSA UNIVERSITY, HAWASSA, ETHIOPIA,
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HAWASSA, ETHIOPIA

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ADVISORS' APPROVAL SHEET

(Submission Sheet - 1)

This is to notify that the research paper entitled "**Evaluation of Wound Healing Activities of 80% Methanol Extract of *Clematis Hirsuta (Perr* and *Guill*) Leaves in Mice**, submitted in partial fulfillment of the requirements for the degree of Master's in pharmacology specialization, the post-Graduate Program, School of Pharmacy, and was carried out by Woyesa Elema ID. No. **GPPhaR/0011/14** under our direct supervision.

Therefore, we recommend that the student has fulfilled the requirements, and hence, can submit the thesis to the department.

1. Alemayehu Toma (PhD).

Signature_____ Date: _____

2. Mr.; Hailu Chare (Asst. Professor)

Signature_____ Date: _____

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(Submission Sheet - 2)

We, the undersigned, members of the Board of Examiners of the final open defense by **Woyesa Elema**, have read, and evaluated his thesis entitled "**Evaluation Of Wound Healing Activities Of 80% Methanol Extract Of *Clematis Hirsuta (Perr, and Guill)* Leaves In Mice**", and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the School of Graduate Studies (SGS) through the School Graduate Committee (SGC) of the candidate's department.

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Declaration Statement

I, **Woyesa Elema**, hereby declare that this MSc thesis, with the title "**Evaluation of wound healing activities of 80% methanol extract of *Clematis hirsuta (perr and guill)* Leaves in mice,**" is my original work. I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis, and compilation of the thesis. It has not been presented for the MSc degree at any other university, and all scholarly sources of material included in this thesis have been duly acknowledged.

Woyesa Elema (B.Pharm)	_____	_____
Name of Principal Investigator	Signature	Date

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List of Acronyms and Abbreviations

AIDS: Acquired Immune Disease Syndrome

ANOVA: Analysis of Variance

BFGF: Basic Fibroblast Growth Factor

BP: British Pharmacopoeia

CE: Crude Extract

CEO: Crude Extract Ointment

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

FGF: Fibroblast Growth Factor.

IHRERC: Institutional Health Research Ethics Review Committee

IL: Interleukin

IRB: Institutional Review Board

NFZ: Nitrofurazone

NSAID: Non-Steroidal Anti-Inflammatory Drug

OECD: Organization of Economic Corporations, and Development

PDGF: Platelet-Derived Growth Factor

ROS: Reactive Oxygen Species

SEM: Standard Error of the Mean

SO: Simple Ointment

TGF-β: Transforming Growth Factor Beta 4

TM: Traditional Medicine

TMPs: Traditional Medicine Practitioners

TS: Tensile of Strengths

WHO: World Health Organization

Abstract

Background: Wound healing is one of the most challenging health problems that needs correction and efficient wound management. The leaves of *Clematis hirsuta* (Perr and Guill) have been used to treat wounds as traditional medicine in different local communities.But no scientific investigation was conducted on the wound healing activities of *Clematis hirsuta* leaves using an in vivo study model. The aim of this study was to evaluate the wound healing activity of an 80% methanol crude extract of *Clematis hirsuta* (Perr and Guill) leaves in mice.

Methods: The leaves of *Clematis hirsuta* were crushed, dried, and macerated in 80% methanol. The resulting extract was concentrated, dried, and formulated into ointments at strengths of 5% w/w and 10% w/w. Acute dermal toxicity was evaluated in female mice at a dose of 2000 mg/kg using the 10% w/w extract. Wound healing activities were assessed using excision, burn, and incision wound models, comparing the 5% w/w and 10% w/w extracts, 0.2% w/w nitrofurazone, and a simple ointment. Parameters such as epithelialization period, wound contraction, histopathological analysis, and tensile strength were measured. Statistical analysis was conducted using a one-way ANOVA followed by a Tukey test, and significance was considered at $p < 0.05$.

Results: In mice, a 2000 mg/kg limit test dose of 10% w/w CE was safe. Topical application of 5% w/w CE and 10% w/w CE of *Clematis hirsuta* leaves possessed potential wound healing activity at ($p<0.037$) and ($p<0.001$), respectively, by increasing wound contraction rate in the excision wound model, while at ($p<0.04$) and ($p<0.001$) in the burn wound model compared to the negative control. Both 5% w/w CE and 10% w/w CE reduced the epithelialization period and possessed collagen deposit, angiogenesis, and fibroblast proliferation in both excision and burn wound models. In the incision wound model, both 5% w/w CE and 10% w/w CE significantly increased tensile strength ($p <0.001$) when compared to the negative control.

Conclusion: The 80% methanol crude extract of Clematis hirsuta (Perr and Guill) leaves showed significant wound healing activities in mice, validating its traditional use for wound treatment.

Keyword*: Burn wound model, Clematis hirsuta, Excision wound model, Incision wound model, wound model.*

1. Introduction

1.1. Background

Wound is a break in the epithelial integrity of the skin, causing loss of cellular, and anatomical or functional continuity of living tissue *(*Segewkal *et al.*, 2013; Mulisa *et al*., 2015*).* Dermal wounds are frequently brought by surgery, trauma, chemicals, or illnesses including diabetes, and gastric ulcers (Befekadu *et al*., 2022). Intentionally inflicted dermal wounds can manifest as either excisional or incisional injuries. Excisional wounds involve the removal of a portion of the skin, while incisional wounds result from surgical incisions made with a scalpel. Additionally, dermal wounds can also be caused by various factors such as physical trauma, exposure to chemicals, microbial agents, or immunological responses (Obagu & Ajiboso, 2023).

Wounds represent significant global health concerns and are often associated with costly and inadequate treatment approaches. Based on estimates, approximately 8.2 million individuals worldwide suffer from wounds, including excisions, burns, and cuts, which, if left untreated, can potentially lead to fatal outcomes (Praveen *et al.*, 2022). Open, closed, puncture, and burn wounds are among the different classifications of wounds, based on the type of injury that caused them. Wounds are further categorized as either acute or chronic, depending on the duration required for healing. Chronic wounds are frequently associated with underlying pathological conditions that hinder and prolong the recovery process, whereas acute wounds typically heal through the normal processes of wound healing (Abeje, 2022).

The annual estimated number of burn victims in the United States exceeds 1.25 million, while the population suffering from non-healing wounds surpasses 5 million. Individuals with chronic wounds commonly face shared challenges, including infection, dehiscence, and problematic scarring (Tessema, 2021)**.** Globally, chronic wounds are more common and cost so much money annually that most of the developing countries can’t afford their treatments *(*Jean Dieu *et al*., 2023).

The biological process of wound healing is a complex event encompassing inflammation, new tissue formation, and remodeling. This process aims to restore the skin to its normal state of balance and integrity. It involves a series of intricate mechanisms that work together to promote healing and maintain the barrier function of the skin *(*Zeghad *et al*., 2023).

The financial burden of non-healing or chronic wounds is substantial, reaching a staggering annual cost of over $3 billion in healthcare expenses. As the global population continues to age, the prevalence of wounds such as cuts, pressure sores, diabetes-related ulcers, burns, gastric ulcers, and duodenal ulcers persists. These wounds pose ongoing challenges and necessitate effective management strategies to address their impact on individuals' health and healthcare systems (Borges *et al.,* 2021) Wounds have a significant impact on healthcare costs for patients, families, and healthcare organizations. Effective wound management is essential to mitigate these financial burdens (Befekadu *et al*., 2022). Although many wounds heal on their own, other wounds, such as those that are large or have a lot of necrotic tissue and infection, require medical intervention (Negash *et al.*, 2020).

According to the World Health Organization (WHO), plant-derived remedies have been utilized for centuries by 80% of the global population, particularly in developing nations, for the treatment of wounds (Andargie *et al*., 2022). Compared to approximately 1–3% of modern medications, almost one-third of all traditional medicines were used to treat wounds and skin conditions (Borges *et al.*, 2021).

The presence of natural components within medicinal plants has the potential to modulate various aspects of wound healing, including coagulation, inflammation, epithelialization, collagen formation, and wound contraction. Additionally, these compounds exhibit immune-regulatory properties and help regulate inflammatory responses (Liang *et al*., 2020). Currently, there is a significant focus in medicinal herbal research to identify molecules that possess a broad spectrum of pharmacological actions, emphasizing their potential therapeutic value in wound healing *(*Nigussie *et al.*, 2021). Polyphenol components such as flavonoids and tannins play a beneficial role in wound healing by reducing lipid peroxidation, thereby preventing cellular damage and enhancing the survival of collagen fibers (Vitale *et al.*, 2022).

Numerous studies suggest that the wound healing properties of traditional medicine may be attributed to its antioxidant, antibacterial, anti-inflammatory, DNA and protein synthesis promotion, epithelial cell proliferation, collagen synthesis, and angiogenesis stimulation effects TM (Thao., 2020). Ethiopia, with its vast array of plant species (over 7,000 higher plant species, 12% of which are endemic), serves as a valuable source of plant extracts that hold potential health benefits *(*Nigussie *et al.*, 2020*)*. The majority of conventional medications currently

available for treating various disorders are derived from natural sources that are relatively non-toxic. These medications are composed of complex molecules that can interact with multiple targets (Nigussie *et al.*, 2021).

1.2. Wound Healing Cascade

Wound healing remains a challenging clinical concern, encompassing distinct and interconnected phases: hemostasis, inflammation, proliferation, and remodeling. Therefore, effective wound treatment is of paramount importance. The field of wound care has witnessed significant attention focused on the advancement of innovative therapeutic modalities and technologies to address both acute and chronic wounds *(*El-elimat *et al*., 2023*).*

The wound-healing process is complex and involves a variety of specialized cells, such as macrophages, platelets, fibroblasts, and epithelial and endothelial cells. It involves a series of interactions between different cell types, glycoproteins, proteins, chemokines, growth factors, cytokines, enzymes, and the extracellular matrix (ECM) (Ayman Alhazmi *et al*., 2022).

1.2.1. Hemostasis

Following an injury, the initial stage of wound healing, known as hemostasis, occurs immediately. Platelets are activated, leading to adhesion and vasoconstriction at the site of injury, effectively stopping bleeding. When exposed to extravascular collagen, platelets become activated. The resulting clot not only acts as a temporary barrier against pathogens and fluid loss but also serves as a reservoir for bioactive substances and antimicrobials. It provides a temporary extracellular matrix that supports immune cell migration and infiltration, initiating the tissue repair process. Hageman Factor XII, in addition to limiting blood loss, triggers the coagulation cascade. Growth factors like platelet-derived growth factor (PDGF), transforming growth factor beta 4 (TGF-β4), fibroblast growth factor (FGF), and epidermal growth factor (EGF) secreted by platelets are crucial for the migration of neutrophils and monocytes to the wound site, initiating the healing process during the inflammatory phase (Agyare *et al*., 2015; Schultz *et al*., 2018).

1.2.2. Inflammatory Phase

After hemostasis, the inflammatory phase commences, typically lasting for 0-3 days. This phase serves to halt further blood loss through vasoconstriction. Once bleeding ceases, blood vessels in the wound dilate, facilitating the entry of fluid-containing cells involved in the healing process.

Common signs of inflammation, such as warmth, swelling, redness, and pain, become apparent around the wound approximately 10 to 15 minutes after the incident. These symptoms trigger the activation of prostaglandins and histamine at the site of injury (Obagu, E. & Ajiboso, 2023*)*

In response to damage signaling, various immune cells including neutrophils, macrophages, monocytes, and lymphocytes migrate to the wound site to prevent microbial infection. This phase is characterized by the release of growth factors and cytokines such as tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor (TGF), as well as interleukins (IL-1, IL-6, IL-17). Additionally, antimicrobial species are released, and immune cells secrete signaling molecules that amplify immune responses. Activation and induction of keratinocytes, along with the production of damage-associated molecular patterns, free radicals, and reactive molecular species, play crucial roles in recruiting immune cells to the site of injury *(*Praveen *et al.*, 2022).

1.2.3. Proliferative Phase

In the proliferative phase, which can span from 3 to 10 days, the formation of granulation tissue occurs, providing a foundation for epithelialization. This phase of wound healing focuses on three main objectives: generating tissue granulation, rebuilding the vascular network, and covering the wounded area. Angiogenesis, the development of granulation tissue, epithelialization, and wound contraction, are key features characterizing the proliferative stage of wound healing (Matsuete *et al*., 2023). Granulation tissue consists of fibroblasts and newly formed blood vessels. To initiate neovascularization, growth factors bind to receptors on endothelial cells of existing blood vessels, triggering an intracellular signaling cascade. During this process, endothelial cells proliferate and migrate, commonly known as "sprouting." The newly formed sprouts form small tubular channels that connect with each other, forming a vascular loop. Eventually, pericytes and smooth muscle cells are recruited to stabilize the vessel wall as the nascent vessels continue to develop and mature (Reinke JM, 2012*).*

1.2.4. Remodeling phase

The last phase of the healing process is remodeling, where granulation tissue undergoes transformation into scar tissue, acquiring increased tensile strength. Maturation of granulation tissue involves a reduction in capillaries as they merge into larger vessels. Additionally, there are alterations in the type, quantity, and organization of collagen, further enhancing the tissue's

tensile strength. Initially, there is an abundant production of type III collagen, but eventually, type I collagen, which is the predominant fibrillar collagen in the skin, surpasses it (Pratibha *et al*., 2023).

During the remodeling phase, a significant aspect is the restructuring of the extracellular matrix (ECM) to resemble the architecture of normal tissue. Simultaneously, the wound undergoes physical contraction, a process believed to be facilitated by contractile fibroblasts known as myofibroblasts that arise during the wound healing process (Ayse ARZU *et al*.,2023*)*.

1.3. Factors Affecting Wound Healing

Numerous variables can disrupt one or more stages of the wound healing cascade, leading to inadequate tissue restoration. Local factors, such as infection, oxygenation, foreign bodies, and poor venous drainage, can contribute to this disruption. Additionally, systemic factors, including stress, age, gender, sex hormones, ischemia, certain diseases (such as keloids, hereditary healing disorders, uremia, jaundice, diabetes, and fibrosis), obesity, medication (such as glucocorticoid steroids, NSAIDs, chemotherapy, etc.), alcoholism, smoking, nutrition, and immune-compromised conditions (such as cancer, radiation therapy, and AIDS), can also impact wound healing (Tazeze et al., 2021) .

1.4. *Clematis hirsuta (Perr and Guill)*

Clematis hirsuta (Perr and Guill) is one of the Ranunculaceae families. It is a woody climber that can grow to reach up to 4 meters long. It has leaves that are placed in opposition. White or yellow flowers in panicles are produced (Figure 2). *C. hirsuta* has different local names in Ethiopia, such as "Azo Hareg" in Amharic and "fiiti" in Afaan Oromo, and it is found in many countries, including Ethiopia. A topical application of a mixture containing crushed and powdered *Clematis hirsuta* leaves combined with water was administered to the affected area (Bitew *et al*., 2019; Giday *et al*., 2007). This plant has several secondary metabolites, including saponins, triterpenoids, alkaloids, glycosides, flavonoids, phenylpropanoids, phenolic acids, and tannins (Abdisa and Kenea, 2020). *C. hirsuta* was one of the medicinal plants claimed in different parts of the world due to its activities as an antioxidant and an antibacterial. *(*Abdisa and Kenea, 2020), anti-inflammatory (Abdel-kader *et al*., 2008), anti-leishmaniasis, hemorrhoids (Teklehaymanot *et al*., 2007*)*, anti-anthrax, and herpes zoster (Hishe and Asfaw, 2015) antifungal *(*Nigussie D, *et al*., 2021), anti-diarrheal (Gahamanyi *et al*., 2021), Bone and tissue

cancer, gland tuberculosis bone and tissue cancer, gland tuberculosis (Eshete *et al.*, 2016), Analgesics and an anti-rheumatic (Al-taweel *et al.*, 2007), respiratory tract problems, cataracts, and wounds as folk medicine *(*Bitew *et al.*, 2019; Giday *et al.*, 2007). No experimental evidence has previously validated the wound-healing potential of *C. hirsuta* leaves in animal models. This study investigates the in vivo wound healing activities of a methanol extract derived from C. hirsuta leaves, utilizing excision, incision, and burn wound models in mice.

1.5. Statements of Problem

Approximately 14 million individuals are affected by wounds and burns every year, with more than 80% of these cases occurring in low and middle-income countries. This alarming statistic emphasizes the significant impact and burden placed on these nations when it comes to managing and providing treatment for such injuries (Mulatu et al. , 2021). Promotion of wound healing has become a key objective of medical treatment, especially in the aging population of developing countries (Casado-d *et al*., 2022*)*. The high rates of chronification, recurrence, and morbidity associated with skin injuries impose a significant burden on health-related quality of life. This burden leads to increased personal and societal expenses, creating challenges for public health systems worldwide. The annual cost attributed to these factors ranges from $28.1 billion to $96.8 billion. Among various types of skin injuries, burn wounds are particularly prevalent in all communities (Hamdoon A. *et al.*, 2022).

In developing countries, burns are recognized as a significant health issue due to the high incidence of severe complications and limited financial resources. These challenges often result in delays in wound healing, increasing the risk of infection, inadequate recovery, and undesirable scarring. As a consequence, addressing these problems becomes crucial to ensure optimal outcomes for burn patients in resource-constrained settings (Badr *et al*., 2023).

The presence of sclerotic tissue commonly characterizes the borders and surrounding areas of chronic wounds. This poses a challenge as conventional liquid antiseptics often prove inadequate and unsustainable in effectively addressing these wounds. Recent studies have demonstrated that treating infected skin wounds in humans with tissue-tolerable plasma resulted in a remarkable 74% reduction in bacterial load. Consequently, wound treatment remains a significant and growing concern worldwide (Praveen *et al.*, 2022).

Each of the various Western medications employed for wound treatment has numerous drawbacks and limitations. However, several studies have indicated that certain disinfectants, such as hydrogen peroxide, acetic acid, and betadine, exhibit toxicity towards essential cells involved in wound healing, including fibroblasts and lymphocytes (Goorani *et al.*, 2019*).*

Medicinal plants have been utilized as medicine since ancient times and are renowned for their capacity to facilitate wound healing. Additionally, they offer the advantage of preventing infection without causing significant side effects, which stands in contrast to the limited efficacy and potential serious adverse effects associated with many modern therapeutic agents *(*Jean Dieu *et al.*, 2023). Consequently, the ethno-medicinal utilization of plants for wound treatment, in various forms such as teas, decoctions, tinctures, syrups, oils, ointments, poultices, and infusions, not only provides affordability and accessibility but also ensures a reliable natural source of therapeutic compounds. Research on medicinal plants has demonstrated that herbal medicines exhibit fewer adverse effects compared to their chemical counterparts, making them a more cost-effective option (Zeghad *et al.*, 2023). The scientific evaluation of *C. hirsuta (Perr and Guill)* leaves for their wound healing efficacy, both in vitro and in vivo, has not been conducted thus far.

1.6. Significance of the study

In vivo evaluation of this plant is important to validate its safety and efficacy for continuous use in the community. It can serve as a starting point for scientific communities to conduct further investigations into the development of effective and safe drugs for wound treatments. Although no scientific evidence regarding the wound healing properties of *C. hirsuta (Perr and Guill)* in animal models was found in the literature survey, this study will serve as a reference for future researchers. Furthermore, the current study will contribute to justifying the traditional use of *C. hirsuta (Perr and Guill)* leaves for wound healing purposes.

1.7. Objective of Study

1.7.1. General Objective

To evaluate the wound healing activity of an 80% methanol crude extract of *C. hirsuta (Perr and Guill)* leaves in mice

1.7.2. Specific Objectives

1. To investigate the acute dermal toxicity of 80% methanol crude leaf extracts of *C.hirsuta* in mice.
2. To assess the wound healing activity of an 80% methanol crude leaf extract of *C. hirsuta using an* excision wound model in mice.
3. To assess the wound healing activity of an 80% methanol crude leaf extract of *C. hirsuta* using incision wound model in mice.
4. To assess the wound healing activity of an 80% methanol crude leaf extract of *C. hirsuta using a* burn wound model in mice
5. To investigate histopathological analysis for excision and burn wound models.
6. To perform phytochemical constituents of *C.hirsuta (Perr and Guill)* leaves.

2. Literature review

2.1. Prevalence of wound

The global incidence of chronic wounds has reached 6 million cases annually. The National Health Service has estimated an annual growth rate of 12% for chronic wounds. In developed countries, approximately 1-2% of the population will experience a chronic wound at some point in their lives The rising prevalence of chronic illnesses such as metabolic disorders, vascular diseases, and the aging population has led to an increase in healthcare costs associated with chronic wounds. For instance, research by (Tessema *et al.*, 2019) found that 800 out of 100,000 individuals were aged over 75 years old, contributing to the burden of chronic wounds. Additionally, chronic wounds accounted for 2-4% of the healthcare budget. Globally, approximately 8.2 million people suffer from chronic wounds, resulting in an annual cost of over 28 billion dollars. In the United Kingdom alone, 2.2 million individuals require wound care, amounting to a cost of 5.3 billion pounds per year (Befekadu et al., 2022).

Although there is limited epidemiological data available, it is anticipated that the incidence of chronic wounds will increase in developed countries due to a high prevalence of accidental traumatic injuries and ulcerations. The challenges associated with non-healing wounds in developing countries have been a contributing factor to the shortcomings in healthcare services. As highlighted by the World Health Organization (WHO), failures in healthcare delivery can lead to a lack of social trust, increased poverty, and overall morbidity. Unreliable follow-up care may result in internal displacement and exacerbate the burden of chronic wounds in these regions (Taye, *et al*., 2011).

2.2. Pharmacological Managements of the Wound

The kind, origin, and extent of the injury all affect how it is treated overall. The treatment of wounds involves a comprehensive approach that includes examination, treatment, and dressing of recent wounds. There are various therapeutic options available for wound care, such as analgesics, antibiotics, and NSAIDs. However, it is important to note that chronic wounds create an environment favorable for microbial growth. As a result, the use of antibiotics becomes crucial. Examples of antibiotics commonly employed in wound treatment include bacitracin, chloramphenicol, clindamycin, gentamicin, cloxacillin, silver sulfadiazine, amikacin (in gel or

cream form), nitrofurazone (cream), mupirocin, and polymyxin B. These medications help combat infection and promote healing in the wound site *(*Getahun *et al.*, 2021*).*

Topical antimicrobials are applied sparingly and aseptically to the wound. Their use is discontinued once healthy granulation is noted within the wound. On the other hand; nitrofurazone has a broad gram-positive spectrum, and have a negative impact on epithelialization (Krahwinkel and Boothe, 2006). However wound healing in Nitrofurazone treated patients was superior to that of patient treated with silver sulfadiazine: crusts separated more rapidly, wounds were dryer, tissue granulation began sooner, and the amount of healing at two weeks was greater (Webber et al., 1977). Again significantly Epithelialization occurred earlier in animals treated with the nitrofurazone group ($P < .05$) than in group treated with silver sulfadiazine with no difference towards histopathological results (Abbas et al., 2015).

Not only are most of these medications expensive, but they also come with various undesirable side effects (G/giorgis et al., 2022). Over the past few years, there has been a growing interest in utilizing herbal medicine for the management of wounds. Numerous studies have been conducted in this area, highlighting the potential of natural therapies in effectively treating wounds as an alternative to conventional synthetic drugs. These herbal treatments have shown promising results and have demonstrated their efficacy in wound healing (Yassine *et al.*, 2021).

2.3. Medicinal plant used to treat the wound

Aloe vera, a fully grown medicinal plant, possesses substantial potential for the treatment of skin wounds. It encompasses a diverse range of over 75 compounds, including essential vitamins such as A, C, E, and B12; enzymes like amylase and catalase; minerals like zinc, copper, and selenium; sugars like glucomannans and polymannans; polyphenols like anthraquinones; and sterols like lupeol and campesterol. These constituents contribute to the therapeutic properties of aloe vera and make it a valuable natural resource for wound healing purposes (Vitale *et al*., 2022). *Aloe vera* treatment dramatically enhanced the number of fibrocytes, collagen mass density when compared to the control group, according to histopathological data (Yazarlu *et al*., 2021).

In vivo and in vitro, aloe gel and aloe extract can both speed up the healing of wounds. Studies conducted on animals have revealed that glycoproteins can speed up human keratinocyte proliferation and encourage the growth of epidermal tissue. Concerning the various stages of

wound healing, such as fibroplasia, collagen production, and contraction, resulted in faster healing compared to untreated animals (Liang *et al*., 2020; Oguntibeju, 2019*)*.

Achyranthes aspera, known as "Telenge" or "ambulale," is a traditional plant used for wound treatment in Ethiopia and India. Topical application of A. aspera leaf ointment (2.5%, 5%, and 10% w/w) significantly improved wound contraction, breaking strength, and epithelization compared to the control group. Histological analysis showed enhanced tissue regeneration, including a well-organized epidermal layer, increased fibrocytes, neovascularization, and epithelialization (Boakye *et al.*, 2016).

The utilization of honey as a treatment for wounds can be traced back to 2000 BC. For centuries, honey has been employed to cleanse wounds and promote faster healing. However, the scientific understanding of its effectiveness remained unclear until recent times. Honey exhibits various biological effects on wounds, including reducing inflammatory edema, stimulating the migration of macrophages, accelerating the removal of necrotic tissue, providing a source of cellular energy, forming a protective protein layer over the wound, and promoting the development of a healthy granulation bed. These properties contribute to the therapeutic benefits of honey in wound healing (Yilmaz and Aygin, 2020).

In a study on guinea pigs, a 96% ethanol leaves extract of Vernonia scorpioides was evaluated for wound treatment. The application of a hydrogel containing 50% of the extract for 30 days did not accelerate wound closure time. However, it significantly improved tissue organization and regeneration compared to the control group. Histological analysis revealed that the treated group had well-structured connective tissue surrounded by granulation tissue, while the control group exhibited inflammation and necrosis with higher monocyte levels. Despite similar overall healing time, the extract treatment demonstrated beneficial effects on tissue healing and inflammation reduction (S.N. Leite *et al.*, 2002).

Falcaria vulgaris aqueous extract ointment resulted in a significant decrease ($p < 0.05$) in the levels of the wound area, total cells, lymphocyte, neutrophil, and macrophage, and a significant increase ($p < 0.05$) in the levels of wound contracture, hydroxyproline, hexosamine, fibrocyte, and fibroblast compared to negative control. The findings show that *F. vulgaris* aqueous extract ointment is effective in treating cutaneous wounds *(*Goorani *et al*., 2019).

Report presented by (Lambebo *et al*.,2021) methanolic crude extract and solvent fractionation of *Vernonia auriculifera* leaves 10% w/w CE formulation was showed considerable wound contraction ($P < 0.001$) from days 4 to 18 compared to the negative control in excision wound model. Periods of epithelialization for 5% w/w and 10% w/w CE ointment was statistically significant at ($P < 0.001$) when compared to negative control in excision and also increase tensile strength in the incision wound model. Ethyl acetate and aqueous fraction ointments showed a statistically significant ($P < 0.001$) compared to negative control in both excision and incision wound model.

The study conducted on the crude extract of Brassica carinata revealed the presence of various compounds, including alkaloids, flavonoids, phenols, terpenoids, saponins, tannins, steroids, and glycosides. Acute cutaneous toxicity testing in mice demonstrated the safety of the extract at a maximum dose of 2000 mg/kg. The formulation of the crude extract at a concentration of 10% w/w significantly reduced the epithelialization period (by day 14; $P < 0.001$) and resulted in notable wound compression from days 6 to 16. Furthermore, a 5% ointment of the crude extract significantly influenced both the length of the epithelialization period (by day 16, $P < 0.01$) and wound contraction (from day 8 onwards, $P < 0.01$). Additionally, the ethyl acetate and aqueous fractions, when incorporated into a 10% w/w ointment, demonstrated significant reduction in wound size starting from the fourth day of treatment *(*Alemu *et al*., 2020). The 80% methanol leaves extract of *Achyranthes aspera L* .(Amaranthaceae) result showed that the wound contraction rate was much higher, and the epithelialization time was shorter with the 10% w/w chloroform fraction ointment ($p < 0.01$) (Teklie *et al*., 2021).

Brucea antidysenterica leaf extract in methanol (80%) was fractionated using water, n-butanol, and chloroform and contains secondary metabolites like; alkaloids, tannins, saponins, flavonoids, terpenoids, phenols, steroids, and glycosides in both solvents. The 2%, and 4% AF (aqueous fraction) demonstrated enhanced tensile strength in comparison to untreated wounds, and wound contraction of 97.5%, and 98.39%, respectively, with ($p < 0.001$) compared to the negative control. 2% AF ointment did not result in a statistically significant difference in the time of epithelialization, while 4% AF significantly ($p < 0.001$) reduced the time of epithelialization compared to control *(*Befekadu *et al*., 2022*)*.

In another study conducted by *(*Ayal *et al*., 2019*)* on *Calpurnia aurea* (Ait.), from day 4 to day 8 after wounding, the 5% w/w, and 10% w/w crude extract ointments revealed a substantial ($p < 0.001$) wound contraction. But the 10% w/w crude extract ointment showed a substantial ($p < 0.01$) reduction of the epithelialization duration. In comparison to the negative control, the 5%, and 10% weight-per-weight crude extract ointments both demonstrated a substantial ($p < 0.001$) increase in tensile strength. The 10% weight-to-weight aqueous fraction ointment showed a high percentage of wound contraction, and a short ($p < 0.001$) epithelialization duration.

According to the study conducted on leaves gel powder of *Aloe trigonantha* in comparison to controls, the wound treatment with ointments at 5%, and 10% (w/w) strength of the gel resulted in significantly faster wound contraction rates, quicker epithelialization times, and stronger skin-breaking strengths. This may be due to its containing secondary metabolites like, tannins, phenols, flavonoids, glycosides, and glycosides (Tazeze *et al*., 2021).

According to the study done on *Zehneria scabra* air-dried leaf, was safe for acute dermal toxicity test. In comparison to the simple ointment, and untreated groups, the 5%, and 10% extract ointments considerably ($p < 0.05$) increased the tensile strength of the healing wound. In comparison to both of these groups, treatment with the standard agent 0.2% (w/w) NFZ showed a considerably ($p < 0.01$) higher tensile strength. The 0.2% (w/w) NF-treated group displayed the highest percentage of the tensile strength (72%). The percentage of wound contraction and tensile strength were both raised with both 5%, and 10% (w/w) extract ointment formulations, and the epithelialization period was decreased (Tekleyes *et al*., 2021).

The results study conducted on 80% methanol extract of *Justicia schimperiana* leaves using the burn, excision, and incision wound models, show Significant ($P < 0.05$) improvements in wound contraction rate (44.84%) after applying 5% (w/w), and 10% (w/w) ointment formulations. On the sixth, and eighth days after wounding, the standard treatment (nitrofurazone ointment, 0.2% (w/w)), compared to the negative control, which was 48.25%, and 53.4%, respectively, showed the highest rate of wound contraction ($p < 0.001$) (G/giorgis et al., 2022).

According to the study conducted on *Stephania abyssinica* roots crude extract as compared to the simple ointment (SO) treated group on excision wounds, the 10% w/w crude extract ointment produced significant ($p < 0.001$) wound contraction from the fourth to the sixteenth post

wounding days, and the 5% w/w CE were significant ($p < 0.01$) wound contraction on the tenth post wounding day. From the sixth post-wounding day, the CEO displayed highly significant ($p < 0.001$) behavior on burn wound models. Compared to the untreated group, and the SO group, the mice treated with CE had considerably higher tensile strength ($p < 0.001$) (Yiblet *et al*., 2022*).*

3. Materials and Methods

3.1. Drugs and Chemicals Supplies

Drugs and chemicals like wool fat (Lab tech chemicals), hard paraffin (Lab tech chemicals), white petrolatum (Lab tech chemicals), cetostearyl alcohol (Lab tech chemicals), methanol (kilitch estrobiotech), distilled water, nitrofurazone skin ointment 0.2% (shanghai general pharmaceutical CO., LTD, china); ketamine hydrochloride injection (neon laboratories limited, India), diazepam injection (Gland pharm limited, India), 70% alcohol, normal saline, 10% formalin, and bee wax (Ethiopian Pharmaceuticals Manufacturing), hematoxylin and eosin (Alpha Chemika Maharashtra, India), Glacial acetic acid (Fisher Scientific, UK), Chloroform (Loba Chemie, India), Hager's reagents, Hydrochloric acid (Fisher Scientific, UK), Sulfuric acid, sodium hydroxide and Ferric chloride solution (Finkem laboratory reagent, India). All chemicals and reagents were analytical grade and purchased from their respective vendors and whole sealer.

3.2. Instruments, and Apparatus

Instruments like Whitman filter paper number 1 (Whatman Ltd., England), conical flask, beaker, water bath, orbital shaker (IKAvibrex Italy), digital balance (KERN-ALJ 220-4, England B428788209), rotary evaporator, ovum, deep freezer, light, vacuum pump, desiccator, mini orbital shaker, electrical hair clipper, mortar, and pestle, spatula, ointment slab, mini orbital stirrer, sharp sterilized scissors, surgical threads with curved needles, forceps, insulin syringe, surgical scalpel blade, adhesive plaster, gloves, thermometer, cotton swab, metal cylinder, transparent polythene sheet, graph paper, and permanent marker were used.

3.3. Study Design

A completely randomized control study design was used.

3.4. Sample Size Determination

From a previously conducted study by calculating the means and standard deviation, the effect size was 1.76 (Mulatu *et al*, 2021). Based on this value the sample size was calculated by using G Power Software version 3.1.7. The t-test is used to compare the differences between two

dependent means (matched pairs). The numbers of animals per group were 6 and, based on this value 81 total number of animals were used in this experiment.

The list of different values was mentioned herein below;

- ✓ Signal =30, Noise= 17, Power=0.9, Significance level =0.05, DF=5
- ✓ Critical t=2.57058
- ✓ Alternative hypothesis=2-sided t-test
- ✓ $\frac{\text{Signal}}{\text{noise ratio}}$=30\17= effect size is 1.76
- ✓ Required sample size 6/group

3.4.1 Sampling Technique

For this experiment, a total of 81 healthy adult Swiss albino mice, weighing between 25-35 g and aged 6-8 weeks, were selected using a simple random sampling method.

3.4.2. Experimental unit

A total of 81 healthy adult Swiss albino mice, weighing between 25 and 35 g and aged 6 to 8 weeks, of both sexes, were included in this experiment. The mice were categorized into a negative control group, a positive control group, and two treatment groups.

3.5. Plant Material Collection

The leaves of *C. hirsuta (Perr and Guill)* were collected from the Arda Jila district, located near Mea Boko town in the Guji zone of the Oromia regional state in Ethiopia. The collection site is approximately 420 km east of the southern part of Addis Ababa. Taxonomic identification of a *C. hirsuta* specimen was performed by a botanist at the Department of Biology, College of Natural and Computational Science, Addis Ababa University. The specimen was assigned a reference number of WE001 (Figure 2).

Figure 2: Clematis hirsute leaves (picture taken by Woyesa Elema on April 20, 2023)

3.6. Experimental Animals.

A total of 78 healthy adult Swiss albino mice, weighing between 25 and 35 g and aged 6 to 8 weeks, of either sex, were utilized for the main experiment. Additionally, three healthy female adult Swiss albino mice were used to determine the acute dermal toxicity test of the crude extract. The animals were obtained from the Animal House of the Department of Pharmacology, School of Pharmacy, College of Medicine and Health Sciences, Wolaita Sodo University, Sodo, Ethiopia. The mice were housed in sanitary polyethylene plastic cages with wire mesh tops, equipped with a hygienic bed of coarse sawdust that was changed every three days. The cages were placed in a well-ventilated room with a humidity level of 50-60% and a 12-hour light-dark cycle. The mice were provided with standard pellet diets obtained from local vendors, along with access to clean drinking water. Prior to the experiment, all mice were given a one-week acclimation period to adapt to the laboratory conditions.

The handling and care of the mice followed internationally recognized standards and recommendations for laboratory animals (Ogden et al., 2017). At the conclusion of the experiment, all animals were euthanized by administering a high dose of ketamine injection (OECD 404, 2015). Furthermore, the research was conducted in accordance with the ethical clearance granted by the Ethical Board Committee of Hawassa University College of Medicine and Health Science.

3.7. Preparation of the Hydromethanolic Crude Extract.

C. hirsuta leaves were rinsed in water, dried in the shade at room temperature to prevent impurities and debris, and hand-compressed into coarse powder to increase the penetration of

extraction solvents. Until extraction, plant powder was stored in a container that was tightly closed. Eight liters of 80% (v/v) methanol were macerated with one kilogram of the coarse powder at room temperature in Erlenmeyer flasks (G/giorgis et al., 2022). The flasks were covered with aluminum foil. Following that, it was held for 72 hours with occasional shaking. After 72 hours, the extract was separated from marc by muslin cloth and then filtered using What-Man filter paper (No. 1). To increase the yield, the marc was three times re-macerated in new, identical volumes of 80% methanol, extracted, and filtered in the same way. To remove the methanol component, each filtrate was mixed and condensed using a rotary evaporator set at 40°C. The residual watery mixture was frozen overnight in a deep freezer (-20°C) before being dried using an ovum at 40°C (Figure 3). The dried extract was weighed, put in an airtight container, and kept until it was utilized for the planned experiments *(*Demilew *et al*., 2018*)***.**

$$\text{Percent yield} = \frac{\text{weight of the extract}}{\text{weight of the plant material}} \times 100$$

Figure 3: Hydro-methanolic crude extraction picture.

3.8. Preliminary Phytochemical Screening

The crude extract was screened for the existence of secondary metabolites such as alkaloids, tannis, saponins, flavonoids, terpenoids, steroids, cardiac glycosides, and phenols by using standard phytochemical screening procedures (Asnakech *et al.*, 2019).

Test for Saponins

5 ml of distilled water was added to 0.25g of crude extract. Then, the solution was shaken strongly and detected for a stable persistence of forth. The presence of saponins was indicated by the formation of a stable froth that persisted for about half an hour.

Terpenoids Test

2 mL of chloroform was added to 0.25g of crude extract. Then, 3 mL of concentrated sulfuric acid was carefully added to form a layer. The presence of terpenoids was indicated by a reddish-brown coloration of the interface.

Test for Tannins

In a test tube, about 0.25g of crude extract was boiled in 10 mL of water and then filtered with filter paper (What-Man No. 1). A few drops of ferric chloride (0.1%) were added to the filtrate. A brownish-green or blue-black precipitate color indicates the presence of tannins.

Test for Flavonoids

0.5g of crude extract was combined with distilled water and heated for 5 minutes in a water bath. The mixture was still hot. Then, to 1 mL of cooled filtrate, a few drops of a 20% sodium hydroxide solution were added. The presence of flavonoids was shown by the formation of a yellow tint that changed to colorless when treated with acid.

Test for phenols: 0.5g of crude extract was combined with a solution of 5% ferric chloride, and the mixture was carefully examined for the formation of a deep blue or black color, which indicates the presence of phenols.

Test for Cardiac Glycosides

0.25 g of crude extract was diluted with 5 mL of water. Then it was added to 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 mL of concentrated sulfuric acid. The presence of a deoxysugar characteristic of cardenolides was indicated by the formation of a brown ring at the interface.

Test for Steroids

To 0.25g of crude extract, 2 mL of acetic anhydride was added with 2 mL of sulfuric acid. The color change from violet to blue or green indicated the presence of steroids.

Test for Alkaloids

A freshly prepared Hager's reagents A few drops were added to 0.5g of crude extract. The presence of alkaloids was indicated by the formation of a yellow precipitated color (Babakura *et al*., 2019).

3.9. Ointment Formulation.

According to the British Pharmacopeia, simple ointments and 80% hydro-methanolic crude extract ointments were made for each wound model on the day of the experiments (British Pharmacopoeia 2016). To prepare 200 g of simple ointment base, 10 g of hard paraffin was placed in a beaker and melted on the electrical heater. Then, 10 g, 170 g, and 10 g of cetostearyl alcohol, white petrolatum, and wool fat were added in descending order of their temperature, respectively (Abeje, 2022; Teklie *et al*., 2021). All the ingredients were melted using an electrical heater while stirring continuously until they reached a homogeneous state. Subsequently, the mixture was taken off the heat source and stirred until it cooled down. Levigation technique was employed to prepare ointments with consistent consistency and smooth texture. A negative control ointment, consisting of 200 g of simple ointment base without the active component, was prepared (Table 1). To prepare the medicated ointment, 10 g and 20 g of the 80% methanol extract were mixed with 190 g and 180 g of the ointment bases, respectively, resulting in 5% and 10% medicated ointments. (Mulisa *et al*., 2015). Lastly, a hygienic, closed bottle was used to transport the hydromethanolic crude extract ointments and a simple ointment base for topical administration during the experiment (Asmare et al., 2019).

Table 1: Simple ointment preparation

Ingredients	Master formula (MF) (g)	Reduced formula (g)
Wool fat	50	10
Hard paraffin	50	10
Cetostearyl alcohol	50	10
White petrolatum	850	170
Total	1000	200

3.10. Acute Dermal Toxicity

Following OECD guideline 404, "limit test at dose of 2000 mg/kg," an acute cutaneous toxicity test was conducted (OECD *404*, *2015*). Three female mice with normal skin texture were randomly chosen, housed in independent cages, and given five days to get acclimated to the lab environment before the test (Yiblet *et al*., 2022). The mice were then given an intra-peritoneal 80 mg/kg ketamine injection to induce anesthesia, and 24 hours before the study, the dorsal portion of the trunk had around 10% of its body surface area fur removed. In the initial experiment, a single mouse received a single application of *C. hirsuta (Perr and Guill)* leaves 10% w/w crude extract at a concentration of 2000 mg/kg (the highest allowable dose). The response of the mice was examined and documented within 60 minutes of the ointment application. After 24 hours following the initial test observation, the other two mice were undergoing a confirmatory test. For 14 days following the removal of the leftover ointment, the mice were monitored daily for the emergence of any unfavorable skin reactions, including edema and erythema. According to the OECD 404 grade, erythema and edema were assessed and scored (G/giorgis et al., 2022; OECD 404, 2015).

3.11. Grouping and Dosing of Animals

The animals were randomly separated into four groups in burn and excision wound models, each with six mice. In both burn and excision wound models, the first group received simple ointment treatment. The second and third groups received ointments containing hydromethanolic crude extract at 5% w/w and 10% w/w concentrations, respectively. Then 0.2% w/w nitrofurazone ointment was used to treat the fourth group. The animals were randomly divided into five

groups, each with six rats, for the incision wound model. The grouping and dosing were the same as for the excision wound model, except for the fifth group left untreated.

3.12. Wound Healing Evaluation

3.12.1. Excision Wound Model

The excision wound model was employed following the procedure outlined by earlier investigators (Demilew *et al*., 2018; Sharma *et al*., 2011). Ketamine (80 mg/kg) and diazepam (5 mg/kg) were injected intraperitoneally to anesthetize the mice. After being cleansed with 70% alcohol, the skin of the dorsolateral flank was shaved with a shaving machine at a distance of 5cm from the ear and 1 to 1.5cm from either side of the vertebral column. With a thin permanent marker, the predicted 314mm2 circular wound was marked, and a 2mm depth excision wound was carefully made following the marks using forceps and tiny, sharp sterilized scissors. By dabbing the wound with a cotton swab soaked in regular saline, hemostasis was accomplished. The wound was left completely open. The day of the injury was counted as day 0, and the treatments were started on day one. The therapies were applied topically once daily until the treatment group's wound was fully healed. Group 1 mice received simple ointment. The second and third groups received ointments containing hydro-methanol crude extract at 5% w/w and 10% w/w concentrations, respectively. Then 0.2% w/w nitrofurazone ointment was used to treat the fourth group. The percentages of wound contraction (taken every other day using graph paper (1 mm2 and a transparent sheet), the length of epithelialization, and histopathology examinations were used to evaluate the wound healing process. Measurements were taken on the 2nd, 4th, 6th, 8th, 10th, 12th, and 16th days following wounding to determine the wound closure rate, which was indicated as a reduction in the percentage of the original wound size. The following formula was used to determine the crude extract contribution to wound healing as assessed by percentages of wound contraction (G/giorgis et al., 2022)

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

Where n is the number of days post-wounding

Period of Epithelialization

The epithelialization period refers to the number of days it takes for dead tissue remains to fall off without leaving a raw wound, which was evaluated at the end of the study (G/giorgis et al., 2022; Yiblet et al., 2022).

Histopathological Analysis

Histopathological analysis was performed blindly by a pathologist for excision and burn wound models. After the mice were killed by anesthetic overdose on days 16 and 18 in the excision and burn wound models, respectively, skin samples from each group were collected. The samples were processed, blocked with paraffin, and then sectioned at thicknesses of 3 μm, mounted on glass slides. Finally, it was stained with hematoxylin and eosin after being preserved in 10% buffered formalin. The tissues were examined under a microscope and ranked as mild (+), moderate (++), and high (+++) in terms of fibroblast proliferation, mononuclear, neovascularization, and collagen deposition concentration in the dermis (Mulisa *et al.*, 2015; Yiblet *et al.*, 2022).

3.12.2. Incision Wound Model

The experimental animals in this model were sedated in the same manner as in the excision wound model. Each mouse was shaved on its dorsal fur after being massaged with 70% alcohol. At a distance of 1.5cm from the dorsal midline, a longitudinal paravertebral incision 3 cm long and 2 millimeters deep was created through the shaved skin on either side. Surgical sutures (No. 000) and a curved needle were used to close wounds with interrupted sutures spaced 1cm apart. After being stitched, wounds are left uncovered. The day of the injury was counted as day 0. According to the grouping and dosing in the above section, the animals in Groups I through IV received the corresponding topically administered therapies, with the exception of Group V, which left untreated. Until the ninth day after the development of the wound, medicines were administered daily. The mice in Group V hadn't received any treatment and were serving as an untreated negative control. The sutures were removed on day eight, and a continuous water flow technique was used to test the skin-breaking strength of the healed wound on day ten after wounding *(*Thakur *et al.*, 2011*)*. Using the following formulas, the percentages of tensile strengths (% TS) of the groups were determined.

$$\%\text{TS of extract} = \frac{\text{TS of extract} - \text{TS of vehicle}}{\text{TS of vehicle}} \times 100$$

$$\%\text{TS of reference} = \frac{\text{TS of reference} - \text{TS of vehicle}}{\text{TS of vehicle}} \times 100$$

$$\%\text{TS of vehicle} = \frac{\text{TS of vehicle} - \text{TS of the group left untreated}}{\text{TS of the group left untreated}} \times 100$$

3.12.3. Burn Wound Model

A total of 24 mice were anesthetized and shaved in the same manner as in excision and incision wound models. To create a partial-thickness burn wound, heated, molten beeswax at 80°C was poured into a metal cylinder with 300 mm2 circular perforations that were placed on the mice's shaved area and left there for 10 minutes while the wax solidified. The metal cylinder containing the wax that stuck to the skin was taken off when it had set. The mice in each group received daily treatments of simple ointment, CE, and nitrofurazone, respectively, as in the excision wound model, and the measurements were taken in the same manner. The measuring parameters used in the excision wound model were also used to evaluate burn wound healing (Yiblet *et al*., 2022).

3.13. Statistical Analysis

Raw data was expressed as the mean ± SEM (standard error of the mean). The results were statistically analyzed using one-way analysis of variance (ANOVA), followed by post-hoc Tukey tests using SPSS version 25. All data were presented as tables and figures. Data were considered statistically significant at a confidence level of 95% and a p-value < 0.05.

3.14. Ethical Consideration

Ethical clearance was obtained from the Institutional Health Research Ethics Review Committee (IHRERC) of Hawassa University College of Medicine and Health Sciences with reference number IRB/153/14. During the study, the care and handling of the mice were in line with the guidelines for the care and use of laboratory animals (Ogden et al., 2017). A supporting letter was written to the Wolaita Sodo University College of Medicine and Health Science to conduct the study.

4. Results

4.1. Percentage Yields of plant extracts

Percentages of yields for crude extract were calculated according to the formula used by (Abdisa and Kenea, 2020) which was 125g (12.5%).

4.2. Phytochemicals screening

The methanol crude extract of C. hirsuta leaves was found to contain various phytochemicals, including alkaloids, tannins, saponins, flavonoids, terpenoids, phenols, and steroids. However, the presence of cardiac glycosides was not detected in the extract (table 2 and figure 4).

Table 2: Phytochemicals screening results

Phytochemicals	80% methanol crude extract
Alkaloid	+
Tannis	+
Saponin	+
Flavonoids	+
Terpenoids	+
Steroid	+
Cardiac glycosides	-
Phenols	+

Note: +presence – absence

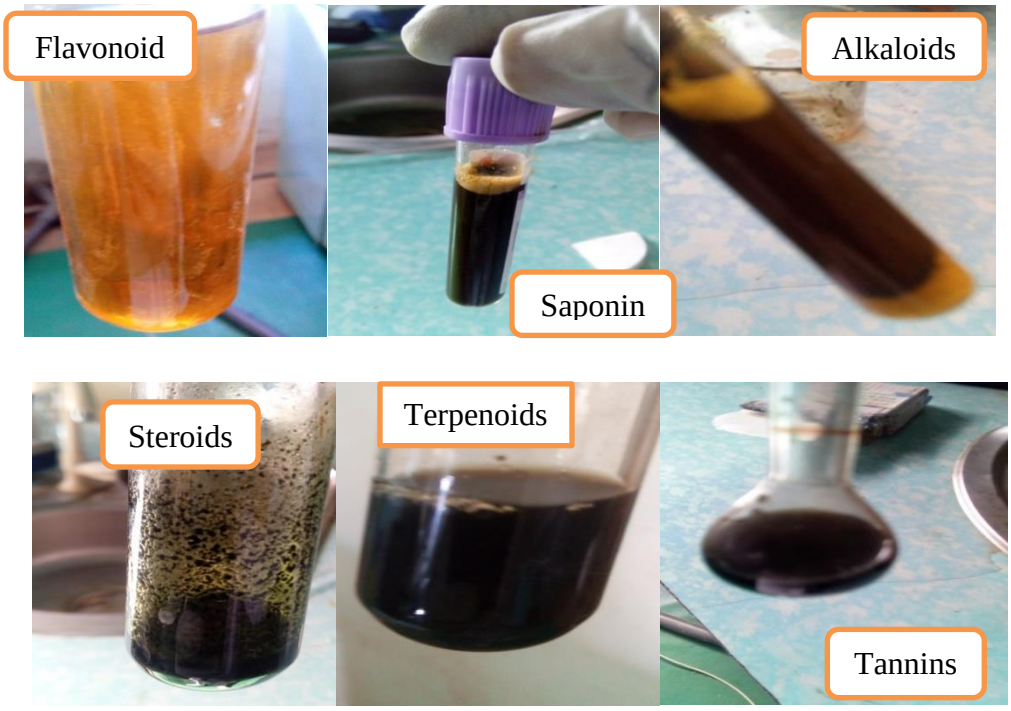


Figure 4: Phytochemical screening results in color change.

4.3. Acute dermal toxicity

The topical application of hydro-methanol crude extracts at a maximum concentration of 10% w/w, administered at a limit dose of 2000 mg/kg of body weight, was found to be safe. No signs of erythema, edema, dermal toxicity, or any other symptoms were observed during the 14-day observation period. Additionally, no cases of mortality were recorded.

4.4. Excision wound Model

Wound contraction

The topical application of hydro-methanol crude extract of *C. hirsuta* leaves ointments demonstrated notable wound healing activities in mice. Both the 5% w/w and 10% w/w concentrations of the extract exhibited significant wound healing effects, starting from day four. In comparison to the negative control, the group treated with 10% w/w, 5% w/w CEO, and 0.2% nitrofurazone showed a significant increase in wound contraction on day four, with p-values of <0.017, <0.037, and <0.015, respectively. These findings highlight the potential of *C. hirsuta* extract as a wound healing agent. In both the 10% w/w and 0.2% nitrofurazone treatment groups, significant wound contractions ($p < 0.001$) were observed starting from day six, compared to the

negative control group. When compared to the group treated with a 5% w/w concentration of hydro-methanol crude extract (CEO), both the 10% w/w CEO and 0.2% nitrofurazone treatment groups only exhibited statistically significant wound contractions on day eight ($p<0.001$). However, when comparing the 10% CEO and nitrofurazone groups to each other, no statistical significance was observed during the study period. These results indicate that the 10% CEO and the standard drug nitrofurazone did not demonstrate a significant difference in promoting wound contraction within the timeframe of this study.

On the 18th day, the group treated with a simple ointment showed a wound contraction of 98.57%, while the groups treated with 5% w/w CEO, 10% w/w CEO, and nitrofurazone achieved 100% wound contraction (table 3, and figure 5).

Table 3: Effects of topical application of 80% methanol crude extract of *Clematis hirsuta* leaves on wound contraction of excision wound models

Days	**Wound area in mm^2 ±SEM (% contraction)**			
	SO	5% w/w CEO	10% w/w CEO	0.2% w/w NFZ
Day 2	306.5±2.32(2.38)	303±1.94(3.5)	302.33±2.99 (3.71)	302.167±3.56 (3.76)
Day 4	279.75±3.16(10.9)	269.33±1.97 (14.2) [a*]	268±2.435 (14.64) [a**]	267.83±2.28 (14.7) [a***]
Day 6	258.25 ± 2.75 (17.75)	225.59±1.93(28.15) [a] ø	206.25±9.4(34.31) [a]*α	205.467±3.577(34.56) [a]*α
Day 8	195.78±.89(37.64)	133±3.75(57.64) [a]*α	112.15±1.85(64.28) [a]*α [b]*α	109.5±2.5 (65.12)[a]*α [b]*α
Day 10	170.235±.284(45.78)	95.91±5.96 (69.45) [a]*α	82.08±2.31 (73.85) [a]*α	79.25±2.55 (74.76) [a]*α
Day 12	141.33±.849(54.99)	45.16±3.07(85.61) [a]*α	35.32±1.275(88.75) [a]*α	34.8717±1.73 (88.89) [a]*α
Day 14	100.528±.375(67.96)	23.92±1.4(92.37) [a]*α	18.1617±.761(94.21) [a]*α	17.53±.917 (94.41) [a]*α
Day 16	69.33±1.45(77.9)	5 ±.96(98.4) [a]*α	3.9167±.583(98.75) [a]*α	3.5±.76(98.88) [a]*α
Day 18	4.63±.514(98.57)	.000±.000(100) [a]*α	.00±.00(100) [a]*α	.00±.00(100) [a]*α

All values were expressed as mean ± SEM (n= 6 mice per group), [a] compared to against negative control group, [b:] compared to against 5% crude extract, * p<0.037, **p<0.017, *** P<0.015. ø p< 0.002, *α p< 0.001. Numbers from 2 to 18 indicate the numbers of days that wound contraction measurements were taken. CEO; Crude Extract Ointment, SO; Simple Ointment, NFZ; Nitrofurazone.

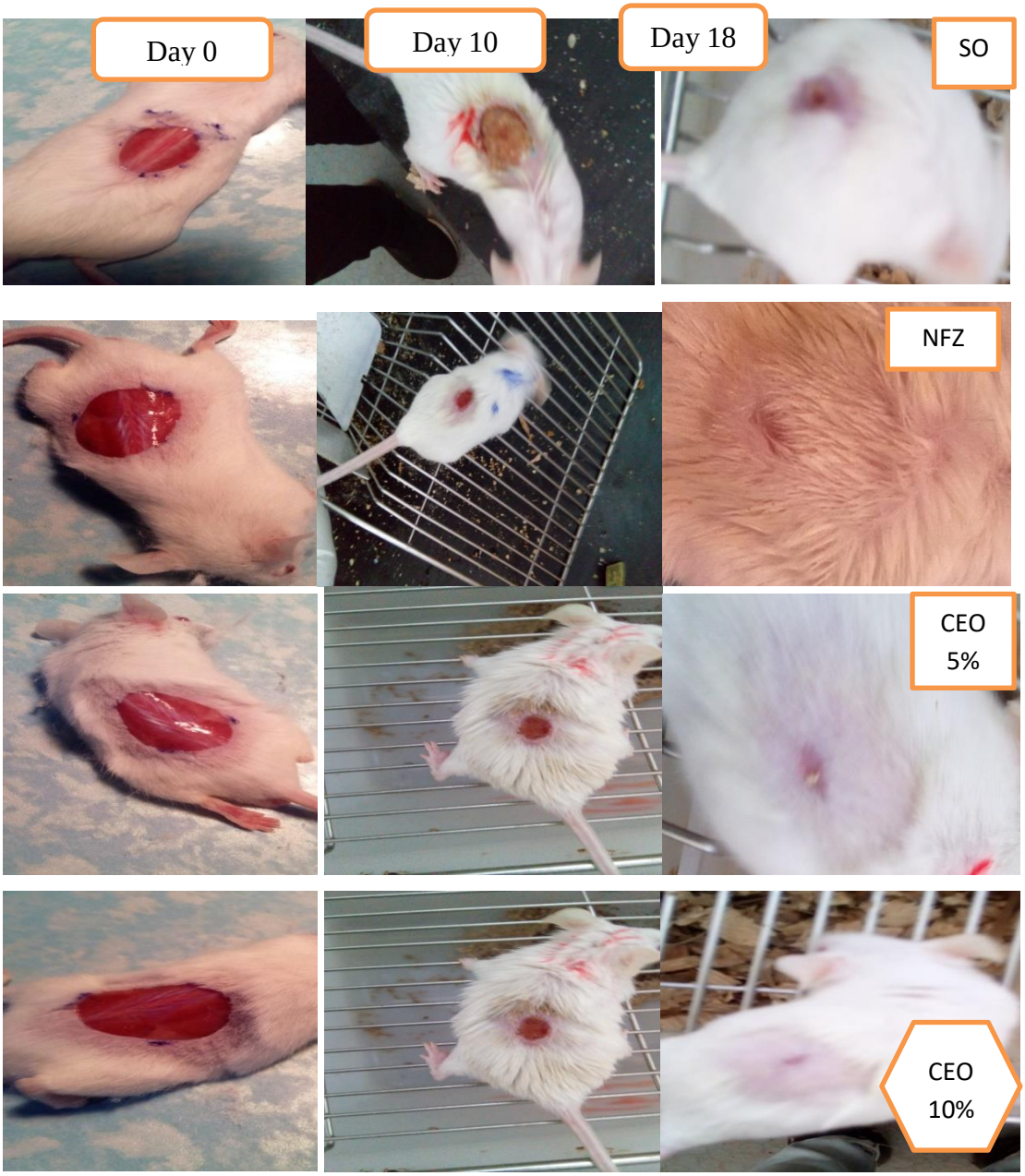


Figure 5: Excision wound model picture.

CEO; Crude Extract Ointment, NFZ; Nitrofurazone, SO; Simple Ointment.

Period of epithelialization

Upon comparing the groups that received simple ointments, it was observed that both the groups receiving 10% w/w crude extract and the standard drug exhibited a significant reduction in the epithelialization period ($p < 0.001$). Additionally, in the groups that received 5% w/w crude extract, a statistically significant reduction in the epithelialization period was also noted ($p < 0.041$) when compared to the negative control. Contrary to expectations, there was no statistically significant reduction in the epithelialization period between the groups treated with the standard drug nitrofurazone and the crude extract. This implies that the crude extracts were not effective in decreasing the epithelialization periods when compared to the standard drug. The percentages of reduction in epithelialization periods was 12.6%, 20%, and 20.9% in 5% w/w CE, 10% w/w CE, and nitrofurazone, respectively (figure 6).

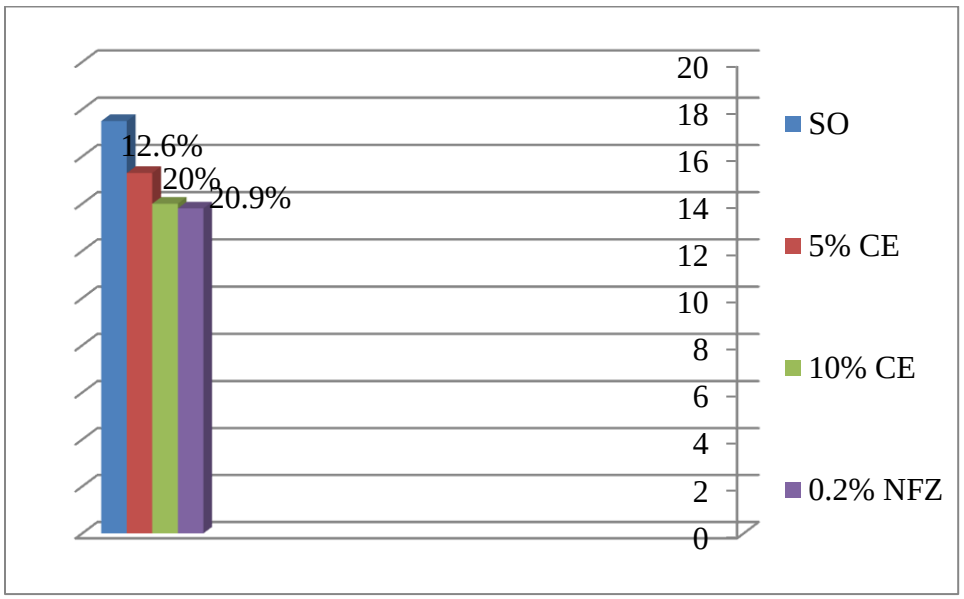


Figure 6: Effect of topical application of crude leaves extract on epithelialization period of excision wound model. NFZ; nitrofurazone, CE; crude extract, SO; simple ointment.

Histopathological analysis:

Histopathological examinations were performed on all groups after 16 days post-wound creation, focusing on parameters such as collagen deposition, fibroblast proliferation, mononuclear cell presence, and neovascularization. The histopathology results revealed distinct differences between the treatment groups.

In the groups treated with simple ointment, a higher presence of mononuclear cells was observed, along with the presence of fibrovascular spindles extending into the dermal stroma, composed of stellate cells. However, when compared to the groups treated with 5% w/w CE, 10% w/w CE, and 0.2% w/w nitrofurazone, these findings were less prominent.

On the other hand, the groups treated with 5% w/w CE, 10% w/w CE, and 0.2% w/w nitrofurazone exhibited significantly higher expressions of collagen deposition, fibroblast proliferation, and neovascularization compared to the negative control group (Table 4 and Figure 10).

Table 4: histopathology examination results for a methanol crude extract of *Clematis hirsuta* leaves on an excision wound model

Parameters	SO	5% w/w CE	10% w/w CE	0.2% NFZ
Mononuclear Cell	+++	+	+	+
Collagen Deposit,	+	++	+++	+++
Neovascularization	++	+++	+++	+++
Fibroblast Proliferations	++	+++	+++	+++

Note: NFZ; Nitrofurazone, CE; crude extract. – Absence + low concentration ++moderate concentration, +++ high concentration.

4.5. Incision wound Model

In comparison to the group treated with simple ointment and the untreated group, all other groups that received 5% w/w CEO, 10% w/w CEO, and 0.2% w/w nitrofurazone exhibited a significant increase in skin breaking strength, as measured by water flow techniques ($p < 0.001$). Additionally, there were statistically significant differences observed when comparing the groups treated with simple ointment to the untreated groups ($p < 0.04$).

However, when comparing the groups treated with 5% w/w CEO, 10% w/w CEO, and nitrofurazone, there was no statistical significance in terms of skin breaking strength. The percentage increases in tensile strength were 7.35% for the group treated with simple ointment, 52.24% for the group treated with 5% w/w CEO, 54.48% for the group treated with 10% w/w CEO, and 55.16% for the group treated with 0.2% w/w nitrofurazone (table 5 and Figure 7).

Table 5: Effects of topical application of an 80% methanol crude extract of *Clematis hirsuta* leaf ointment in an incision wound model

Treatment group	Tensile of strength	% of tensile strength
Left untreated	265.17± 4.12	_____
SO	284.67± 5.56[a*]	7.35%
5% w/ w CEO	432.3± 3.85a**b**	52.24%
10% w/w CEO	439.± 3.7[a**b**]	54.48%
0.2w/w NFZ	440.83± 5.08[a**b**]	55.16%

All values were expressed as mean ± SEM (n= 6 mice per group), [a] compared to against left untreated group, [b] compared to against simple ointment group, * p<0.04, **p<0.001. CEO; crude extract ointment, NFZ; nitrofurazone, SO: simple ointment

Figure 7: Tensile strength measurement for incision wound model.

4.6. Burn wound model

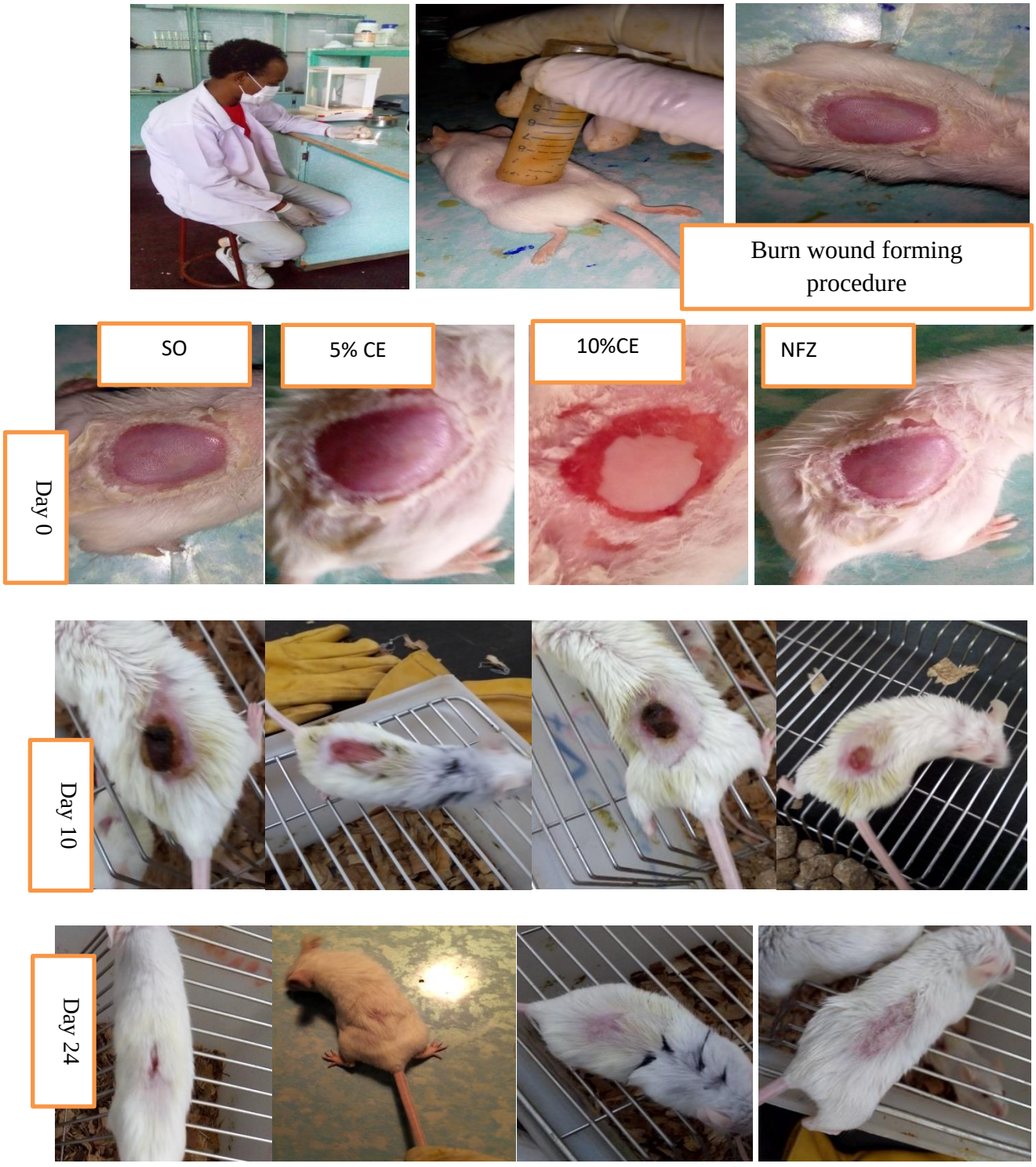
Wound contraction

In this particular investigation, it was observed that both the crude extracts and standard drugs demonstrated higher rates of wound contraction in comparison to the simple ointment. Notably, when compared to the group that received the simple ointment, the group treated with 10% w/w CE and the standard drug nitrofurazone exhibited significant wound contraction ($p < 0.037$, $p < 0.028$), respectively, on the sixth day of the post-wounding period. Additionally, the groups treated with 10% w/w CE and nitrofurazone displayed a substantially increased rate of wound contraction ($p < 0.001$) starting from the 10th day of the post-wounding period, when compared to the negative control. During the course of this experimental observation, it was observed that both the groups treated with 10% w/w CE and nitrofurazone exhibited significant wound contraction ($p = 0.017$ and $p < 0.004$, respectively) on the 14th day, in comparison to the group treated with 5% w/w CE. However, it is worth noting that despite these findings, the groups administered with 10% w/w CE and nitrofurazone did not achieve statistically significant wound contractions throughout the entire 24-day observation period. On the other hand, the groups treated with 5% w/w crude extract demonstrated significant wound contraction ($p < 0.04$) on the 8th day and starting from the 12th day ($p < 0.001$) of the post-wounding period, when compared to the negative control group. Notably, complete wound closure (100%) was achieved on the 20th day for the 10% w/w CE and nitrofurazone groups, and on the 22nd day for the 5% w/w CE group. Furthermore, the group that received the simple ointment exhibited a wound contraction percentage of 97.7% on the 24th (table 6 and figure 8).

Table 6: Effects of topical application of an 80% methanol crude extract of *Clematis hirsuta* leaves on wound contraction in burn wound models

Days	Wound area in mm^2 ±SEM (% contraction)			
	SO	5% w/w CEO	10% w/w CEO	0.2% w/w NFZ
Day 2	290.3± 1.34 (3.23)	288.8± 1.3 (3.95)	288.2± 1.8 (3.94)	288.1± 1.4 (3.96)
Day 4	278.96± 1.04 (7.01)	275± 1.67 (8.3)	274.3± 2.3 (8.56)	273.8± 2.4 (8.73)
Day 6	252.33± 1.34 (15.89)	241.3± 4.8(19.56)	237.2± 3.67 (20.9) [a*]	236.5± 3.8 (21.3) [a**]
Day 8	223.94± 1.8 (25.3)	209.67± 4.6 (30.1)[a] ***	204.9± 3.6 (31.7) [a++]	203.17± 3.2 (32.3) [a++]
Day 10	196.4± 3.79 (34.5)	177± 4.74 (41) [a++]	167 ± 4.9 (43.3) [a]*α	166.3± 4.6 (44.6) [a]*α
Day 12	163.67± 4.0 (45.4)	138.8± 4.8 (53.7) [a] *α	123.6± 7.2 (58.7) [a]*α	122.67± 5.9 (59.1) [a]*α
Day 14	139.67± 4.34 (53.4)	108.9± 3.8 (63.7) [a] *α	87.6± 7.1 (70.8) [a]*α [b]α	83.38± 3.9 (72.2) [a]*α [b]¥
Day 16	100.17± 6.4 (66.6)	70.69± 3.7 (76.4) *α	47.4± 7.9 (84.2) [a]*α	42.17± 4.77 (85.9) [a]*α
Day 18	58.17± 6.84 (80.6)	19± 4.9 (93.6)[a]*α	16.± 5.01 (94.6) [a]*α	15.17± 3.4 (94.9) [a]*α
Day 20	30.5± 4.89 (89.8)	7.0± 2.2 (97.6) [a]*α	0±0 (100) [a]*α	.0± .0 (100) [a]*α
Day 22	16.6± 3.57 (94.4)	.0± .0 (100) [a]*α	.0± .0 (100) [a]*α	.0± .0 (100) [a]*α
Day 24	6.83± 2.0 3(97.7)	.00± .08 (100) [a]*α	.0± .0 (100) [a]*α	.0± .0 (100) [a]*α

All values were expressed as mean ± SEM (n= 6 mice per group), [a] compared to against negative control group, [b:] compared to against 5% crude extract, * p<0.037, **p<0.028, *** p<0.04, ++ p<0.05, ¥ p<0.004, α p<0.017 *α p< 0.001. CEO; crude extract ointment, SO; simple ointment, NFZ; nitrofurazone. Numbers from 2 to 18 indicate the numbers of days that wound contraction measurements were taken.



Figure; 8: Burn wound model picture.

CE; crude extract, NFZ; nitrofurazone, SO simple ointment

Periods of epithelialization

In the burn wound model, similar to the excision wound model, the treatment groups exhibited a reduction in the epithelialization period. Specifically, in the group treated with 5% w/w CEO, 10% w/w CEO, and the standard drug 0.2% w/w NFZ, there was a significant decrease in the epithelialization period ($p < 0.027$, $p < 0.005$, and $p < 0.001$, respectively) when compared to the group treated with the simple ointment. Although the differences did not reach statistical significance, the epithelialization period of the 10% w/w CE and 0.2% w/w NFZ groups showed a shorter duration compared to that of the 5% w/w CE group. However, it is important to note that the crude extracts were unable to expedite the epithelialization period more effectively than the standard drug, 0.2% w/w NFZ. The average duration of epithelialization for the group treated with 5% w/w CE, 10% w/w CE, and 0.2% w/w NFZ was 17.83, 17.6, and 16.6 days, respectively, in contrast to 20.5 days for the group treated with the simple ointment. The percentages of reduction in epithelialization were calculated as 13%, 17%, and 19% for the 5% w/w CE, 10% w/w CE, and 0.2% w/w NFZ groups, respectively (figure 9).

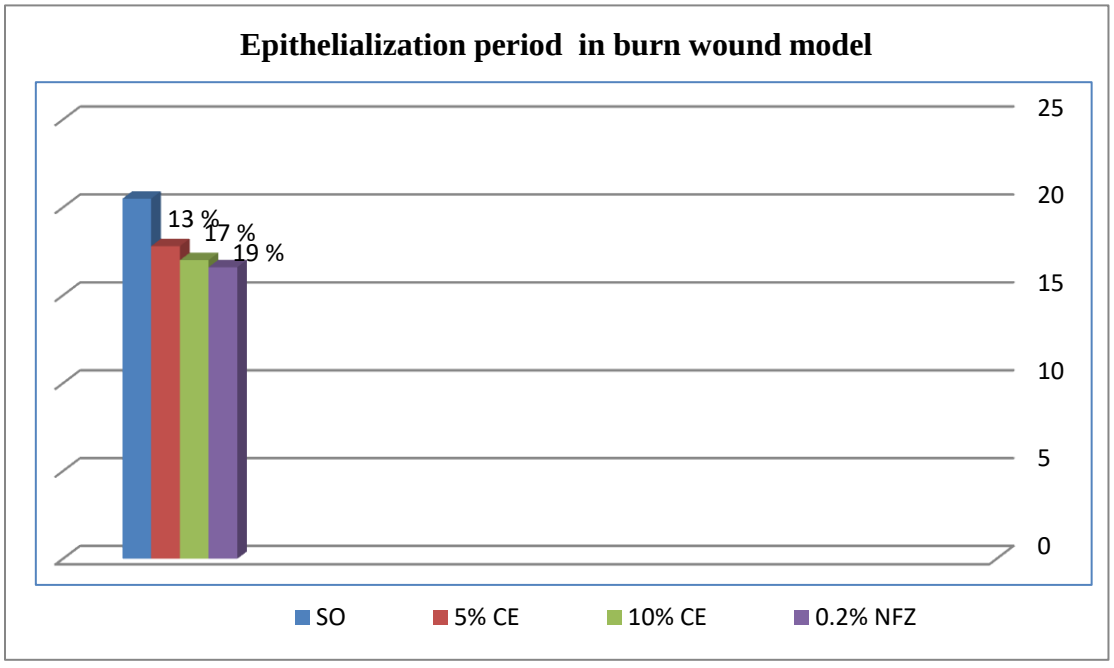


Figure 9: Effect of topical application ointment of 80% methanol crude extract of *Clematis hirsuta* leaves on epithelialization period of burn wound model.

NFZ; Nitrofurazone, CE; Crude Extract, SO; Simple Ointment.

Histopathological analysis:

Histopathological examinations, including assessments of collagen deposition, fibroblast proliferation, mononuclear cell infiltration, neovascularization, and wound contraction, were carried out on the 20th day post-wounding for all groups. The histopathological results demonstrated that the groups treated with 5% w/w CE, 10% w/w CE, and 0.2% w/w NFZ exhibited reduced mononuclear cell infiltration, fibrovascular spindle formation, and ulceration compared to the group treated with the simple ointment. In addition, these treatment groups displayed higher levels of collagen deposition, fibroblast proliferation, neovascularization, and wound contraction when compared to the negative control group. Overall, the histopathological findings indicated improved healing outcomes in the groups treated with 5% w/w CE, 10% w/w CE, and 0.2% w/w NFZ, as evidenced by enhanced tissue remodeling and wound contraction (Table 7 and Figure 10).

Table 7: Histopathology examination results for a methanol crude extract of Clematis hirsuta leaves in a burn wound model

parameters	SO	5% w/w CE	10% w/w CE	0.2% NFZ
Mononuclear Cell	+++	+	+	+
Collagen Deposit	+	++	+++	+++
Neovascularization	+	+++	+++	+++
Fibroblast Proliferation	++	+++	+++	+++

Note: SO; simple ointment, NFZ; Nitrofurazone, CE; crude extract. – Absence + low concentration ++moderate concentration, +++ high concentration.

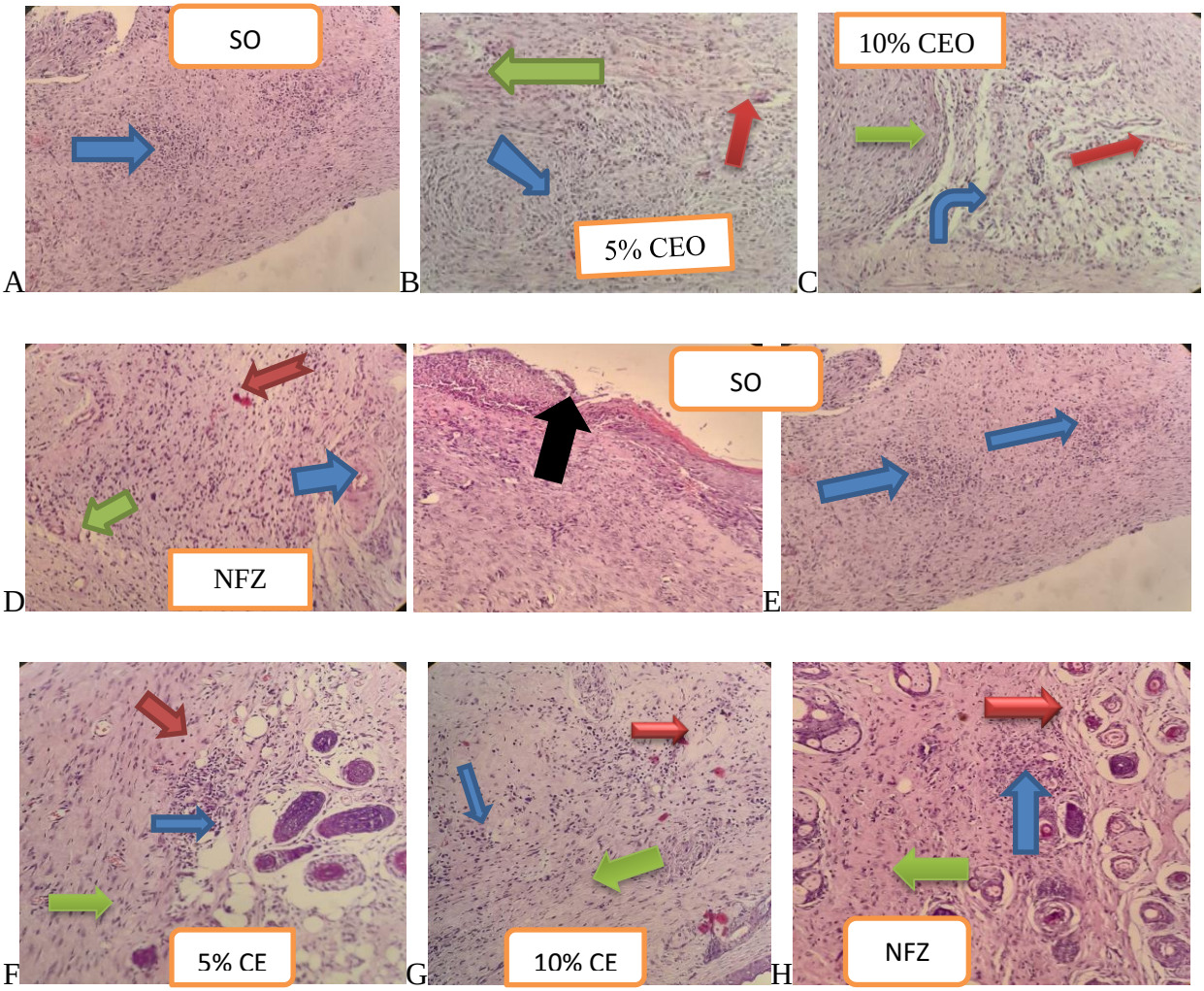


Figure 10: Histopathology examination results for the excision and burn wound models. Picture A-D is for excision, while picture E-H is for burn.
Green rays: fibrocytes with collagen deposition; blue rays: mononuclear inflammatory cell infiltrates; red rays: angiogenesis; and black rays: ulceration. CE: crude extract; NFZ: nitrofurazone; SO: simple ointment

5. Discussion

Wound healing activities of *C. hirsuta (Perr and Guill*) leaves have been reported in a range of ethnobotanical studies. The present study was intended to evaluate the wound healing activities of the methanol extract of *C. hirsuta (Perr and Guill)* leaves. Wound healing is the complex cascade interaction of cellular, and biochemical action leading to the restoration of structural, and functional integrity with regain of the strength of injured tissues (Lucas *et al.,* 2021). It also involves varies overlapping phases like inflammation, contraction of wounded area, tissue re-epithelialization, remodeling, and tissue granulation with angiogenesis (Shewaye *et al*., 2023).

In comparison to the negative control group, the treatment with the methanol extract of *C. hirsuta* leaves exhibited faster wound healing, indicating a significant improvement in wound healing progression with *C. hisruta* They regenerate the lost tissue and induce wound healing by different mechanisms since they contain different secondary metabolites (Badr *et al*., 2023*).* The application of leaf extract has the potential to exert a modulatory effect on one or more phases of the wound healing process, thereby resulting in several beneficial outcomes. These include enhanced wound contraction, accelerated epithelialization, increased biosynthesis and deposition of new collagen, and improved scar formation. These unique mechanisms of action associated with the use of leaf extract make it a distinctive and noteworthy approach, as it influences multiple aspects of wound healing. Consequently, the application of leaf extract holds promise in promoting a more efficient and effective healing process. To obtain a comprehensive understanding of wound healing processes, it is crucial to employ multiple in vivo models rather than relying solely on a single model or in vitro studies (Mulisa et al., 2015). In this study, three distinct animal wound models (excision, incision, and burn) were employed to evaluate the in vivo wound healing potential of the 80% methanol extract obtained from *C. hirsuta* leaves in mice. This comprehensive approach enabled a thorough assessment of the therapeutic effects and efficacy of the plant extract in promoting wound healing. Notably, *C. hirsuta* leaves have a longstanding tradition of use for their wound healing properties, often involving the direct application of leaf juice to the affected area (Bitew et al., 2019). Therefore, this study aimed to evaluate the wound healing activities of the methanol extract of *C. hirsuta* leaves using excision, incision, and burn wound models in mice.

The leaves were extracted using the maceration technique with 80% methanol to isolate the active components from the cellular residue. Subsequently, the resulting extract was incorporated into simple ointment bases to ensure sustained release at the site of application, facilitating the desired therapeutic effect. This approach enabled a controlled and targeted investigation of the wound healing potential of *C. hirsuta* leaf extract. Methanol was selected as the extraction solvent due to its higher polarity, which effectively extracts polar secondary metabolites. The maceration technique was chosen for its practicality and cost-effectiveness in small and medium-scale plant extractions, allowing for efficient extraction of desired compounds from the plant material (Babakura et al., 2019).

To enhance ease of application and minimize systemic toxicity, topical skin ointments were formulated using crude extracts. The ointment base incorporated ingredients such as hard paraffin and white soft paraffin to maintain skin moisture, as well as cetostearyl alcohol and wool fat to provide ointment thickness and stability (Puratchikody, C.Devi, 2006). Then the 5% w/w and 10% w/w crude extract concentrations were made from the simple ointment base and kept in a closed container.

Throughout the 14-day observation period, no signs or symptoms of acute dermal toxicity were observed, confirming the safe use of C. hirsuta leaves in the actual experiments. In the excision and burn wound models, the measured parameters included wound contraction area, epithelialization period, histopathological analysis, and tensile strength in the incision wound model. Both 5% w/w and 10% w/w crude extracts demonstrated dose-dependent improvements in wound contraction rate and reduced healing time in both excision and burn wound models. This effect might be attributed to the concentration of bioactive constituents present in the extract. Specifically, the group receiving the 10% w/w crude extract exhibited enhanced wound contraction rates starting on day 4 ($p < 0.017$) and day 6 ($p < 0.037$) in the excision and burn wound models, respectively. This was comparable to the study reported by (Teklie *et al.*, 2021) on the leaf extract of *Achyranthes aspera L*. However, the findings of this study differ from those presented by (Befekadu *et al.*, 2022) regarding the wound healing activity of *Brucea antidysenterica* leaf extract. Their study demonstrated significant wound healing activity within a shorter timeframe and at a lower concentration. This variation in results could be attributed to the higher potency of *Brucea antidysenterica* in promoting wound healing. In the excision wound model, the 5% w/w crude extract (CE) demonstrated an improved rate of wound contraction

starting on day 4 ($p < 0.037$), while in the burn wound model, the improvement was observed from day 8 ($p < 0.04$). Similarly, the 10% w/w CE exhibited a significantly higher wound contraction rate ($p < 0.001$) starting from day 6 in the excision wound model and from day 10 in the burn wound model. In contrast, the groups receiving the 5% w/w CE showed a comparable onset of observable wound healing, starting from day 9 in the excision wound model and day 11 in the burn wound model. It is worth noting that the negative control group displayed observable wound healing starting from day 9 in the excision wound model and day 11 in the burn wound model, which may be attributed to the supplementary effect of the simple ointment on the natural wound healing process.

In the excision wound model, the epithelialization period was reduced from 17.5 days to 15.3 days with the 5% w/w CE and to 14 days with the 10% w/w CE. These results are comparable to those reported by (Tessema, 2021). In the burn wound model, the periods of epithelialization were decreased from 20.5 days to 17.83 days with the 5% w/w CE and to 17 days with the 10% w/w CE, compared to the negative control group. Therefore, the crude extract derived from *C. hirsuta* leaves demonstrated a significantly shorter epithelialization period compared to the negative control group (Teshome et al., 2022) which is 16.80 and 16.00 in excision. This might be due to plant effectiveness or a difference in procedure. Overall, contraction in the wounded area decreases the extra-cellular matrix extent required to repair the defect and helps re-epithelialization by reducing the distance moved by migrating keratinocytes (Tekleyes *et al*., 2021*).*

This effect can be attributed to the individual or synergistic activity of bioactive secondary metabolites such as alkaloids, tannins, saponins, flavonoids, terpenoids, steroids, and phenols. These compounds were identified during the phytochemical analysis (table 2) and are known to have wound healing properties. According to a study conducted by (Abdisa and Kenea, 2020), *C. hirsuta* demonstrates significant antioxidant activity and is rich in polyphenolic compounds, including flavonoids, phenols, tannins, and saponins. These findings align with the results obtained from our phytochemical screening study. The presence of polyphenolic compounds in *C. hirsuta* is believed to contribute to the wound healing process through their antioxidant effects. (Şakul *et al*., 2023), anti-inflammatory, antimicrobial, angiogenesis, and stimulation of cell proliferation activity (Thao, 2020).

Another previous study (Segewkal *et al.*, 2013) revealed that clematis species like *C. longicauda, C. burgensis*, and the genus *clematis* (Ranunculaveae) (Chawla *et al.*, 2012*),* possess wound-healing activity due to the presence of the above secondary metabolites. Reactive oxygen species (ROS) have the potential to induce cell necrosis and tissue damage, which can lead to delays or impairments in the wound healing process. However, the presence of polyphenolic compounds in *C. hirsuta* plays a beneficial role by scavenging ROS, neutralizing free radicals, and promoting wound healing, thereby reducing the healing time. Tannins, known for their astringent effects, contribute to wound healing by reducing protein oxidation through chelation with free radicals and ROS. This mechanism promotes wound contraction, facilitates the formation of fibroblasts and capillary vessels, and supports the overall healing process (Alemu *et al*., 2020).

Terpenoids and alkaloids contribute to rapid wound healing by promoting wound contraction and reducing the epithelialization period. These bioactive compounds exert their effects through various mechanisms, including antimicrobial activity (disruption of bacterial membranes), astringent properties, anti-inflammatory effects (suppression of inflammatory mediator synthesis from arachidonic acid), and antioxidant activities. These actions facilitate the maturation phase of the wound, leading to accelerated healing (G/giorgis et al., 2022).

According to the report presented by (Mekonnen, 2017), *C. hirsuta* has anti-bacterial activities against common pathogens like Staphylococcus aureus, Salmonella thyphi, Escherichia coli, Pseudomonas aeruginosa, and Shigela boydii. and antifungal (Nigussie *et al*., 2021). The antimicrobial activity of *C. hirsuta* leaf extract plays a crucial role in maintaining a clean wound environment, preventing infection, and reducing the accumulation of cellular debris. Bacterial endotoxins can prolong the wound healing process and contribute to local necrosis by enhancing the inflammatory phase. By exhibiting antimicrobial properties, *C. hirsuta* leaf extract helps reduce bacterial loads in the vicinity of the wound. This, in turn, promotes epithelial cell proliferation and enhances fibroblast activity, leading to improved wound contraction and a shorter epithelialization period in both the excision and burn wound models. These antimicrobial effects of *C. hirsuta* complement its other mechanisms of action in facilitating the wound healing process.

Based on the histopathological results, the observed rapid wound contraction and shortened epithelialization periods in both excision and burn wounds may be attributed to the increased expression of collagen deposition, fibroblast proliferation, neovascularization, and reduced

presence of mononuclear cells in the treatment groups compared to the negative control groups (Figure 10). These findings are consistent with a study conducted by (Abeje, 2022) on *Urtica simensis* leaves. Wound healing mainly depends on synthesis, collage deposit, and their consequent maturation (Kore *et al*., 2023), which provides strength and integrity for the matrix of the tissue (Nagar *et al.,* 2016). Angiogenesis is required to promote wound healing by providing metabolites, oxygen, and nutrients (Yiblet *et al*., 2022), to the newly formed tissue to remove the damaged tissue during the repair process (Hou *et al*., 2016). The increased expression of collagen deposition, fibroblast proliferation, neovascularization, and decreased presence of mononuclear cells in the treatment group and positive control group were crucial for achieving rapid wound healing and reducing the epithelialization period.

In groups II, III, and IV, there was a lower presence of mononuclear cells and a more prominent fibroblast proliferation compared to group I. These findings suggest that the crude extracts possess anti-inflammatory activity. This study's results align with previous reports b (Negash *et al*., 2020), further supporting the anti-inflammatory properties of the tested crude extracts.

In the incision wound model, the measurement of skin-breaking strength using the water flow technique demonstrated a significantly higher resistance of the skin in the groups treated with 5% w/w CE, 10% w/w CE, and nitrofurazone ($p < 0.001$) compared to the untreated group and the group that received a simple ointment. However, there was no statistically significant difference between the crude extract and nitrofurazone groups, which may be attributed to their comparable efficacy in promoting wound healing activities.

The skin-breaking strength percentages were measured as 52.24%, 54.48%, and 55.16% in the groups treated with 5% w/w CE, 10% w/w CE, and nitrofurazone, respectively, while it was 7.35% in the groups treated with a simple ointment. These results from our study are comparable to the findings reported by (Beshir et al., 2016) regarding the leaf extract of *Becium grandiflorum.* The effect may be due to the wound healing properties of secondary metabolites, which aid in collagen maturation. This process strengthens the tissue matrix and promotes stable inter- and intra-crosslinking (Shewaye *et al*., 2023). The observed increase in tensile strength can be attributed to an elevated concentration of collagen synthesis, stabilization of fibers, and angiogenesis. These factors collectively support essential wound healing processes (Arzu *et al*., 2023).

5.1. Limitation of the Study: The major limitations faced during the experimental period were: lack of sufficient chemicals and materials; risks of bias since many procedures were done by one individual; and the unchecked effectiveness of solvent fractionations.

6. Conclusion

The topical application of an 80% methanol crude extract of *C. hirsuta* leaves at a limit test dose of 2000 mg/kg was found to be safe. Furthermore, the topical application of ointment formulations containing 5% w/w and 10% w/w concentrations showed promising wound healing activity. These formulations demonstrated increased wound contraction, improved skin breaking strength, reduced inflammation, and shortened epithelialization periods. These effects were comparable to the standard drug, 0.2% w/w nitrofurazone, in all the excision, incision, and burn wound models. The herb also exhibited positive effects on collagen deposition, fibroblast proliferation, and angiogenesis, which are markers of an enhanced wound healing process. These effects are likely attributed to the presence of bioactive secondary metabolites that promote rapid wound healing. The findings of the present study support the traditional claim of C. hirsuta leaves possessing wound healing activities.

7. Recommendations

Based on the present finding, it was suggested that further

- Investigation of wound healing and anti-inflammatory activity of different solvent fractionations of *C. hirsuta*
- Perform sub-acute and chronic toxicity studies.
- Perform quantitative phytochemical screening of *C.hirsuta* leaves.

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