1. INTRODUCTION

The wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues [Nalwaya et al., 2009] Because the skin serves as a protective barrier against the outside world, and any break in it must be rapidly and efficiently mended [Martin, 1997]. Healing of wounds is an important biological process involving tissue repairs and regeneration [Esimone et al., 2009]. Proper healing of wound is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin [Annan and Dickson, 2008]. Current estimates indicate that nearly 6 million people suffer from chronic wounds worldwide [Sasidharan et al., 2010].

A conducted by the WHO reports that more than 80% of the world’s population still depends upon the traditional medicines for various diseases [Patel et al., 2009]. Some medicinal plants have been employed in folk medicine for wound care. Some of these plants either possess pro-wound healing activities or exhibit antimicrobial and other related properties that are beneficial in overall wound care [Esimone et al., 2009]. With a view to the increase in the wide spectrum of medicinal usages, the present day requires a new biologically active ointment which exhibit wound healing activity when applied locally [Roy et al., 2009].

Traditional herbal medicine practitioners, throughout the world, have described the healing power and usage of various wild plants. The presence of various life sustaining constituents in plants have urged scientists to examine those plants for potential wound healing properties. The wounds normally progress through the four distinct but overlapping phases during healing: homeostasis, inflammation, proliferation and remodeling [Kumaraswamy et al., 2007]. The complex process of wound healing includes acute inflammation by wounding, regeneration of parenchyma cells, proliferation of connective tissue cells, synthesis of extracellular matrix, remodeling of connective tissue, and subsequent covering of wound bed by the epithelial cells and acquisition of wound strength [Ramzi et al., 1999]. A major hurdle in the improved treatment of wound is the lack of objective, biochemical and physiological parameters, unwanted side effect of the drugs used, and the assessment of wound status. The process of wound healing is reported to promote by plant products such as tannins [Padmaja et al.
131

1.2. Wound healing and the healing cascades
Wound healing involves sequences of events, which is triggered by tissue injury and ends in either partial or complete regeneration or more commonly by repair. The healing cascade begins immediately following injury when the platelets come in contact with exposed collagen [Nayak, 2006]. Wound healing can be classified into any of three types depending on the nature of the edges of the healed wounds. Primary wound healing or healing by first intention occurs within hours of repairing a full-thickness surgical incision. In wounds healed by the first intention, the edges are smoothly closed that no scar is left [Esimone et al., 2005]. Wound healing by second intention involves formation of granulation tissues, which fill up the gaps between the wound edges and is associated with significant loss of tissue, leaving little scars [Esimone et al., 2005]. In a third type of healing, a full-thickness wound is allowed to close and heal. It results in an inflammatory response that is more intense than with primary wound healing. Most skin lesions are healed rapidly and efficiently within a week or two. However, the product is neither aesthetically nor functionally perfect. The healing process involves four phases namely, clot formation, inflammation, granulation and remodeling [Shetty et al., 2006].

1.2.1. Clot formation
The formation of a clot is the immediate response to any trauma. The clot has two functions; firstly it temporarily protects the uncovered tissues and it serves as a provisional matrix for cell migration [Polimeni et al., 2006].

1.2.2. Inflammation
Within hours of injury, inflammatory cells populate the clot and cleanse the wound from bacteria and necrotic. Macrophages migrate into the wound area and, in addition to wound debridement, secrete polypeptide mediators targeting cells during wound-healing process [Polimeni et al., 2006]. Growth factors and
cytokines secreted by macrophages are involved in the proliferation and migration of fibroblasts, endothelial cells, and smooth muscle cells into the wound area [Polimeni et al., 2006]. If the inflammatory phase is prolonged, degradation of collagen will exceed its synthesis [Sasidharan et al., 2010].

1.2.3. Granulation
The formation of new vasculature requires extracellular matrix and basement membrane degradation followed by migration, mitosis, and maturation of endothelial cells. Epithelialization of the wound is initiated within hours of injury. Epithelial cells from the basal layer proliferate and migrate through the fibrin clot and eventually the breach in the epithelium is sealed [Polimeni et al., 2006].

1.2.4. Remodeling
Remodeling can last for years after the initial injury occurred. In this phase the wound undergoes contraction resulting in a smaller amount of apparent scar tissue [James and Friday, 2010]. Maximal tensile strength of the wound is achieved by the 12th week, and the ultimate resultant scar has only 80% of the tensile strength of the original skin. Whether the damaged tissues heal by regeneration or repair depends upon two crucial factors: the availability of cell type(s) needed; and the presence or absence of signals necessary to recruit and stimulate these cells [Polimeni et al., 2006].

*Pedilanthus tithymaloides* (L.) Poit. (family Euphorbiaceae) is a low tropical wild shrub grown in different parts of India, and in the Indian system of medicine it is used as antiviral, antibacterial, emetic, antihemorrhagic, antitumor, abortive [Renne, 1996], anticancer and anti-inflammatory [Bunyapraphatsara and Chokchaichareonporn, 2000] while the aerial part can cure skin disorder [Kumar and Chaturvedi, 2010]. Earlier reports showed the plant contain several bioactive principles including long-chain alcohol, triterpenes [Misra and Khastgir, 1969; Mukherjee et al., 1989; Mukherjee et al., 1992], azafrin [Upadhyay and Hecker, 1974], diterpene pedilstatin [Pettit et al., 2002], kaempferol 3-O-β-D-glucopyranoside-6-(3-hydroxy-3-methylglutarate), quercitrin, isoquercitrin and scopoletin [Abreu et al., 2008], a protease pedilanthain having oral
anti-inflammatory activity [Dhar et al., 1988] and a galactose-specific lectin having mitogenic [Seshagirirao, 1995], antidiabetic activity [Nagda and Deshmukh, 1998] and antitubercular activity [Ankush et al., 2003]. However, a recent study showed that the ethanol extract of leaves and some of its phytoconstituents have antimicrobial activity [Vidottia et al., 2006]. Traditionally tribal peoples used a handful of leaves warmed on fire and tied around the affected wound and fire burns for relief and healing [Sandhya et al., 2006]; while its latex (known as Vilayti-sher), is used in Maharashtra for healing wound [Patil et al., 2009]. The contemporary literature reveals that ethanol extract of *P. tithymaloides* was useful on excision wound in mice [Sriwiroch et al., 2010]. However, there is no systematic scientific or clinical evaluation of the wound healing property of the crude extract of the plant leaves or its phytoconstituents. Thus, the present study for the first time, aims to assess the wound healing activity of *P. tithymaloides* leaves and its isolated constituents in ointment form, compared to the standard formulation povidion iodide ointment.

2. EXPERIMENTAL

2.1. Plant material
Methanol extract (ME) preparation as well as isolation of phytoconstituents from *Pedilanthus tithymaloides* (PT) leaf extract described in chapter 2. Thus, ME and isolated compound(s) from bioactive extract were used to evaluate the wound healing activity.

2.2. Preparation of formulations
Formulations were prepared to evaluate the efficacy of extract (as used traditionally) and its constituents, in comparison with standard Povidion-iodine ointment, USP. Ointment base was prepared by mixing the ingredients (wool fat 5g, hard paraffin 5g, cetostearyl alcohol 5g, soft white paraffin 85g) as per British Pharmacopoeia; 1980, in a beaker and heat at 65°C in a water bath. After cooling, the mixture was homogenized by a homogenizer at 1500 rpm for 10-15 min. The most stable ointment base was selected for the preparation of five formulations (four test groups and one control). The stability was again evaluated at accelerated conditions, to get the most stable formulations for further study. Two types of topical formulations were prepared, one with methanol extract (2.5g
Chapter 6

and 5.0g) of PT leaf and another with isolated compound 5 and 6 (0.25g) using the above ointment base. Thus, formulation containing 2.5 g and 5 g of methanol extract with 100 g of ointment base yielded 2.5% and 5% w/w of active extract in ointment formulation, respectively. Similarly, 25 mg of isolated constituents were incorporated with 10g of ointment base to get 0.25% w/w isolated active compound(s) in ointment formulation. Then, the ointment base was mixed with extract or isolated compound and stirred to get homogeneous ointment preparation [Cooper, 1987]. The drug formulations were freshly prepared on every 5th day.

2.3. Stability of formulation

Stability of the prepared ointment formulations was evaluated according to the guidelines of the International Conference on Harmonization (ICH), 1993; in terms of physical parameters that may affect the stability and acceptability of the formulations. The physical stability of ointment formulations were evaluated by testing its physical changes like phase separation, color, odour, and consistency. Samples of the ointment formulations were kept at different temperature (40°C, 37°C and room temperature) for 45 days and periodically observed for changes like phase separation, development of objectionable color, odour, etc. Centrifugation is used to evaluate accelerated deterioration of ointments. Stability of formulated ointments to centrifugation was determined at 10,000 rpm for 10 min with an Eppendorf centrifuge as described by Cockton and Wynn, 1952 [Cockton and Wynn, 1952]. The formulations, which were resistant towards centrifugation, were selected for spreadability. The spreadability was determined by modified wooden block apparatus consisted of a wooden block with fixed glass slide and a pulley at one end, and a pan attached to another glass slide (movable) with a string at other end. To determine the spreadability, a measured amount of ointment(s) was placed in the fixed glass slides, and the movable slide with the pan was placed over the fixed slide, so that the ointments were sandwiched between the two slides for 5 min. A 50 g weight was placed on the top of two plates and the time required by the top plate to cover a distance of 10 cm was recorded [Prasad and Dorle, 2006]. The spreadability was determined by the formula: \( S = \frac{M}{T} \), where \( S \) is the spreadability in g/s,
Chapter 6

$M$ is the mass in grams and $T$ is the time in seconds. A shorter interval indicates better spreadability [Chakole et al., 2009].

2.4. Experimental animals
Male Wistar albino rats of either sex, weighing about 150–180 g were selected for the study. Animals were maintained in polypropylene cages and given free access to normal food and water ad libitum. All Animals were weighted before and after experiments. The study was carried out with the approval of Institutional Animal Ethics Committee (Reg. No. 0367/01/C/CPCSEA) and was in accordance with the guidelines of CPCSEA. Healthy Swiss albino mice of either sex weighing 20-25 g were used to determine the toxicity and safety profile of the extract and compound(s) as described in chapter 3.

2.5. Acute skin irritation test
The study was carried out by the method of Gfeller et al., 1985, on rats. An area of about 500 mm$^2$ on the dorsal fur of the animals was shaved. The shaved area was cleaned and the prepared ointments were applied separately to different groups of animals. After 4 h, the skins were observed for signs of inflammation.

2.6. Evaluation of Wound-healing activity
Excision, incision and dead space wound models were used to evaluate the wound-healing activity of methanol extract of PT leaves and its isolated constituents. The rats were divided into six groups, each containing six animals, for excision and incision wound models. Fifty milligrams of formulated ointments were applied topically to each animal, once a day. The animals of group I received ointment base (control), while group II were treated with a 5% w/w povidone-iodine ointment. The animals of group III and IV were treated with 2.5% and 5% w/w of methanol extract ointments, while group V and VI were treated with 0.25% w/w ointment of the isolated compound 5 and 6 respectively. For the dead space wound model, four groups, each containing six animals, were used. The animals of group I (control) was treated tropically with simple ointment base. Group II (reference standard) was treated with 5% w/w povidone-iodine ointment USP; while group III and IV were treated with methanol extract ointments 2.5% and 5%
w/w respectively. The rats were anaesthetized using ketamine hydrochloride (100 mg/kg) prior to and during infliction of the experimental wounds. All the animals were closely observed for any infection, so that the animals showing any sign of infection can be excluded from the study.

2.6.1. Excision wound

In the excision wound model [Nayak et al., 2009] animals were anaesthetized prior to and during the creation of experimental wounds, with intravenous ketamine hydrochloride (100 mg/kg body wt.). Rats are inflicted with excision wound as described by Morton and Malon, 1972. The dorsal fur of the animals was shaved with electric clipper and full thickness of excision wound of 500 mm$^2$ was created along the marking using toothed forceps, a surgical blade and pointed scissor. The entire wound was left open [Diwan, 1982]. All groups of animals were treated in the similar manner as mentioned above. The healing of wound was assessed by tracing the wound on 1$^{st}$, 3$^{rd}$, 6$^{th}$, 9$^{th}$, 12$^{th}$, 15$^{th}$, 18$^{th}$, 21$^{st}$ post wounding days using transparency paper and a marker, and the recorded wound areas were measured graphically.

2.6.1.1. Rate of wound contraction

The rate of wound contraction was measured as percentage reduction of wound size at every 2 days interval. Progressive decrease in the wound size was monitored periodically using transparency paper and a marker, and the wound area was assessed graphically to monitor the percentage of wound closure, that indicates the formation of new epithelial tissue to cover the wound. Wound contraction was expressed as reduction in percentage of the original wound size. The Percentage (%) wound contraction= (Wound area on day 0 × Wound area on day n)/Wound area on day 0×100.

2.6.1.2. Epithelialization time

Falling of eschar leaving no raw wound area was considered as complete healing of wound and the number of days required for falling of eschar without any residual raw wound, was calculated as period of epithelialization.
2.6.2. Incision wound

In the incision wound model [Udupa et al., 1994] rats were anaesthetized with ketamine hydrochloride (100 mg/kg) prior to and during the creation of experimental wounds. The dorsal fur of the animals was shaved with electric clipper and two paravertebral long incision of 6 cm length were made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals as described earlier [Ehrlich and Hunt, 1968]. After the incision, the parted skin was stitched together at intervals of one centimeter using surgical thread (No. 000) and curved needle (No.11). The wounds were left undressed. All groups of animals were treated with the same manner as explained above. On 8th post-wounding day sutures were removed and treatment was continued. The skin breaking strength of healed wound was measured on the 10th day by the method of Lee, 1968. The breaking strength is the strength of a healing wound which is measured by the amount of force required to disrupt it.

2.6.2.1. Determination of wound Breaking Strength

Tensile strength is the resistance to breaking the wound under tension, measured as load per unit of cross-sectional area at rupture, while breaking strength (progressive increase in biochemical strength) is the load required to break a wound. Breaking strength is the most crucial phase in dermal wound healing, and restoration of the mechanical properties of tissue strength is a critical outcome of repair process. However, insufficient wound strength may lead to the failure of wound healing, and factors that modulate wound repair can be evaluated on the basis of their influence of development of wound strength. The mechanical properties of the skin are attributed to the collagen structure and elastic fiber networks of dermis. Breaking strength of the healed wound is measured as the minimum force required to break the incision apart, and skin breaking strength provides quantifiable estimates of the efficacy of the aggregate healing process which indicate the tensile strength of wound tissues and the degree of healing [Shetty et al., 2008]. Tensile strength is associated with the organization, content and physical properties of the collagen fibril network, and indicates how much the repaired tissue resists breaking under tension and the quality of the repaired tissue. After removal of skin sutures on post-operative Day 7, gradually increasing weight was applied to one side of the wound while
the other side was fixed. The weight that completely separated the wound from the incision line is considered to be the breaking strength. The sutures were removed on the 8th day after wounding and the breaking strength was measured on 10th day. The mean breaking strength on the two para-vertebral incisions on both sides of the animals were taken as the measures of the breaking strength of the wound of the individual animal.

2.6.3. Dead space wound model

Dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm × 0.3 cm), one on either side of the lumbar region on the ventral surface of each rat [Turner, 1965], and all groups of animals were treated similarly. On the 10th post wounding day, animals from each group were sacrificed under ketamine hydrochloride (100mg/kg body weight, i.m.) anaesthesia, and the granulation (wound) tissues formed on the grass piths were excised carefully. The wet weight of the granulation tissues was recorded and the tissue samples were dried (at 60°C for 12h in an oven) to obtain constant dry weight, expressed as mg/100g body weight [Dispasquale and Meli, 1965]. Simultaneously, dried granulation tissue was hydrolyzed with 5 ml of 6N HCl for 24h at 110°C in sealed glass tubes. The hydrolysate was neutralized to pH 7. The neutralized acid hydrolysate of dry tissue was then subjected to hydroxyproline content estimation as described by Neuman and Logan; 1950. About 200µl of sample were mixed with 1ml of 0.01M copper sulfate solution followed by the addition of 1ml of 2.5N sodium hydroxide and then 1ml of 6% hydrogen peroxide. The solution was mixed with occasionally shaking for 5min. All the tubes were incubated with shaking at 80°C for 5min, then cooled and 4ml of 3N H₂SO₄ was added to each tube with agitation. Finally, 2ml of 5% p-dimethylaminobenzaldehyde was added. The samples were then incubated at 70°C for 16 min, cooled at 20°C, and the absorbance was measured at 540nm in a colorimeter. The amount of hydroxyproline present in the sample(s) was calculated using a standard curve prepared with pure L-hydroxyproline [Gurung and Skalko-Basnet, 2009]. On 10th day, the animals of each group were sacrificed and the granulation tissues formed on the grass piths were excised carefully. A part of wet granulation tissue was preserved in 10% formalin for histopathological evaluation of the effect of the formulations on collagen formation [Sharath et al., 2010]. The preserved tissue were
embedded on 5-6mm thin paraffin blocks, sectioned and stained by Van Geison’s stain [Gal et al., 2006]. The stained sections were examined microscopically for inflammatory cells, blood vessels-marker of near complete healing, and quantification of collagen.

2.7. Statistical analysis
The results were expressed as mean±SE. The statistical significance was evaluated by one-way ANOVA followed by Dennett’s’t’ test (compared differences between experimental groups with control) using the “INSTAT 3” statistic computer program, and P<0.001 and P<0.05 was considered statistically significant.

3. RESULTS
3.1. Stability of the formulation
The evaluation of stability parameters showed that there was no phase separation, objectionable odour or any physical instability. The effect on storage at varying temperature on spreadability of ointments is presented in Table 6.1 and the results indicate that all the formulated ointments are nearly same in terms of applicability or spreading capability. Storage of the formulations, at accelerated stability conditions does not influence the stability of formulation. Thus the formulations are satisfactory as far as physical parameters are concerned.

3.2. Acute skin irritation test
The formulated ointments of methanol extract of PT leaves and its isolated constituents did not show any type of irritation and noticeable inflammation, swelling or any other change on the skin.

3.3. Wound healing study
To evaluate the wound healing ability of the prepared formulations, we measured the following parameters: rate of wound contraction and epithelialisation time (excision wound), tensile strength of newly formed tissue (incision wound), hydroxyproline content in newly formed tissue and histopathological studies of healed tissues (Dead space wound model).
3.3.1. Wound contraction and epithelization time

Wound contraction indicates the rate of reduction of unhealed area during the healing process, i.e., the fast rate of wound closer indicate the better efficacy of medication. The progressive reduction in wound area of the different groups over 18 days, by methanol extract of PT leaves and its isolated constituents is presented in Fig. 6.1 and 6.2. The fastest healing of wound was observed in animals which received ointment of compound 5 and 6, along with complete healing (with 100% wound contraction) within 18 days, as compared with the animals treated with standard 5% w/w povidone-iodine ointment. On the 18th post-wounding day, 5% extract treated animals showed significant reduction in wound area (95.88%) with faster rate of epithelialisation (19.5±0.39) compared to the animal treated with 2.5% extract, which showed moderate degree of healing (93.85% wound area, 20.0±0.52 epithelialization time). Within 18th post-wounding day the complete epithelialisation was noticed in the animals treated with standard drug (5% w/w povidone-iodine) having 100% wound contraction. The lowest rate of wound healing with highest epithelization time (22 days) was seen in control group, which received no drug treatment. While in methanol extract ointment treated group wound remained unhealed even after 15th day of treatment, although the unhealed area was smaller than the control group, with moderate degree of healing. Interestingly, the methanol extract ointment helped in healing of wound, but the treatment with ointment formulation of isolated compounds not only heals the wound but the rate of healing was faster (epithelization time 17.25-17.16 days), and close to the standard ointment treated group (Table 6.2).

3.3.2. Measurement of tensile strength or wound Breaking Strength

The wound healing evaluated by the measurement of tensile strength of the healing skin, treated with different formulations on 10th days revealed that the wound treated with ointment base had the minimum strength (372.13); while the tensile strength of the tissue treated with other formulations was substantially higher than control group (512 to 565.10). It is interesting to note that 5% and 2.5% extract ointment exert nearly similar result on the tensile strength of the healing tissue (Table 6.2). The tensile strength of wound treated with povidone-iodine ointment is comparable (572.50) to the group of
animals that received ointment formulations containing compound 5 (565.10) and 6 (561.12). This observation confirms that methanol extract as well as isolated phytoconstituents possesses excellent wound healing property.

3.3.3. Determination of hydroxyproline content and histopathology of healed tissues

Collagen is the predominant extracellular protein in the granulation tissue and hydroxyproline is an integral part of collagen fibre. Increase in hydroxyproline content indicates increased collagen synthesis, which in turn leads to enhanced wound healing. Table 6.3 represent the wet weight (g), dry weight (g) and hydroxyproline content (mg/g) of the granulation tissue of the animals received different formulations up to 10 days. The dry and wet weight of control animals (13 and 85 g) was increased in 5% extract ointment treated group (19.1 and 177.66 g), whereas the standard drug treated group showed maximum weight (19.1 and 121.15 g). Significant increase in hydroxyproline content was seen in animals which received standard povidone-iodine (207.66 mg/g) and nearly comparable values were observed in animals that received the 5% extract ointment (192.33 mg/g). Thus, these results confirmed the data obtained in wound contraction test. The animals which received only ointment base had lowest content of hydroxyproline content (163.6 mg/g).

Histopathology of granulation tissue, collected on 10th post-wounding day, was examined for cellular epithelial regeneration and matrix organization. The results provide a good evidence of suitability of the formulations in promoting healing of wound (Fig. 6.3). The granulation tissue section of control animals showed decreased epithelialization, fibrosis and more aggregation of macrophages with less collagen fibers, indicating incomplete healing of wounds (Fig. 6.3A). The tissue section from animals treated with povidone-iodine ointment showed complete healing with most prominent epithelialisation and increased collagenation and fibroblast (Fig. 6.3B). While the tissue sections from animals treated with 5% and 2.5% extract ointment showed proliferation of epithelial tissue covering the wound area (Fig. 6.3C, 6.3D) with multiplication of fibrous connective tissue, without proper union, in dermis region. Interestingly the animals treated with ointment formulations of compound 5 and 6 showed fibrous connective tissue with well-collagenation and complete healing (Fig. 6.3E, 6.3F).
Table 6.1. Spreadability of ointment formulations on different days at various temperatures

<table>
<thead>
<tr>
<th>Day</th>
<th>Temperature(°C)</th>
<th>2.5% extract ointment</th>
<th>5% Extract ointment</th>
<th>Compound 5 ointment</th>
<th>Compound 6 ointment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spreadibility(g/s)</td>
<td>Spreadibility(g/s)</td>
<td>Spreadibility(g/s)</td>
<td>Spreadibility(g/s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0th</td>
<td>34</td>
<td>5.314</td>
<td>5.523</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5.526</td>
<td>5.267</td>
<td>5.320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>5.190</td>
<td>5.449</td>
<td>5.120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7th</td>
<td>34</td>
<td>5.220</td>
<td>5.310</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5.189</td>
<td>4.921</td>
<td>5.234</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>5.265</td>
<td>5.120</td>
<td>5.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15th</td>
<td>34</td>
<td>6.212</td>
<td>5.224</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5.721</td>
<td>5.127</td>
<td>5.189</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>5.449</td>
<td>5.342</td>
<td>4.983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30th</td>
<td>34</td>
<td>5.810</td>
<td>6.027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5.891</td>
<td>5.537</td>
<td>5.543</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>5.127</td>
<td>5.278</td>
<td>5.157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45th</td>
<td>34</td>
<td>5.627</td>
<td>5.452</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>6.293</td>
<td>5.104</td>
<td>5.219</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>6.130</td>
<td>5.235</td>
<td>5.234</td>
</tr>
</tbody>
</table>

Table 6.2. Effect of topical application of methanol extract of *P. tithymaloides* and its constituents on period of epithelialization and Breaking strength.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Excision wound Epithelialization time (days)</th>
<th>Incision wound Breaking strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Simple ointment base)</td>
<td>22.00±0.1</td>
<td>372.13±3.23</td>
</tr>
<tr>
<td>Standard ointment (5% povidone-iodine)</td>
<td>15.50±0.28*</td>
<td>572.50±3.4*</td>
</tr>
<tr>
<td>5% w/w Extract ointment</td>
<td>19.5±0.52*</td>
<td>512.00±5.77*</td>
</tr>
<tr>
<td>2.5% w/w Extract ointment</td>
<td>20.0±0.39*</td>
<td>525.33±4.32*</td>
</tr>
<tr>
<td>Compound-5 ointment</td>
<td>17.16±0.4*</td>
<td>565.10±3.1*</td>
</tr>
<tr>
<td>Compound-6 ointment</td>
<td>17.25±0.25*</td>
<td>561.12±3.9*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SE, *P* < 0.05 versus control (One–way ANOVA, followed by Dennett’s ‘t’ test).
Table 6.3. Effect of topical application of methanol extract of *P. tithymaloides* on healing of dead space wound.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wet Weight (g)</th>
<th>Dry weight (g)</th>
<th>Hydroxyproline content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Simple ointment base)</td>
<td>85± 2.30</td>
<td>13± 2.30</td>
<td>163.6± 2.90</td>
</tr>
<tr>
<td>Standard (5% povidone-iodine)</td>
<td>121.15± 1.85*</td>
<td>19.1±0.55*</td>
<td>207.66± 1.45*</td>
</tr>
<tr>
<td>5% w/w Extract ointment</td>
<td>117.66± 1.07*</td>
<td>15.86± 0.59*</td>
<td>192.33± 1.45*</td>
</tr>
<tr>
<td>2.5% w/w Extract ointment</td>
<td>107.33± 2.90*</td>
<td>85± 2.30</td>
<td>156.6 ± 3.10*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SE. *P< 0.001 versus control (One –way ANOVA, followed by Dennett’s t’ test).

Fig. 6.1. Effect of topical application of methanol extract of *P. tithymaloides* and its constituents expressed as percentage wound contraction. Values are expressed as mean ±SE, of 6 animals in each group. *P< 0.001 and ** P< 0.05 versus control (One –way ANOVA, followed by Dennett’s t’ test).
Fig. 6.2. Excision wound (on day 1 and day 18) of animals treated with (A) simple ointment base; (B) 5% standard Povidone-iodide; (C) 5% extract ointment; (D) 2.5% extract ointment; (E) 0.25% compound 5; (F) 0.25% compound 6.
Fig. 6.3. Histology of granulation tissue (A) control animal (B) standard Povidone-iodine ointment treated animal (C) and (D) 5% and 2.5% extract treated animal (E) and (F) with compound 5 and 6 ointment treated animal.
Chapter 6

4. DISCUSSION

Wound healing is a complex and dynamic process of restoring tissue structure in damaged tissue as closely as possible to its normal state. Healing mainly depends upon the repairing ability of the tissue, type and extent of damage, and general state of the host’s health. It is characterized by hemostasis, re-epithelialization, granulation, remodeling of the extracellular matrix and scar formation [Mary et al., 2002]. The aim of the present work is to verify, for the first time, the traditional use *P. tithymaloides* leaves for wound healing and the ability of healing wounds by the formulation containing the PT leaf extract as well as its isolated constituents. No single method is adequate to represent collectively the various components of the wound healing process as a whole [Shirwaikar et al., 2003], thus, the present work aimed to evaluate whether the formulations containing methanol extract or its isolated constituents can act as topical ointment to help in wound healing process in three different models.

Herbal ointment containing different medicinal plants are used in the folk practice and have been reported to be beneficial in wound care, promoting the rate of wound healing with minimum pain, discomfort and scarring of the patient [MacKay and Miller, 2003; Odimegwu et al., 2008]. Ointment is the obvious choice of dosage form due to its convenience of topical application. Thus, to ensure the stability of the ointment during storage, before any formulation, we studied the stability parameters like spreadability and consistency and found that the prepared formulation as per British Pharmacopoeia, 1980, is stable and suitable for topical application.

The process of shrinkage of wound area depends on the repairing abilities of the tissue type, extent of the damage and state of the tissue health [Priya et al., 2004; Anuar et al., 2008]. *In vivo* studies showed enhanced rate of wound contraction in animals treated with ointment containing isolated compounds from *P. tithymaloides* leaves, as compared to control group, which might be due to enhanced epithelization in shorter time. This is because the plant promoted epithelization either by facilitating the proliferation of epithelial cells or by increasing the viability of epithelial cells.

The breaking strength is the strength of healing wound which can be measured by the amount of force required to disrupt it. In the beginning of healing process a wound have little breaking strength, but as it heals breaking strength increases rapidly due to
synthesise of collagen molecules and formation of stable intra- and intermolecular cross linking [Madden and Peacock, 1968]. An increase in skin breaking strength of the animals treated with the PT leaf extract and its constituent, explained that probably the PT extract or its phytoconstituents help in the increase in aldehyde groups of collagen fibres responsible for forming cross linkages which results in greater tensile strength [Chithra et al., 1998].

The granulation tissue formed in the proliferative phase primarily consists of fibroblast, collagen, inflammatory cells and small blood vessels. Wound contraction is a fibroblast dependent method involving the deposition and maturation of collagen. The tensile strength of the granulation tissue increases proportionally with the collagen deposition. The increase in granulation tissue weight in PT leaf extract or its constituents treated animals suggests higher protein content as well as enhances collagen maturation due to increase cross-linking of collagen fibres [Azad, 2002]. However, a number of healing phases like coagulation, inflammation, macrophagia, fibroplasias, collagenation, contraction and epithelization are intimately interlinked. Therefore, a treatment could influence the healing process by intervening one or more phases of healing. Here, the methanol extract of PT leaves and its phytoconstituents treated animals showed a significantly increased hydroxyproline content of granulation tissue, indicating increase collagen turnover. Collagen is the predominant extra cellular protein in the granulation tissue of wounds [Chithra et al., 1998] and hydroxyproline is an integral part of collagen fibre, used as a biochemical marker [Kumar et al., 2006]. The present study, between two test groups, showed that the wound healing activity of the phytoconstituents from the methanol extract of PT leaves is higher than the methanol extract alone. In the methanol extract treated group the lesser activity is due to some other chemical constituents of PT leaves that hindered each other’s activity.

The chemical analysis of bioactive extract of PT leaves yielded a flavone 2-(3,4-Dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one, isolated for the first time from this plant, while the another compound is a tetradecanediol, sodium salt, isolated for the first time from a plant source. The isolated flavone is a yellow crystalline powder, sparingly soluble in water, also known as luteolin is reported to have anti-inflammatory [Jang et al., 2008], antioxidant, antimicrobial and immunomodulating activities. Luteolin exerts its
anti-inflammatory activity by inhibiting thromboxane and leukotriene enzyme of arachidonic acid pathway, along with scavenging of hydrogen peroxide, due to ortho-dihydroxy groups at its B ring and OH substitution at C-5 position of A ring [Odontuya et al., 2005]. It is also reported that luteolin had bacteriostatic activity against *Staphylococcus aureus* [Liu and Matsuzaki, 1995; Tsuchiya et al., 1996], *Helicobacter pylori*, and *Neisseria gonorrhoeae* due to the inhibition of arylamine N-acetyltransferase [Tsou et al., 2001]. Moreover, the ability of luteolin to inhibit IL-6 [Jang et al., 2008], phosphodiesterase [Yu et al., 2010], and multiple sclerosis [Theoharides, 2009], neuroprotection through rebalancing of pro-oxidant-antioxidant level [Zhao et al., 2012] and the activation of monoamine transporter [Zhao et al., 2010] may contribute to the faster wound healing potential of this plant. Modulation of ROS, inhibition of topoisomerases I and II, reduction of NFkB, stabilization of p53, and inhibition of PI3K, STAT3, IGF1R, and HER2 are possible mechanisms for the putative bioactivities of luteolin [Lopez-Lazaro, 2009]. While the second compound tetradecanediol, sodium salt is not reported from plant yet, but the synthetic tetradecanediol have detergent activity [Piepmeyer, 1966]. Thus, the observed wound healing activity of the isolated flavone from methanol extract of PT leaves is probably due to the activation of multiple factors related to the wound healing process and inflammatory pathways.

5. CONCLUDING REMARKS
The present study for the first time demonstrated that topical application of methanol extract of *Pedilanthus tithymaloides* leaves and its isolated compounds 2-(3,4-Dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one and Tetradecanediol, 1-(hydrogen sulfate), sodium salt may promote wound healing activity in rats, probably due to their ability to scavenge free radicals, inhibite of several mediators of inflammatory pathway, reduction of NFkB [Theoharides, 2009], along with its antibacterial activity [Tsuchiya et al., 1996, Vidottiia et al., 2006]. Thus, the result of the present study offers pharmacological evidence of the folk use of *P. tithymaloides* for wound healing.