CHAPTER 6

IN VITRO WOUND HEALING ACTIVITY OF Lobelia trigona Roxb ETHANOLIC EXTRACT.

6.1. INTRODUCTION

The process of wound healing involves three partly overlapping phases: inflammation (1st phase), proliferation (2nd phase), granulation tissue formation and remodeling of new tissue (3rd phase). Fibroblast and keratinocytes are the two cell types which plays a major role in wound healing process (Gurtner et al. 2008, Martin 1997, Schafer and Werner 2007).

The process of re-epithelialization are observed in the initial stages of tissue formation that occurs with migration of keratinocytes of the injured epidermis and hair follicles followed by proliferation of these cells at the wound edge. Fibroblasts plays a major role in the repair mechanism of the injured dermal wound and it proliferate and migrate into the wound area and synthesize new ECM (Broughton et al. 2006, Schafer and Werner 2007, Singer and Clark 1999, Gurtner et al. 2008).

Medicinal plants and preparations were traditionally used by ancient people for wound healing (Schmidt et al. 2009, Reuter et al. 2009). In vitro scratch assay or cell migration assay is an inexpensive tool to check and evaluate how plant extracts can influence the formation of new tissues (Van Horssen et al. 2006; Liang et al. 2007).
The present study was designed to use an assay to investigate the migration of HaCaT keratinocytes cells to an artificial scratch wound with the objective to evaluate the pharmacological effects of a herbal preparation: *Lobelia trigona Roxb* ethanolic extract on cytotoxicity and wound healing.

6.2. MATERIAL AND METHODS

6.2.1. Cell lines used

HaCaT cell line was obtained from NCCS Pune and maintained in DMEM (Dulbecco’s modified eagles media) supplemented with 10% Fetal Bovine Serum (FBS) and grown to confluency at 37°C in 5 % CO₂ in a CO₂ incubator. The keratinocyte cells (HaCaT) was trypsinized for 30 seconds and passaged to T25 flasks in complete aseptic environment.

6.2.2. *In vitro* cytotoxicity assay

HaCaT cells (keratinocytes) was cultured in 96-well tissue culture plates (1 × 10⁴ cells/ml) and treated with various concentrations (6.25–100μg/ml) of *Lobelia trigona Roxb* ethanolic extract respectively. The cytotoxic activity of ethanolic extract of *Lobelia trigona Roxb* was estimated by using MTT assay at 540 nm using the microplate reader (Mosmann 1983).

6.2.3. *In vitro* scratch assay of *Lobelia trigona Roxb* ethanolic extract

The effect of *Lobelia trigona Roxb* ethanolic extract on wound closure was determined by using a 12-well plate. Exponentially growing HaCaT cells in DMEM (1% FBS) was trypsinized and then seeded at a density of 200,000 cells
into a 12-well plate and cultured overnight to allow adhering and reaching a 90% confluence.

The scratch wound was made by a sterile 1 mL pipette tip through a pre-marked line. The cell monolayer was subsequently rinsed three times with PBS followed by incubation with indicated concentrations of Lobelia trigona Roxb ethanolic extract (10, 50, 100 µg/mL) for 30 minutes and incubated at 37°C for 24 hours in a CO₂ incubator. DMEM medium with dimethyl sulfoxide was used as control. The effect of Lobelia trigona ethanolic extract on wound closure was determined microscopically (20X magnification, Olympus CKX41). The experiment was performed at least in triplicate. The wound area was photographed and analyzed using CellID imaging software (Selinummi et al. 2005). For the statistical analysis, all data was standardized to the density of confluent cells (100%) and expressed as

Percentage of cells in wounded area (%) = (test compound/confluent area) × 100.

6.2.4. Statistical analysis

Values are represented as Mean ± SEM with triplicate estimations and P < 0.001 was considered as significant.

6.3. RESULTS

6.3.1. In vitro cytotoxic effect of Lobelia trigona Roxb ethanolic extract

As cytotoxic activity can influence function of wound healing process, the effect of Lobelia trigona Roxb ethanolic extract on cell viability after 24 h treatment in the logarithmic growth phase was measured by using the MTT
assay. *Lobelia trigona Roxb* ethanolic extract was found to be non-toxic to the immortalized human keratinocyte cell lines.

![Graph showing cell viability (%) against Concentration (µg/mL)]

**Figure 6.1** Cytotoxic effect of *Lobelia trigona Roxb* ethanolic extract in HaCaT keratinocyte cells after 24 hours post incubation. Values are represented as Mean ± SEM with triplicate estimations and ** -P<0.001 was considered as significant.

6.3.2. Effect of *Lobelia trigona Roxb* ethanolic extract on cell migration

The effect of *Lobelia trigona Roxb* ethanolic extract on cell migratory activity of HaCaT keratinocytes was investigated and analyzed using a scratch assay. Result of scratch assay was expressed as percentage of cells in wounded area (Figure 6.3). *Lobelia trigona Roxb* ethanolic extract at concentrations 10, 50 and 100 µg/mL significantly promoted the migration of HaCaT cells thereby leading to wound closure (Figure 6.3).
Figure 6.2 Microscope image of evaluating in vitro wound healing effect of Lobelia trigona Roxb ethanolic extract in the scratch assay.

Stimulation of HaCaT keratinocytes migration by Lobelia trigona Roxb ethanolic extract was found effective at concentrations of 100 $\mu$g/mL (90.13%, $P < 0.001$) followed by 50 $\mu$g/mL (75.06%, $P < 0.001$) and 10 $\mu$g/mL (45.41%, $P < 0.001$) respectively. The in vitro wound healing effect of Lobelia trigona Roxb ethanolic extract is illustrated in a dose-dependent manner (Figure 6.2).
Figure 6.3 Digital image showing the effect of *Lobelia trigona* ethanolic extract on the migration of HaCaT cells (keratinocytes) in the scratch assay after 24 hours of incubation. Mean values ± SEM of three experiments are expressed as percent of cell numbers in the wounded area compared to the control.

6.4. DISCUSSION

Wound healing is the effort of tissues to restore normal function and structure after injury. This process is necessary to reform barriers to fluid loss and infection, limit further entry of foreign organisms and material and restores the mechanical integrity of the injured system.

Cell migration is one of the important steps during the second phase (proliferative phase) of wound healing and remodeling of the extracellular matrix process is under the responsibility of the keratinocytes and fibroblasts. Migration of keratinocytes into the wound bed is one of the main factors contributing to epidermal wound healing.
HaCaT keratinocytes cell lines are commonly used for investigating *in vitro* wound healing activity of medicinal plant extract because collagen production, cell proliferation and cell migration of keratinocytes and fibroblast plays a major role in the process of wound healing.

In the present study the investigation was done for the effect of *Lobelia trigona* Roxb ethanolic extract on *in vitro* wound healing. The *in vitro* effects of the ethanolic extract of *Lobelia trigona* Roxb on the skin cells rationalized the remedial effect in wound healing. It was observed that cell migration of *Lobelia trigona* Roxb ethanolic extract occurs in a dose-dependent manner (10, 50 and 100 μg/mL). The effective migration was observed with 100 μg/mL of *Lobelia trigona* Roxb ethanolic extract. A major valuable finding in this study was the stimulatory effect of *Lobelia trigona* Roxb ethanolic extract on the migratory activity of HaCaT keratinocytes leading to an enhanced wound closure. The faster wound closure in *Lobelia trigona* Roxb ethanolic extract treated wounds might also be associated with the increased keratinocyte proliferation and their migration to the surface.

The ethanolic extract of *Lobelia trigona* Roxb exhibited almost a promising effect in the *in vitro* scratch assay, in which the results showed a better activity even at low concentrations. The scratch assay concentrates on the proliferative phase of wound healing process which is characterized by cell migration and cell proliferation of either keratinocytes or fibroblasts (Schafer and Werner 2007, Martin 1997, Gurtner et al. 2008). However the scratch assay couldn’t be an alternate to *in vivo* models. This prompted to extend the study on *in vivo* wound healing effects using the *Lobelia trigona* Roxb ethanolic extract.