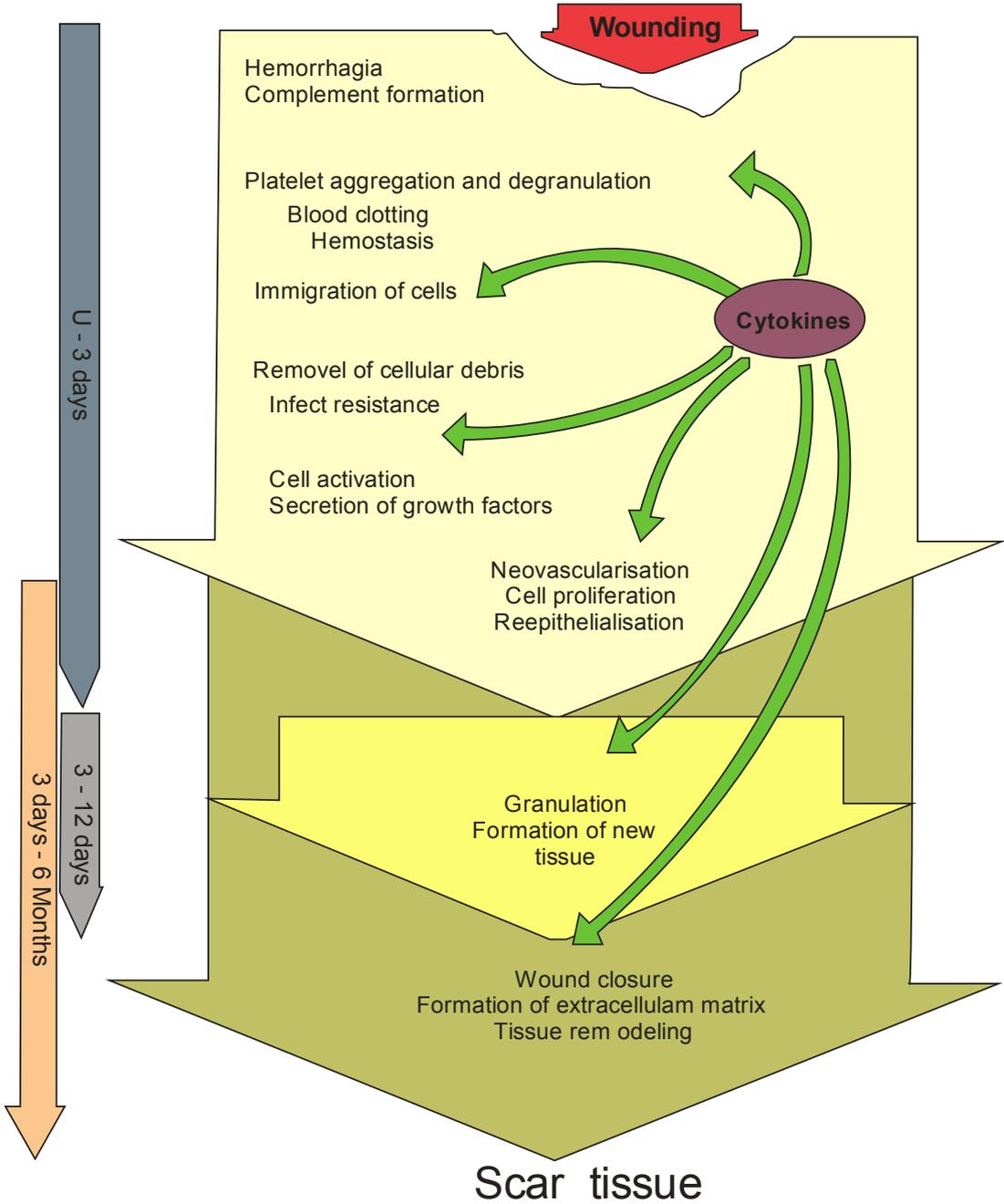# CHAPTER 9

# WOUND HEALING ACTIVITY

## 9.1 INTRODUCTION

The skin is the largest organ of our body and represents a barrier to the environment. It protects the organism from water loss and penetration of harmful substances. The barrier can be damaged by mechanical and thermal action or by UV light and different diseases, such as psoriasis and atopic dermatitis [169, 170]. If the skin barrier is disturbed, it starts repair mechanisms [171]. The kinetic of the wound healing processes is influenced by endogenous and exogenous factors [172]. The analysis of the kinetic is important for therapy control and for the development of efficient drugs and cosmetic products, stimulating the wound healing [173].

Wound is defined simply as the disruption of the cellular and anatomic continuity of a tissue [174].Wound may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue. The process of wound healing consists of integrated cellular and biochemical events leading to reestablishment of structural and functional integrity with regain of strength of injured tissue. Clinically, an individual often encounters non-healing, under-healing or over healing. Therefore the aim of treating a wound is to either shorten the time required for healing or to minimize the undesired consequences [175]. Attention should be directed towards discovering an agent, which will accelerate wound healing either when it is progressing normally [176] or when it is suppressed by various agents like corticosteroids [177], anti-neoplastics [178] or non steroidal anti-inflammatory agents.

Wound healing is a complex process, which is commonly divided into three phases includes the invasion of inflammatory cells, proliferation of tissue-repairing cell and tissue remodelling [179]. The rate of wound healing depends upon many factors including the size of the wound, blood supply to the area, presence of foreign bodies, microorganisms, age, health of the patient, nutritional status of an individual, use of drugs and a variety of other systemic diseases [180].

Various growth factors like platelet derived growth factor, macrophage derived growth factor, monocyte derived growth factor etc. are necessary for the initiation and promotion of wound healing (Figure 9.1). Many substances like tissue extracts [181] vitamins, minerals and a number of plant products [182] have been reported by various workers, to possess pro-healing effects. Wound healing herbals encourage blood clotting, fight infection and accelerate the healing of wounds. Plants or chemical entities derived from plants need to be identified and formulated for treatment and management of wounds. In this direction a number of herbal products are being investigated at present. Various herbal products have been used in management and treatment of wounds over the years.

Medical treatment of wound includes administration of drugs either locally (topical) or systemically (oral or parenteral) in an attempt to aid wound repair [183]. The topical agents used include antibiotics and antiseptics [184], sloughing agents (chemical debridement, e.g. hydrogen peroxide, eusol and collagenase ointment), wound healing promoters (e.g.Tretinoin, honey, comfrey, benzoyl peroxide, dexpanthenol, tetrachlordecaxide solution, clostebol acetate and the experimental cytokines) [185].

**Wounding**

Hemorrhagia
Complement formation

Platelet aggregation and degranulation
Blood clotting
Hemostasis

Immigration of cells

Cytokines

Removel of cellular debris
Infect resistance

Cell activation
Secretion of growth factors

Neovascularisation
Cell proliferation
Reepithelialisation

Granulation
Formation of new
tissue

Wound closure
Formation of extracellulam matrix
Tissue rem odeling

U - 3 days

3 - 12 days

3 days - 6 Months

Scar tissue

**Figure 9.1 Various stages in Wound Healing**

### 9.1.1 Plants Used in the Treatment of Wounds (Natural Wound Healers)

Some of the crude drugs with Wound Healing activity are:

*Acalypha indica* L. [186], *Areca catechu* L. [187], *Anacardium occidentale* L. [188], *Calotropis gigantea* (L.) R. Br. [189], *Cleome viscosa* L. [190], *Euphorbia nerifolia* [191], *Ficus bengalensis* L., *Ficus racemosa* L., *Jatropha gossypifolia* L., *Pongamia pinnata* (L.) Pierre, L., *Ixora coccinia Smilax zeylanica* L., [192], *Scoparia dulcis* L., [193] *Terminalia bellirica* (Gaertn.) Roxb., [194], *Themeda triandra* Forssk., *Trichodesma zeylanicum* (Burm. f.) R. Br., Boraginaceae and *Vitex altissima* L. [195].

### 9.2 MATERIALS AND METHODS

Animal wound healing models are important biological tools to understand basic processes of tissue repair and to develop and validate strategies for clinical treatment. Human wound healing has many unique aspects that relate to the physiology, age and environment of the species, but the opportunity to carry out controlled, clinical experimentation on the mechanism and therapy of wounds is limited.

In general, animal models (with the exception of some transgenic and targeted gene deletions) attempt to reflect human wound healing problems, dehiscence, ischemia, ulceration, infection, and scarring. Henceforth the models utilised for evaluating wound healing activity in experimental animals for MELA depicting three different types of wounds are as follows.

**a) Chemicals Required**

Simple ointment, Corn oil, Nitrofurazone ointment, Anaesthetic ether, Phosphate buffer, 10% formalin, Ethanol, Xylene, Paraffin, Haematoxylin, Eosin.

### 9.2.1 Drug Formulation

0.5 g of MELA was mixed with 9.5 gm of simple ointment in a porcelain tile and transferred to a tightly closed amber colored container. Two types of drug formulations were prepared for topical administration 5% w/w ointment was prepared in simple ointment base. For oral administration, 200 and 400mg/kg suspensions of the extract was prepared in corn oil. The drugs were administered orally by an intra-gastric tube.

For assessment of wound healing activity, excision, incision and dead space wound models were used. The animals were divided into nine groups of 6 animals each, with three groups each used for excision, incision and dead space wound models, respectively.

### 9.2.2 Excision Wound

In the Excision wound model the animals were grouped as follows:

**Group I** : served as control corn oil, 1ml/kg, topically for 21 days.

**Group II** : received topical application of 5 % Nitrofurazone I.P. twice a day for both excision and incision models for 21 days.

**Group III** : received topical application of 5 %w/w of the extract in Simple Ointment base I.P. twice a day for both excision and incision models for 21 days.

The animals were anaesthetized under light ether anaesthesia and then the skin of the impressed area was excised to full thickness to obtain a wound area of about 500 mm$^2$. The drugs were topically applied once a day until complete epithelisation. The parameters studied were wound closure (measured at regular intervals of time to determine percent wound closure) and epithelisation time (indicated by the formation of new epithelial tissue to

cover the wound). The wounded areas were later evaluated and Wound contraction was calculated as a percentage of the reduction in wounded area on 4th, 8 th, 12 th, 16 th, 20 th and 21st days until complete re-epithelialization was achieved. (The day the scar peeled off without leaving any residual raw wound was considered the day complete epithelialization was attained).The following parameters was studied,

### 9.2.2.1    Epithelization Period

It was monitored by observing the number of days required for Escher to fall away, leaving no raw wound behind.

### 9.2.2.2    Wound Contraction

To monitor this, progressive changes in wound area were followed planimetrically. Leaving the wounding day, wounds were traced on a transparent paper on alternate days. The animal was restrained in proper position during tracing. The tracings were then transferred to 1 mm$^2$ graph sheet. From this, wound areas were read and the percent of wound contraction was calculated taking the initial size of wound (100 mm$^2$) as 100%. Percentage wound closure can be calculated using the formula as follows (9.1)

$$Percentage\ wound\ closure = \frac{Initial\ area\ of\ wound - nth\ day\ area\ of\ wound}{(Initial\ area\ of\ wound)} \times 100$$

(9.1)

### 9.2.3    Incision Wound Model

In the incision model [196], the rats were anaesthetized by anaesthetic ether and two longitudinal paravertebral incisions of 6 cm length were made through the skin and cutaneous muscle at a distance of about 1.5 cm from the midline on each side of the depilated back. After the incision, the

parted skin was sutured 1 cm apart using surgical thread (no. 000) and curved needle (no. 11).

**Group I** : served as Simple ointment base topically twice a day for 10 days.

**Group II** : received topical application of 5 % Nitrofurazone ointment twice a day for 10 days.

**Group III** : received topical application of 5 %w/w of the extract in Simple Ointment base I.P. twice a day for 10 days.

The wounds were left undressed. The drugs were topically applied to the wound twice a day until complete healing occurred. The sutures were removed on the $10^{th}$ (post wound) day. On day 11, all the animals were sacrificed under anaesthesia. One linear paravertebral incised skin of each animal was measured for tensile strength using a fabricated tensilometer.

**9.2.4     Determination of Tensile Strength**

The Tensile strength (skin breaking strength) of the 10-day old wound was measured by the method of Lee et al [197]. The rats were secured to the operating table and a line was drawn on either side of the wound 3 mm away from the wound. Two allice forceps were firmly applied to the line facing each other. One of the forcep was fixed, while the other was connected to a freely suspended lightweight polypropylene graduated container through a string run over to a pulley. Water was allowed to flow from the reservoir slowly and steadily into the container. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. The moment the wound just opened up, the water flow was arrested and the volume of water collected in the container (approximately equal to its weight) was noted. Three readings were recorded for a given incision wound and the procedure was repeated on the wound on the contra lateral side. The mean reading of the

group was taken as the breaking strength for a given group in the incision model.

### 9.2.5 Dead Space Wounds

Dead space wounds were created by implanting two pre-weighed sterilized polypropylene tube (2.5 length x 0.25 cm diameter) beneath the dorsal Para-vertebral skin of the anaesthetized rats.

**a)Group Separation**

After this procedure, the animals were divided into three groups of six animals each.

Group I    :  served as control and the animals received vehicle only for 10 days.

Group II   :  received MELA at a dose of (200 mg/kg) for 10 days.

Group III  :  received MELA at a dose of (400 mg/kg) for 10 days.

On the $10^{th}$ (post-wound) day, the granulation tissues formed on the implanted tubes were carefully detached from surfaces of the tubes. The wet weight of the granulation tissue was noted. The breaking strength of granulation tissue was measured by the method of Lee et al. Thereafter, the granulation tissues were collected dried at $60^{o}$ C for 24 h and their dry weights were noted. The dried tissue was added to 5 ml 6 M HCl and kept at $110^{o}$C for 24 h. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline [198].

Granulation tissue from the second tube was collected in phosphate-buffer saline to estimate antioxidant enzymes -Superoxide dismutase (SOD) [86] and Catalase [87], while a piece of the wet granulation tissue was preserved in 10% formalin for histopathological studies.

**Estimation of SOD and Catalase**

Estimation of Catalase and Superoxide dismutase were determined as per the procedure followed in the hepatoprotective activity of MELA.

**Estimation of Hydroxyproline**

Dry granulation tissue from both control and test groups were used for the estimation of hydroxyproline. Hydroxyproline present in the neutralized acid hydrolysate was oxidized by sodium peroxide in presence of copper sulphate and subsequently complexed with P-dimethylaminobenzaldehyde to develop a pink colour that was measured spectrophotometrically at 540 nm.

### 9.2.6    Histopathological Evaluation of Wounded Tissues

A portion of the granulation tissue was subjected to histopathological studies. The tissues were fixed in 10% neutral formalin solution for 24 h and dehydrated with a sequence of ethanol–xylene solution series [199]. The materials were filtered and embedded with paraffin $(40 - 60^0 \text{ C})$ and a microtome section of 5 $\mu$ thickness was taken. The sections were again processed with ethanol-xylene solvent series and stained with haematoxylin-eosin dye. The histopathological changes were observed and photographed using a compound light Microscope (Novex, USA).Ulceration, necrosis, epithelisation, congestion, oedema, Polymorpholeukocytes (PML), mononuclear cells, fibroblasts and vascularisation were evaluated in the skin tissues.

### 9.2.7    Statistical Analysis

The results were expressed as mean ± SEM of 6 animals in each group. The data were statistically evaluated by one-way ANOVA, followed by Dunnet's t-test for comparison of test groups with control. Values of $p<0.05$ were considered statistically significant.

## 9.3      RESULTS

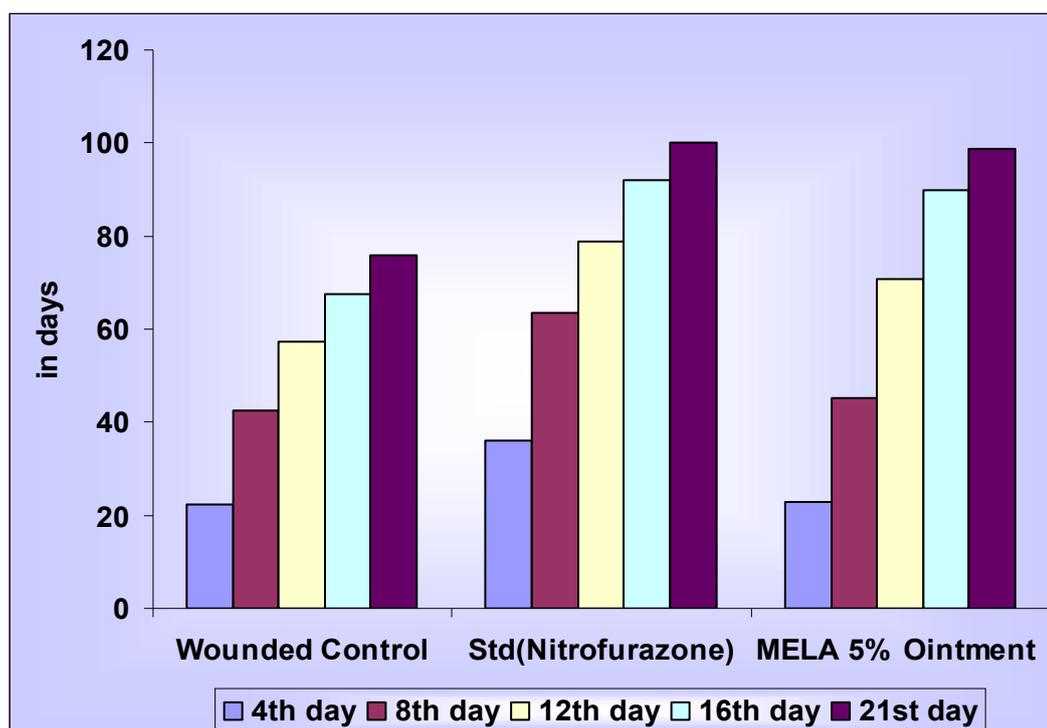### Effect of MELA on Excision Model

In the excision wound model, (Table 9.1) the wound contracted progressively when treated with the extracts and the reference drug which required a mean period of $16.0 \pm 0.8$ days for optimum healing as evidenced by the shorter period required for Escher dropping. Thus the extracts promoted wound contraction significantly when compared with the control group. The mean period of epithelialization of the control group was $20 \pm 0.86$ days. It was significantly ($P<.001$) reduced to $16.6 \pm 0.33$ days in *L.acidissima* treated group. The mean period of epithelialization in Nitrofurazone treated group was $15 \pm 0.06$ days which was significantly ($P<0.001$) reduced when compared with that of the control group.

**Table 9.1     Effect of MELA on percent wound contraction and epithelialization period**

| Treatment | 4th day | 8th day | 12th day | 16th day | 21st day | Epithelialization in Days |
|---|---|---|---|---|---|---|
| Normal control | 22.4± 2.8 | 42.4± 1.2 | 57.23± 0.6 | 67.4± 0.5 | 75.8± 0.69 | 20±0.86 |
| Wounded control | 36± 0.72 | 63.6± 0.98 | 78.8±0.39 | 92± 0.61*** | 100±0*** | 15±0.06 |
| MELA treated | 23± 0.54 | 45.2± 0.82 | 70.7± 0.52 | 89.8± 0.6 | 98.8±0.35 | 16.6±0.33 |

Values are mean ± SEM expressed as (n=6) *P* *<0.05; **<0.01; ***<0.001; as compared with Group II.

Statistical evaluation by one-way ANOVA, followed by Dunnet's t-test for comparison of test groups with control.

**Figure 9.2 Effect of MELA on wound contraction**
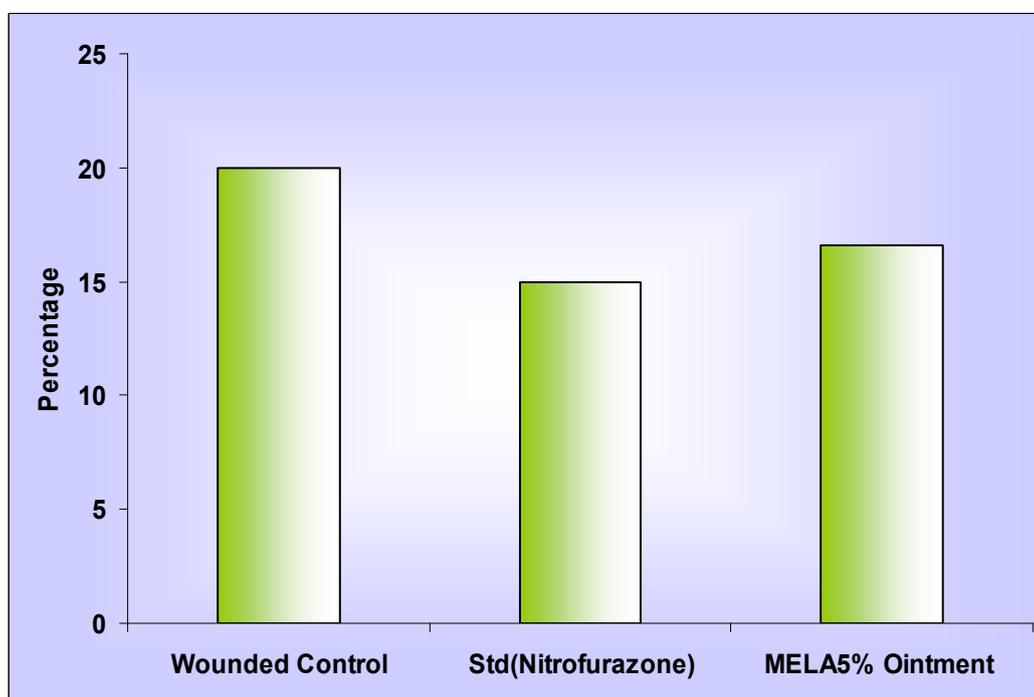


**Figure 9.3 Effect of MELA on Epithelialization period**

**9.3.2    Effect of MELA on Incision Model**

The wounding healing results for the incision and dead space models are indicated in Table 9.2. In the incision wound model, wound breaking strength (WBS) was measured. It was significantly increased in the MELA-treated group compared with control and both the extracts and with both the doses. A significant increase was observed in the skin tensile strength of the methanol extract-treated group on the tenth post-wounding day, at both dose levels (Table 9.1 Figure 9.2).

**Table 9.2   Effect of MELA on incision and dead space wound model and antioxidant enzymes**

| Treatment | Breaking strength(gm) | Hydroxyproline (mg/g tissue) | Dry tissue weight(mg/ 100g rat) | SOD (U/mg Protein) | Catalase (U/mg Protein) |
|---|---|---|---|---|---|
| Wounded Control | 275.14±22.89 | 15.46±0.5 | 30.5±0.21 | 1.8±0.51 | 0.01±0.018 |
| Standard Nitrofurazone ointment | 427.4±30.3*** | - | - | - | - |
| MELA 200 mg/kg | 380 ± 18.5** | 55.2±0.25 | 44.6±0.31 | 4.92±0.81 | 0.43±0.013 |
| MELA 400 mg/kg | 418 ± 16.5** | 67.4±0.31 | 51.52±0 | 4.92±0.81 | 0.58±0.02 |

Values are mean ± SEM expressed as (n=6) $P$ *<0.05; **<0.01; ***<0.001; as compared with Group II.

Statistically evaluated by one-way ANOVA, followed by Dunnet's t-test for comparison of test groups with control.

**9.3.4      Effect of MELA on Dead Space Model**

The MELA treated animals of the dead-space wound model showed a significant increase in dry granuloma weight, granuloma breaking strength and the level of hydroxyproline content at both dose levels.Hydroxyproline content , granulation tissue weight, as well as SOD and Catalase all increased significantly ($p < 0.05$), following treatment with the extract and standard drug, when compared with the control group. Thus, the extract not only promoted wound healing but also exerted anti-oxidant activity.
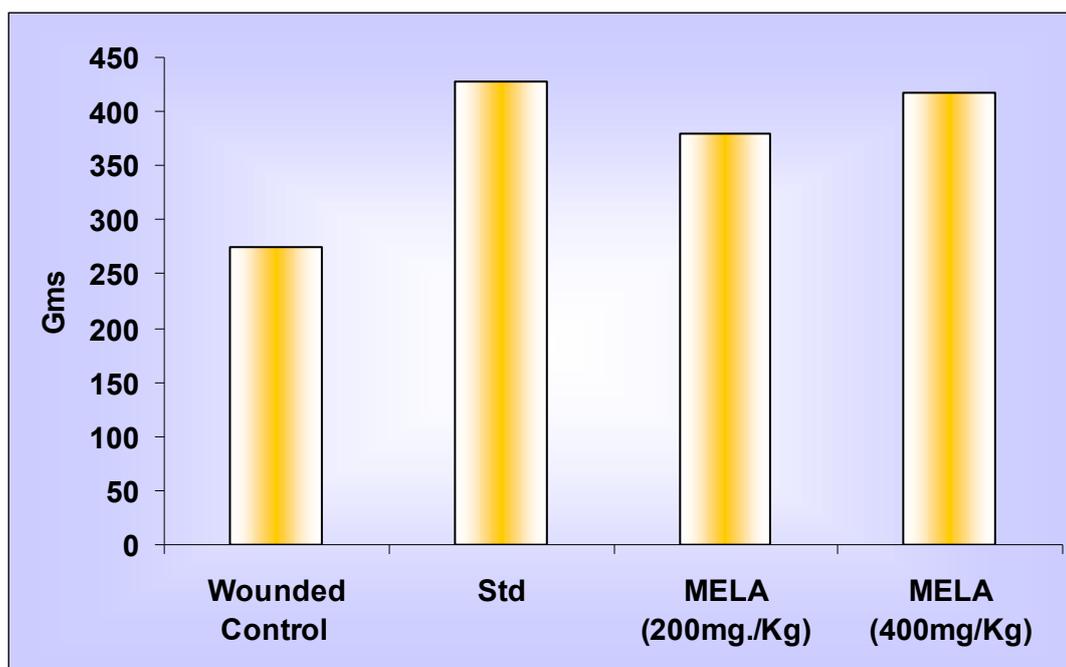


**Figure 9.4 Effect of MELA on Wound breaking strength**
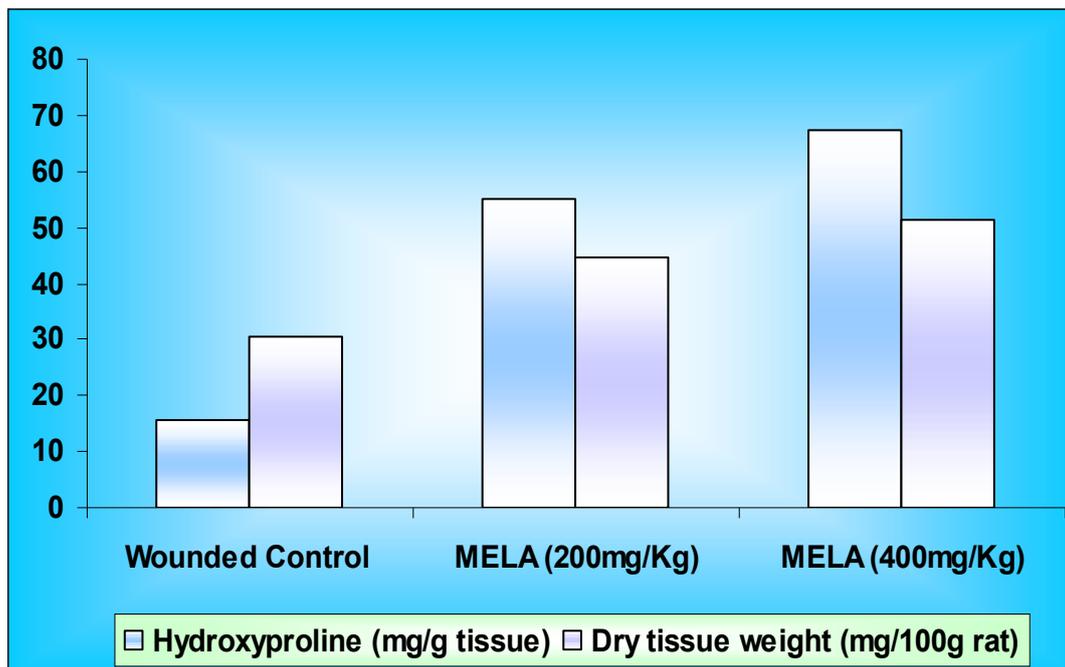
**Figure 9.5 Effect of MELA on Hydroxy proline content and Dry Tissue weight**
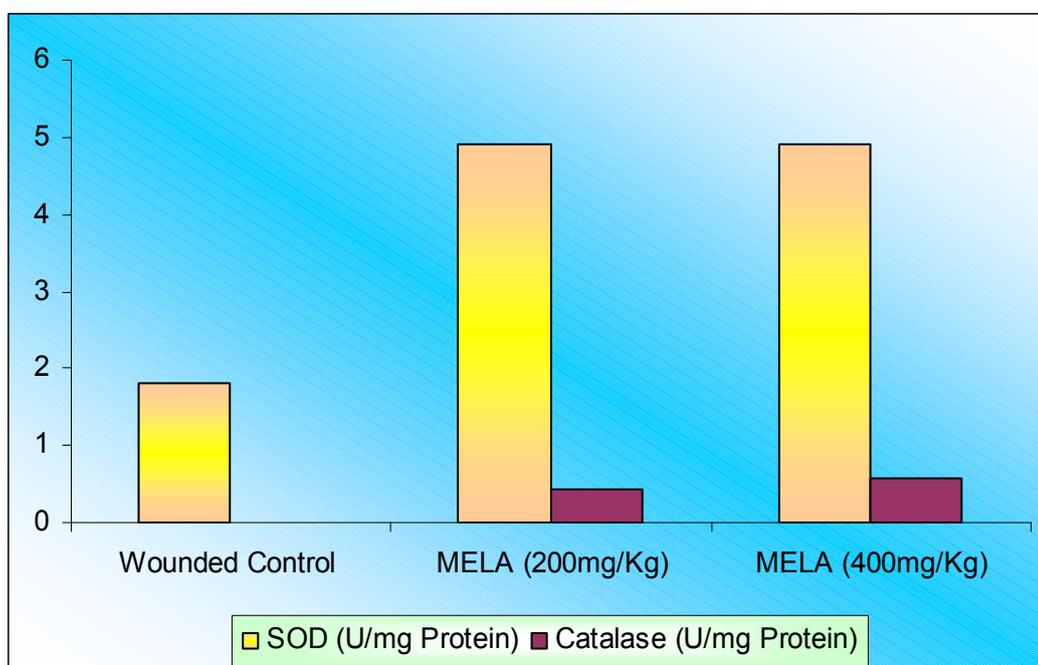


**Figure 9.6 Effect of MELA on antioxidant enzymes**

### 9.3.5      Effect of MELA on Histopathological Evaluation

The photomicrographs of the granulation tissues obtained from the animal wounds are shown in Fig 8. The images revealed that while increased number of fibroblasts can be seen in animals in the control group Figure 9.7 (a), the collagen fibres are indistinguishable. On the other hand, animals treated with 200 mg/kg of the extract showed moderate collagenation and fibrosis Figure 9.7 (b). Animals treated with 400 mg/kg of the extract revealed higher fibrosis and well-formed collagen fibres Figure 9.7 (c).

(a)



(b)



(c)
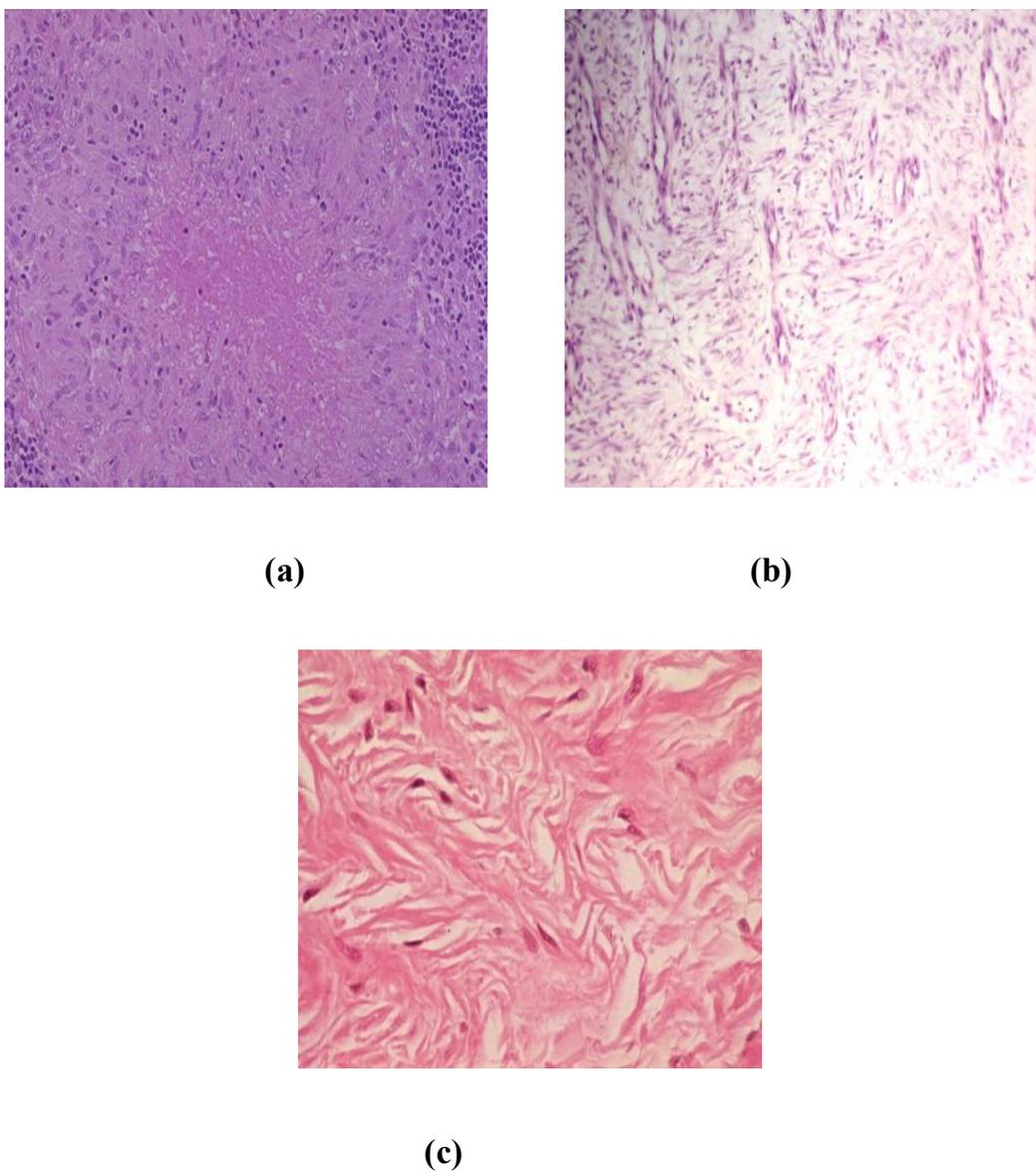
**Figure 9.7 Histopathology of granulation tissue**

(a) Control animals, showing increased number of fibroblasts and indistinguishable collagen fibres.

(b) MELA (200 mg/kg) animals, showing moderate collagenation and fibrosis.

(c) MELA (400mg/kg) animals, showing a high level of fibrosis as well as well-formed collagen fibres.

**Effect of MELA on Biochemical Parameters**

In the dead space wound model, the granulation tissue, hydroxyproline level was significantly increased in MELA 400 mg/kg ($P < 0.01$), and in Nitrofurazone treated groups are compared with that of the control group ($P < 0.001$). There was no significant difference among different doses of methanolic extract. When compared with methanolic extract 200 mg/kg, there was significant increase in hydroxyproline level in the extract 400 mg/kg ($P < 0.01$) (Table 9.2, Figure 9.5).

**Effect of MELA on Antioxidant Parameters**

SOD activity in granulation tissue was significantly increased in the case of rats treated with methanolic extract 200 mg/kg ($P < 0.05$), 400 mg/kg ($P < 0.05$) when compared with control (Table 9.2, Figure 9.6). Catalase level in granulation tissue was significantly increased in the case of methanolic extract 200 mg/kg ($P < 0.05$), methanolic extract 400 mg/kg ($P < 0.05$) when compared with control.

## 9.4 DISCUSSION

Wounds are common clinical entities in day-to-day life, which may be major or minor. The process of wound healing can be classified into five phases - cellular phase (granulation), narrowing of wound area (wound contraction), collagen deposition (collagenation), epithelial covering (Epithelialisation) and scar remodelling (cicatrisation).These phases are concurrent but independent of each other. Any agent which accelerates the process is a promoter of wound healing [200].

Wound healing or repair is a natural process of regenerating dermal and epidermal tissue and may be categorized into three phases viz, inflammation, proliferation and remodelling phase. In the inflammation phase, various growth factors such as tumour necrosis factor (TNF), interleukins (IL) are released to initiate the proliferation phase. The latter is characterized by angiogenesis, collagen deposition, granular tissue formation, epithelialization and wound contraction [201]. In the last phase, the levels of collagen production and degradation equalize, after which disorganized fibres are rearranged thus increasing the tensile strength of the wound. The capacity of wound to heal depends, in part, on its depth, as well as on the overall health and nutritional status of the individual. Following injury, inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial tissue is regenerated. It is well known that, stages in healing namely, coagulation, inflammation, microphasia, fibroblasts formation and collagenation, are intimately interlinked [202].

The wound breaking strength is determined by the rate of collagen synthesis and more so by the maturation process where there is a covalent binding of collagen fibrils through inter and intra molecular cross linking. In our study, dead space wound model showed a significant increase in hydroxyproline concentration and also the dry weight of the granulation tissue was significantly increased in MELA treated group. By this we can assume that MELA might not have increased the collagen content but probably have altered the maturation process, by affecting the cross linking of collagen or improving the quality of collagen fibrils. The increase in weight by Nitrofurazone treated group could be due to high protein concentration and collagen bundle formation.

In the present investigation, MELA at the doses of 200 and 400 mg/kg showed significantly increased healing by wound contraction, when compared to the control group. Increase in the breaking strength of the wound is indicative of improved collagenation which contributes to healing, enhances epithelization and promotes wound contraction by increasing granulation tissue weight due to infiltration of macrophages [203].

Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area. This centripetal movement of wound margin is believed to be due to the activity of myofibroblasts [204]. During wound contraction, the wound is made smaller by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. In the maturation and remodelling phase, collagen is remodelled and realigned along tension lines and cells that are no longer needed to be removed by apoptosis. MELA–based ointment has significant influence on one or some of the stages resulting in faster rate of wound closure when compared to the control group. Since MELA enhanced wound contraction, it would have either enhanced contractile property of myofibroblasts or increases the number of myofibroblasts recruited into the wound area. The wound is eventually closed by a combination of all these process.

In excision wound model, *L.acidissima* hastened the period of epithelialization significantly. The process of wound contraction and epithelisation is separate and independent. The activity of fibroblast is responsible for wound contraction and involves movement of entire dermis. Epithelization involves migration and proliferation of cells. It is known that stabilization of lysosomal membranes, inhibition of cellular migration and inhibition of fibroblast contraction are responsible for their anti-healing effects [205]. Thus, intervention in any one of these phases by drugs would

eventually lead to either promotion or depression of collagenation, wound contraction and epithelisation [206]. In studies using the excision wound model, animals treated with MELA showed a significant decrease in the epithelisation period, as evidenced by the shorter period for the fall of escher compared to control. The extract also facilitated the epithelisation period significantly at both dose levels (Table 9.1 and Figure 9.3). The faster wound contraction rate of the extract may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes. It may increase the gap junctional intracellular communication in cultured fibroblasts and induces a more rapid maturation of granulation tissue.

Collagen is a major protein of the extracellular matrix and is the component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptide. Therefore, measurement of hydroxyproline could be used as an index for determining collagen turnover [207]. The extract-treated groups showed significant increases in the level of hydroxyproline, which is a reflection of increased collagen content. This was confirmed by histopathological examination of wound granulation tissue which showed a well-developed matrix in extract treated group with the collagen was well-organized and bundles formed between the cells. There was also better neovascularisation in the extract treated groups than in the control group.

Elimination of reactive oxygen species is reported to be an important strategy to improve healing of wounds. Many plant extracts and medicinal herbs have shown potent antioxidant activity. Research into the role of antioxidants from plant extracts in wound healing has been published widely [208].Reactive oxygen species (ROS) play a vital role in wound

healing and can trigger various beneficial oxygen free radicals. While antioxidants improve healing in ischemic skin wounds [209]. Elevated lipid peroxide levels have also been demonstrated in certain inflammatory skin lesions such as wound and dermatitis. Therefore, if a compound has antioxidant potential, it can be a good therapeutic agent for enhancing the wound healing process. Hence, estimation of antioxidants like SOD, Catalase in granulation tissues is also relevant because these antioxidants hasten the process of wound healing by destroying the free radicals [210].

An increase in the levels of anti-oxidant enzymes (SOD and Catalase) was observed in the granulation tissue of the extract treated groups may also have contributed to the wound healing effect of the extract. Studies on the estimation of antioxidant enzyme revealed that the extract significantly increased the levels of superoxide dismutase and Catalase, the two powerful antioxidant enzymes of the body that are known to quench Superoxide radicals (Table 9.2,Figure 9.6).The antioxidant enzymes are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals. So in this study, scavenging effect might be one of the most important components of wound healing which may be responsible to support wound healing property. Thus the enhanced wound healing may be due to the free radical scavenging action of the plant as well as enhanced antioxidant enzyme level in granuloma tissues [211].

Several antioxidants such as Ascorbic acid, Catalase was found to improve healing. Ascorbic acid has a role in both the formation and maintenance of collagen in healing wounds. As the extract of *L.acidissima* contains rich source of Vitamin - C, this also may attribute to the antioxidant potential and in turn significant wound healing effect. Catalase was found to detoxify hydrogen peroxide which can otherwise inflict severe damage to

regenerating cells. In condensation, the results indicated the beneficial effects of MELA by reduced lipid peroxide levels in treated wounds, which may in turn be responsible for acceleration of the healing process.

## 9.5    CONCLUSION

The present study reveals the wound healing and antioxidant activities of MELA in experimental animal model against incision,excision and dead space models which was proved by theevaluated parameters and the antioxidant enzyme levels.Further works are being carried out to isolate and identify the active principle involved in the wound healing and antioxidant activities of this plant extract.Thus MELA can be utilised as an economical therapeutic agent for wound management as a pro-healer, as well as to control abnormal healing on proper formulation.