CHAPTER 6

WOUND HEALING ACTIVITY

6.1 INTRODUCTION

6.1.1 Wound Healing

Injury to tissue may result in cell death and tissue destruction. Healing on the other hand, is the body's response to injury in an attempt to restore normal structure and function. Also wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue (Puratchikody, 2006).

Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of modern drugs (Mantle *et al.*, 2001).

Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelization and wound contraction are abundant in the scientific literature (Ulubelen *et al.*, 1995; Hemmati *et al.*, 2000; Choi *et al.*, 2001; Bairy, 2002). Still, one should keep in mind that plants have not only beneficial effects in promoting the healing process of wounds and burns or protecting the skin from fungal and bacterial infection or anti-tumor activity against skin cancer, but can also be involved in different allergic, photoallergic and irritant skin reactions (Mantle *et al.*, 2001). Many traditional remedies are based on systematic observations and methodologies and have been time-tested but for many of them, scientific evidence is lacking. There are only few prospective randomized controlled trials that have proved the clinical efficacy of these traditional wound healing agents. **Table 6.1** Includes some plants used in the process of wound healing.

Wounds are referred to as disruption of the normal anatomic structure and function (Gerald *et al.*, 1994). Skin wounds could happen through several causes like physical injuries resulting in opening and breaking of the skin. The most common symptoms of wounds are bleeding, loss of feeling or function below the wound site, heat and redness around the wound, painful or throbbing sensation, swelling of the tissues in the area and puslike drainage (Rashed *et al.*, 2003).

Wound healing is a very complex, multifactor sequence of events involving several cellular and biochemical processes. The aims in these processes are to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin to injury, initiates immediately after wounding and occurs in four stages. The first phase is coagulation, which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridgement of the wound followed by re-epithelialization, which includes proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the

final stage of the healing process collagen deposition and remodeling occurs within the dermis (Chettibi *et al.*, 1997; Hj Baie *et al.*, 2000).

Table 6.1 Use of following plants is reported in literature for the wound healing process: (George *et al.*, 1999; Hakeem *et al.*, 1989)

Plants	Family	Parts Used
1. *Curcuma longa*	Zingiberaceae	Rhizome
2. *Lawsonia inermis*	Lythraceae	Leaves juice
3. *Terminalia-chebula*	Combretaceae	Flowers
4. *Mallotus philippinensis*	Euphorbiaceae	Fruit
5. *Bambusa arundinaceae*	Graminae	Bamboo
6. *Agave Americana*	Amaryllidaceae	Leaves
7. *Aleo vera*	Lillaeceae	Leaves
8. *Artocarpus communis*	Integrifolia	Fruit
9. *Mirabillis jalapa*	Convolvulaceae	Root
10. *Solanum nigram*	Solanaceae	Leaves
11. *Sterculia urens*	Sterciliaceae	Root and bark
12. *Hibiscus rosa sinensis*	Malavaceae	Flower
13. *Daucus carota*	Umbelliferae	Rhizome
14. *Geum urbanum*	Rosaceae	Plant
15. *Olea europaea*	Oleaceae	Oil
16. *Glycyrrhiza glabra*	Leguminoseae	Root
17. *Plantago major*	Plantaginaceae	Leaves
18. *Ficus carica*	Moraceae	Friuts and leaves
19. *Cinchona officinalis*	Rubiaceae	Bark
20. *Solanum dulcamara*	Solanaceae	Plant
21. *Abutilon indicum*	Malvaceae	Whole plant
22. *Ricinus communis*	Euphorbiaceae	Oil and seeds
23. *Calendula officinalis*	Compositae	Whole plant
24. *Pistacia lentiscus*	Anacardiaceae	Resin
25. *Shorea robusta*	Dipterocarpaceae	Resin
26. *Blessed thistle*	Compositae	Whole plant
27. *Tridax procumbens*	Asteraceae	Whole plant
28. *Areca catechu*	Palmaceae	Seed
29. *Sausurrea lappa*	Sausurreae	Root

6.1.2 Tissue injuries

The injuries are of various types:

(1) Diabetic foot and leg ulcerations, including neuropathic ulcerations, decubitus lesions, and necrobiosis lipoidica diabeticorum (Michell *et al.*, 1997).

(2) Vascular ulcerations, including venous stasis ulceration, arterial ulcerations, varicose vein ulcerations, post-thrombotic ulcerations, atrophie blanche ulcerations, congenital absence of veins/ulcerations, congenital or traumatic arteriovenous anastomosis, temporal arteritis, atherosclerosis, hypertension (Martorell's ulcerations), thrombosis, embolism, platelet agglutination, ankle blow-out syndrome, or hemangiomas.

(3) Decubitus ulcers or pressure sores (e.g., on lying on bed for long time)

(4) Traumatic ulcerations, such as those caused by external injuries, burns, scalds, chemical injuries, post-surgical injuries, self-inflicted injuries, lesions at an injection site, neonatal or perinatal trauma, or sucking blisters.

(5) Infestations and bites, such as those caused by spiders, scorpions, snakes, or fly larvae (mydriasis).

(6) Cold injury, such as perniosis (erythrocyanosis frigida), or cryoglobulinemic ulcerations.

(7) Neoplastic ulceration, such as those caused by basal cell carcinomas, squamous cell carcinomas, malignant melanomas, lymphoma, leukemia, Kaposi's sarcoma, tumor erosion, midline lethal granuloma, or Wegener's granulomatosis.

(8) Blood diseases with ulcerations, such as polycythemia, spherocytosis, or sickle cell anemia.

(9) Skin diseases with ulcerations, such as tinea, psoriasis, pemphigoid, pemphigus, neurotic excoriations, trichotillomania, erosive lichen planus, or chronic bullous dermatosis of childhood.

(10) Metabolic disease ulcerations, such as those associated with diabetes mellitus or gout (hyperuricemia).

(11) Neuropathic ulcerations, such as those associated with diabetes mellitus, tabes dorsalis, or syringomyelia.

(12) Ischemic ulcerations, such as those associated with scars, fibrosis, or radiation dermatitis.

(13) Vasculitis ulcerations, such as those associated with lupus erythematosus, rheumatoid arthritis, scleroderma, immune complex disease, pyoderma gangrenosum, or ulceration associated with lipodermatosclerosis.

(14) Infectious ulcerations, such as: (a) viral ulcerations, e.g. those associated with Herpes simplex or Herpes zoster in an immunocompromised or normal individual; (b) bacterial infections with ulcerations, such as those associated with tuberculosis, leprosy, swimming pool granuloma, ulceration over osteomyelitis, Buruli ulcer, gas gangrene, Meleny's ulcer, bacterial gangrene associated with other bacterial infection (e.g., streptococcal infection), scalded skin syndrome, ecthyma gangrenosum (such as occur in children infected with Pseudomonas aeruginosa), and toxic epidermal necrolysis; (c) mycotic ulcerations, such as those associated with superficial fungal infection or deep fungal infection; (d) spirochetal ulcerations, such as those associated with syphilis or yaws: (e) leishmaniasis; (f) mydriasis; or (g) cellulites.

(15) Surgical ulcerations, such as those associated with closed incisions or excisions, open incisions or excisions, stab wounds, necrotic incisions or excisions, skin grafts, or donor sites.

(16) Other ulcerations, such as those associated with skin tears (traumatic ulcerations), fistula, peristomal ulcerations, ulcerations associated with aplasia cutis congenita, ulcerations associated with epidermolysis bullosa, ulcerations associated with ectodermal dysplasias, ulcerations associated with congenital protein deficiency, ulcerations associated with

congenital erosive and vesicular dermatosis, ulcerations associated with acrodermatitis enteropathica, and amputation stump ulcerations.

Wound healing is a complex biological process that differs according to the wound type: acute or chronic. The principal elements of wound repair are the immediate events of hemostasis and stimulus for inflammation, then inflammation and cell proliferation and migration, followed by molecular synthesis, collagen polymerization and cross-linking, remodeling, and wound contraction. Inflammation is characterized by vasodilation, increased vascular permeability, leukocyte infiltration, bacterial killing, and macrophage-based stimulation of cellular proliferation and protein synthesis.

In cell proliferation and migration, fibroblasts appear within 2-3 days and dominate wound cell population during the first week. For the initial 2-3 days, their activity is confined to fibroblast replication and migration. At days 4-5, fibroblasts begin to synthesize and secrete extracellular collagen. Fibroblasts produce collagen.

Angiogenesis is regulated by a complex cascade of cellular and molecular events and it is essential to wound repair and scar formation (Aplin *et al.,* 2008). Capillary proliferation is required to support fibroblast migration into wound and fibroblast metabolic requirements. In the absence of angiogenesis, such as in ischemic ulcers or arteriosclerosis obliterans, fibroblast migration arrests and wound healing fails to proceed.

Angiogenesis has the stages of cell attachment, basement membrane degradation and migration, proliferation, and differentiation, and is associated with epithelial cell migration.

Molecular synthesis includes collagen synthesis and proteoglycans synthesis. Collagen synthesis begins with the intracellular phase of monomer synthesis. Secretion into the extracellular space then occurs, followed by polymerization into collagen fibers and cross-linking to increase tensile strength.

Remodeling typically begins 3 weeks after injury. Wound remodeling begins and continues for 2 years. There is a progressive increase in tensile strength as Collagen III is replaced by Collagen I. Epithelialization is the hallmark of successful wound repair and occurs in four phases: mobilization, migration, mitosis, and cellular differentiation.

Granulation tissue contains numerous capillaries and has a support matrix rich in fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and periocytes. If vascular endothelial

growth factor (VEGF) is removed, there is an absence of granulation tissue, and wound angiogenesis and the wound healing process cease.

In chronic wound healing, there is typically an absence of epithelial migration, excessive granulation tissue, and fibrosis, with scarring and impaired function possibly being present.

Although many advances have been made in the understanding of wound healing, the healing of wounds still presents a considerable challenge to the clinician. This is particularly true in patients who are diabetic, who have impaired circulation to the skin, or who are susceptible to infection, such as the result of being in an immunocompromised condition. Additionally, when such wounds do heal, they frequently heal with cosmetically undesirable consequences such as scars or discoloration.

Accordingly, there is a need for an improved method of wound healing that is particularly suitable for application in patients with diabetes, who have poor circulation in the skin, or who are immune compromised. There is a further need for treatments and methods that can reduce or eliminate the consequences that can occur from wound healing, such as scars and discoloration. There is an additional need for factors that are well-tolerated and can be used with other treatments in such patients. (Zaveri, 2007)

The process of healing involves two distinct processes. Regeneration when healing takes place by proliferation of parenchyma cells and usually results in complete restoration of original tissues. Repair, when the healing takes place by proliferation of connective tissue elements resulting in fibrosis and scarring.

6.1.3 Regeneration

Some parenchymal cells are short lived while others have a longer life span. In order to maintain proper structure of tissues, these cells are under the constant regulatory control of their cell cycle. Cell cycle is defined as the period between 2 successive cell divisions and is divided in to 4 unequal phases. The regeneration process include growth factors such as epidermal growth factor, fibroblast growth factor, platelet derived growth factor, endothelial growth factor, transforming growth factor 13.

6.1.4 Repair

Repair is the replacement of injured tissue by fibrous tissue. Two processes are involved in repair (Ali *et al.*, 2005):

1) Granulation tissue formation; and 2) Contraction of wounds.

Repair response takes place by participation of mesenchymal cells (consisting of connective tissue stem cells, fibrocytes and histiocytes), endothelial cells, macrophages, platelets and the parenchymal cells of the injured organ.

6.1.4.1 Granulation Tissue Formation

The term granulation tissue derives its name from slightly granular and pink appearance of the tissue. Each granule corresponds histologically to proliferation of new blood vessels which are slightly lifted on the surface by thin covering of fibroblasts and young collagen. The following three phases are observed in the formation of granulation tissue.

1. **Phase of Inflammation:** Following trauma, blood clots at the site of injury. There is acute inflammatory response with exudation of plasma, neutrophils and some monocytes within 24 hours.

2. **Phase of Clearance:** Combination of photolytic enzymes liberated from Neutrophils, autolytic enzymes from dead tissue cells and phagocytic activity of macrophages clear off the necrotic tissue, debris and red blood cells.

3. **Phase of ingrowth of Granulation Tissue:** This phase consist of two main processes- angiogenesis or neovascularisation and formation of fibrous tissue.

a] Angiogenesis (neovascularisation): Formation of new blood vessels at the site of injury takes place by proliferation of endothelial cells from the margins of severed blood vessels. Initially, the proliferated endothelial cells are solid buds but within a few hours they develop a lumen and start carrying blood. The newly formed blood vessels are leakier accounting for the oedematous appearance of new granulation tissue. Soon, these blood vessels differentiate into muscular arterioles, thin walled venules and true capillaries.
The process of angiogenesis takes place under the influence of the following:

i) Endothelial cell growth factors, which act as positive stimuli and appear in granulation tissue.

ii) Some components of matrix like type IV Collagen, which acts as negative stimuli and appear late in the granulation tissue formation.

b] Fibrous tissue formation: The newly formed blood vessels are present in an amorphous ground substance or matrix. The new fibroblasts originate from fibrocytes as well as by mitotic division of fibroblast. Some of these fibroblasts have morphologic and functional characteristics of smooth muscle cells (myofibroblasts).

Collagen fibrils begin to appear by about 6th day. As maturation proceeds, more and more of the collagen is formed while the number of the active fibroblasts and new blood vessels decrease. The results in formation of inactive looking scar known as *cicatrisation.*

6.1.4.2. Contraction of Wounds

The wound starts contraction after 2-3 days and the process completes by the 14th day. During this period, the wound is reduced by approximately 80% of its original size. Contracted wound results in rapid healing since lesser surface area of the injured tissue has to be replaced.

In order to explain the mechanism of wound contraction following factors have been proposed.

1. Dehydration as a result of removal of fluid by drying of wound was first suggested but without being substantiated.

2. Contraction of collagen was thought to be responsible for contraction but wound contraction proceeds at a stage when the collagen content of granulation tissue is very small.

3. Myofibroblasts appearing in active granulation tissue has resolved the controversy surrounding the mechanism of wound contraction. These cells have features intermediate between fibroblasts and smooth muscle cells. Evidences support that migration of fibroblasts in to the wound area and their active contraction decreases the size of the defect. Morphological as well as functional characteristics of modified fibroblasts or myofibroblasts as follows:

a] Fibrils present in the cytoplasm of these cells resemble those seen in smooth muscle cells.

b] These cells contain actin-myosin similar to that found in non-striated muscle cells.

c] The nuclei of these cells have in folding of nuclear membrane like in smooth muscle cells.

d] These cells have basement membrane and demosomes which are not seen in ordinary fibroblasts.

e] The cytoplasm of these modified cells demonstrates immunofluorescent labeling with anti-smooth muscle antibodies.

f] The drug response of granulation tissue is similar to that of smooth muscle.

6.1.5 Mechanism of wound healing

Healing of skin wounds provides a classical example of combination of regeneration and repair described above. This can be accomplished in one of the two ways.

- Healing by first intention (primary union) and
- Healing by second intention (secondary union).

6.1.5.1 Healing by First Intention (Primary Union)

This is healing of wound with the following characteristics:

1. Clean and uninfected
2. Surgically incised
3. Without much loss of cells and tissue, and
4. Edges of wound are approximated by surgical sutures.

The sequence of events in primary union is as described below.

1. Initial hemorrhage: Immediately after the injury, the space between the approximated surfaces of incised wound is filled with blood which then clots and seals the wound against dehydration and infection.

2. Acute inflammatory response: This occurs within 24 hours with appearance of polymorphs from the margins of incision. By 3rd day, polymorphs are replaced by macrophages.

3. Epithelial changes: The basal of epidermis from both the cut margins starts proliferation and migration towards incision space in the form of epithelial spurs. A well- approximated wound is covered by a layer of epithelium in 48 hours. The migrated epidermal cells separate the underlying viable dermis from the overlying necrotic material and blood clot, forming scab. The basal cells from the margins continue to divide. By 5th day a multilayered new epidermis is formed which differentiates into superficial in deeper layer.

4. Organization: On 3rd day, fibroblasts invade the wound area and on 5th day, new collagen fibrils start forming which dominate till healing completes. Within 4 weeks, the scar tissue along with scanty cellular element, vascular elements, a few inflammatory cells and epithelialised surface is formed.

5. Suture tracks: Each suture is a separate wound and incites the same phenomena as in healing of primary wound i.e. filling the space with hemorrhage, some inflammatory cell reaction, epithelial cell proliferation along the suture track is avulsed and remaining epithelial tissue in the track is absorbed. However, the epithelial cells may persist in the track (implantation or epidermal cysts).

Thus the scars formed in sutured wound are neat due to close apposition of the margins of the wound. The use of adhesive avoids removal of sutures and its complications.

6.1.5.2 Healing by Second Intention (Secondary Union)

This is healing of wound having following characteristics (Harsh, 2000):

1. Open with a large tissue defect.
2. Having extensive loss of cells and tissues and
3. The wound is not approximated by surgical sutures but left open.

The basic events in secondary union are similar but differ in having a larger tissue defect, which has to be bridged. Hence healing takes place from the base upwards as well as from the margins inwards. The healing by second intention is slow and results in large, ugly scar as compared to rapid healing and neat scar of primary union.

The sequence of events in secondary union is illustrated as below:

1. Initial hemorrhage: As a result of injury, the wound space fills with blood and fibrin clot which helps drying.
2. Proliferation epithelial cells do not cover the surface fully until granulation tissue.
3. Inflammatory phase: There is an initial acute inflammatory response followed by appearance of macrophages which clears off the debris as in primary union.
4. Epithelial changes: As in primary healing, the epidermal cells from both the margins of wound proliferate and migrate into the wound in the form of epithelial spurs till they meet in the middle and re-epithelise the gap completely. However, from the base has started healing the wound space. In this way pre-existing viable connective tissue is separated from necrotic material and blood clot, forming scab. Regenerated epidermis may become stratified and keratinized.

5. Granulation tissue: The main bulk of secondary healing is by granulations. Granulation tissue is formed by proliferation of fibroblasts and neovascularisation from the adjoining viable elements. The newly- formed granulation tissue is deep red, granular and very fragile. With time, the scar on maturation becomes pale and white due to increase in collagen and decrease in vascularity. The specialized strictures of skin like hair follicles and sweat glands are not replaced unless their viable residues remain, which may regenerate.

6. Wound contraction: contraction of wound is an important feature of secondary healing, which is not seen in primary healing. Due to the action of myofibroblasts present in granulation tissue, the wound contracts to ⅓rd to ¼th of its original size. Wound contraction occurs at a time when active granulation tissue is being formed.

6.1.6 Complication of wound healing

During the course of healing, following complications may occur:

1. Infection of wound: Due to entry of micro-organisms delay the healing.

2. Implantation: Epidermal cyst formation may occur due to persistence of epithelial cells in the wound after healing.

3. Pigmentation: Healed wounds may have rust – like colour due to staining with haemosiderin. Some colored particulate material left in the wound may persist and impart colour to the healed wound.

4. The deficient scar formation: may occur due to inadequate formation of granulation tissue.

5. Incisional hernia: A weak scar, especially after a laparotomy, may be the sight of bursting open of a wound (wound dehiscence) or an incisional hernia.

6. Hypertrophiea scars and keloid formation: At times the scar formed is excessive, ugly and painful. Excessive formation of collagen in healing may result in keloid (claw like) formation, seen more commonly in blacks. Hypertrophied scars differ from keloid in that they are confined to the borders of the initial wound while keloids have tumor-like projection of connective tissue.

7. Excessive contraction: An exaggeration of wound contraction may result in formation of contractures or cicatrisation e.g. Dupuytren's (palmar) contracture, plantar contracture and Peyronie's disease (contraction of the cavernous tissue of penis)

8. Neoplasia: Rarely scar may be the site for development of carcinoma at later stage e.g. squamous cell carcinoma in scar.

6.1.7 Extracellular matrix (wound strength)

The wound is strengthened by proliferation of fibroblasts and myofibroblasts which gets structural support from the extracellular matrix (ECM). In addition to providing structural support, ECM can direct cell migration, attachment, differentiation and organization.

ECM has five main components:

(1) Collagen (2) Adhesive glycoproteins (3) Basement membrane (4) Elastic fibers and (5) Proteoglycans.

1. Collagen

The collagens are a family of proteins which provide structural support to the multicellular organism. It is the main component of tissue such as fibrous tissue, bone, cartilage, valves of heart, cornea, basement membrane etc.

Collagen is synthesized and secreted by a complex biochemical mechanism of ribosomes. The collagen synthesis is stimulated by various growth factors and degraded by collagenase. Regulation of collagen synthesis and degradation takes place by various local and systemic factors so that the collagen content of normal organs remains constant. On the other hand, defective regulation of collagen synthesis leads to hypertrophied scar, fibrosys and dysfunction.

Depending upon the biochemical composition, 18 types of collagen have been identified called collagen type I to XVIII any of which are unique for specific tissues. Type I, III and V are true fibril collagen which form the main portion of the connective tissue during healing of the wounds in scars. Other types of collagen are non-fibril and amorphous material seen as component of the basement membrane.

Morphologically the smallest units of the collagen are collagen fibrils, which align together into parallel bundles to form collagen fibres and then collagen bundles.

2. Adhesive glycoprotein

Various adhesive glycoproteins acting as glue for the ECM and the cells consist of fibronectin, tenascin (cytotacin) and thrombospondin.

a] Fibronectin (nectere=to bind) is the best characterized glycoprotein in ECM and has binding properties to other cells and ECM. It is of two types: plasma and tissue fibronectin.

b] Tenascin or cytotactin is the glycoprotein associated with fibroblasts and appears in wound about 48 hours after injury.

c] Thrombospondin is mainly synthesized by granules of platelets. It functions as adhesive protein for keratinocytes and platelets but is inhibitory to attachment of fibroblasts and endothelial cells.

3. Basement membrane

Basement membranes are periodic acid Schiff (PAS) positive amorphous structure that lies underneath epithelia of different organs and endothelial cells. They consist of collagen type IV and laminin.

4. Elastic fibres

While the tensile strength in tissue is built up in collagen; the ability is recoil by elastic fibres. Elastic fibres consist of two components; elastic glycoprotein and elastic microfibril. Elastases degrades the elastic tissue e. g. in inflammation, emphysema etc.

5. Proteoglycans:

These are a group of molecules having two components; an essential carbohydrate polymer (called polysaccharide or glycosaminoglycan), and a protein bound to it, hence the name Proteoglycans. Various proteoglycans are distributed in different tissue they are as follows.

1. Chondroitin Sulphate- abundant in cartilage, dermis.
2. Heparin Sulphate- abundant in basement membrane.
3. Dermatan Sulphate- abundant in dermis.
4. Keratan Sulphate- abundant in cartilage.
5. Hyaluronic acid – abundant in cartilage and dermis.

The strength of wound also depends upon the factors like the site of injury, depth of incision and area of wound. After removal of stitches on around 7^{th} day, the wound strength is approximately 10% which reaches 80% in about 3 months.

6.1.8 Factors influencing healing

Two types of factors influence the wound healing, those acting locally and those acting in systemic.

A. Local factors

These include the following factors:

1] Infection is the most important factor acting locally which delays the process of healing.

2] Poor blood supply to wound slows healing e.g. injuries to face heal quickly due to rich blood supply while injury to leg with varicose ulcers having poor blood supply heals slowly.

3] Foreign bodies including sutures interfere with healing and cause intense inflammatory reaction and infection.

4] Movement delays wound healing.

5] Exposure to ionizing radiation delays granulation tissue formation.

6] Exposure to ultraviolet rays facilitates healing.

7] Type, size and location of injury determines healing takes place by resolution or organization.

B. Systemic factors

These include:

1] Age: Wound healing is rapid in young and somewhat slow in aged and debilitated people due to poor blood supply to the injured area in the latter.

2] Nutrition: Deficiency of constituents like protein, vitamin C (scurvy) and zinc delays the wound healing.

3] Systemic infection delays wound healing.

4] Uncontrolled diabetics are more prone to develop infection and hence delay in healing.

5] Haematologic abnormalities like defects of neutrophil functions (chemotoxis and phagocytosis), neutropenia and bleeding disorders slow the process of wound healing.

6.1.9 Screening Models for wound healing activity

1) Resutured incision wound models

These models are employed to asses the skin breaking strength in rats. In these models, animals are divided in to groups. Two para-vertebral straight incision of 6 cm each is made through entire thickness of skin on either side at least 1cm lateral to the vertebral column. Wound is sutured with catgut, sutures are removed on 1st post wounding day and the breaking strength is estimated on 10th post wound day by continuous constant water flow technique (Lee *et al.*, 1968, Ali *et al.*, 2005).

2) Excision wound models

The model is employed to study the rat wound contraction and epithelization. In this model, animals are randomly assigned with groups. A round seal of 2.5cm in diameter is impressed on the dorsal thoracic central region, 5cm away from the ears. The entire thickness of the skin from demarked area is excised to get 500mm^2 wound areas. Animals are subjected to the treatment from 0 day till the wound completely healed or up to 21st post wounding day, whichever is earlier. The observations of percentage wound contraction are made on 4th, 5th, 12th and 16th post wounding days, starting from day 2.

3) Dead space wound models

The models usually employed for assessing the extent of collagenation. In this model, rats are divided in to groups. Wound is created by implanting subcutaneously 2.5 X 0.5cm polypropylene tubes in the lumber region on dorsl side of the animal. Animals receive drugs from 0 day to 9 post wounding day. On the 10^{th} day granulation tissue developed around the tube is harvested. The tubular granulation tissue is further cut into approximately equal pieces. The breaking strength is generally measured by continuous constant water flow technique.

Research on wound healing drugs is a developing area in modern biomedical sciences. Scientists who are trying to develop newer drugs from natural resources are looking towards the Ayurveda, the Indian traditional system of medicine. Several drugs of plant, mineral and animal origin are described in the Ayurveda for their wound healing properties under the term *Vranaropaka.* Most of these drugs are derived from plant origin. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and patients, but the potential of most remains unexplored. In a few cases, active chemical constituents were identified. Some Ayurvedic medicinal plants, namely, *Ficus bengalensis, Cynodon dactyln, Symplocos racemosa, Rubia cordifolia, Pterocarpus santalinus, Ficus racemosa, Glycyrrhiza glabra, Berberis aristata, Curcuma longa, Centella asiatica, Euphorbia nerifolia, and Aloe vera,* were found to be effective in experimental models (Biswas, 2003). In the present study all the three plants i.e. *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* are selected to establish their ethnomedicinal claims for wound healing activity.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

The fresh leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)*and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133).

6.2.2 Preparation of extracts

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were extracted with methanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. In case of *Morinda citrifolia* the clean roots are shade dried for twenty days and then subjected to soxhelation

using methanol as the solvent. The extract yield was found to be 5% for *U. parviflora,* 7.5% for *C. arborea* and 11.0% for *M. citrifolia.*

6.2.3 Animal study

Healthy male albino rats weighing between 160-220gm were used in the study. They were individually housed in aseptic condition and maintained on normal diet and water. They were kept in plastic cages at 23 ± 1^0C in 12:12 hr dark: light cycle. All experiments were carried out between 10:00 and 16:00 hrs. The animal experiments were conducted as per protocol approved by the *Institutional Animal Ethics Committee (IAEC) No.* HPI/07/60/IAEC/0005.

6.2.4 Wound Models

6.2.4.1 Excision wound model

For excision wound study, the male albino rats were divided into eight groups, each comprising six animals. They were starved for 12 hrs prior to wounding. Under light ether anaesthesia, wounding was performed aseptically. A circular wound of about 2.5 cm diameters was made on depilated dorsal thoracic region, washed with normal saline and observed during the study. Wounds were traced on 1mm^2 graph paper on the day of wounding and subsequently on alternative days until healing processes were complete. Changes in wound area were calculated, giving an indication of the rate of wound contraction. The alcoholic extracts (5% w/w) were formulated as an ointment prepared by IP method.

The prepared ointments (500 mg) were applied on the wound, once daily for 18 days, starting from the day of wounding in groups VI, VII and VIII. The extracts were orally fed to the animals in the dose of 300 mg/kg in a form of slurry with the help of oral feeders in group III, IV and V. No medication other than the extracts was given to the test groups. The standard group was treated with Framycetin (1%) ointment (Soframycin skin ointment, Aventis). While the control group only received the vehicle (2% gum acacia) orally. The percentage of wound closure was observed on 2nd to 18th post wounding days. The period of epithelialization was calculated as the number of days required for falling of the dead tissue without any residual raw wound (Manjunath, 2005).

6.2.4.2 Incision wound model

Two paravertebral incisions of 6 cm length were made in the skin on either sides of the vertebral column with the help of a sharp blade. The liner wounds are at least 1cm away from the vertebral column. The wounds were sutured using 4-0 number silk thread using a (No11) bend needle. The sutures are spaced 5 mm apart. On 8th day the sutures were removed and breaking strength was determined on 10th post wounding day. The breaking strength was

measured with a manually operated instrument in terms of weight (Nayak *et al.*, 2006). The animals were treated with drugs as in **6.2.4.1** except that the treatment was given up to 9th day only in case of Incision wound model.

6.2.4.3 Dead space wounds

The wounds were made in the region of axilla and groin under light ether anaesthesia where sterilized grass piths of 2.5 cm length and 0.3 cm diameter were introduced in each side to induce granuloma formation. The wounds were sutured and mopped with a saline swab. The animals were treated with drugs except the control group for 9 days from the day of wounding. Granuloma tissues formed on implanted piths were dissected out on the 10th post wounding day. One of the pith was used to determine the tensile strength by manually operated instrument in terms of weight, while the other pith containing the granuloma tissue was used for estimation of hydroxyproline content by Woessner method (Woessner, 1963).

6.2.4.4 Determination of wound breaking strength

The anesthetized animal was secured to the table, and a line was drawn on either side of the wound 3 mm away from the line. This line was gripped using forceps one at each end opposed to each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light weight metal plate. Weight was added slowly and the gradual increase in weight, pulling apart the wound edges. As the wound just opened up, addition of weight was stopped and the weights added was noted as a measure of breaking strength in grams. Three readings ere recorded for a given incision wound, and the procedure was repeated on the contralateral wound. The mean reading for the group was taken as an individual value of breaking strength. The mean value gives the breaking strength for a given group (Nayak *et al.*, 2006).

6.2.5 Histopathology Study

The histopathology study was carried on the section of granuloma tissue to observe the stages of keratinization, fibrosis, collagenation, epithelization and neovascularisation. The tissues were fixed in 10% formalin and dehydrated with 90% ethanol, embedded in paraffin, made in to sections of 7µm thickness, stained with haematoxyline-eosin dye and subjected to microscopy. The results are evaluated by numbering 1 to 5. '1' indicates least and '5' indicates maximum similarity with normal tissue in all the groups.

6.2.6 Statistical Analysis:

Values are expressed as Mean ± SEM. Statistical analysis (Graph Pad Prism Software) was made by using Tukey-Kramer Comparisons ANOVA test at different time intervals. $P<0.001$ was taken as significant compared to control.

6.3 RESULTS OF THE WOUND HEALING ACTIVITY

6.3.1 Excision wound model

The result of the excision wound healing model is presented in **Table 6.2.** From the result it is revealed that all the six groups of animals who received the methanolic extracts and ointments of the three plants as mentioned in the method (**6.2.4.1**) showed increased wound contraction continuously from 2nd day to 18th day. The animals of group VII and VIII who received ointment of MECA and MEMC the healing of wound was found to be completed within 14 days in contrast to group V and VI who received MEMC orally and ointment of MEUP respectively whose healing was completed on day 16 only. The group III and IV who received MEUP and MECA orally have shown complete wound contraction on day 18 whereas the control group I failed to show the healing up to 18 days. The groups VII and VIII showed complete healing within 14 days similar to the group II, which received the standard drug Framycetin ointment. Hence the ointments of the extract of *C. arborea* and *M. citrifolia* are comparable to the standard drug Framycetin in healing of wound. The epithelization period was also found to be less in group V (12.6 days) and in group VII (12.9 days) which is similar to the standard drug (Framycetin) treated group (12.5 days)

6.3.2 Incision wound model

The effect of the test drugs in incision wound model is presented in **Table 6.3.** The breaking strength was found to be maximum in group VIII who received ointment of MEMC (710.00 ± 4.22) and is similar to standard drug Framycetin group (Group II) (712.23 ± 2.84). The other two groups who received ointment of MEUP and MECA (group VI and VII) also showed better breaking strength as compared to orally fed drugs groups i.e. group-III, IV and V.

6.3.3 Dead space wound model

The result of dead space wound model is presented in **Table 6.3.** The three parameters namely dry granuloma weight, breaking strength and estimation of hydroxyproline were examined in this model. The two grass piths collected from each of the animal were air dried for two hours and then subjected to the above mentioned tests. Animals of group VIII treated with ointment of MEMC showed maximum dry granuloma tissue weight (72.01 ± 1.19 mg/100g) which is much higher than that of the standard drug Framycetin treated group (62.12 ± 0.38 mg/100g).The MEUP ointment treated group (Group VI) showed the same dry

granuloma tissue weight with that of the standard drug group i.e. (62.12 ± 0.38 mg/100g).The control group showed the lowest dry granuloma tissue weight (26.32 ± 0.41 mg/100g).

The MEMC ointment treated group (group VIII) also showed maximum breaking strength (600.13 ± 4.36g) amongst all the test groups and is comparable to standard drug treated group (group II) which is found to be 612.13 ± 2.31g) The MEMC ointment treated group also showed maximum amount of hydroxyproline (2397.24 ± 2.01µg/100g) which is comparable to the results found in the standard drug treated group of animals (2439.61 ± 0.87µg/100g) which reveals that the extract of the roots of *M. citrifolia* is found to be the most effective in healing of wound among the three plant drugs used in this study.

6.3.4 Histopathology

The results of the histopathological examinations were recorded in five parameters and are presented in **Table 6.4** and **Figure 6.1** to **6.4.** The MECA and MEMC treated groups (groups VII and VIII) showed similar stage of keratinization (4.1 ± 0.09 and 4.1 ± 0.03 respectively) which is comparable to the effect of the standard drug Framycetin in group II (4.2±0.05). Similarly the MEMC ointment treated group showed maximum epithelization (4.2 ± 0.26) comparable to the standard drug treated group (4.3 ± 0.14).

The stage of fibrosis was also found to be of very high value in case of the two plants i.e. *U. parvifolia* and *C. arborea* (4.0 ± 0.13 and 4.0 ± 0.12) but it was maximum with the ointment of the extract of the plant *M.citrifolia* (4.1± 0.32) which is 4.2 ± 0.15 in case of the standard drug Framycetin.

The collagen formation was maximum in group VII (4.4± 0.16), which received MECA ointment amongst the test groups and is comparable to the standard drug treated group i.e. group II (4.5± 0.17). The stage of neovascularization in MECA ointment treated group was found to be 4.4 0.07 which is similar to the standard drug treated group (4.4 0.09). The value in MEUP ointment treated group is also very appreciable i.e. 4.3±0.08, comparable to the standard drug treated group and is much higher than that of the control group (0.6+0.07). Thus the ointment forms of the extracts of two plants *U. parvifolia* and *C. arborea* were found to have maximum wound healing activity.

Table 6.2 Effect of extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* on excision wound model.

(Group) Treatment	Epithelization Period (days)	Excision wound model (% of wound contraction by day)								
		2	4	6	8	10	12	14	16	18
(Gr I) Control	17.4 ±0.81	15.22 ±0.07	32.14 ±0.28	40.73 ±0.41	59.38 ±1.23	79.11 ±1.86	85.66 ±2.78	91.37 ±2.84	95.23 ±2.92	98.46 ±2.89
(Gr II) Framycetin	12.5 ±0.43	19.28 ±0.06	39.32 ±0.19	79.16 ±0.29	86.44 ±1.01	90.21 ±2.81	98.91 ±2.68	100.00 ±2.55	–	–
(Gr III) MEUP 300 mg/kg p.o	14.1 ±0.69	17.01 ±0.11	34.29 ±0.18	54.16 ±0.37	70.98 ±1.98	83.78 ±2.73	89.23 ±2.91	95.66 ±3.02	99.82 ±2.79	100.00 ±2.13
(Gr IV) MECA 300 mg/kg p.o	13.8 ±0.62	16.56 ±0.08	34.97 ±0.22	56.27 ±0.46	72.14 ±2.14	84.19 ±2.05	91.17 ±2.84	94.92 ±2.93	98.71 ±2.62	100.00 ±1.97
(Gr V) MEMC 300 mg/kg p.o	12.6 ±0.72	18.35 ±0.07	37.00 ±0.25	61.11 ±0.39	78.19 ±2.44	87.09 ±2.21	95.13 ±3.01	98.01 ±3.12	100.00 ±2.81	–
(Gr VI) MEUP 5%w/w	13.2 ±0.48	17.91 ±0.09	36.78 ±0.26	71.23 ±0.49	78.53 ±1.69	86.74 ±1.98	95.34 ±2.32	99.33 ±2.05	100.00 ±1.93	–
(Gr VII) MECA 5%w/w	12.9 ±0.51	18.39 ±0.09	37.81 ±0.28	74.18 ±0.47	80.35 ±1.72	88.35 ±2.00	97.62 ±1.93	100.00 ±1.86	–	–
(Gr VIII) MEMC 5%w/w	13.0 ±0.52	18.99 ±0.77	38.23 ±1.31	75.01 ±0.71	83.24 ±2.99	90.00 ±2.97	98.01 ±3.01	100.00 ±3.69	–	–
One way ANOVA F df P	8.967 7, 40 <0.0001	23.052 7, 40 <0.0001	21.003 7, 40 <0.0001	807.21 7, 40 <0.0001	18.279 7, 40 <0.0001	2.557 7, 40 =850.02	3.050 7, 40 =0.0114	1.26395. 7, 40 =0.2932	0.6671 4, 25 0.5986^{N}	0.8693 2, 15 0.1414^{N}

Superscripted N= Not Significant. Other 'P' values are significant, n = 6.

MEUP = Methanolic Extract of *U. parviflora*
MECA = Methanolic Extract of *C. arborea*
MEMC = Methanolic Extract of *M. citrifolia*

Table 6.3 Effect of extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* on wound healing in incision and dead space wound models.

(Group) Treatment	Incision	Dead space		
	breaking strength(g)	Dry granuloma weight(mg/100g)	Breaking strength(g)	Hydroxyproline (µg/100g)
(Gr I) Control	389.87±3.86	26.32±0.41	380.44±1.12	1401.22±0.98
(Gr II) Standard (Framycetin)	712.23±2.84	62.12±0.38	612.13±2.31	2439.61±0.87
(Gr III) MEUP 300mg/kg p.o	634.47±3.91	44.39±0.53	481.37±3.02	1958.12±1.09
(Gr IV) MECA 300mg/kg p.o	656.81±3.89	49.53±0.49	498.11±3.57	1979.26±1.03
(Gr V) MEMC 3000 mg/kg p.o	677.94±4.68	56.12±1.33	541.34±4.58	1997.42±3.01
(Gr VI) MEUP 5%w/w	700.31±3.67	62.12±0.42	587.19±3.46	2198.78±1.20
(Gr VII) MECA5%w/w	709.19±3.54	68.16±0.44	592.49±3.08	2310.16±1.19
(Gr VIII) MEMC 5%w/w	710.00±4.22	72.01±1.19	600.13±4.36	2397.24±2.01
One way ANOVA				
F	789.11	91.460	563.98	69671
df	7,40;47	7,40;47	7,40;47	7,40;47
P	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are mean ±SE of 6 replicant

Tukey-Kramer Multiple Comparisons Test

P values are extremely significant.

Table 6.4 Histopathological examinations of wound treated with the aqueous and methanolic extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* at the end of 10 days.

(Group) Treatment	Parameters				
	Keratinization	Epithelization	Fibrosis	Collagen	Neovascula - risation
(Gr I) Control	0.4±0.09	1.6±0.15	2.5±0.17	2.6±0.17	0.6±0.07
(Gr II) Standard (Framycetin)	4.2±0.05	4.3±0.14	4.2±0.15	4.5±0.17	4.4±0.09
(Gr III) MEUP 300mg/kg p.o	3.6±0.16	3.9±0.17	3.7±0.08	4.0±0.15	3.8±0.10
(Gr IV) MECA 300mg/kg p.o	3.8±0.13	3.9±0.18	3.8±0.09	4.1±0.14	3.9±0.09
(Gr V) MEMC 300mg/kg p.o	4.0±0.47	4.0±1.07	3.9±1.88	4.0±0.86	4.1±1.76
(Gr VI) MEUP 5%w/w	4.0±0.08	4.0±0.09	4.0±0.13	4.3±0.14	4.3±0.08
(Gr VII) MECA 5%w/w	4.1±0.09	4.1±0.08	4.0±0.12	4.4±0.16	4.4±0.07
(Gr VIII) MEMC 5%w/w	4.1±1.03	4.2±0.26	4.1±0.32	4.2±0.54	4.0±0.38
Oneway ANOVA					
F	9.680	4.656	0.6228	2.437	3.914
df	7,40;47	5,30;35	7,40;47	7,40;47	7,40;47
P	< 0.0001^{s}	< 0.0007^{s}	< 0.7339^{s}	< 0.0353^{s}	< 0.0024^{s}

Values are expressed ± SEM, n = 6.
Tukey-Kramer Multiple Comparisons Test
's' indicates P values are considered very significant.

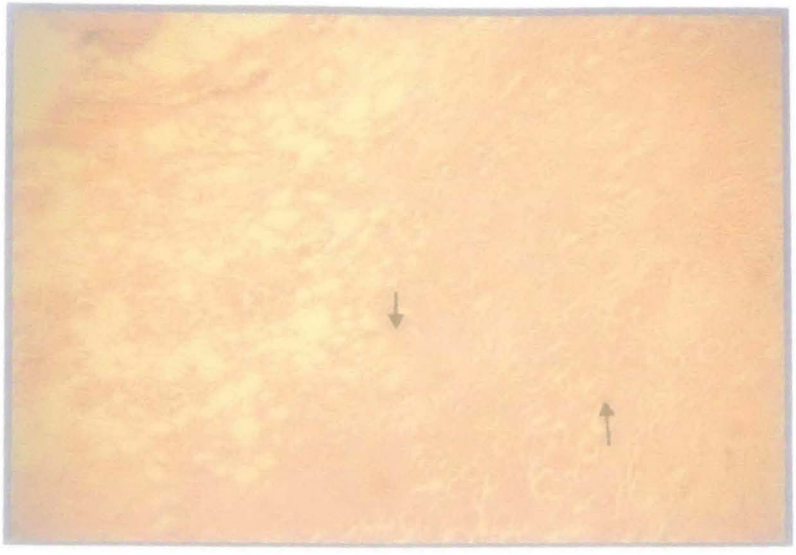

Fig 6.1 Histological section of Granulation tissue of control animal showing more macrophages and less collagen. (M x 400)

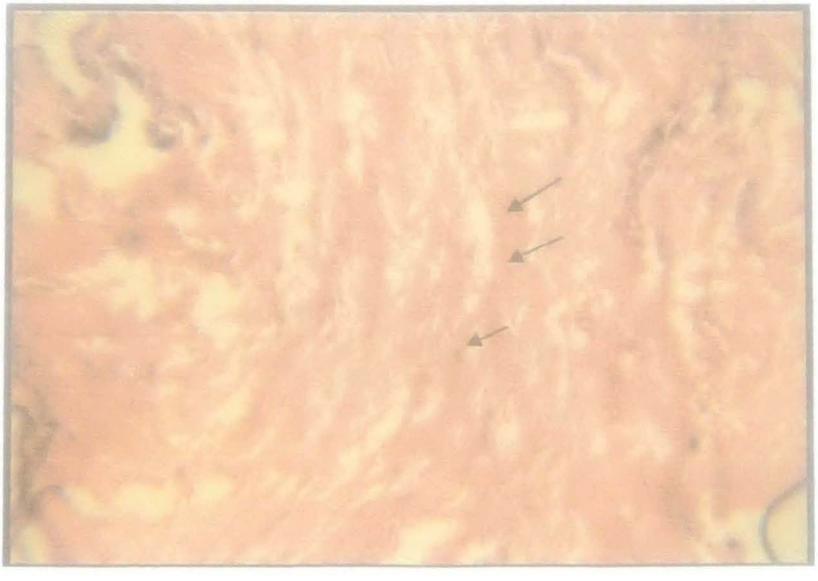

Fig 6.2 Histological section of Granulation tissue of the EEUP ointment treated animal showing very few macrophages and increased collagen deposition. (M x 400)

Fig 6.1 Histological section of Granulation tissue of control animal showing more macrophages and less collagen. (M x 400)

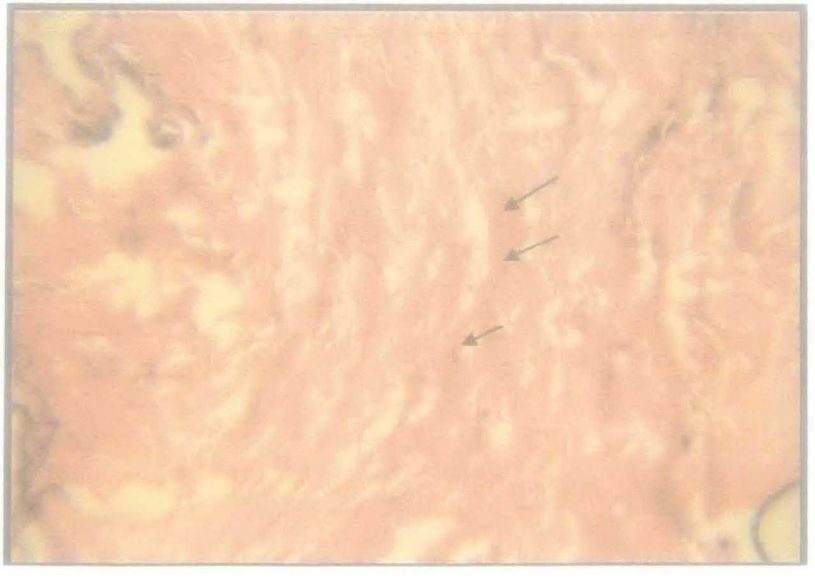

Fig 6.2 Histological section of Granulation tissue of the EEUP ointment treated animal showing very few macrophages and increased collagen deposition. (M x 400)

Fig 6.3 Histological section of Granulation tissue of the EECA ointment treated animal showing collagen fibres well organized into distinct bundles with increased cellularity. (M x 400)

Fig 6.4 Histological section of Granulation tissue of the MEMC ointment treated animal showing collagen fibres well organized into distinct bundles with increased cellularity, joined bundles of collagen fibres with minimal cellularity and increased vascularity. (M x 400)

REFERENCES

Ali, S. and Purohit, M. G. Review on wound healing activity, *Adv Pharmacol Toxicol,* 2005, 6, 2, 31.

Aplin, A. C., Fogel, E., Zorzi, P. and Nicosia, R.F. The aortic ring model of angiogenesis, *Methods Enzymol*, 2008, 443, 119-36.

Bairy, K. L. Wound healing potential of plant products, *J Nat Rem,* 2002, 2, 11–20.

Biswas, T. K. Plant medicines of Indian origin for wound healing activity: A review, *The Int J of Lower Extremity Wounds,* 2003, 2, 1, 25-39.

Brzezinska, S. E. Fever induced oxidative stress: The effect on thyroid status and the 5'-mono deiodinase activity, protective role of selenium and vitamin E, *J Physiol Pharmacol,* 2001, 52, 275-284.

Chettibi, S. and Ferguson, M. W. J. Wound repair—an overview. In: Gallin, J. I., Snyderman, R. (Eds.), Inflammation—Basic Principles and Clinical Correlates. Lippincot, Williams and Wilkins, Philadelphia, 1997, 865–881.

Choi, S. W., Son, B. W., Son, Y. S., Park, Y. I., Lee, S. K. and Chung, M. H. The wound healing effect of a glycoprotein fraction isolated from *Aloe vera, Brit J Dermatol*, 2001, 145, 535–545.

Ehrlich, H. P. and Hunt, T. K. The effects of cortisone and Anabolic steroids on the tensile strength of healing wounds, *Ann Surg*, 1969,170, 2, 203-206.

George, D. and Pamplona R. M. D. Encyclopedia of Medicinal plants. Graficas Reunids;mar Tirreno: Marid, 1999, 361.

Gerald, S. L., Diane, M. C., David, R. K., David, J. M., Roger, E. P., George, R. and Martin, C. Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair and Regen*, 1994, 2, 165–170.

Ghosh, M. N. Fundamentals of Experimental Pharmacology, 2nd Ed, Scientific Book Agency, Calcutta, 1984, 156.

Hakeem, M. Z. H. Mineral Remedies, Islamic organization for medical sciences of Kuwait 1989. 141.

Harsh, M. Text book of Pathology, 4th Edn. Jaypee Brothers, Medical Publishers Pvt. Ltd., 2000, 152-157.

Hemmati, A. A. and Mohammadian, F. An investigation into the effects of mucilage of quince seeds onwound healing in rabbit, *J Herbs Spices and Med Plants*, 2000, 7, 41–46.

Hj Baie, S. and Sheikh, K. A. The wound healing properties of Channa striatus-cetrimide cream-wound contraction and glycosaminoglycan measurement, *J Ethnopharmacol*, 2000, 73, 15–30.

Lee, K. H. Study on the mechanism of action of salicylates II, Retardation on wound healing by aspirin, *J Pharma Sc*, 1968, 57, 6, 1195-1197.

Mantle, D., Gok, M. A., Lennard, T. W. J. Adverse and beneficial effects of plant extracts on skin and skin disorders, *Adv Drug Reactions and Tox Rev*, 2001, 20, 2, 89–103.

Michell, R.N. and Cotran, R.S. acute and chronic inflammation, Basic pathology, Edited by Kumar, Cotran and Robbins, 6th Ed, Harcourt Asia Pvt Ltd, 1997, 49.

Morton, J. J. P. and Malone, M. H. Evaluation of vulnerary activity by an open wound procedure in rats, *Arch Int Pharmacodyn Ther*, 1972, 196, 117.

Nayak, B. S. and Pereira, L. M. P. *Catharanthus roseus* flower extract has wound-healing activity in Sprague Dawley rats, *BMC Compl Alt Med*, 2006, 6, 41.

Puratchikody, A., Nithya Devi, C. and Nagalakshmi, G. Wound Healing Activity of *Cyperus rotundus* Linn., *Ind J Pharm Sci*, 2006, 68, 1, 97-101.

Rao, R. R., Babu, R. M., Rao, M. R. V. and Babu, M. G. V. Studies on antipyretic, analgesicand hypoglycemic activities of root of *Gynandropsis gynandra* Linn., *Ind Drugs*, 1997, 34, 690.

Rashed, A. N., Afifi, F. U. and Disi, A. M. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. growing in Jordan on *Mus musculus* JVI-1. *J Ethnopharmacol*, 2003, 88, 131–136.

Turner, R. A. Screening methods in Pharmacology, New Academic Press, New York, 1965, 61.

Ulubelen, A., Topcu, G., Tan, N., Olcal, S., Johansson, C., Ucer, M., Birman, H. and Tamer, S. Biological activities of a Turkish medicinal plant, *Prangos platychlaena*, *J Ethnopharmacol*, 1995, 93–197.

Woessner, J. F. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid, *Arch Biochem*, 1963, 93, 440.

Zaveri and Chanda, Peptides with wound healing activity, www.okpatent.us.