CHAPTER 7

IN VIVO WOUND HEALING ACTIVITY OF *Lobelia trigona* Roxb
ETHANOLIC EXTRACT.

7.1. INTRODUCTION

Wound healing process comprises of suppression of inflammation, cell proliferation and collagen tissue contraction (Houghton et al. 2005). Wound healing is a complex but a natural process characterized by 3 phases: inflammation, Proliferative (tissue formation) and Maturation (tissue remodeling) phase (Sidhu et al. 1999). Burns are known to be one of the most common forms of injury which became an important cause of disability and death in the world. More than a million people suffer from burn injuries annually (Ezzati et al. 2009; Upadhyay et al. 2011). There is always a need for an effective and local wound healing treatment. In burn injuries, allergic reactions and skin irritations are the most important adverse effects of topical antiseptic agents and disinfectants, which reduce the rate of skin repair and increase the rehabilitation period (Upadhyay et al. 2011). Numerous research have been carried out in order to diminish the microbial infection in wounds, promoting re-epithelialization, decreasing fluid imbalance and reducing the occurrence of scar tissue.

Traditional formulation of wound medicine, especially the herbal and other medicinal products deployed worldwide, are still under scientific evaluation for their properties in the treatment of wounds. Herbal products could be extensively preferable due to their widespread accessibility and efficacy with less or no toxicity and are cost effective than allopathy drugs (Upadhyay et al. 2011).
However, modern scientific methods should be applied to confirm the claims about the therapeutic effects of herbal compounds (Chaudhari and Mengi 2006).

7.2. MATERIALS AND METHODS

7.2.1. Animals Used

Adult male wistar rats weighing approximately 150g - 200g body weight were used in the study. The rats were acclimatized to the laboratory environment for a period of 10 days. The protocol was approved by IAEC (Institutional Animal Ethical Committee) (IAEC/KU/BT/14/23).

7.2.2. Acute dermal toxicity

Acute dermal toxicity of Lobelia trigona Roxb was carried out in order to determine the dose as per OECD guidelines No. 402 (OECD guidelines 1987). Lobelia trigona Roxb ointments with highest concentration (2000 mg/kg) was applied on the shaved dorsal region of the rats.

7.2.3. Grouping of animals and drug administration

The rats were divided into fifteen groups of six animals in each group (n=6).

Group I : Normal control animals left untreated.
Group II : Control animals treated topically with simple ointment base (SOB). [Simple ointment base is a common vehicle for topical
application of drugs containing white soft paraffin (85gm), wool fat (5gm), cetosteryl alcohol (5gm) and hard paraffin (5gm)]

Group III: Animals treated with Standard ointment containing 1% w/w Silver Sulphadiazine.

Group IV: Animals treated topically with 5% w/w Lobelia trigona Roxb ointment (5% LT).

Group V: Animals treated topically with 10% w/w Lobelia trigona Roxb ointment (10% LT).

All the above mentioned treatments were done by applying 1g of ointment once daily. Three wound models were used in the study: Incision wound model, Excision wound model and Burn wound model.

7.2.4. Incision wound model

After wound area preparation with 70% alcohol, a longitudinal paravertebral incision was made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on either depilated side of the vertebral column with a sterile sharp surgical blade. Each incision made was 5 cm in length, and after complete hemostasis, the parted skin is stitched with interrupted sutures, 1.0 cm apart using black braided silk surgical thread (no. 000) and a curved needle (no. 11). The continuous threads on both wound edges are tightened for good closure of the wound. The wounds were left undressed and mopped with a cotton swab. The ointments were applied topically to the animals of all the groups until 9th day starting from the day of operation. The sutures were removed and the skin breaking strength of the healed wound is measured on 10th day (Ehrlich and Hunt 1968, Udupa et al. 1995, Krishnaveni et al. 2009, Asif et al. 2007, Hemalatha et al. 2001).
7.2.5. Excision wound model

Excision wounds are inflicted on the dorsal thoracic region 1–1.5 cm away from the vertebral column on either side and 5 cm away from the ear. After wound area preparation with 70% alcohol, using a surgical blade the skin from the predetermined area on the depilated back of the animal was excised to its full thickness to obtain a wound area of about 100 mm diameter. Hemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. The ointments were applied topically to the animals of all the groups until complete epithelialization starting from the day of operation (Morton and Melone 1972, Kakali et al. 1997, Nayak et al. 2007, Shenoy et al. 2009).

7.2.6. Burn wound model

Burn wounds are inflicted on the dorsal thoracic region 1–1.5 cm away from the vertebral column on either side and 5 cm away from the ear. After wound area preparation with 70% alcohol, using a surgical blade the skin from the predetermined area on the depilated back of the animal. A special metal plate 1×1 cm with holder was heated to 100°C and applied to the dorsal area of the animals for 30 seconds to induce full thickness burn wound. Animals were placed in individual cages after recovery from anesthesia. The ointments were applied topically to the animals of all the groups until the day of scab falling starting from the day of operation (Wannarat et al. 2009).

7.2.7. Tensile strength of incision wound model

Tensile strength measurement was performed (Lee 1968) on the 10th day using tenisiometer, an Instron type 6021 with 1 kN piezo resistive load cell to measure the tensile load and an acquisition frequency of 5000 Hz, with the signal
filtered to 750-800 Hz ensuring a smooth output. The skin specimens were clamped using special custom made anti slip clamps so as to counteract any slipping. The cage length was set at 30 mm and the velocity of the cross-head was 100 mm/min. The tensile strength of wounds treated with 5% and 10% w/w *Lobelia trigona* Roxb ethanolic extract ointments were compared with standard and control groups. Tensiometer measures the breaking strength in N (Newton) and then converted to grams.

7.2.8. Percentage of wound contraction

The percentage of the excision and burn wound contraction was measured (Sadaf et al. 2006) and calculated by using the formula

\[
\% \text{ of wound contraction} = \left[ \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \right] \times 100.
\]

7.2.9. Period of Epithelialization

It was measured in days from 0\textsuperscript{th} day till the Eschar totally separated itself with the raw wound left behind.

7.2.10. Biochemical assays evaluated for wound healing

Initial wet weight and final dry weight of tissues on different days of different groups were weighed. The amount of uronic acid (Schiller et al. 1961), hexosamine (Elson and Morgan 1993), protein (Lowry et al. 1951) and collagen (hydroxyproline) (Woessner 1961) were estimated for all the wound tissue of all the groups. All the values were determined and expressed as mg/g of wound tissue.
7.2.11. Histopathological examination

On the 16\textsuperscript{th} day the tissues were stained with haematoxylin and eosin (H&E) and Masson’s trichrome (MT). It was then observed under a light microscope. (Ehrlich et al. 1973; Hunt and Mueller 1994).

7.2.12. Statistical analysis

Results have been expressed as Mean ± SEM. The data was evaluated and assessed by using one way ANOVA and then by Dunnett’s T-Test. (P < 0.05) and (P < 0.01) were considered as significant.

7.3. RESULTS

Comparison of the tensile strength of animals from different groups shown in Figure 7.1. Less tensile strength was observed in normal untreated group (430.37±17.47 g). Among the other groups the tissues treated with standard ointment and 5\% w/w Lobelia trigona Roxb ointment exerts (713.67±14.59 g) and (711.62±12.90 g) respectively which is more or less the same strength. The groups treated with 10\% w/w Lobelia trigona Roxb ointment have the highest tensile strength. (837.36±16.37 g). By noticing the results of tensile strength it can be claimed that 10\% extract of Lobelia trigona Roxb have great wound healing property when compared to standard ointment.
Figure 7.1 Tensile strength of incision wound model. Value are represented as Mean ± SEM with triplicate estimations (** -P<0.01).

In excision wound, the reduction of wound area of different groups until the 16th day was calculated and depicted in Figure 7.2 and 7.3. Control untreated groups showed very less percentage of wound healing (70.35±0.13%) followed by SOB treated group (77.5±0.67%) and Standard ointment group (89.29±0.63%). Group treated with 5% w/w ointments of Lobelia trigona Roxb showed wound contraction rate at (93.55±0.24%). Wound healing was faster (99.33±0.55%) in group treated with 10% w/w ointment of Lobelia trigona Roxb. The epithelialization period was found to be lesser or quicker in 10% w/w Lobelia trigona Roxb ointment group (16.10 days) followed by 5% w/w Lobelia trigona Roxb ointment (17.10 days) and Reference standard treated group (17.91 days) (Figure 7.4). Epithelialization time of control group was 22.74 days which is higher followed by SOB treated group (20.64 days).
Figure 7.2 Macroscopic appearance of excision wound on day 4 to day 16 treated with 5% and 10% w/w Lobelia trigona Roxb ointments compared with the reference standard (Silverex containing Silver Sulphadiazine 1% w/w)
Figure 7.3 Percentage of wound healing using *Lobelia trigona* Roxb ointments (5% and 10%) on excision wound model. Values are represented as Mean ± SEM with triplicate estimations (** -P<0.01).

Figure 7.4 Period of epithelialization of *Lobelia trigona* Roxb ointments (5% and 10%) on excision wound model in days by group.
Table 7.1 Wet weight and dry weight of tissues of different groups on different days- Excision wound model.

<table>
<thead>
<tr>
<th>Groups</th>
<th>4th Day</th>
<th>8th Day</th>
<th>12th Day</th>
<th>16th Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WW (g)</td>
<td>DW (g)</td>
<td>WW (g)</td>
<td>DW (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.222</td>
<td>0.102</td>
<td>0.155</td>
<td>0.079</td>
</tr>
<tr>
<td>Standard</td>
<td>0.238</td>
<td>0.110</td>
<td>0.150</td>
<td>0.087</td>
</tr>
<tr>
<td>SOB</td>
<td>0.229</td>
<td>0.119</td>
<td>0.143</td>
<td>0.077</td>
</tr>
<tr>
<td>5% LT</td>
<td>0.309</td>
<td>0.151</td>
<td>0.135</td>
<td>0.069</td>
</tr>
<tr>
<td>10% LT</td>
<td>0.269</td>
<td>0.144</td>
<td>0.168</td>
<td>0.080</td>
</tr>
</tbody>
</table>

WW: Wet weight; DW: Dry weight

Table 7.1 shows the initial wet weight and final dry weight of different groups of excision and burn wound tissues on different days.

The biochemical estimation of collagen (hydroxyproline), uronic acid, protein and hexosamine in the granulation tissues of all the animal groups of excision and burn wound model are depicted in Figure 7.5, 7.6, 7.7, 7.8.

Increasing amount of collagen (hydroxyproline), uronic acid, protein and hexosamine from day 4 – day 12 was observed in groups treated with Lobelia trigona Roxb ointments. There after gradual decrease was observed until the 16th day.
**Figure 7.5** Effect of ointment (5% LT and 10% LT) on total non-collagenous protein content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (**P<0.01)**.

**Figure 7.6** Effect of ointment (5% LT and 10% LT) on hexosamine content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (**P<0.01)**.
**Figure 7.7** Effect of ointment (5% LT and 10% LT) on uronic acid content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).

**Figure 7.8** Effect of ointment (5% LT and 10% LT) on total collagen content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).
Hematoxylin and eosin (H&E) staining was done (Figure 7.9) to evaluate the excision and burn wound healing activity of *Lobelia trigona* Roxb ethanolic extract.

**Figure 7.9** Photographic representation of histopathology of wounded tissue on day 16 stained with H&E (hematoxylin and eosin).
Masson’s trichrome staining (MTS) method differentiate and assess the morphological parameters for wound healing (collagen deposition).

<table>
<thead>
<tr>
<th></th>
<th>4 X</th>
<th>10 X</th>
<th>40 X</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>SOB</strong></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>STANDARD</strong></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>5 % LT</strong></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>10 % LT</strong></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 7.10** Photographic representation of histopathology of wounded tissue on day 16 stained with MTS (Massson’s trichrome stain).
The results exhibited ulcerated epithelium, sub epithelium with dense infiltrates, edema, and proliferation of thin walled vessels with the areas of hemorrhages. Fibro collagenous stroma and adipose tissue were seen in untreated control groups, which show that the wound was not healed. Whereas in section of 5% and 10% *Lobelia trigona* Roxb treated groups, the granulation tissue shows neovascularization, re-epithelialization, with the proliferation of fibroblasts, neutrophilic infiltration and the presence of more collagen with less inflammation in the dermis indicating the healed wound.

The results of the MTS method (Figure 7.10 and Figure 7.19) confirmed the presence of maximum collagen density (greenish blue) in groups treated with 5% and 10% w/w *Lobelia trigona* Roxb ointment than the control group.

In burn wound, the reduction of wound area of different groups until the 16th day was calculated and depicted in Figure 7.11 and 7.12. Control untreated groups showed very less percentage of wound healing (71.31±0.58%) followed by SOB treated group (84.6±0.75%) and standard ointment group (90.57±1.28%). Group treated with 5% w/w ointments of *Lobelia trigona* Roxb showed wound contraction rate at (99.43±0.28%) which is almost a healed wound. Wound was healed almost in day 12 in group treated with 10% w/w ointment of *Lobelia trigona* Roxb. It showed wound contraction rate of (98.06±0.17%) and the wound healed completely (100±0.00%) on day 16. The epithelialization time was found to be lesser or quicker in group treated with ointment containing 10% w/w *Lobelia trigona* Roxb (between 13-16 days) than the group treated with 5% w/w *Lobelia trigona* Roxb ointment (16.09 days) and Reference standard treated group (17.66 days). Epithelialization time of control group was 20.69 days which was higher followed by SOB treated group (18.91 days).
Figure 7.11 Pictorial representation of burn wound on day 4 to day 16 treated with 5% and 10 % w/w *Lobelia trigona* Roxb ointments compared with the reference standard (Silverex containing Silver Sulphadiazine 1% w/w)
**Figure 7.12** Percentage of burn wound closure by group. Values are represented as Mean ± SEM with triplicate estimations (** -P<0.01).

**Figure 7.13** Epithelialization period of burn wounds in days by group.
**Table 7.2** Wet weight and dry weight of tissues of different groups on different days - Burn wound model.

<table>
<thead>
<tr>
<th>Groups</th>
<th>4th Day</th>
<th>8th Day</th>
<th>12th Day</th>
<th>16th Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WW (g)</td>
<td>DW (g)</td>
<td>WW (g)</td>
<td>DW (g)</td>
</tr>
<tr>
<td>Control</td>
<td>0.278</td>
<td>0.101</td>
<td>0.458</td>
<td>0.322</td>
</tr>
<tr>
<td></td>
<td>0.287</td>
<td>0.128</td>
<td>0.187</td>
<td>0.090</td>
</tr>
<tr>
<td>Standard</td>
<td>0.266</td>
<td>0.109</td>
<td>0.478</td>
<td>0.290</td>
</tr>
<tr>
<td></td>
<td>0.278</td>
<td>0.122</td>
<td>0.110</td>
<td>0.054</td>
</tr>
<tr>
<td>SOB</td>
<td>0.247</td>
<td>0.122</td>
<td>0.459</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>0.267</td>
<td>0.129</td>
<td>0.178</td>
<td>0.093</td>
</tr>
<tr>
<td>5% LT</td>
<td>0.285</td>
<td>0.141</td>
<td>0.549</td>
<td>0.303</td>
</tr>
<tr>
<td></td>
<td>0.327</td>
<td>0.152</td>
<td>0.210</td>
<td>0.102</td>
</tr>
<tr>
<td>10% LT</td>
<td>0.249</td>
<td>0.122</td>
<td>0.427</td>
<td>0.202</td>
</tr>
<tr>
<td></td>
<td>0.208</td>
<td>0.107</td>
<td>0.103</td>
<td>0.057</td>
</tr>
</tbody>
</table>

WW: Wet weight; DW: Dry weight

Table 7.2 shows the initial wet weight and final dry weight of different groups of excision and burn wound tissues on different days.

The biochemical estimation of collagen (hydroxyproline), uronic acid, protein and hexosamine in the granulation tissues of all the animal groups of excision and burn wound model are depicted in Figure 7.14, 7.15, 7.16, and 7.17.

Increasing amount of collagen (hydroxyproline), uronic acid, protein and hexosamine from day 4 – day 12 was observed in groups treated with *Lobelia trigona* Roxb ointments. There after gradual decrease was observed until the 16th day.
Figure 7.14 Effect of ointment (5% LT and 10% LT) on total non-collagenous protein content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).

Figure 7.15 Effect of ointment (5% LT and 10% LT) on hexosamine content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).
Figure 7.16 Effect of ointment (5% LT and 10% LT) on uronic acid content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).

Figure 7.17 Effect of ointment (5% LT and 10% LT) on total collagen content of wound tissue. Values are represented as Mean ± SEM with triplicate estimations (** P<0.01).
Haematoxylin and eosin (H&E) staining was done (Figure 7.18) to evaluate the excision and burn wound healing activity of *Lobelia trigona Roxb* ethanolic extract.

The results exhibited ulcerated epithelium, sub epithelium with dense infiltrates, edema, and proliferation of thin walled vessels with the areas of hemorrhages. Fibro collagenous stroma and adipose tissue were seen in untreated control groups, which show that the wound was not healed.

Whereas in section of 5 % and 10% *Lobelia trigona Roxb* treated groups, the granulation tissue shows neovascularization, re-epithelialization, with the proliferation of fibroblasts, neutrophilic infiltration and the presence of more collagen with less inflammation in the dermis indicating the healed wound.

Masson’s trichrome staining (MTS) method differentiate and assess the morphological parameters for wound healing (collagen deposition).

The results of the MTS method (Figure 7.19) confirmed the presence of maximum collagen density (greenish blue) in groups treated with 5 % and 10 % w/w *Lobelia trigona Roxb* ointment than the control group.

As the results showed that groups treated with 10 % *Lobelia trigona Roxb* healed faster showed less scar, less inflammation and more fibroblasts and collagen.
Figure 7.18 Photographic representation of histopathology of wounded tissue on day 16 stained with H&E (hematoxylin and eosin).
Figure 7.19 Photographic representation of histopathology of wounded tissue on day 16 stained with MTS (Massson’s trichrome stain).
7.4. DISCUSSION

Three wound models (incision, excision and burn wound) were used to evaluate the wound healing activity of the ethanolic extract of *Lobelia trigona Roxb*.

The increase in tensile strength of wounds treated with *Lobelia trigona Roxb* ethanolic extract ointments might be due to the increase in the concentration of hexosamine, collagen, uronic acid and protein content (Udupa et al. 1995). Uronic acid and hexosamine act as ground substances whereas the collagen plays a major role in integrity to the tissue matrix and in epithelialization.

The topical ointment of 10% w/w *Lobelia trigona Roxb* ethanolic extract significantly increased the fibroblast growth which plays a major role in anatomic integrity of excision and burn wound healing, collagen synthesis, and thus the healing process by accelerating the rate of burn wound healing.

Biochemical evaluation revealed that *Lobelia trigona Roxb* ethanolic extract has helped in its proper deposition and alignment of collagen. In *Lobelia trigona Roxb* treated wounds, the levels of uronic acid and hexosamine gradually increased from day 4 until day 8 post wounding and gradually decreased thereafter which helped in collagen synthesis and deposition.

Haematoxylin and eosin (H&E) staining was done to evaluate the excision and burn wound healing activity of *Lobelia trigona Roxb* ethanolic extract. Masson’s trichrome staining (MTS) method differentiate and assess the morphological parameters for wound healing (collagen deposition). Based on the histopathology report, we propose that the ointment of *Lobelia trigona Roxb*
ethanolic extract might have enhanced the excision and burn wound healing time and process by increasing the rate of all the wound healing phases, such as cell proliferation and collagen formation.

The outcome of the in vivo wound models demonstrated that the wound healing ability of the ethanolic extract ointment of Lobelia trigona Roxb on an incision, excision and burn wound was much greater than that of the standard ointment that contains 1% w/w Silver Sulphadiazine.

It has been observed that phytochemicals like triterpenoids (Scortichini et al. 1991), flavonoids (Tsuchiya et al. 1996), and tannins (Rane et al. 2003) are known to have wound healing properties and it significantly accelerate the wound healing process of excision and burn wound. And also free radical scavenging mechanisms by medicinal plant extracts may act either individually or in combination to enhance the wound healing process (Somashekar et al. 2008).

The wound healing action of Lobelia trigona Roxb might have occurred because of the rich content of wound healing phytochemical components (tannin, terpenoid, phenolic and flavonoid content) present in it. The chemical constituents of Lobelia trigona Roxb includes polyphenols, flavonoids, alkaloids, steroids, saponins, tannins, glycosides, terpenoids, reducing sugars, pholabatannins, carbohydrates, lactones, resins, cardenolides, vitamin C, proteins and amino acids (Rex et al. 2015). All of the above mentioned phytochemical constituents play a major role in wound healing and also in improving and maintaining human health (Trevisanato 2000).