Ethnopharmacological Approaches for the Investigation of Wound Healing Potential of Medicinal Plants– A Comprehensive Review

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ABSTRACT

Wound healing activity is a complex and dynamic biological process involving a cellular, molecular, and biochemical events aimed at restoring the integrity of injured tissue. This process typically encompasses four overlapping phases. Medicinal plants have long been utilized in traditional systems of medicine for treating wounds due to their antimicrobial, anti-inflammatory, antioxidant, and collagen-promoting properties. To validate the therapeutic efficacy of such herbal drugs, *In vitro, Ex vivo, and In vivo* screening methods have been employed. These screening methods serve as critical tools for assessing different aspects of wound repair, including cell proliferation and migration, collagen synthesis, tensile strength, and histopathological changes. Common approaches include the scratch assay (cell migration),

excision and incision wound models *(In vivo),* the chorioallantoic membrane (CAM) assay, and biochemical estimations like hydroxyproline and antioxidant assays. This review not only elucidate the wound healing process and mechanism of action of herbal extracts but also supports the development of evidence-based phytotherapeutic products.

KEYWORDS: Wound healing, Epidemiology, Assay methods, Medicinal plants, Overlapping phases.

INTRODUCTION

An injury to living tissue or a rupture in the epithelial integrity of the epidermis' outermost layer is referred to as a wound. Based on the underlying reason of wound creation, wounds are classified as either open or closed. Additionally, wound healing physiology determines whether a wound is acute or chronic. Acute wounds heal spontaneously within 5–10 days or within 30 days using the usual healing channel; chronic wounds, on the other hand, do not heal using the normal healing road. When a wound is open, blood flows from the body and the bleeding is obvious. Blood exits the vascular system but stays in the body in a closed wound **(Renuka Verma *et al.,* 2019).**

In humans and animals, wound healing is a significant but complex process that is regulated by a number of overlapping but sequential phases, including as hemostasis, inflammation, proliferation, and remodeling. The capacity to heal wounds without causing them to heal excessively is clearly age-dependent. The greater the age, the greater the likelihood of excessive wound healing. The differences between the wound healing processes of fetal and adult skin can be demonstrated by at least four mechanisms. While neutrophil and macrophage migration and an inflammatory response are hallmarks of the early stages of adult recovery, fetal inflammation is not visible. Research indicates that compared to adult wounds, neonatal wounds contain fewer inflammatory cells **(Peng-Hui Wang *et al.*, 2018).**

PHASES OF WOUND HEALING (Werner *et al*., 2008)

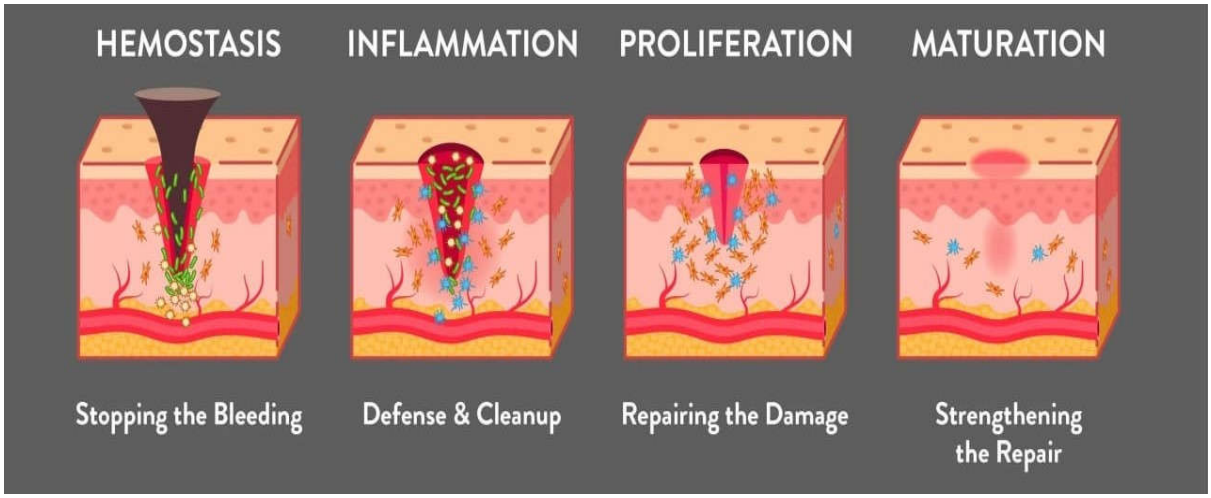

Figure. 1: Four stages of wound healing

- ✓ **Hemostasis Phase** (Duration: Few hours): The clot is mostly made up of platelets, fibrin, and trapped red blood cells. Incomplete clot formation and decreased growth factor release are possible in some disease situations (such as diabetes).
- ✓ **Inflammatory Phase** (Duration: 1–3 days): Macrophages are big cells with frothy cytoplasm. appearance of pus in infected wounds. The presence of plasma cells and lymphocytes indicates chronic inflammation. delayed inflammation in wounds caused by ischemia or immunological suppression.
- ✓ **Proliferative Phase** (Duration: Three to ten days): Granulation tissue manifests as: Early, highly cellular, vascularized tissue with little matrix. Later, fewer inflammatory cells and more collagen were seen.
- ✓ **Phase of Remodelling and Maturation** (Duration: Weeks to Months): Scar tissue: hypocellular, avascular, collagen-rich. It might display: Excessive, disordered collagen bundles and contractures are characteristics of keloid or hypertrophic scars, which are found in deep wounds. Unusual renovation could manifest as: Chronic inflammation, fibrosis, and ulceration (in diabetic wounds or pressure sores) **(Gurtner GC *et al.*, 2008).**

ETIOLOGY

Every wound has the potential to develop into a chronic one. They are divided into four groups based on their etiology: arterial, diabetic, pressure, and venous ulcers.

Arterial Ulcer: Often called ischemic ulcers, arterial ulcers are lesions that refuse to heal because of insufficient arterial blood flow or low perfusion pressure to the lower extremity tissues **(Ajay K. Khanna *et al.*, 2016).**

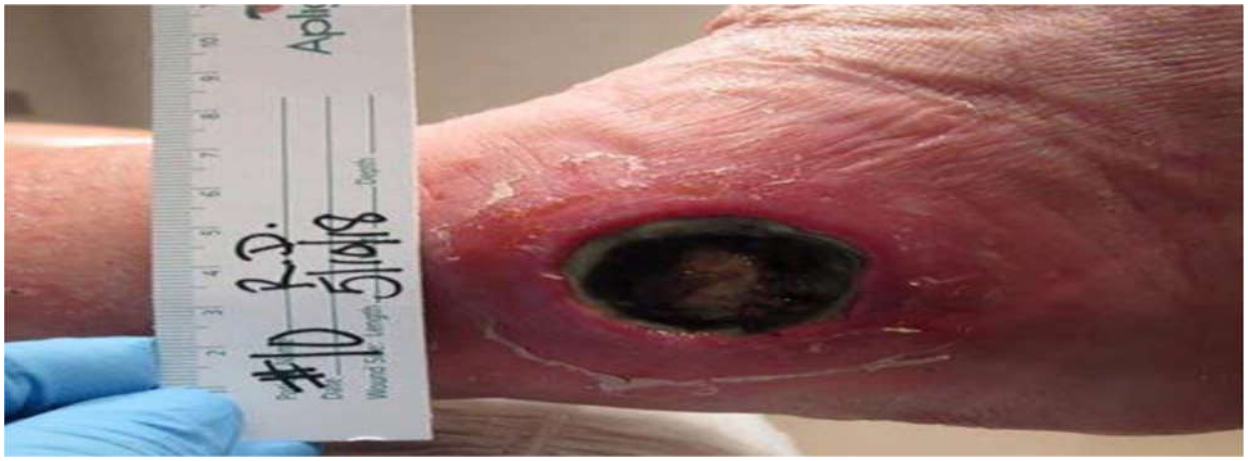

Figure.2: Arterial Ulcer

Diabetic Foot Ulcer: Diabetic foot ulcer (DFU) is a serious and incapacitating sign of uncontrolled diabetes that typically appears as an ulcer on the plantar part of the foot. One of these ulcers will eventually develop in about 15% of people with diabetes, and of those people, 14%–24% will need to have the ulcerated foot amputated because of a bone infection or other ulcer-related problems**(Joel M Raja *et al*., 2023).**

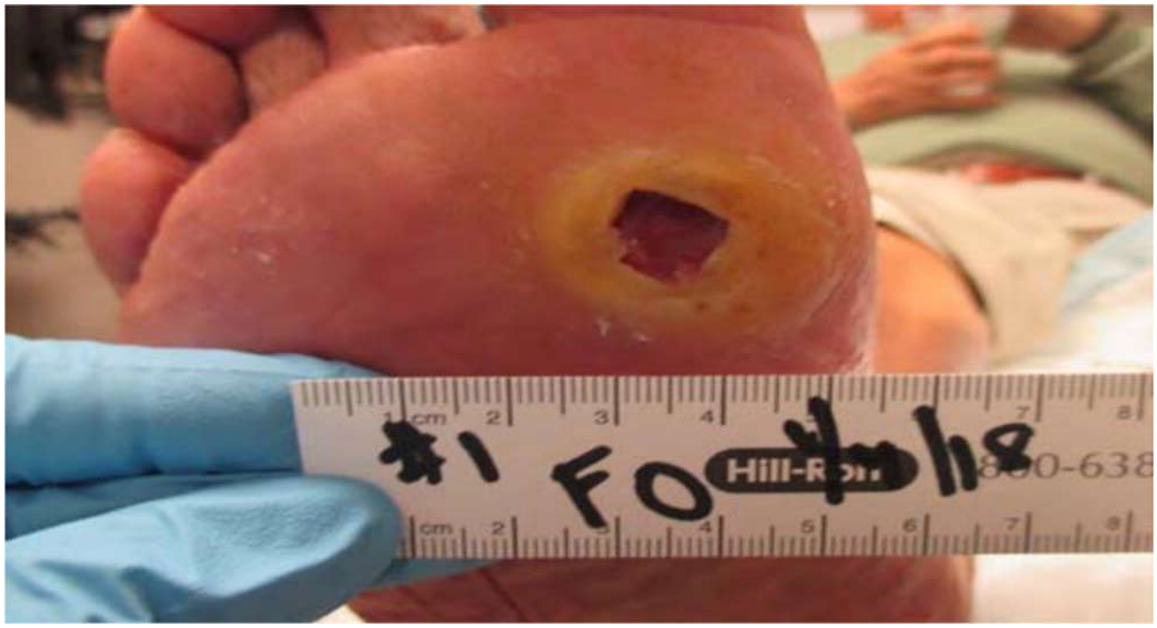

Figure.3: Diabetic foot ulcer

Pressure Ulcer: Pressure ulcers (PUs), sometimes referred to as bedsores or decubitus ulcers, are localized patches of tissue and skin that have degraded as a result of ongoing pressure and friction, especially in the body's bony parts **(Julie Zuniga *et al.*, 2024).**

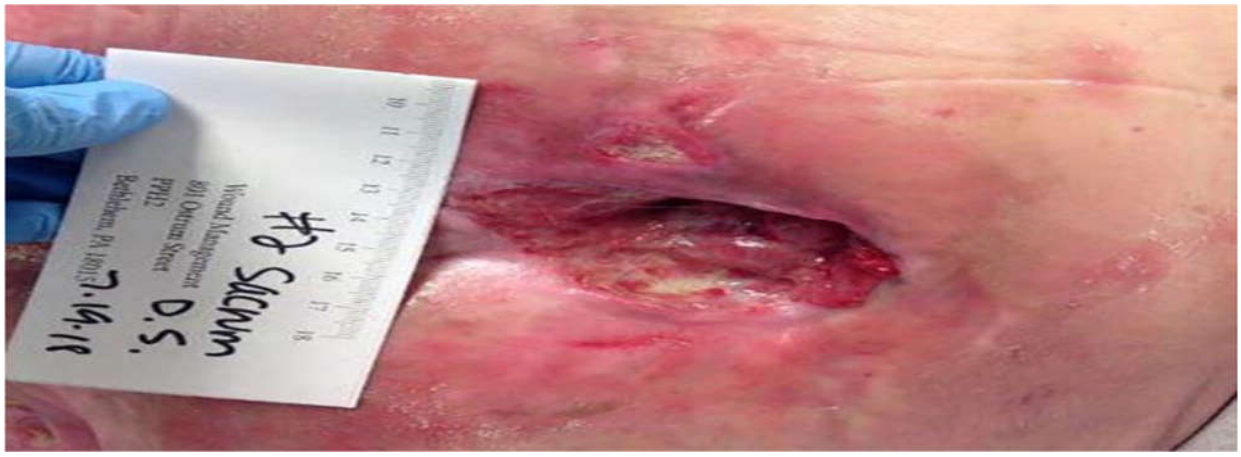

Figure.4: Pressure ulcer

Venous Ulcer: Long-term chronic venous disease (CVD), sometimes known as chronic venous insufficiency (CVI) in the more advanced phases of the condition, is characterized by VLUs. This is characterized as venous valvular incompetence leading to an improperly functioning venous system **(Stéphanie F Bernatchez *et al.*, 2021).**

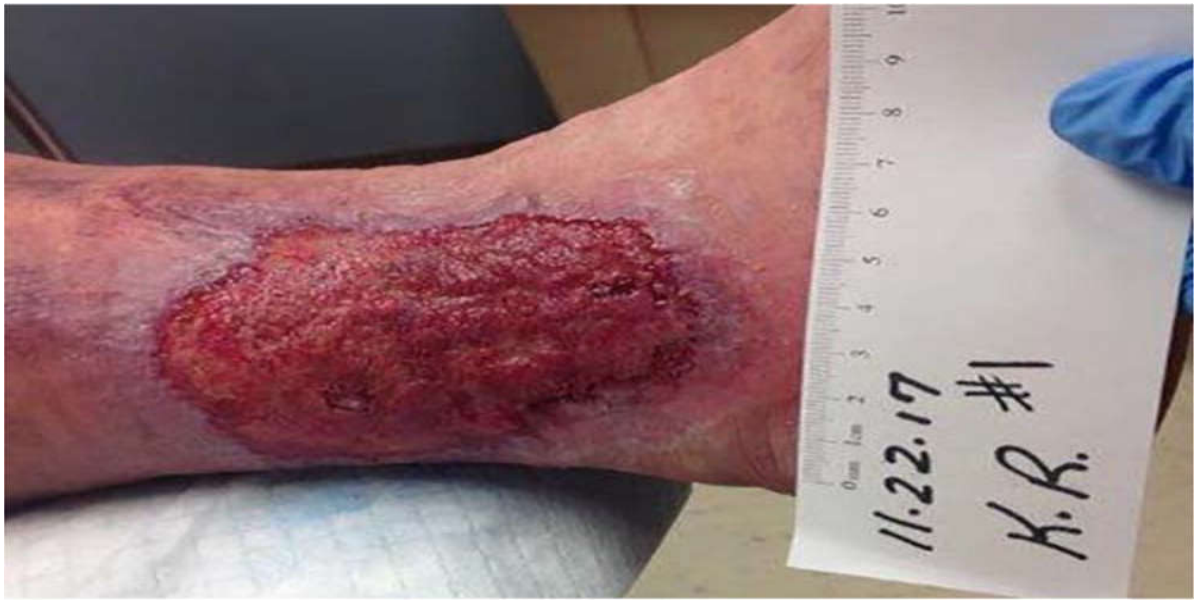

Figure.5: Venous ulcer

FACTORS AFFECTING WOUND HEALING

Local Factors that Influence Healing

Oxygenation: Oxygen is essential for almost all wound healing processes and for cell metabolism, particularly the ATP-based energy production process. It stops wounds from getting infected, stimulates angiogenesis, boosts keratinocyte migration, differentiation, and

re-epithelialization, increases collagen synthesis and fibroblast proliferation, and encourages wound contraction **(S. Guo *et al.*, 2009).**

Desiccation: Compared to a dry environment, where cells usually dehydrate and die, a moist environment promotes faster and less painful wound healing. This hinders healing by causing a crust or scab to grow over the wound site. Using a moisture-retentive dressing to keep the wound hydrated promotes epidermal cell migration and epithelialization **(Cathy Thomas Hess *et al.*, 2011).**

Infection or abnormal bacterial presence: To identify the causing bacteria and direct antibiotic treatment, a wound culture should be taken if there is purulent drainage or exudate, induration, erythema, or fever, which are signs of an infection **(Cathy Thomas Hess *et al.*, 2011).**

Trauma and edema: In an environment where they are frequently traumatized or where edema prevents them from receiving a local blood supply, wounds heal poorly or not at all **(Cathy Thomas Hess *et al.*, 2011).**

SYSTEMIC FACTORS THAT INFLUENCE WOUND HEALING

Age: One of the main risk factors for poor wound healing is aging. The epidermal layer thins with age, and metabolic and systemic changes might result from becoming older. The inflammatory response of the aged is altered in a number of ways, including delayed leukocyte recruitment to the region, decreased growth factor/cytokine release, and diminished macrophage activity as phagocytosis **(Fahrun Nur Rosyid *et al*., 2022).**

Sex: Age-related deficiencies in wound healing are influenced by sex hormones. It has been demonstrated that older guys recover acute wounds more slowly than older females. The fact that male androgens (testosterone and 5α-dihydrotestosterone, DHT), female estrogens (estrone and 17β-estradiol), and their steroid precursor dehydroepiandrosterone (DHEA) seem to have a major impact on the wound-healing process may help to explain this **(S. Guo *et al.*, 2009).**

Stress: Through the dysregulation of endocrine hormones, stress can impede the healing of wounds and a number of systemic disorders. Stress can increase the release of hormones because it affects the hypothalamus and neurological system **(Fahrun Nur Rosyid *et al.*, 2022).**

Diabetes: Hundreds of millions of people worldwide suffer with diabetes. The ability of diabetics to heal acute wounds has been shown to be compromised. Additionally, 15% of people with diabetes are thought to acquire chronic non-healing diabetic foot ulcers (DFUs), which are more common in this population **(S. Guo *et al.,* 2009).**

Alcohol consumption: Alcohol exposure reduces wound healing and raises the risk of infection, according to clinical data and animal studies **(S. Guo *et al.*, 2009).**

Smoking: Smoking has been linked to a higher risk of a number of illnesses, including slowed wound healing. Additionally, research indicates that the substances included in tobacco cigarettes—nicotine, carbon monoxide, and hydrogen cyanide—can alter the processes by which wounds heal **(Fahrun Nur Rosyid *et al.,* 2022).**

EPIDEMIOLOGY OF WOUND HEALING

Since many medical conditions also have a tendency to develop hypertrophic scars and keloid formation due to hereditary penetrance, recent epidemiologic studies have demonstrated that certain medical conditions, such as hypertension (endothelial cell dysfunction), are also linked to excessive scar formation. This supports the evidence that genetic and epigenetic traits play a significant role in scar formation. Nevertheless, there is still much to learn about the genetic component of keloid formation because of the limitations of genetic research on keloid scarring **(Peng-Hui Wang *et al.,* 2018).**

PREVENTION

Early detection and the start of therapy following surgery or trauma are the initial steps in treating excessive wound healing. In order to prevent infection, careful tissue handling, suturing, and wound care are essential. To lessen scar hyperpigmentation, sun protection is crucial. Preventive measures, such as silicone gel sheeting or ointments, hypoallergenic microporous tape, and concomitant intralesional steroid injection, are beneficial for patients who are more likely to experience excessive wound healing. The only proven treatment for hypertrophic scars is silicone gel sheeting, which is used extensively. For more than two decades, silicone gel sheeting has been used safely and effectively, as evidenced by numerous randomized controlled trials **(Peng-Hui Wang *et al.,* 2018).**

SCREENING METHODS ON WOUND HEALING ACTIVITY

Traditional medicine has employed medicinal herbs to treat wounds. To assess their potential for healing, scientific screening techniques are crucial.

1. *In vitro* method

a. Scratch assay

b. MTT assay

c. Collagen assay

2. *Ex vivo* method

a. Chorio allantoic membrane (CAM) assay

3. *In vivo* method

a. Excision wound model

b. Incision wound model

c. Burn wound model

d. Dead space wound model

4. Histopathological evaluation

This helps determine the effectiveness and quality of the healing process.

5. Biochemical analysis

a. Hydroxyproline estimation

b. Antioxidant & inflammatory markers

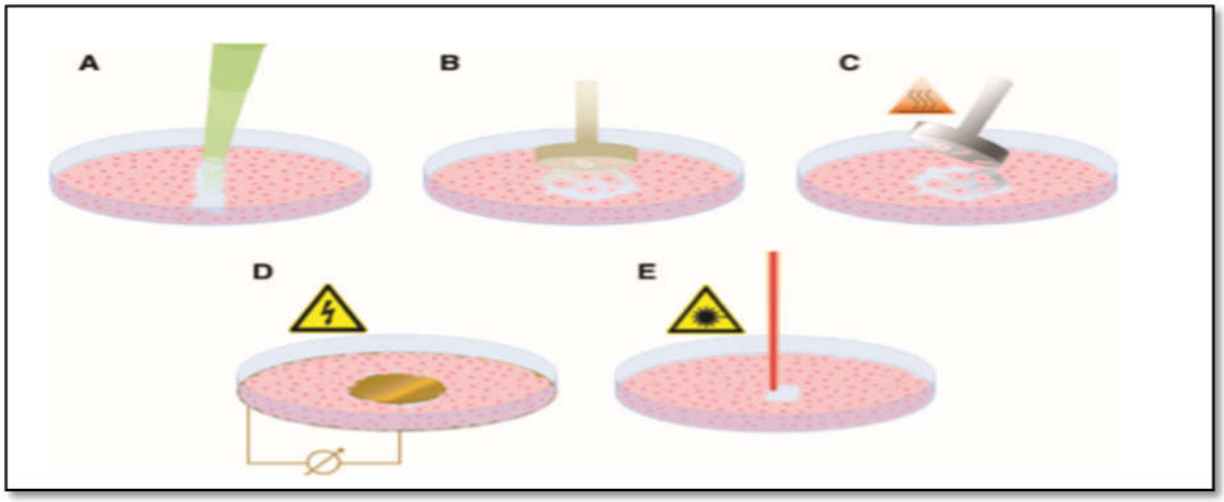

Figure. 6: Wound healing assay: (A) scratch assay, (B) stamp assay, (C) thermal wounding, (D) electrical wounding, (E) optical wounding using laser

***In vitro* assay methods**

The Latin phrase "the technique which is performed outside the living animal" is the source of the term "*in-vitro*." The tissue from the animal's body is taken out for this kind of research and kept in the appropriate growth medium for a few days to several months. Antimicrobial effects and compounds that promote healing can be determined with the help of the *in-vitro* assay **(Renuka Verma *et al.*, 2019).**

a) Scratch assay

By using a previously reported technique to conduct in vitro cell migration tests on L929 cells, the wound healing potential of the sample extract was evaluated. In short, 6-well plates were seeded with $2x10^5$ cells/ml and cultivated throughout the entire night. Following a wash with Delbucco's Phosphate Buffered Saline (DPBS), a sterile 200µL tip was used to make a scratch on the cells.

The cells were washed with DPBS to get rid of the detached cells and other cellular debris. The cells were cultured for 24 hours after being treated with 125 µg/mL of extract and 5 µg/mL with Cipladine, a positive control. Cipladine is a common medication used to treat wounds. Negative control cells were untreated. Images captured by an inverted microscope with a digital camera showed the morphological alterations and cell migration. Three duplicates of each experiment were conducted. Image J software was used to examine the width of the scratch and wound closure at various time intervals (0, 12, 24, and 48 hours) **(Srinivasa Rao Bolla *et al.*, 2019).**

b) MTT assay

Cell viability following extract treatment was assessed using the MTT test, which was carried out in accordance with the manufacturer's instructions. In a 96-well plate, 3×10^3 cells were cultivated in each well. A 22µm sterile syringe filter was used to filter the extract. Each well was filled with varying quantities of the fungal extract (1250, 625, 312.5, and 156.25 µg/ml), and the wells were incubated for 48 hours at 37°C with 5% carbon dioxide. The negative control wells were those that were not subjected to the extract treatment. Each well received MTT reagent after 48 hours, and an Eliza reader set to 540nm was used to measure the plate's absorbance **(Seyedeh Kiana Teymoorian *et al.*, 2024).**

c) Collagen assay

Fibroblast cells (such as L929 or NIH 3T3) should be cultured. Use different quantities of medicinal plant extract as a treatment. After 24-72 hours of incubation, gather the cells or

medium. Collagen is broken down by acid hydrolysis, which yields hydroxyproline. Ehrlich's reagent and chloramine-T react with hydroxyproline to form a pink chromophore. Make use of a spectrophotometer to measure absorbance at 560nm. To measure the amount of collagen, compare with a standard curve **(Reddy GK *et al.*, 1996).**

***Ex vivo* assay methods**

An organ or tissue that has been separated from an entire animal is used in the investigation. *Ex-vivo* models are useful for assessing the pace of epithelization following treatment and for gathering comprehensive molecular data regarding wound healing **(Renuka Verma *et al.*, 2019).**

a) Chorioallantoic membrane (CAM) assay

The angiogenic activity of different plant fractions was evaluated using the chick chorioallantoic membrane (CAM) model. For the study, nine-day-old fertilized chick eggs were chosen, and they were incubated in an incubator set at 37°C and 80% relative humidity. To prevent infections, 70% ethanol was used to wipe the egg shells. A little 1.0 cm^2 window formed in the shell after 72 hours. A tiny hole was drilled in the air space to lower the membrane, and a rubber bulb was used to extract the air. A sterile disk of methylcellulose loaded with varying amounts (10–40 mg) of chloroform, ethyl acetate, and ethanol residues was placed at the intersection of two large vessels after the window was opened. After that, the window was taped shut once more, and the eggs were incubated for 72 hours at 37ºC. Next, the eggs were cracked open. The creation of new vessels was noted and contrasted with that found in eggs that contained disks devoid of test substances. The impact of different plant fractions on chick embryo chorioallantoic models is documented in comparison to the control **(Vijay S. Borkar *et al.*, 2015).**

***In vivo* assay methods**

It is characterized by the fact that the test is conducted on living organisms, such as mice, rats, rabbits, etc. Because the pharmacological effects in humans and animals are identical, nonclinical research in animals is necessary before administering medication to humans **(Vijay S. Borkar *et al.*, 2015).**

a) Excision wound model

Morton and Malone (1972) reported that this was created in rats under light ether anesthesia. A wound area of roughly 500 mm^2 was obtained by excising the epidermis of the impressed

area to its full thickness. Scar area, epithelization time, and wound closure were the parameters under investigation. At day zero, day four, day eight, day twelve, and day sixteen, the percentage of wound closure was noted. Planimetric measurements were made after the scar's area and shape were traced **(A. Mathew *et al.*, 2008).**

b) Incision wound model

Rats were given light ether anesthesia to create an incision wound, as Ehrlich and Hunt (1969) described. On the eighth post-wounding day, the interrupted sutures used to close the wound were taken out. The 10-day-old wound's breaking strength was assessed **(A. Mathew *et al.*, 2008).**

c) Burn wound model

Beginning immediately after the induction of a burn wound, rats were split into five groups: 1% SSD cream as the reference standard, eucerin as the control, and 5%, 10%, and 20% AE flower extract ointments as the treatment groups. For 14 days, ointments were applied topically to the wounds each day. Adobe Photoshop CS5 was used to compute the wound areas after they were cleansed and captured on digital camera.

This formula was used to measure the rate of wound contraction: 100 × [(first day wound area - specified wound area)/first day wound area] is the wound contraction percentage. In order to assess the histological alterations, the animals were killed on day 14 (the end of the experiment), and the granulated tissues were gathered and stored in 10% buffered formalin. For every sample, a series of slices with a thickness of 3–4 μm were produced, stained with hematoxylin/eosin, and photographed under a × 400 magnification **(Mohammed Sirajuddin Khan *et al.*, 2018).**

d) Dead space wound model

The granuloma tissue's mechanical and physical alterations are examined in this model. By creating a pouch through a tiny skin nick, the subcutaneous dead space wounds are applied to each animal's ventral region, one on each side of the axilla and groin. The pouch is filled with sterile cotton pellets (5–10 mg each) or cylindrical grass piths (2.5 × 0.3 cm). Two cotton pellets or grass piths were given to each animal at a different area.

A sterile, shallow, metallic ring (2.5 × 0.3 cm) called a cylindrical pith or polypropylene tube (2.5 × 0.5 cm) is subcutaneously implanted on each side beneath the dorsal paravertebral lumbar skin surface to form the dead space wound. The wounds are then sutured. For ten days in a row, the animals in each group receive the appropriate therapeutic medication topically or orally. This model is used to study the physical alterations in the granuloma tissue. In the dead

space wound model, animals treated with deoxyelephantopin also showed a substantial increase in granulation tissue weight and breaking strength **(Mohammed Sirajuddin Khan *et al*., 2018).**

HISTOPATHOLOGICAL EVALUATION

All five animal groups' tissue-healing samples were collected and submitted for histological examination. Hematoxylin and eosin-stained samples were formally preserved, put on slides, and seen using an automated illumination microscope. Documentation included ulcer scores, fibroblastic aggregation, neovascularization, hyperpolarization, inflammatory cells, scars, and epidermal hyperplasia **(Samiullah Allahbaksh Auti *et al*., 2021).**

Biochemical analysis

The quantitative assessment of biochemical markers in wound tissue, such as collagen content, antioxidant enzyme levels, inflammatory mediators, and oxidative stress indicators, is known as biochemical analysis on wound healing activity.

a) Estimation of Hydroxyproline

On day 4, 8 and 16 of the post surgery of excision, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content, which is basic constituent of collagen. Tissues were hydrolyzed in 6 N HCl at 130 °C for 4 hours in a sealed tube after being dried to constant weight in a hot air oven at 60 to 70 °C. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine T oxidation for 20 min, the reaction was terminated by addition of 0.4 M per chloric acid and color was developed with the help of Ehrlich reagent at 60 °C and measured at 557 nm using UV/Vis spectrophotomete **(Deepak Dwivedi *et al.,* 2016).**

b) **Antioxidant & inflammatory markers**

Antioxidant biomarkers

Superoxide Dismutase (SOD)

Reduces oxidative stress by converting superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2). H_2O_2 is broken down by catalase (CAT) into oxygen and water. A non-enzymatic antioxidant that combats free radicals and supports redox equilibrium is glutathione (GSH). Vitamins C and E, Scavenge free radicals and encourage the production of collagen. Improved healing and less tissue damage are indicated by increased antioxidant enzyme activity **(Eming *et al*., 2007).**

Inflammatory Biomarkers

The second stage of wound healing, inflammation, is essential for removing infections and dead cells. Acute-phase proteins and cytokines, which function as indicators of inflammation, control it. Tumor necrosis factor-alpha, or TNF-α, stimulates neutrophils and macrophages and starts inflammation. Interleukin-1 beta, or IL-1β, promotes the synthesis of enzymes involved in tissue remodeling. Interleukin-6, or IL-6, controls the development of immune cells and facilitates tissue healing. C-reactive protein, or CRP, is a sign of systemic inflammation that is higher in wounds that are infected or chronic. Tissue regeneration results from the proper regulation of these indicators, which guarantees the shift from inflammation to proliferation **(Eming *et al.*, 2007).**

Table. 1: Medicinal plants exhibit wound healing activity

S.No	Medicinal plant	Part and extract	Method	Reference
1.	*Alternanthera brasiliana* Kuntz	Methanolic extract of leaves	*In vivo* and *in vitro* method	Burua *et al.,* 2009
2.	*Wedelia trilobata* (L.)	Ethanolic extract of leaves	Column chromatography	Neelam Balekar *et al.,* 2012
3.	*Phyllanthus muellerianus*	Aqueous aerial part extract	Excision and Incision wound models	Yaw D Boakye *et al.,* 2018
4.	*Berlinia confusa*	Ethanolic extract with stem bark	Dermal Excision model	Silas Adjei *et al.,* 2025
5.	*Anogeissus leiocarpus*	Methanolic extracts with leaves	Phytochemical screening method	Stephen Ayaba *et al.,* 2013
6.	*Propolis* and Honey	Ethanolic extract of mixture	*In vitro* scratch assay	Alexandra M. Afonso *et al.,* 2020

7.	*Aristolochia saccata*	Methanolic leaf extract	*In vitro* scratch assay	Srinivasa Rao Bolla *et al.,* 2019
8.	*Gentiana lutea*	Alcohol and petroleum ether extract of rhizome	Excision and incision wound model assay	A. Mathew *et al.,* 2008
9.	*Aglaia elaeagnoidea*	Ethanolic leaf extract	Dead space and burn wound assay	Mohammed Sirajuddin Khan *et al.,* 2018
10.	*Echinochloa colona*	Ethanolic extract of whole plant	*In vivo* and *in vitro* assay	Vijay S. Borkar *et al.,* 2015
11.	*Pongamia pinnata*	Methanolic leaf extract	Hydroxyproline assay	Deepak Dwivedi *et al.,* 2016
12.	*Glycyrrhiza Glabra*	Ethanolic extract of stem	*In Vitro* scratch assay	Ishika roy et al., 2023
13.	*Centella Asiatica*	Alcoholic extract of whole plant	*In vitro and in vivo method*	Elena Arribas-López et al., 2022
14.	*Aloe vera*	Ethanolic leaf extract	*Invivo method*	Nadia Mohamed Said Arafa *et al.,* 2025

CONCLUSION:

Wound healing is a complex process influenced by the type of wound, underlying conditions like diabetes or hypertension, and factors such as oxygenation, infection, age, and nutrition. Epidemiological evidence shows that genetic and lifestyle factors also impact healing outcomes, including risks of keloids or chronic ulcers. Healing is both an art and a science — and medicinal plants beautifully sit at the intersection of both. Through diverse screening methods, from scratch assays to animal models, we gain not just data, but a deeper understanding of how nature supports tissue repair. As research advances, refining these models will be key to unlocking the full potential of phytomedicine in wound care — where tradition meets technology for better, natural recovery.

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